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2010. Embryonated Eggs as an Alternative Infection  
Model To Investigate *Aspergillus fumigatus* Virulence.  
*Infect. Immun.* 78(7):2995-3006.  
doi:10.1128/IAI.00268-10.

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# Embryonated Eggs as an Alternative Infection Model To Investigate *Aspergillus fumigatus* Virulence<sup>∇</sup>

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Received 18 March 2010/Returned for modification 9 April 2010/Accepted 16 April 2010

**Infection models are essential tools for studying microbial pathogenesis. Murine models are considered the “gold standard” for studying *in vivo* infections caused by *Aspergillus* species, such as *A. fumigatus*. Recently developed molecular protocols allow rapid construction of high numbers of fungal deletion mutants, and alternative infection models based on cell culture or invertebrates are widely used for screening such mutants to reduce the number of rodents in animal experiments. To bridge the gap between invertebrate models and mice, we have developed an alternative, low-cost, and easy-to-use infection model for *Aspergillus* species based on embryonated eggs. The outcome of infections in the egg model is dose and age dependent and highly reproducible. We show that the age of the embryos affects the susceptibility to *A. fumigatus* and that increased resistance coincides with altered chemokine production after infection. The progress of disease in the model can be monitored by using egg survival and histology. Based on pathological analyses, we hypothesize that invasion of embryonic membranes and blood vessels leads to embryonic death. Defined deletion mutant strains previously shown to be fully virulent or partially or strongly attenuated in a mouse model of bronchopulmonary aspergillosis showed comparable degrees of attenuation in the egg model. Addition of nutrients restored the reduced virulence of a mutant lacking a biosynthetic gene, and variations of the infectious route can be used to further analyze the role of distinct genes in our model. Our results suggest that embryonated eggs can be a very useful alternative infection model to study *A. fumigatus* virulence and pathogenicity.**

*Aspergillus fumigatus* is a ubiquitous mold with the ability to cause life-threatening disease in immunocompromised human patients (19). Despite the development of novel antimycotics, the lethality of invasive aspergillosis in human patients is still high and can exceed 50% (14, 57). Thus, a better understanding of the infectious process and the fungal factors involved in pathogenesis is essential for development of more effective treatments. One approach to identify virulence factors is to study fungal gene deletion mutants in appropriate infection models. The most commonly used infection models for *A. fumigatus* are laboratory rodents, especially mice. These models are well characterized and have been critical for understanding host-pathogen interactions, as well as for developing better therapeutic approaches (11). However, ethical considerations and legal restrictions limit the use of mammals for infection studies. Additionally, animal experiments are costly and require specialized facilities and specially trained personnel, which further limits the availability of mammalian models to researchers. Therefore, several alternative infection models for fungal pathogens have been developed in recent years, ranging from cell lines and tissue cultures to invertebrate hosts. Insect models, like the fruit fly (*Drosophila melanogaster*) (28, 29) and larvae of the greater wax moth (*Galleria mellonella*) (21, 30, 37,

42, 43), have been successfully used to investigate virulence traits of *A. fumigatus* and the efficacy of antimycotic treatment. However, these models are limited compared to mammalian hosts; the limitations include, for example, the low temperature required for experiments, the route of infection, and certain aspects of the immune response. One possible way to bridge the gap between insect and mammalian hosts is to use embryonated eggs as an alternative infection model.

Birds of all ages are susceptible to aspergillosis, and this disease has been described for a variety of avian species, both birds in captivity and wild birds (54). Aspergillosis outbreaks can cause significant economic losses in the poultry industry (41) and have prompted the development of vaccination strategies to prevent *Aspergillus* infections (46). The avian immune response to *Aspergillus* infections is similar to the mammalian response with respect to the beneficial effect of a type 1 response, the role of macrophages and heterophils (avian immune cells similar to mammalian neutrophils), and the production of specific antibodies (10, 27, 33). Although these features suggest that birds could be an interesting and potential animal model for studying aspergillosis (11, 47), the same ethical, legal, financial, and logistical restrictions that apply to experiments with mammals apply to experiments with birds. However, embryonated bird eggs could provide an alternative model, which would fulfill the demand for refinement of animal models. Fertilized eggs are readily available from commercial breeders at low cost, are easy to handle, and require little specialized equipment and no specialized facilities or personnel. Since embryonated eggs have been successfully used to study virulence features of various bacteria (2, 55) and the fungal

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<sup>∇</sup> Published ahead of print on 26 April 2010.

pathogen *Candida albicans* (16, 17), we investigated the potential of embryonated eggs as an alternative model for studying the virulence of *A. fumigatus*.

## MATERIALS AND METHODS

***A. fumigatus* strains and preparation of conidia for infection.** *A. fumigatus* wild-type strains CBS 144.89 (Centralbureau voor Schimmelcultures, Utrecht, Netherlands), ATCC 46645 (American Type Culture Collection, Manassas, VA), and Af293 (Fungal Research Trust) ([www.aspergillus.man.ac.uk](http://www.aspergillus.man.ac.uk)), as well as laboratory strains CEA17 $\Delta$ akuB (13) and AfS35 (24), which are deficient for nonhomologous recombination, were used in this study.  $\Delta$ acuD,  $\Delta$ mcsA,  $\Delta$ hcsA, and  $\Delta$ gliP deletion mutants and corresponding complemented strains have been described previously (20, 25, 48, 49). Malt agar slants or plates were inoculated with conidia and incubated at room temperature for 7 days. Conidia were suspended in sterile phosphate-buffered saline (PBS) containing 0.1% Tween 20 (AppliChem GmbH, Darmstadt, Germany) and filtered through a 40- $\mu$ m cell strainer (BD Bioscience, Heidelberg, Germany). After the conidia were counted, the suspension was diluted with PBS to obtain the desired concentration and used within a few hours for egg infection. PBS alone was used as a negative control for infection experiments.

**Preparation of eggs and infection of the CAM.** Fertilized "weiße Leghorn" chicken eggs were obtained from a local producer and stored at 8°C for a maximum of 7 days prior to incubation. Incubation was performed at 37.6°C and 50 to 60% relative humidity in a specialized incubator (BSS 300; Grumbach, Germany). From the fourth day of incubation onward, the eggs were turned four times a day until they were infected. Vitality was assessed daily by candling. Infection of the chorioallantoic membrane (CAM) was performed as described by Härtl et al. (17). Briefly, after the shell was wiped with a disinfectant (Braunol; Braun, Melsungen, Germany), it was perforated at the blunt end and on the side using a dentist's drill. An artificial air chamber on the side was then formed by applying negative pressure at the hole in the blunt end. After perforation of the shell membrane, a 0.1-ml inoculum was applied via the artificial air chamber to the CAM using a sterile 1-ml syringe. The holes were then sealed with paraffin. Survival was monitored for up to 8 days by candling. Unless indicated otherwise, eggs were infected on day 10 of incubation ("developmental day 10"). Twenty eggs were infected for each group, and experiments were repeated at least two times. Survival data were plotted as Kaplan-Meier curves and were analyzed statistically by a log rank test using Graph Pad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA).

**Lysine supplementation.** For lysine supplementation experiments, harvested conidia were diluted to obtain the desired concentration in PBS containing either 2.5 mM or 5 mM lysine. Additional lysine doses on days 1 to 4 postinfection (p.i.) were applied using 100  $\mu$ l 5 mM lysine in PBS via the hole used for infection with a sterile needle and syringe.

**i.p. infection of chicken embryos.** For intraperitoneal (i.p.) infection, an artificial air chamber was constructed as described above. A small circular saw blade on a dentist's drill was then used to carefully cut a 1- by 1.5-cm window into the shell on the side of the egg without damaging the CAM. The cut shell fragment was removed with sterile forceps. The embryo was located through the intact CAM and carefully grabbed with atraumatic sterile forceps to bring it into a position which allowed intraperitoneal injection through the intact CAM. The window was covered with UV-sterilized Parafilm, which was fixed to the shell with adhesive tape. Twenty eggs per group were infected, and the experiments were repeated two times. Survival data were plotted as Kaplan-Meier curves and were analyzed statistically by a log rank test using Graph Pad Prism version 5.00 for Windows.

**Reisolation.** Dead eggs that were identified by candling or eggs that were humanely killed by chilling on ice for 1 h followed by decapitation and exsanguination were randomly selected from various experiments. Following disinfection of the surface of an egg with 70% ethanol, the shell was opened with sterile scissors. The CAM surrounding the shell perforation site was removed with sterile forceps and swabbed onto malt agar plates. The embryo was then carefully separated from its surrounding membranes and placed in 70% ethanol for 2 to 3 min. Using sterile scissors and forceps, the abdominal cavity was opened. The liver was removed and swapped onto malt agar plates. The plates were incubated at room temperature for 54 h prior to macroscopic evaluation of fungal growth.

**Histological examination of CAM and liver.** For sample preparation, embryos were killed by chilling the eggs on ice for 1 h. The egg surface was disinfected with 70% ethanol. Using sharp scissors, the upper half of each shell was cut and removed, leaving the CAM attached to the removed shell fragment. The CAM was then cut into samples that were suitable sizes and fixed in 10% formalin. The

liver was removed during postmortem examination and fixed in 10% formalin. Samples were embedded in paraffin and cut into 5- $\mu$ m sections. Mounted sections were stained with either hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS) stain using standard protocols and were examined using bright-field microscopy at the magnification indicated below.

**RNA isolation and transcription analysis using quantitative real-time RT-PCR.** Embryos were infected on the CAM as described above with  $10^3$  conidia of strain CEA17 $\Delta$ akuB on either developmental day 10 or developmental day 12. At different time points, the embryos were killed by chilling the eggs on ice for 1 h, and the eggs were opened as described above. For PBS controls and infected eggs up to 24 h p.i., a round piece of the CAM (2 to 2.5 cm in diameter) surrounding the drilled hole was removed. At later time points, when fungal mycelia were visible, an 8-mm biopsy punch was used to excise and remove CAM adjacent to mycelia. Spleens were removed at postmortem examination. All samples were placed into RNAlater RNA stabilization reagent (Qiagen, Hilden, Germany) immediately after removal and incubated at 4°C overnight on a tube rotator prior to storage at -20°C. For total RNA isolation, 20 to 30 mg (CAM) or 5 mg (spleen) of tissue was used. Due to the small size of the spleens, two or three spleens were pooled for one RNA preparation. Sample lysis, removal of genomic DNA, and RNA isolation were performed with QIAshredder, RNase-free DNase Set, and an RNeasy minikit (all obtained from Qiagen) used according to the manufacturer's recommendations. The quality and quantity of eluted RNA were determined by spectral analysis using a NanoDrop ND-100 spectrophotometer (peqlab, Erlangen, Germany). Only samples with  $A_{260}/A_{280}$  and  $A_{260}/A_{230}$  ratios of about 2 were used in subsequent analyses. The concentration of RNA was adjusted to 100 ng/ $\mu$ l immediately before reverse transcription-PCR (RT-PCR) was performed. Previously described primers and temperature protocols for K60, inducible nitric oxide synthase (iNOS), gamma interferon (IFN- $\gamma$ ), and the internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (5, 7, 40) were used. For macrophage inflammatory protein 1 $\beta$  (MIP-1 $\beta$ ) the following primers were used with an annealing temperature of 59°C: forward primer 5'-TCCTGCTGCTTCACCTACATCT-3' and reverse primer 5'-ATG AACACAACACCAGCATGAG-3'. Interleukin-1 $\beta$  (IL-1 $\beta$ ) was analyzed using an annealing temperature of 57°C with the following primers: forward primer 5'-TTTITGAGCCGTCACCTTC-3' and reverse primer 5'-TACA GCGCAATGTTGAGCCTC-3'. Quantitative real-time RT-PCRs were performed using a one-step quantitative RT-PCR MasterMix Plus for SYBR green I kit (Eurogentec, Seraing, Belgium) according to the manufacturer's instructions. Amplification and detection of specific products were performed with a 7300 real-time PCR system (Applied Biosystems, Foster City, CA) using the dissociation curve mode to check the specificity of amplification products. Specific mRNA levels were quantified using the threshold method (38) and were normalized using the changes in the cycle threshold ( $\Delta C_T$  values) for GAPDH. The results were expressed as fold changes compared to the PBS control determined by calculating  $2^{-\Delta\Delta C_T}$ . Two groups at a given time point were compared by the unpaired, two-tailed *t* test using Graph Pad Prism version 5.00 for Windows.

## RESULTS

**Dose-dependent mortality and comparison of different wild-type and laboratory *A. fumigatus* strains.** In order to determine the appropriate infectious dose and to determine whether *A. fumigatus* kills embryos in a dose-dependent manner, eggs were infected on developmental day 10 with  $10^7$  to  $10^2$  conidia of wild-type and laboratory strains (CBS 144.89, ATCC 46645, Af293, CEA17 $\Delta$ akuB, and AfS35). Developmental day 10 was chosen as the time of infection since at this age viable embryos can be identified reliably and because initial experiments showed that embryos that are this age tolerate manipulation of the shell and the natural air space (data not shown). Infectious doses of  $10^4$  conidia per egg or higher resulted in 100% lethality within 2 to 4 days in all individual experiments, irrespective of the strain used. Infection with  $10^3$  conidia per egg resulted in a delay in killing, but there was still 90 to 100% lethality. Reducing the infectious dose to  $10^2$  conidia per egg further delayed and reduced lethality. This dose dependence was ob-

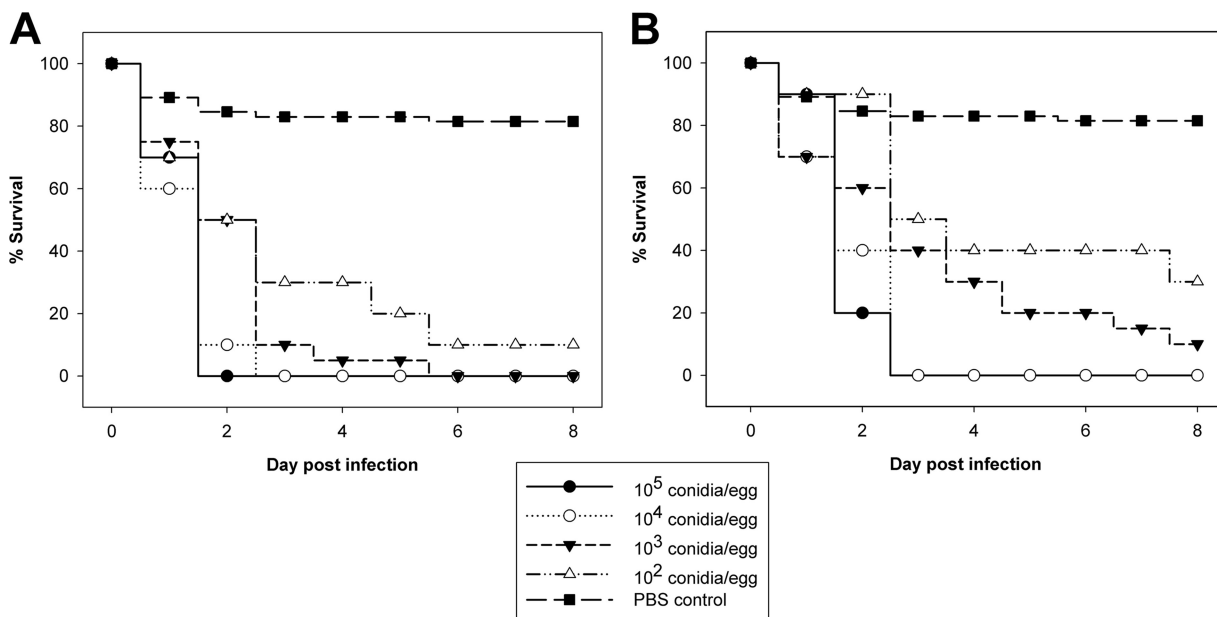


FIG. 1. Dose-dependent mortality of embryonated eggs infected with *A. fumigatus* CEA17ΔakuB (A) and *A. fumigatus* ATCC 46645 (B). Eggs were infected with the doses indicated on developmental day 10 ( $n = 20$  for  $10^5$  and  $10^4$  conidia/egg;  $n = 40$  for  $10^3$  and  $10^2$  conidia/egg). Experiments were performed twice. Survival was monitored daily for 8 days, and the results are expressed as Kaplan-Meier curves. The log rank test for trend was significant for both strains ( $P < 0.0001$ ) (the results for the PBS control were not included in the statistical analysis).

served for all strains, was statistically significant ( $P < 0.0001$ , log rank test for trend), and is shown for CEA17ΔakuB and ATCC 46645 in Fig. 1A and B, respectively.

Treatment with all wild-type and laboratory strains tested (CEA17ΔakuB, CBS 144.89, ATCC 46645, Af293, and AfS35) resulted in indistinguishable lethality rates if eggs were infected with  $10^4$  or more conidia (data not shown) and similar mortality rates after infection with  $10^3$  and  $10^2$  conidia (Fig. 2A

and B); 80 to 95% of PBS mock-infected embryos survived until the end of the observation period. Embryonic death in the control group could be attributed to manipulation since the embryos died mainly 1 to 2 days after infection. It could also have been caused by genetic or developmental defects independent of the experimental procedure. The variability with an infectious dose of  $10^3$  conidia per egg was found to be very low, and 85 to 100% mortality was observed consistently on day 3 or

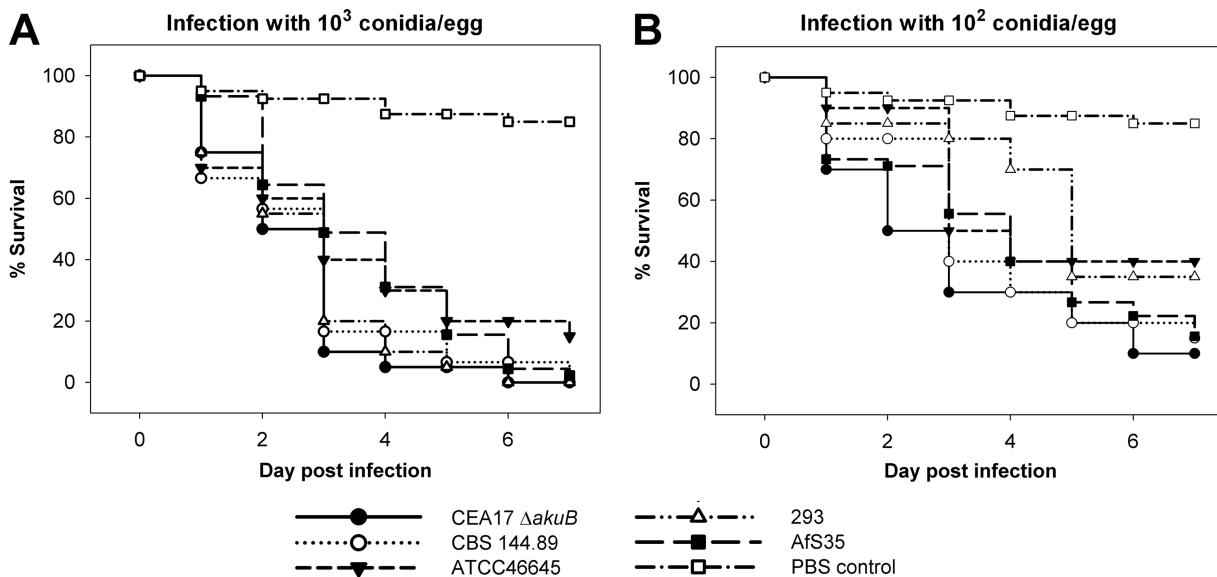


FIG. 2. Virulence of wild-type and laboratory *A. fumigatus* strains in embryonated eggs. Embryonated eggs were inoculated with  $10^3$  (A) or  $10^2$  (B) conidia per egg on developmental day 10 (20 eggs per group per experiment; three independent experiments per strain per infectious dose). Survival was monitored daily for 8 days, and the results are expressed as Kaplan-Meier curves. The log rank test did not indicate that there were significant differences between strains ( $P > 0.05$ ).

4 p.i. These variations were within the viability range for PBS control eggs.

#### Macroscopic and histological alterations during infection.

In order to analyze morphological modifications of the CAM and to determine fungal growth, eggs containing viable embryos were selected randomly on days 1, 2, 4, and 6 p.i. Ten eggs per day per group (PBS control eggs and eggs infected with  $10^2$  conidia of *A. fumigatus* CEA17 $\Delta$ *akuB*) were analyzed. In the PBS control eggs, the entire CAM was uniformly nerved with well-defined branched blood vessels. No signs of trauma from the application process were observed at the site of inoculation (Fig. 3A to D). In infected eggs, mycelia were macroscopically visible in most eggs from day 2 p.i. onward (Fig. 3F to H). The size of the area covered by mycelium increased over time to a maximum diameter of ca. 3 cm. Sporulation was frequently observed on days 4 and 6 in eggs in which the artificial air chamber formed at the time of infection did not recede; in contrast, sporulation was rarely seen in eggs in which the artificial air chamber receded. To determine whether a visible mycelium and sporulation were correlated with death and virulence, we simultaneously investigated dead eggs and eggs which had survived 7 days of infection in survival experiments with different wild-type and mutant strains. However, irrespective of the strain investigated and the viability of the embryo, ~70 to 80% of the eggs contained mycelium, and in 50 to 70% of these eggs there was sporulation. Thus, the presence of macroscopically visible mycelium and sporulation did not correlate with viability.

To elucidate whether *A. fumigatus* was present on CAM with no macroscopically visible mycelia, culturing of CAM was performed for a total of 154 infected eggs and 20 PBS control eggs. No microorganisms were isolated from any of the PBS control eggs. In contrast, *A. fumigatus* was readily reisolated from the CAM of 134 infected eggs, demonstrating that fungal cells were present in the CAM in the absence of visible mycelium. The 20 remaining infected eggs, for which fungal growth was not detected on agar plates, generally were eggs that were infected with low numbers of conidia ( $10^2$  conidia). Since only a small part of the CAM was excised, the infected regions might have been missed in these eggs. Alternatively, the immune system might have been able to clear the infection when very low doses of conidia were used.

A typical feature observed in most (>90%) infected eggs and all eggs containing macroscopically visible mycelium was areas where there were altered blood vessel patterns in the CAM. In the vicinity of hyphae, blood vessels either were completely absent (Fig. 3G, K, and M) or appeared to be very thin (Fig. 3H) or blotchy (Fig. 3I, L, and M). Alterations were observed mainly for smaller vessels, while larger vessels appeared to be unchanged (Fig. 3H and K). Invasion of *A. fumigatus* hyphae into blood vessels was confirmed by histological analysis of infected CAM. Hyphae grew through the outer epithelium of the CAM, formed a tight mycelium in the inner layer of connective tissue, and penetrated blood vessels (Fig. 3O).

The invasion of blood vessels by hyphae prompted us to investigate whether *A. fumigatus* could disseminate via the CAM into internal organs of the embryo, as *A. fumigatus* is commonly found in the livers of infected birds (22, 32, 54). We performed necropsies of 40 dead embryos infected with  $10^2$

$10^5$  conidia of CEA17 $\Delta$ *akuB* and cultured liver tissue from these embryos. *A. fumigatus* was reisolated from 7 of the 40 livers; all culture-positive livers originated from embryos infected with  $10^4$  or  $10^5$  conidia. In general, no gross alterations of internal organs were observed upon necropsy; however, some livers of *A. fumigatus*-infected embryos appeared to be darker. Thus, we analyzed embryonic livers from embryos that were alive on days 3 and 6 after infection with  $10^2$  conidia histologically. The livers of infected embryos had enlarged blood vessels with an abundance of erythrocytes, indicating that there was liver congestion (Fig. 3Q).

As nutrient acquisition by a fungus during growth on the CAM might impair nutrient availability for the embryo, we weighed surviving uninfected and infected embryos on days 1, 2, 4, 6, and 8 p.i. (10 embryos for each day and group). No significant differences in weight were observed.

**Influence of developmental age on the outcome of infection and the immune response.** Since the embryonic immune system matures during development, we investigated whether the age of embryos at the time of infection influences the outcome of *A. fumigatus* infection. In this analysis eggs were infected on developmental days 10, 12, and 15. Compared to the results for embryos infected on developmental day 10, the onset of mortality was clearly delayed in older embryos, and survival was significantly greater for eggs infected on developmental days 12 and 15 ( $P < 0.05$  for every individual experiment,  $P < 0.0001$  for summarized data shown in Fig. 4). As the experiments had to be terminated on developmental day 20 to avoid full development and hatching of chicks (day 21), eggs infected on developmental day 15 could be monitored for only 5 days p.i. The mortality rate during these 5 days resembled that of eggs infected on developmental day 12. Whether differences in mortality rates could occur later in the time course of infection could not be determined with the experimental design used.

To analyze whether immune maturation during embryogenesis influences the immune response, we determined the levels of transcription of genes encoding the proinflammatory immune mediators K60 (CXCL1), iNOS, IFN- $\gamma$ , MIP-1 $\beta$ , and IL-1 $\beta$  locally in the CAM of PBS controls and infected eggs on either developmental day 10 or developmental day 12. The basal levels of transcription of these factors in PBS control eggs did not vary significantly over time (data not shown). The greatest increase in transcription level after infection with *A. fumigatus* was observed for the K60 chemokine gene. The levels of K60 gene transcription in infected embryos steadily increased over time and were significantly higher 12 h, 24 h, and 72 h p.i. in embryos infected on developmental day 12 than in embryos infected on developmental day 10 (Fig. 5A). Transcription of the proinflammatory cytokine IL-1 $\beta$  gene was moderately increased at 72 h p.i., and the increase was significantly greater for embryos infected when they were older (Fig. 5B). The levels of transcription of the iNOS gene increased little over time, and there was no difference between the groups (Fig. 5C). The levels of transcription of the genes coding for IFN- $\gamma$  (Fig. 5D) and MIP-1 $\beta$  (data not shown) were not influenced by infection.

To assess the systemic immune response, levels of transcription in the spleen were analyzed 24 h and 72 h p.i. In the spleen, no upregulation was observed for the genes encoding IL-1 $\beta$  (Fig. 5B) and MIP-1 $\beta$  (data not shown). The K60 gene

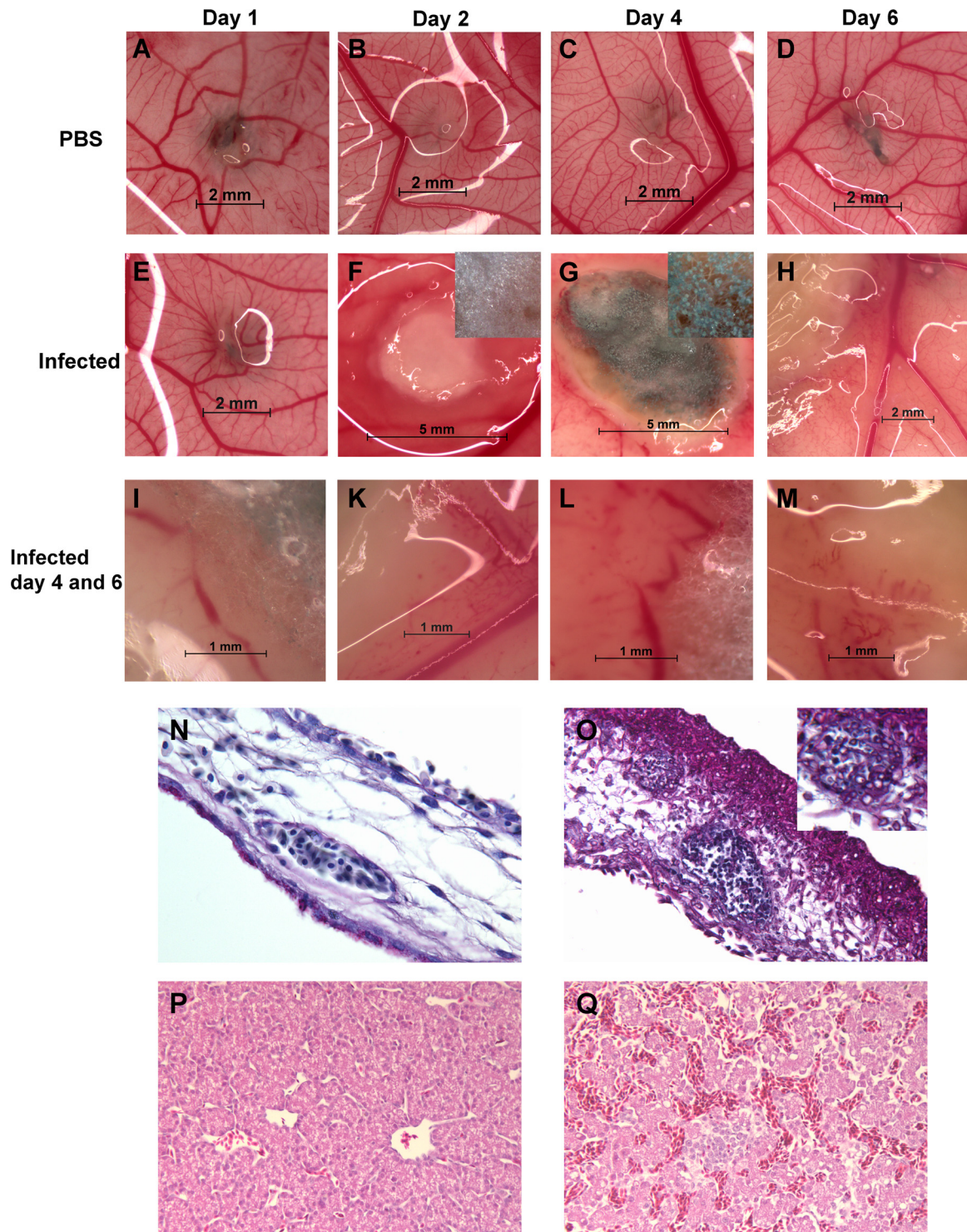


FIG. 3. Macroscopic and microscopic changes in infected embryonated eggs. Eggs were infected on developmental day 10. (A to M) Macroscopic changes on the CAM surrounding the hole drilled for infection (darker area). (A to D) PBS control. The days after infection are indicated at the top. (E to H) Infection with  $10^2$  *A. fumigatus* CEA17 $\Delta$ *akuB* conidia/egg. The insets in panels F and G show higher magnifications of mycelia. (I to M) Higher magnifications of eggs infected with *A. fumigatus* on day 4 after infection (I and K) and on day 6 after infection (L and M). (N to Q) Histological analysis of the CAM (N and O) and livers (P and Q) of infected embryos. (N and O) CAM stained with PAS stain. Magnification,  $\times 63$ . (N) PBS control. (O) CAM infected with  $10^2$  *A. fumigatus* CEA17 $\Delta$ *akuB* conidia/egg (inset, higher magnification of left blood vessel). (P and Q) Liver stained with H&E. Magnification,  $\times 20$ . (P) PBS control. (Q) Liver after infection with  $10^2$  *A. fumigatus* CEA17 $\Delta$ *akuB* conidia/egg.

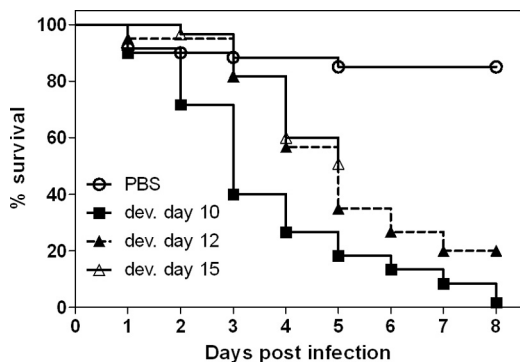


FIG. 4. Influence of embryonic age at the time of infection on mortality. Eggs were infected on developmental (dev.) day 10, 12, or 15 with  $10^2$  *A. fumigatus* CEA17 $\Delta$ *akuB* conidia/egg (20 eggs per age per experiment), and three independent experiments were performed. Survival was monitored daily for 8 days, and the results are expressed as Kaplan-Meier curves. Embryos infected on developmental days 12 and 15 survived significantly longer than less-developed embryos ( $P < 0.001$ , log rank test).

showed earlier upregulation in the spleens of embryos infected on developmental day 10, but the transcript levels at 72 h p.i. were higher in older embryos (Fig. 5A); however, none of the differences were statistically significant. The iNOS transcript levels in the spleens of embryos infected on developmental day 10 were significantly higher than the iNOS transcript levels in the spleens of embryos infected on developmental day 12 (Fig. 5C), but they were only  $\leq 1.5$ -fold higher than the iNOS transcript levels in the spleens of PBS control embryos. Similarly, the IFN- $\gamma$  transcript levels in the spleens were significantly higher in embryos infected on developmental day 10 than in embryos infected on developmental day 12 at 72 h p.i., but they were less than the transcript levels for the PBS controls (Fig. 5D).

Thus, an increased local immune response of embryos at later developmental stages coincided with reduced mortality after *A. fumigatus* infection.

**Infection with *A. fumigatus* deletion mutants.** To determine whether embryonated eggs are a suitable alternative model for detecting differences in the virulence of *A. fumigatus* deletion mutants, mutants with defined virulence potential in cortisone acetate-treated mice were used to infect embryonated eggs on developmental day 10. Based on the results of the previous experiments,  $10^3$  conidia per egg were used to induce 90 to 100% lethality. Since partial virulence defects might not be detectable at a high infectious dose which rapidly causes 100% lethality, we also performed infection experiments with  $10^2$  conidia per egg. The results for the virulence of mutants were highly reproducible in these experiments, and the total mortality rates differed 10 to 15% between experiments.

An *A. fumigatus* mutant with the *acuD* isocitrate lyase gene deleted ( $\Delta$ *acuD* mutant) has previously been shown to be fully virulent in a mouse model (48). Likewise, this mutant was fully virulent in the egg model (Fig. 6A and B). Deletion of the *mcsA* methylcitrate synthase gene results in attenuation of virulence in mice which is more pronounced with a lower infectious dose (20). Analogous to the situation in the murine model, a  $\Delta$ *mcsA* mutant displayed dose-dependent attenuation

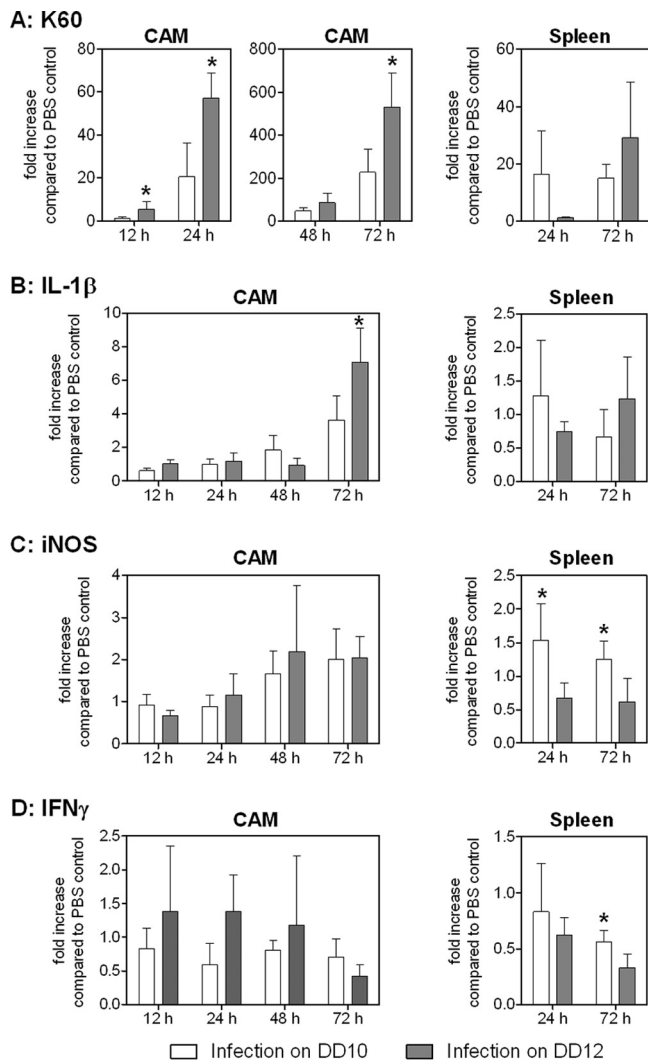


FIG. 5. Levels of transcription of chemokines, cytokines, and iNOS in the CAM and spleen after infection with *A. fumigatus* CEA17 $\Delta$ *akuB*. Eggs were infected on the CAM with  $10^3$  conidia on either developmental day 10 (DD10) or developmental day 12 (DD12). Transcripts were quantified by real-time quantitative RT-PCR, and the results were normalized using GAPDH and are expressed as fold increases compared to age- and time-matched PBS controls (5 eggs per group per time point). The two groups for each time point were compared by using an unpaired, two-tailed *t* test. Asterisks indicate significantly ( $P < 0.05$ ) higher levels of transcription.

in the egg model. At a higher dose the mutant was fully virulent (Fig. 6C), but it showed significant attenuation of virulence at a lower dose (Fig. 6D) ( $P < 0.05$ ). Finally, we found that a homocitrate synthase mutant ( $\Delta$ *hcsA* mutant), which is strongly attenuated in mice infected intranasally (49), was significantly and strongly attenuated in eggs infected via the CAM on developmental day 10, irrespective of the infectious dose (Fig. 6E and F) ( $P < 0.05$ ).

Our results show that there was a significant decrease in mortality in older embryos infected with *A. fumigatus* CEA17 $\Delta$ *akuB* (Fig. 4), which was accompanied by changes in the chemokine and cytokine expression pattern (Fig. 5). These findings might have been related to progression of immune

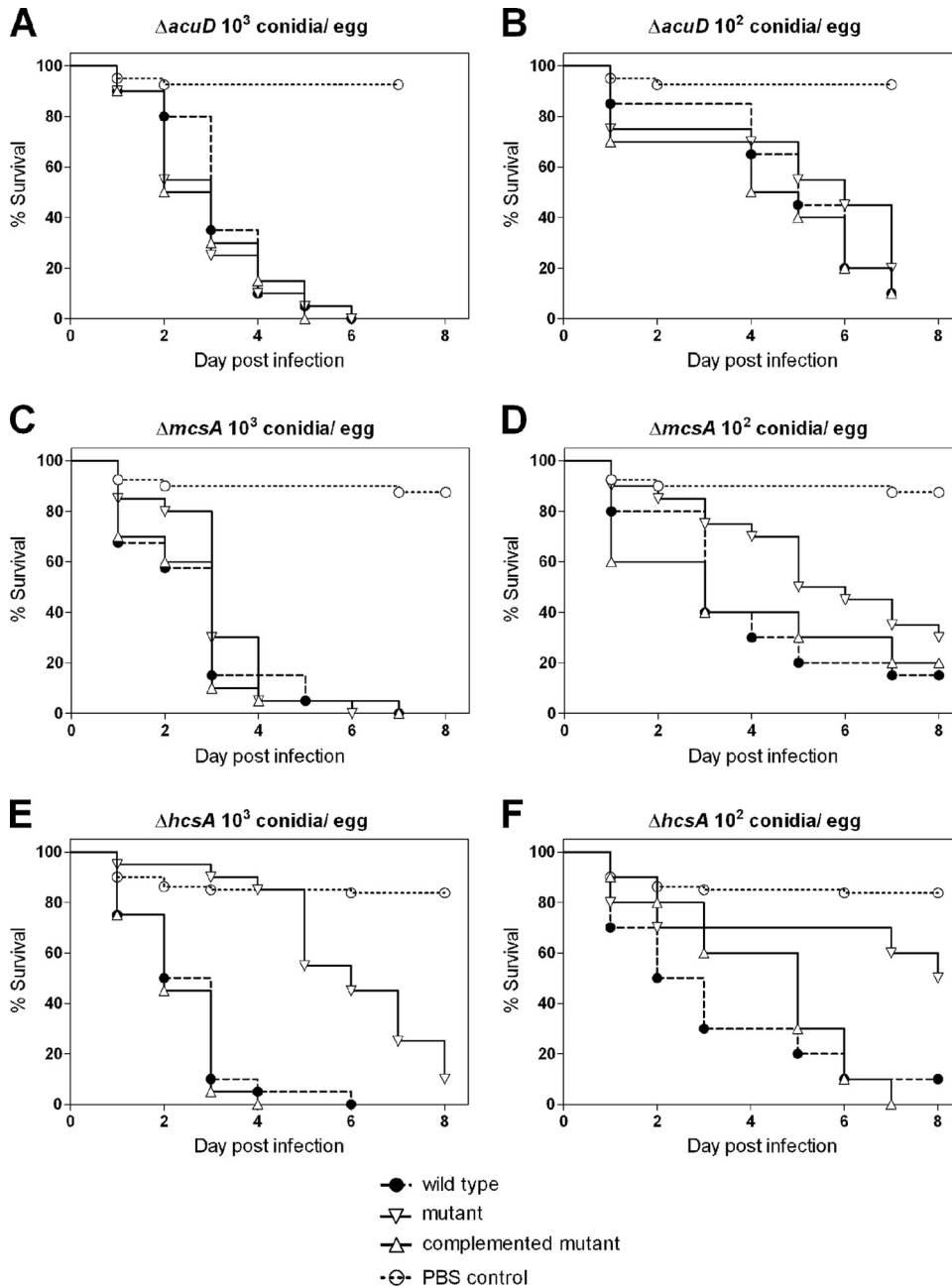


FIG. 6. Virulence of defined *A. fumigatus* deletion mutants in embryonated eggs. Eggs (20 eggs per group per experiment; three independent experiments per strain and infectious dose) were infected on developmental day 10 with  $10^3$  conidia/egg (A, C, and E) or  $10^2$  conidia/egg (B, D, and F). Survival was monitored daily for 8 days, and the results are expressed as Kaplan-Meier curves. (A and B) Isocitrate lyase (*acuD*) mutant. (C and D) Methylcitrate synthase (*mcsA*) mutant. (E and F) Homocitrate synthase (*hcsA*) mutant.

maturation and increased host defense. Therefore, we hypothesized that the partial virulence defect of the *A. fumigatus*  $\Delta mcsA$  mutant might be more pronounced in embryos infected at a later developmental stage. Thus, the  $\Delta mcsA$  mutant was also tested in eggs infected on developmental day 12. In accordance with our hypothesis, this mutant showed significantly reduced virulence ( $P < 0.01$ ) at a higher dose ( $10^3$  conidia per egg) in embryos infected on developmental day 12 (Fig. 7A). The rates of mortality caused by  $10^3$  mutant conidia in older embryos were comparable to the rates of mortality caused by

the wild-type and complemented mutant when a 1-log-lower infection dose was used (Fig. 7A); thus, this outcome closely resembled the outcome of infection with this strain in cortisone acetate-treated mice (20).

To further test the hypothesis that the age of embryos is related to immune maturation and host defense, we tested a gliotoxin-deficient strain ( $\Delta gliP$  mutant) in the egg model. The role of gliotoxin in virulence has been shown to depend on the immune status of the host, as gliotoxin-deficient *A. fumigatus* strains are attenuated in cortisone acetate-treated mice but



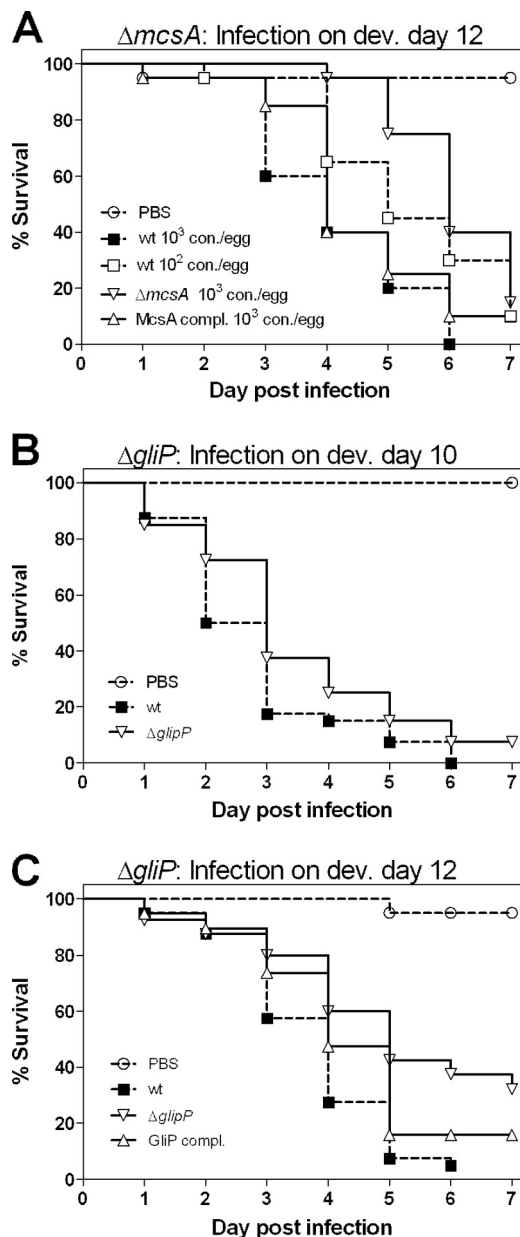


FIG. 7. Virulence of the *A. fumigatus* methylcitrate synthase ( $\Delta mcsA$ ) mutant and the gliotoxin-deficient ( $\Delta gliP$ ) mutant in older embryonated eggs. (A) Methylcitrate synthase mutant. Eggs (20 eggs per group per experiment; two independent experiments) were infected on developmental day 12 (dev. day 12) with  $10^3$  conidia/egg of the mutant and the complemented strain. Infection with the wild-type control was performed for two groups using  $10^3$  and  $10^2$  conidia/egg. wt, wild type; con., conidia; McsA compl., complemented methylcitrate synthase mutant. (B and C) Gliotoxin-deficient mutant. Eggs (20 eggs per group per experiment; two independent experiments) were infected on developmental day 10 (B) or developmental day 12 (C) with  $10^3$  conidia/egg of the wild-type control strain, the mutant, and the complemented strain (data are shown only for developmental day 12). Survival was monitored daily for 7 days, and results are expressed as Kaplan-Meier curves. GliP compl., complemented gliotoxin mutant. Analysis using the log rank test revealed significant attenuation of the  $\Delta mcsA$  mutant ( $P < 0.01$ ) and the  $\Delta gliP$  mutant ( $P < 0.001$ ) in embryos infected on day 12.

fully virulent in leucopenic mice (51). We found that the  $\Delta gliP$  mutant exhibited full virulence in eggs infected on developmental day 10 with either  $10^3$  conidia (Fig. 7B) or a lower dose ( $10^2$  conidia) (data not shown). In contrast, the  $\Delta gliP$  mutant was significantly attenuated ( $P < 0.01$ ) in eggs infected with  $10^3$  conidia on developmental day 12 (Fig. 7C).

We have recently shown that in mice the reduced virulence potential of a  $\Delta hcsA$  lysine auxotrophic mutant can be restored by supplementation of the drinking water with lysine if the water is supplemented continuously after infection (49). To test whether lysine supplementation likewise restores virulence in the egg model, we performed two sets of supplementation experiments with the egg model. Supplementation with 100  $\mu$ l 2.5 mM lysine on the day of infection did not influence the virulence of the  $\Delta hcsA$  mutant (Fig. 8A). However, addition of 100  $\mu$ l 5 mM lysine daily from the day of infection until day 4 p.i. restored the virulence of the  $\Delta hcsA$  mutant (Fig. 8B). Thus, lysine supplementation had comparable results in the murine and egg models.

The route of infection can have a major effect on the outcome of infection experiments. While the *A. fumigatus*  $\Delta hcsA$  mutant shows strong attenuation in a murine pulmonary model, this mutant is fully virulent if it is inoculated systemically into mice (49). To test if similar experiments can be performed in the egg model, we infected embryos on developmental day 12 intraperitoneally with  $10^3$  conidia of the wild type and the  $\Delta hcsA$  mutant. Developmental day 12 was chosen because preliminary experiments revealed that embryos at this stage are more resistant to the necessary manipulations. We still observed approximately 30% mortality in all groups, including the PBS mock-infected embryos, in the first 24 h p.i. This early mortality was probably due to the more extensive manipulations necessary to perform the systemic infection procedure. However, >95% of the PBS-treated embryos that were alive on day 1 p.i. survived until the end of the experiment. Therefore, a survival analysis was performed only for embryos that survived the first 24 h p.i. We reproducibly observed no difference between the mortality after systemic infection with the wild type and the mortality after systemic infection with the  $\Delta hcsA$  mutant (Fig. 8C); these results are comparable to the results obtained for systemic murine infection, in contrast to the results obtained for infection of the CAM (Fig. 6E and F).

## DISCUSSION

Embryonated eggs have been used for decades as a convenient alternative infection model for studying viruses and for determination of bacterial virulence determinants (2, 18, 23, 55). However, to our knowledge, they have never been used as a model to study the pathogenesis of filamentous fungi, such as *A. fumigatus*.

Embryonated eggs can be infected via the CAM, the albumen, the yolk sack, or the allantoic cavity or by direct injection into the embryo or major blood vessels. We decided to infect eggs via the CAM for the following reasons. (i) The CAM is a thin, translucent membrane consisting of two epithelial layers separated by loose connective tissue (44). Pathogens applied to the outer layer of the CAM have to invade the outer epithelial layer to access nutrients, and invasion is an important virulence determinant for *A. fumigatus* in the lung. (ii) The CAM is

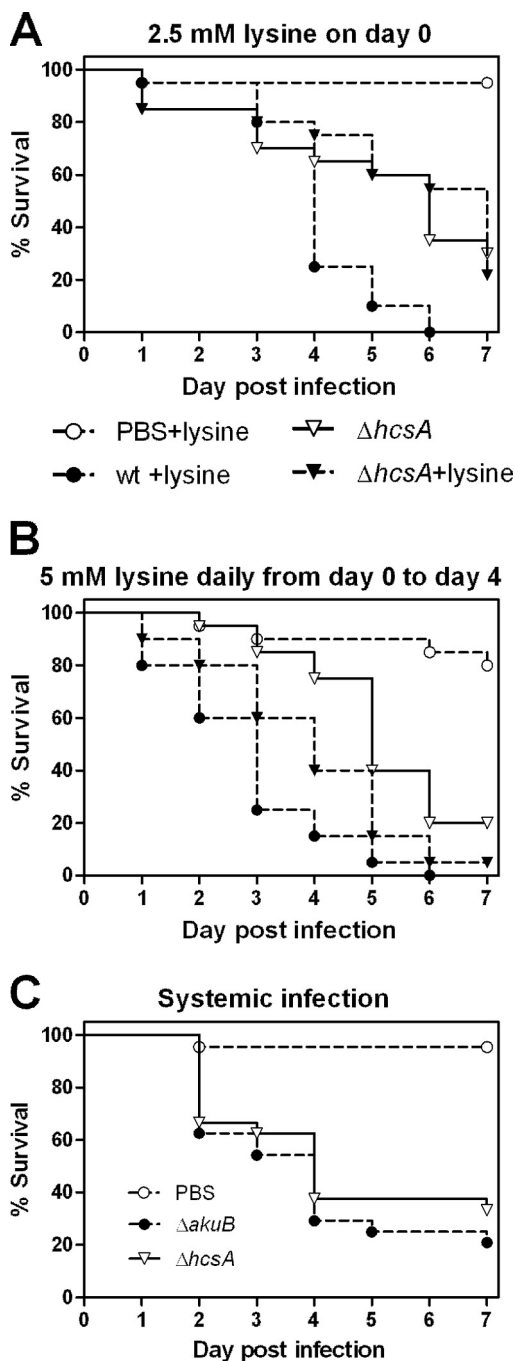


FIG. 8. Effects of lysine supplementation and systemic application on the virulence of the  $\Delta hcsA$  mutant. Eggs (20 eggs per group per experiment; two independent experiments) were infected on developmental day 10 (A and B) or developmental day 12 (C) with  $10^3$  conidia/egg of the mutant and the wild-type strain (wt). In the experiment whose results are shown in panel A 100  $\mu$ l 2.5 mM lysine was added at the time of infection, and in the experiment whose results are shown in panel B 100  $\mu$ l 5 mM lysine was added at the time of infection and daily during the first 4 days p.i. Survival was monitored daily for 7 days, and the results are expressed as Kaplan-Meier curves. (B) The survival of embryos infected with the  $\Delta hcsA$  mutant and grown with 5 mM lysine was significantly different from the survival of embryos infected with the  $\Delta hcsA$  mutant without lysine ( $P < 0.02$ , log rank test) and wild-type strain-infected embryos ( $P < 0.05$ , log rank test). (C) The log rank test did not indicate that there were significant differences between strains after systemic infection ( $P = 0.35$ ).

highly vascularized in order to mediate both nutrient transport from the albumen and yolk sack to the embryo and, importantly, gas exchange during the latter two-thirds of embryonic development (44). High levels of vascularization and gas exchange are also characteristics of mammalian pulmonary tissue. (iii) Aseptic application of solutions onto the CAM is technically straightforward, and the required manipulation is well tolerated by the embryo (56).

Our results demonstrate that embryonated eggs are highly susceptible to *A. fumigatus* infection via the CAM in a dose-dependent manner. This fungus proliferates radially on the CAM, invading the tissue and blood vessels; these findings resemble histological findings for human patients (9, 31) and experimentally infected mice (4, 53). Similar to murine models of pulmonary invasive aspergillosis, in which dissemination to internal organs is rarely observed, dissemination from the CAM to the embryonic liver was rare and occurred only if high infectious doses ( $\geq 10^4$  conidia per egg) were applied. We examined only dissemination to liver, and the fungus might infect other internal organs at a higher rate. However, we observed no gross pathological alterations of internal organs. Additionally, in disseminated aspergillosis in human patients and during natural infections in birds, *A. fumigatus* is found in the liver, demonstrating the general ability of *A. fumigatus* to thrive in the liver (15, 22, 32, 50, 54, 59). Consequently, direct destruction of internal organs of the embryo by invading fungi is unlikely to be the cause of mortality in this model. Since we observed rapid growth of *A. fumigatus* on the CAM, we considered the possibility that nutrient competition might contribute to pathogenesis. Nutrient deprivation should have led to reduced growth rates of infected embryos and reduced weight compared to the results for control embryos. However, the growth rates of infected and control embryos were indistinguishable during the first 4 days of infection, which is the time period when most embryos died. Given that fungal growth on the CAM was associated with macroscopically visible destruction of blood vessels, we hypothesize that impairment of blood flow is the most likely cause of embryonic death during *A. fumigatus* infection. This hypothesis is supported by the histological evidence of liver congestion in infected embryos, indicating abnormal blood flow or pressure.

Sporulation is generally not observed in pulmonary aspergillosis of humans and in infection models with mice and other rodents. In contrast, sporulation is regularly observed in aspergillosis in birds (6, 45, 52). Most likely, sporulation inside the host is favored if the fungus grows on a mucosa-air interface, like that found in the avian lung air sacs (54). The natural and artificial air chamber in fertilized eggs also is an interface between mucosa (CAM) and air and thus promotes sporulation. Therefore, it was not surprising that sporulation was regularly observed in infected eggs. However, the frequency and quantity of sporulation did not correlate with mortality, as we did not observe differences in sporulation between strains with different virulence properties or upon postmortem analysis of dead and surviving embryos. Therefore, sporulation at the outer surface and blood vessel infiltration seem to be unrelated, and the latter process is the main cause of embryonic death.

Immunosuppression is essential for establishment of *A. fumigatus* infections in mammalian models of pulmonary as-

pergilliosis. Due to incomplete maturation of the immune system, chicken embryos can be considered naturally immunocompromised. While the phagocytic system of the innate immunity develops early during ontogenesis and both functional macrophages and heterophils are present on developmental day 10, the spleen is colonized by lymphocytes in several waves which expand into the posthatching period (12). The expression of Toll-like receptors, cytokines, and effector molecules, such as defensins, increases with embryonic age (1, 34). We speculated that if the interaction of *A. fumigatus* with the host's immune system influences the outcome of infection of embryonated eggs, older embryos should display increased resistance to infection. We indeed observed delayed mortality in older embryos, suggesting that the host immune response plays a role in pathogenesis in the embryonated egg model. Additionally, age-dependent differences in the levels of transcription of proinflammatory immune mediators between embryos infected on either developmental day 10 or developmental day 12 were observed. The greatest increase in mRNA levels was observed for K60, a CXCL chemokine with functions orthologous to those of human CXCL8. K60 has been shown to be associated with avian early proinflammatory responses and influx of heterophils after bacterial infection (26, 58). We observed early upregulation of K60 in the CAM of infected embryos and a continuous increase in the transcript level over time. Notably, the transcript levels were significantly higher in older embryos. In mice, the level of neutrophil recruitment mediated by the chemokines KC and MIP-2 has been shown to influence survival. Low levels of KC and MIP-2 were associated with decreased neutrophil recruitment and increased mortality, while overexpression of KC increased resistance of mice to *A. fumigatus* infection (35). Thus, the greater local transcription of K60 in older embryos in the egg model may lead to an increased influx of neutrophils and might therefore explain increased resistance. This hypothesis is supported by our observation that the level of expression of the proinflammatory cytokine IL-1 $\beta$  is significantly higher in the CAM of older embryos.

In contrast to the findings for K60 and IL-1 $\beta$ , the levels of transcription of IFN- $\gamma$  were not elevated in infected embryos compared to the PBS controls at any time, independent of the age at the time of infection. IFN- $\gamma$  has been shown to be involved in murine resistance to *A. fumigatus* infections, as an impaired IFN- $\gamma$  response is associated with mortality in murine models of invasive aspergillosis (3, 8). Upon *A. fumigatus* infection, IFN- $\gamma$  is upregulated in immunocompetent mice but not in immunocompromised mice. Therefore, the absence of an IFN- $\gamma$  response in infected chicken embryos suggests that embryos on developmental day 12 should be considered naturally immunocompromised. It might also partially explain the observed high susceptibility of chicken embryos to *A. fumigatus* infection. We analyzed iNOS transcription, because iNOS has been shown to be upregulated in chickens after infection with invasive *Salmonella* strains (5). However, infection with *A. fumigatus* resulted in only a minor increase in local levels of iNOS gene transcription. While this finding might indicate that there is a general immune deficiency during embryogenesis, the immune deficiency likely does not affect the outcome of infection, as killing of *A. fumigatus* by mammalian macrophages has been shown to be independent of iNOS (39). Fi-

nally, there were no differences in transcription of the macrophage chemokine MIP-1 $\beta$  between infected and noninfected embryos, suggesting that attraction of heterophils by K60 rather than macrophages is the predominant immune response in infected embryos. This resembles the important role of neutrophils in combating *A. fumigatus* infections in murine model systems (36).

Testing the virulence of genetically defined fungal mutants in animal models is a common approach for identifying fungal factors involved in pathogenesis (11). Thus, an important characteristic for testing the validity of alternative models for infection research is that attenuation of mutants detected in the "gold standard" infection model can be similarly detected in the alternative model. Therefore, we tested *A. fumigatus* mutants with defined virulence in mice in the egg model. The mutants were chosen based on their different degrees of attenuation (virulence indistinguishable from wild-type virulence, partially attenuated, and strongly attenuated) and normal growth *in vitro* on rich media. We observed a good correlation between the murine and egg models. Using 20 eggs per group in every independent experiment, we found that the mortality rates were highly reproducible in experiments. However, the limited observation period for infected eggs prior to hatching might hamper detection of subtle differences in virulence in attenuated mutants.

Partial attenuation of the  $\Delta mcsA$  mutant was detected either by using a lower infectious dose or by infecting eggs at a later developmental stage (day 12). As we assume that immune maturation leads to delayed killing in older embryos, we hypothesize that infection of older embryos might correlate best with nonneutropenic murine models, such as treatment with cortisone acetate, while younger embryos seem to resemble neutropenic murine models using cyclophosphamide. Consistent with this hypothesis, we found that a gliotoxin-deficient strain ( $\Delta gliP$  mutant) was fully virulent if embryos were infected on developmental day 10, irrespective of the infectious dose. In older embryos, however, the  $\Delta gliP$  mutant was attenuated. Similarly, a  $\Delta gliP$  mutant has been shown to be fully virulent in neutropenic mice but is attenuated in mice immunosuppressed with corticosteroids (51).

While reduced mortality rates with genetically defined fungal mutants indicate that the deleted or disrupted gene is involved in pathogenesis, mortality rates alone do not provide information about the affected pathogenicity mechanisms. One option to obtain additional information is to perform pathological and histological examinations, like those that we performed with embryos to characterize the egg model. Additionally, the virulence of mutants that show a defect in nutrient acquisition can be restored by supplementation or variation of the route of infection. We have recently used supplementation of the drinking water with lysine to demonstrate that the lysine auxotrophy of a  $\Delta hcsA$  mutant is responsible for the observed attenuation in mice (49). Likewise, we restored virulence in the egg model by supplying additional lysine, thus demonstrating that supplementation experiments are feasible in the alternative model. Attenuation of a  $\Delta hcsA$  mutant in mice was observed only after pulmonary infection and not after systemic infection (49). Similarly, the  $\Delta hcsA$  mutant was fully virulent in the egg model if it was applied systemically. Thus, variation

of the infectious route is possible in the alternative model, and the results closely resemble the results for mice.

In summary, our data show that embryonated eggs provide the basis for a highly reproducible alternative model for studying the pathogenicity of *A. fumigatus*. Since this model is inexpensive and easy to perform and it does not require specialized facilities (only a humidity-controlled incubator and an appropriate drill are needed), infection of embryonated eggs could be used widely. By using embryos at different developmental stages for infection, hosts with different levels of immunocompetence can be used, similar to the different established models of immunosuppression in mice. Additionally, the egg model allows investigation of fungal invasion processes by histological analysis of infected membranes, which is not possible when alternative systems, such as cell lines, are used. The role of factors required for nutrient acquisition can be studied using nutrient supplementation. Furthermore, by using different routes of infection it is possible to clarify whether the factors being studied are locally and/or systemically involved in virulence. Prescreening of selected strains in the egg model and analysis of virulence mechanisms that may be involved might aid in choosing the appropriate mouse model for further testing of mutants. Therefore, the embryonated egg model may help to significantly reduce the numbers of mammals needed for infection studies.

#### ACKNOWLEDGMENTS

This work was partially supported by the “Netzwerk Grundlagenforschung” of the Leibniz Institute for Natural Product Research and Infection Biology—Hans-Knöll-Institut (HKI) and by the Federal Ministry of Education and Health (BMBF).

The *ΔgliP* strain was kindly provided by Axel Brakhage and Thorsten Heinekamp, Department for Molecular and Applied Microbiology, Leibniz Institute for Natural Product Research and Infection Biology, Jena, Germany. We thank Birgit Weber, Ursula Stöckel, and Gudrun Steinmetzer for excellent technical help and Duncan Wilson for critical reading of the manuscript.

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Editor: G. S. Deepe, Jr.