Maturity as a Critical Determinant of Resistance to Fungal Infections: Studies in Murine Blastomycosis

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Marked resistance of 9-week-old mice as compared to that of 5-week-old mice was demonstrated after pulmonary challenge with three strains of Blastomyces dermatitidis of various virulences. Quantitative studies with graded doses of the strain that was intermediate in virulence indicated that the difference in resistance between the two age groups was 1,000-fold. Acquisition of resistance appeared to be gradual between the ages of 5 and 9 weeks. Maturational differences among individual mice appeared to be most crucial at 5 weeks of age. With two fungal strains, susceptibility of the younger mouse was also demonstrated after intraperitoneal challenge; with one of these strains, the differences between the two groups of mice were much smaller, suggesting that maturation of defenses in the peritoneal cavity may develop faster. Studies with serial sacrifice of different groups of mice given pulmonary challenges (of comparable amounts) indicated that the differences are not due to the amount of challenge reaching the lungs or its clearance. The infection-limiting effect in the lungs of older mice occurred within 4 days after challenge. These differences in resistance over this narrow age interval appear to be unique; resistance appears to occur later than resistance to other infectious agents and at a time when most murine immune functions have matured. This model and these observations provide an opportunity for further studies of the mechanism of resistance and are relevant to clinical observations of susceptibility of infants and children to deep mycoses.

The development of a murine model of pulmonary blastomycosis with several desirable features described elsewhere (9) has enabled the study of pathogen factors (C. Brass and D. A. Stevens, Am. Rev. Respir. Dis. [Suppl.] 119:243–245, 1979) and therapy (8) in respiratory mycoses. Preliminary observations have suggested that resistance to infection is age dependent (9). In the present study, we defined this difference and uncovered a remarkable transition from susceptibility to resistance over a narrow time interval of 4 weeks, beginning at 5 weeks of age, when in most previous studies (5–7, 10, 12, 18–20, 22, 23, 25, 27, 28) mice were considered to be immunologically mature. This delayed development of resistance may be unique in infectious models. Chronological studies presented here, performed after infection, will help in focusing further investigations so that the mechanism of resistance can be defined. In this study, we showed that the development of resistance proceeds at different rates in different body compartments, suggesting differences in local cellular function or milieu. The need for further information concerning the susceptibility of human infants to fungal infection has been emphasized (15).

MATERIALS AND METHODS

Fungi and infection. Blastomyces dermatitidis strains were used in the yeast phase as described in previous studies (9). They were kept on replicate brain heart infusion agar slants under sterile water at 4°C before use. The prior histories of the strains used in this study are cited elsewhere (9, 16).

The methods of inoculum preparation, which involves growth in liquid medium and transfer to solid medium before harvest, intranasal (i.n.) inoculation, serial sacrifice of members of a group of mice infected in an identical manner, recovery of fungi in the lungs of mice, and enumeration of colony-forming units (CFU) on agar in samples from various sources were done as previously described (9).

The method of enumeration of CFU in organs made use of the whole lung and an assay (9) of decimal dilutions of ground tissue in saline; therefore, even 1 CFU per organ could theoretically be detected. Intraperitoneal (i.p.) inoculation was accomplished with 0.3 ml of inoculum.

Mice and tissue. Individual experiments consisted of comparisons between groups of 10 to 15 male BALB/c mice (Jackson Laboratories, Bar Harbor, Maine) which had been observed in our facilities for 1

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week before challenge. Initially, mice were requested of the supplier by weight (<15 and >25 g) and were selected from various litters that were 3.5 to 4.5 and 7 to 9 weeks of age, respectively. At the time of challenge, after the 1-week observation period, their weights remained at <15 to 15.5 and >25 g, respectively.

In some studies described below, animals were selected from litters born within 1 to 2 days of each other, regardless of weight. Weight versus time to death in these experiments was analyzed statistically by Alvaro Munoz, Division of Biostatistics, Department of Preventive Medicine, Stanford University Medical School, using the Kendall test (11) of the null hypothesis.

Interpretation of histopathological specimens prepared after Formalin fixation and stained with hematoxylin-eosin or Gomori methenamine silver stain was kindly performed by Robert Archibald, Department of Pathology, Santa Clara Valley Medical Center. The slides were coded before examination so as not to reveal the age of the animal and time after infection.

RESULTS

Relationship of age, resistance, and fungal strain. Harvey et al. (9) have presented detailed evidence to suggest that i.n. challenges with <20 CFU (and possibly as few as 1 CFU) of the virulent strain of B. dermatitidis 26199 (designated strain V) to <15-g BALB/c mice are 100% lethal in 30 days. We confirmed this hypothesis in subsequent studies (C. Brass and D. A. Stevens, Am. Rev. Respir. Dis. [Suppl.]) which also pointed out the marked dependence of the outcome of infection on the ages of the mice.

Briefly, for example, challenges of 10^1, 10^2, and 10^4.2 CFU to >25-g BALB/c mice produced only 10, 50, and 75% lethality, respectively, in 60 days.

It was of interest to learn whether this remarkable change in resistance over several weeks was unique to this strain of Blastomyces. To explore this possibility, a spontaneous mutant of strain V that was attenuated in virulence, designated strain A and described elsewhere (16), was used for i.n. challenge and 60-day experiments (Fig. 1). These studies indicated that the relationship between age and survival is clearly demonstrable even with a less virulent fungus. Although challenges of >10^5 CFU are required for 100% lethality in <15-g mice, cohorts of >25-g mice did not demonstrate any deaths until challenges of almost 10^6 CFU were used; challenges of >10^6 CFU, close to the maximum that can be delivered by this method of challenge, produced only 60% lethality.

Strains V and A are closely related to each other. We wondered whether these differences would be demonstrable with yet other B. dermatitidis strains. Of particular interest was strain GA-1, which had previously been described to be avirulent (4, 9); this finding had been described, however, in experiments in which relatively low-dose challenges or older animals or both were used. Low-dose challenges (e.g., 10^2 CFU) or even high challenges to >25-g mice were not lethal (Fig. 2). However, when large inocula were given i.n., deaths could be produced in <15-g mice with this purportedly avirulent strain. Thus, with all three strains of B. dermatitidis, marked differences in susceptibility were noted over several weeks of maturation.

Histopathological studies of the lungs of <15- and >25-g mice infected i.n. with strain V were performed to study the sequence of fungal growth and host response in both types of mice. We desired to compare infections with similar outcomes to look for differences between the two age groups; the challenge used was the minimal CFU to give a lethal infection at each age. The pathology of fatal challenge in <15-g mice has been described previously (9). In summary, animals sacrificed immediately after challenge showed isolated fungi in alveoli and lower

![FIG. 1. Challenge of <15-g (4.5- to 5.5-week-old) (○) and >25-g (8- to 10-week-old) (●) mice i.n. with B. dermatitidis strain A. Percent mortality (vertical axis) at 60 days is shown.](image1)

![FIG. 2. Challenge of <15-g (4.5- to 5.5-week-old) (▲) and >25-g (8- to 10-week-old) (●) mice i.n. with B. dermatitidis strain GA-1. Percent mortality (vertical axis) at 60 days is shown.](image2)
airways. By 192 h, distinct focal nodules with intervening normal lung were noted (Fig. 3). The nodules contained Blastomyces cells with inflammatory cells (macrophages and polymorphonuclear leukocytes [Fig. 4]). At 288 h, the nodules had enlarged further, and inflammatory cellular debris (without recognizable organisms) was also noted in bronchi. The nodules continued to enlarge until the death of the animal, which is probably owing to replacement of lung tissue. Extrapulmonary dissemination is a terminal event and occurs only in infections that are not rapidly progressive (produced by low challenges). Examination of lungs removed from

FIG. 3. Typical focal pneumonic area noted in a <15-g mouse. Multiple nodules with intervening normal lung were seen. With a higher CFU challenge, the picture in >25-g mice was identical. Hematoxylin and eosin stain; magnification, ×40.
FIG. 4. Focal nodular areas of infection containing many individual yeast cells (black areas) and inflammatory cells filling the alveolus of a <15-g mouse. The picture in >25-g mice was indistinguishable. Gomori methenamine silver stain counterstained with hematoxylin and eosin; magnification, ×800.
>25-g mice serially sacrificed after infection revealed an indistinguishable histopathological sequence. The only differences were in the later appearances of the intrabronchial debris and the nodules of fungi, macrophages, and polymorphonuclear leukocytes. The histopathology of the lung in fatal GA-1 infection is similar to that seen with more virulent strains.

**Relationship among age, susceptibility, and route of challenge.** Experiments were performed to compare the resistance of mice at the ages studied above to challenge by the i.p. route (Fig. 5), using strain V. The results obtained with i.p. challenge were similar to those described above for i.n. challenge with strain V (Fig. 3). Older (>25-g) mice appeared to be more resistant to i.p. challenge than to i.n. challenge only at low-challenge inocula; e.g., a challenge of 10^3 CFU i.p. produced 0% lethality versus 50% lethality produced after the same challenge by the i.n. route.

Similar experiments were performed with strain A. In >25-g mice, this strain was markedly more lethal by the i.p. route as compared with the i.n. route. By the i.p. route, 100% lethality could be produced in >25-g mice, and with challenges of only 10^3 CFU (cf. Fig. 1); similarly, a challenge of 10^2.8 CFU i.p. was 20% lethal, whereas producing 20% lethality i.n. necessitates a challenge of 10^4.2 CFU (Fig. 1). However, the dose-response curve obtained with <15-g mice after i.p. challenge was not distinguishable from that (Fig. 1) obtained with <15-g mice after i.n. challenge. Greater lethality with strain A via the i.p. route in >25-g mice makes the dose-response curves for i.p. challenge of >25- and <15-g mice approximate each other. In fact, at challenge doses of <10^2.4 or >10^2.7, the curves were superimposed (=10 and ≥90% mortality, respectively, representing the results of eight experiments). The only clear separation between the curves in these experiments occurred with one intermediate challenge size used, 10^2.8 CFU i.p., which was fatal to 20 and 80% of the >25- and <15-g mice, respectively.

**Chronological analysis of events in lungs of older (>25-g) and younger (<15-g) mice.** To begin to delineate the sequence of events after infection, the time course of fungal replication was followed in the lungs (Fig. 6) of cohorts of animals whose members were sacrificed sequentially. For this experiment, strain A was used. This strain was chosen because it was deemed necessary to know what the outcome (eventual death or survival) would have been in each mouse sacrificed so as to avoid mixing data at any time point from animals with progressive and resolving infections. To minimize any differences resulting from comparing events after challenges of vastly different sizes, which could result in different patterns of host response, we wished to quantitate the infection after the smallest dose which could produce 100% lethality in <15-g mice and the largest dose which could produce 0% lethality in >25-g

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**Fig. 5.** Challenge of <15-g (4.5- to 5.5-week-old) (○) and >25-g (8- to 10-week-old) (●) mice i.p. with B. dermatitidis strain V. Percent mortality (vertical axis) at 60 days is shown.

**Fig. 6.** Serial sacrifice studies with quantitative recovery of fungi (strain A) from lungs of mice after challenge with similar inocula (see text) producing lethal infection in <15-g (4.5- to 5.5-week-old) mice (○) and nonlethal infection in >25-g (8- to 10-week-old) mice (●). Each point represents the average determination for three mice. Vertical axis, CFU from one lung; horizontal axis, time after challenge. Asterisk, culture results in one of three mice sacrificed on day 14; the other two mice were sterile (as were all three mice sacrificed on day 16 or 18).
mice. From the data presented in Results, it was evident that strain A was most suitable for preparing challenges that were similar in size and also met these criteria (strain V challenges that would meet these criteria would need to differ by several logs, and GA-1 does not produce 100% mortality even in <15-g mice in doses that can be used with our techniques). The doses selected were 7,656 CFU for <15-g mice and 28,067 CFU for >25-g mice (cf. Fig. 1).

The sequence of events as quantitated by fungal CFU in the lung was identical in both ultimately lethal and ultimately nonlethal infections for the first 4 days (Fig. 6): the fungal CFU steadily declined in both cases. A similar phenomenon was described earlier, using lethal challenges of strain V; in this situation, when low dose challenges were used, the number of CFU recoverable from the lung fell below the threshold detectable in the assay, before logarithmic growth and progressive disease occurred (9). This was referred to as an eclipse phase.

However, at 4 days after challenge with strain A, the two types of infections took diverging paths (Fig. 6). In <15-g mice, the CFU recovered began a steep climb which continued and was eventually lethal when replication to approximately 10^7 CFU per lung was reached (data not shown). The older mice continued to clear the infection until viable CFU were no longer present by 10 to 12 days after challenge.

The results from these experiments suggest that the infection-limiting effect present in >25-g mice occurs later than 4 days after challenge.

Intermediate ages. Although the differences demonstrated between mice of approximately 5 and 9 weeks of age appear to be profound when the narrow age interval between the groups is considered, experiments were performed to examine points in this transition interval. For this purpose, the groups of mice were not comprised of animals born within 1 to 2 weeks of each other, as was previously the case, but from different litters within 1 to 2 days of each other. Animals of 5, 8, and 10 weeks of age were challenged i.n. with strain V or A. In 8-week-old mice, the level of susceptibility to infection fell between those of the other two groups, whether measured by median time to death with 100% fatal challenges or percent survival with less lethal challenges (Table 1). These observations indicated that even differences in resistance related to maturation differences of 2 to 3 weeks are detectable over the interval studied.

Maturation differences among mice of the same age. Maturation differences among mice of approximately the same age may have been minimized in the early experiments by selecting subgroups of animals on the basis of weight (<15 and >25 g). Experiments were performed to investigate whether there are even finer gradations in maturation among mice that might be studied for differences in resistance. This was performed by challenging litters of mice that were born on the same day but whose members covered a range of weights and recording the outcome to gain information about the relative contributions of age and weight in selection of our groups. It was also hypothesized that weight differences at a given age might indicate maturation differences, as could be reflected in differences in resistance, rather than mere differences in body habitus.

The results obtained with 5-week-old mice are shown in Fig. 7. There was evident a relationship between resistance as assayed by survival time after challenge with strain A and weight. Analysis by the Kendall test showed a highly significant association (two-sided P value, 0.006). However, similar experiments with strain V and 6-week-old mice and with strain A and 8- and 10-week-old mice did not show such a correlation (data not shown). A repeat experiment in which strain V was used with 5-week-old mice from a different supplier, with a median weight of 16 g (as compared with 15.4 g in the preceding experiment with 5-week-old mice), also did not demonstrate a significant difference (P. A. Morozumi, personal communication).

**DISCUSSION**

These studies show a striking difference between the resistance of mice approximately 9 weeks of age to challenge with *B. dermatitidis* and that of cohorts 4 weeks younger. This difference was shown in relation to pulmonary infection with three strains of Blastomyces. With strains V and GA-1, the extremes of virulence and "avirulence", there was no overlap in the dose-response curves of the two age groups. When a strain intermediate in virulence, A, was used, the data indicated a 1,000-fold difference in the susceptibility of mice between these two ages (e.g., extrapolated 50% lethal dose for each group in Fig. 1). Since strain GA-1 has been described in prior literature as avirulent, these

| Age (wk) | No. of deaths/total challenged | % |
|---------|-------------------------------|---|
| 5       | 21/21                         | 100|
| 8       | 19/22                         | 86 |
| 10      | 13/21                         | 62 |

*1,500 CFU of strain A given i.n. to each mouse was the challenge; animals were observed for 60 days.
results also indicate a caution: such terms need to take into account host factors.

These age-related differences were shown to be independent of the route of challenge. However, some finer distinctions to the general rule became apparent. With strain A, the resistance of older mice compared with that of younger mice was much less with challenges given by the i.p. route (at several challenge doses) as compared with the differences between the two age groups after i.n. challenge. This result suggests that, whatever maturational changes occur during the interval studied, they may proceed at different rates in different milieus and provides clues regarding the study of which cell types in different foci might be related to the differences in resistance. However, this difference may be specific to this strain of Blastomyces, since such relationships were not shown with strain V or GA-I (C. Brass and D. A. Stevens, Am. Rev. Respir. Dis. [Suppl.]); i.e., with these two strains, there was evidence of greater resistance by the i.p. route as compared with the i.n. route.

Studies with other strains of inbred mice indicate that age-related differences in susceptibility to pulmonary infection are not specific to the BALB/c strain (16).

Histological studies of pulmonary infection did not suggest marked difference in cell types or deficiency in cell numbers responding to the progressing disease. These studies do not, however, eliminate the possibility of there being different functional abilities of such cells in restricting infection. Experiments involving serial sacrifice of members of cohorts of infected animals indicated progressive clearing of pulmonary infection in older animals at challenge doses comparable to those producing progressive disease in young ones. These chronological studies suggest that differences in resistance relate to some early events after challenge but not to the percent reaching the lung after i.n. challenge nor to mechanical clearance. We would speculate at this time that the difference relates to nonspecific mucosal immunity, nonspecific phagocytic cells, or other nonspecific killer cells rather than specific humoral or other lymphocyte-mediated immunity, which would require more time for its full expression. Further studies will be directed to this hypothesis.

These studies also suggested a gradual acquisition of resistance over the time interval studied. When animals at the beginning of the maturational period studied were used, their weight prechallenge appeared to be a predictor of the outcome of infection. This suggests that, at that age, weight differences may be related to different rates of maturation, including immunological maturation. Animals 1 to 5 weeks or older did not show such a correlation, suggesting that weight in such animals is a result of food intake or body habitus that does not relate to immunological maturation. Mice younger than 4.5 weeks were not studied in this model, because the original observations in this model were described with strain V, with which 100% lethality was produced with very few CFU; thus, differences in susceptibility of even younger mice would have been difficult to demonstrate. Now, however, with the availability of the attenuated strain A, quantitative dose-response studies extending from both ends of the age range studied here are possible.

Previous studies have suggested that the development of resistance to other infections occurs at ages younger than those studied here. In one recent study of resistance to Klebsiella, 5-week-old mice were considered and referred to as adult mice (20). Thus, mice were shown to be susceptible to measles (19) and hepatitis (28) viruses up to the age of 3 weeks, resistance to Sindbis virus had developed by 2 (6, 12) or 4 (5) weeks, and resistance to amoebae had developed by 6 weeks (7).

Such observations correspond to studies of the time of development of murine immune functions. Thus, the mitogenic response of spleen cells to lipopolysaccharide (22, 23) or pneumococcal polysaccharides I and II (23) has matured by 1 to 2 weeks of age, as has antibody formation to these (23) and other thymic-independent antigens (18). Murine B cells acquire a complete set of markers by 10 days of age (25). Others have shown normal mitogenic responsiveness to concanavalin A of thymus and spleen cells by 3 weeks (27), mitogenic responses of spleen cells to phytohemagglutinin by approximately 6 weeks (27), and normal blasto-
genesis or antibody formation to thymic-dependent antigens such as sheep erythrocytes by 4 to 8 weeks (23). Lymphocyte-mediated natural cytotoxicity peaks by the age of 5 to 8 weeks (10). In contrast to these observations, later development of some functions was suggested by reports that the blastogenic response of mouse spleen to phytohemagglutinin, concanavalin A, and staphylococcal antigen became normal by 8 weeks of age (22), and T-cell influence on antibody to pneumococcus antigen III converted from the suppressor to the helper mode by only 8 to 10 weeks of age (17).

Some of the deficiencies in immunity have been ascribed to suppressor mechanisms, though the suppressor function was usually reversed by the ages studied here. Thus, in various studies, suppression of contact sensitivity had disappeared by the age of 5 days (26), suppression of primary or secondary antibody responses in vitro by nonadherent spleen cells had reversed by the age of 3 weeks (2), and T cells suppressing mixed lymphocyte cultures were gone by the age of 3 weeks (1).

The preceding discussion, which is relevant to murine immunological observations, appears to have parallels in humans. Thus, depressed complement, chemotaxis, phagocytosis, reticuloendothelial clearance, and lymphokine production have been reported (14) in newborns and infants. Leukocyte killing of *Candida* is also impaired, and there is decreased cheluminescence, metabolic burst, and stimulation of the hexose monophosphate shunt (14, 29). Even more relevant to our studies are not only the well-known and unique problems of candidiasis in infancy (15) but also the association of acute progressive disseminated histoplasmosis with childhood (3), increased dissemination of coccidioidomycosis in childhood (13), association of the acute form of paracoccidioidomycosis with the young (24), and a recent report of the severity of acute pulmonary blastomycosis in children (21).

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