Correlation of Susceptibility of Immature Mice to Fungal Infection (Blastomycosis) and Effector Cell Function

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Immature mice are highly susceptible to blastomycosis, which is similar to other mycoses and has parallels in humans. The murine susceptibility is noteworthy in that it persists beyond the development of resistance to other, nonfungal pathogens and the maturation of most immune functions. As the susceptibility to blastomycosis appeared to be related to an early event after infection, primary effector cell function was studied. We found that peritoneal inflammatory cells, enriched for neutrophils, from immature (3-week-old) mice killed nonphagocytizable Blastomyces dermatitidis cells less (25%) than did cells from mature (8-week) mice (70%) (P < 0.01), a defect intrinsic to the neutrophils. This correlated with an impaired immature cell oxidative burst. Killing of phagocytizable Candida albicans was not significantly different, 73 versus 87%. Thiglycolate-elicited cells were more impaired; killing of B. dermatitidis was insignificant, and killing of C. albicans was more impaired in immature (16% killing) than in mature (45%) cells (P < 0.02). Peripheral blood neutrophils from mature animals killed B. dermatitidis (41%) more than did those from immature animals (10%) (P < 0.02); C. albicans was killed efficiently by both. Resting or activated peritoneal macrophages from both types of animals showed no differences in B. dermatitidis killing. These results suggest that the susceptibility of immature mice is related at least in part to the depressed capacity of their neutrophils to kill B. dermatitidis.

Previous studies from our laboratory have shown striking differences in the susceptibilities of immature and mature mice to blastomycosis (8). For example, for challenges via the pulmonary route, 5-week-old mice were 1,000-fold more susceptible than 9-week-old mice. This striking difference in susceptibility was not dependent on the route of challenge. Resistance developed progressively over age 3 to 10 weeks. Studies involving serial sacrifice of infected cohorts revealed that the infection-limiting event in older mice, i.e., the interval before infectious burdens began to diverge in the two age groups, occurred within 4 days of challenge (8). This timing suggested that the difference in the age groups rested in differences in nonspecific immunity, e.g., activity of effector cells, rather than specific humoral or lymphocyte-mediated immunity, which would require more time for its expression. The studies reported here address the possible mechanism of the age-related differences in immunity, by studying effector cell function.

MATERIALS AND METHODS

Animals. Sendai virus-free BALB/cByJIMR (California Institute for Medical Research, San Jose) male mice 3 and 8 weeks old were used throughout these experiments. The average body weights were 15 g for immature mice and 25 g for mature mice. Yeast form inoculum was determined by plating appropriate dilutions on blood agar plates. B. dermatitidis (71), was used throughout these experiments. Yeast form was stored on Sabouraud agar slants at 4°C.

B. dermatitidis isolate ATCC 26199, a virulent isolate in mice (43), was an exemplar used in these studies.

Animals. Sendai virus-free BALB/cByJIMR (California Institute for Medical Research, San Jose) male mice 3 and 8 weeks old were used throughout these experiments. The average body weights were 15 g for immature mice and 25 g for mature mice.

Materials. Media and reagents. Dulbecco's phosphate buffered saline (PBS), minimal essential medium (MEM), RPMI 1640, heat-inactivated fetal bovine serum, penicillin (10,000 U/ml), and streptomycin (10,000 μg/ml) were purchased from Gibco Laboratories, Grand Island, N.Y. Complete tissue culture medium consisted of RPMI 1640, 10% (vol/vol) fetal bovine serum, and 100 U of penicillin plus 100 μg of streptomycin per ml. Histopapae 1077, dextran 300 K, luminol, and concanavalin A (ConA) were obtained from Sigma Co., St. Louis, Mo. Sodium caseinate and thiglycolate liquid medium (BACTO-B256) (Difco Laboratories) were used in these studies.

Peripheral blood. Mice were anesthetized with ether, a pouch of skin was formed between a front leg and body torso by dissection, the brachial artery was severed, and blood was collected with a Pasteur pipette. When blood was used as a source of polymorphonuclear leukocytes (PMN), it was heparinized with preservative-free heparin (30 U/ml) on collection. Fresh mouse serum was collected from clotted blood and was shown previously to have complement activity in a cytotoxicity assay (11).

PEC-PMN. Peritoneal exudate cells (PEC) enriched for PMN were induced by intraperitoneal injection of 1 ml of 1% sodium caseinate (Dilico) or thiglycolate broth (Clinical Standards Laboratory, Caron, Calif.). Four hours later, peritoneal cells were collected by repeated lavage of the peritoneum of each mouse with a total of 10 ml of MEM containing 10 U of preservative-free heparin (American Scientific Products, McGaw Park, Ill.) per ml. PEC were fractionated by density gradient centrifugation on Histopapae 1077 (9), 400 × g for 20 min, at room temperature. The pelleted cells were further enriched for PMN by centrifugation in a metrizamide gradient, 400 × g for 20 min (15). These cells were washed once in MEM, suspended in complete tissue culture medium, and counted with a hemacytometer.

Peripheral blood PMN. Peripheral blood PMN were obtained as follows: (i) layering heparinized blood diluted 1:1 in saline over an equal volume of Histopapae 1077; (ii) centrifugation at 900 × g for 20 min; (iii) suspension of pelleted red blood cells and PMN in an equal volume of saline; (iv) mixing suspended pelleted cells with an equal volume of 3% (wt/vol) dextran 300 in saline and incubating for 1 h at 37°C; (v) collection of buffy coat layers and pelleting of cells by centrifugation, 400 × g, 10 min; (vi) suspension of pelleted cells in 10 ml of 0.85% NaCl to lyse contaminating red blood cells; and (vii) washing of treated cells with MEM followed by suspension in complete tissue culture medium.

Peritoneal macrophages. Resident and elicited peritoneal macrophages were induced by intraperitoneal injection of 0.1 ml of saline alone or containing 100 μg of ConA. Twenty-four hours later, peritoneal cells were collected by repeated peritoneal lavage with a total of 10 ml of MEM containing 10 U of preservative-free heparin per ml. After one wash in this medium, cells were suspended in

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complete tissue culture medium, counted in a hemacytometer, and dispensed (0.2 ml of 5 × 10^6 cells/ml) into flat-bottomed wells of 96-well tissue culture clusters (catalog no. 3596; Costar, Cambridge, Mass.). After incubation at 37°C in 5% CO₂–95% air. Cultures were harvested with distilled water as previously described (17) and plated on blood agar plates. CFU per culture was determined by counting CFU per plate after 2 days (C. albicans) or 4 to 5 days (B. dermatitidis, at 37°C in 5% CO₂–95% air for 4 h. The content of each well was transferred to each coculture and control culture, they were incubated at 37°C for 2 h in complete tissue culture medium. After fresh mouse serum (0.02 ml) was added (Falcon 3072; Becton Dickinson Co., Oxnard, Calif.) and then challenged with tissue culture medium) were dispensed into flat-bottomed Micro Test plate wells (10,000 CFU/ml of PBS), at room temperature, as previously described (46) (10^7 fungi/ml of PBS), at room temperature. Photon emission was measured in a scintillation counter (Mark II; Nuclear-Chicago Corp., Des Plaines, Ill.) at room temperature with the windows set on “manual” and levels set at L-infinity. The manual setting permitted rapid counting of samples, and the counts per minute were calculated by using the counting time (±0.2 min) registered by the scintillation counter.

### RESULTS

#### Fungicidal activity of caseinate-elicited PMN.

Most published data concerning rodent PMN function have utilized cells elicited in the peritoneal cavity. We studied such cells elicited by two different elicitors, caseinate and thioglycolate medium, and compared the activities of cells from mature and immature mice against both B. dermatitidis (not phagocytizable by a single host effector cell) (10) and C. albicans (a phagocytizable target).

We found that PEC enriched for PMN as induced by caseinate in mature mice significantly killed B. dermatitidis ([70 ± 20]%, n = 7; mean ± standard deviation, number of experiments) (Table 1). This was significantly different (P < 0.01) from the killing by caseinate-induced PMN of immature mice ([25 ± 27]%, n = 7). Killing by PMN from immature mice was thus more variable, but there was always a ≥20% difference in percent killed, in favor of mature mice, in each of seven concurrent experiments (P < 0.01, Wilcoxon rank sum test).

This difference was not explained by a different percent PMN in the PEC population between mature and immature mice. PEC from mature mice were 68% PMN, and those from

<table>
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<th>Phagocyte</th>
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<th>Elicitant</th>
<th>Target</th>
<th>Killing (mean ± SD; reduction of inoculum)</th>
<th>No. of expts</th>
<th>P value</th>
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<tr>
<td>PEC (PMN)</td>
<td>Mature</td>
<td>Caseinate</td>
<td>B. dermatitidis</td>
<td>70 ± 20</td>
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*Abbreviations: PB, peripheral blood; Mono, mononuclear cells; PM, peritoneal macrophages; NS, not significant. Further definitions of the cell populations are given in the text.*
immature mice were 70% PMN (mean of two experiments); the remaining cells in both groups were mononuclear.

We showed that the fungicidal activity in the PEC was due to PMN by treating the PEC with anti-PMN antibody plus complement. In this experiment, caseinate-induced PEC from mature mice killed 74% of B. dermatitidis cells; after treatment with anti-PMN antibody plus complement, killing was 0%. Prior studies have shown that this antibody plus complement does not impair macrophage killing of fungi (Candida) (9).

Further evidence that the differences in killing were intrinsic to the cells was demonstrated in three experiments by incubating PEC from immature animals in the exudate of mature animals for 1 h prior to challenge in vitro with B. dermatitidis; the ability of the immature cells to kill was not significantly affected by exposure to the exudate of mature animals (data not shown).

In contrast, caseinate-induced PEC of both mature and immature animals were efficient in killing C. albicans. The cells from mature animals killed (87 ± 7)% (n = 3), and those from immature animals killed (73 ± 5)% (n = 3). The greater killing by mature animals' cells was not significantly different from that by those of immature animals.

**Fungicidal activity of thioglycolate-elicited PMN.** Cells elicited with thioglycolate gave different results. Killing of B. dermatitidis by PEC from mature animals was (8 ± 7)% (n = 4), and killing by PEC from immature animals was 0% (n = 4). Neither result was significant. In contrast, candidacidal activity of cells from mature animals (45 ± 17)% (n = 5) was significantly greater (P < 0.02) than that of cells from immature animals (16 ± 12)% (n = 5).

This difference was not explained by differences in percent PMN in the two PEC populations. The PEC from mature animals were 73% PMN, and those from immature animals were 69% PMN (mean of two experiments); the remainder were mononuclear.

The yield of PEC (and thus, PMN) per gram of body weight was not significantly different between mature and immature mice with either elicitant. The yield of PEC per gram with caseinate was 32 × 10⁴ ± 15 × 10⁴ for mature animals and 25 × 10⁴ ± 17 × 10⁴ for immature animals. With thioglycolate, the yields were 31 × 10⁴ ± 20 × 10⁴ and 22 × 10⁴ ± 10 × 10⁴, respectively.

**Oxidative burst in PMN of mature and immature animals, as assayed by chemiluminescence.** The mechanism of the differences in killing described was studied with caseinate-induced PEC and the chemiluminescence assay. As previously noted with cells from mature animals (19), chemiluminescence was found to be greater after challenge with C. albicans (Fig. 1) than with B. dermatitidis (Fig. 2), with cells of mature and immature animals. It was particularly noteworthy that, at the peak of the oxidative burst, at 15, 30, and 60 min, there was significantly less (P < 0.05, <0.001, and <0.001, respectively) chemiluminescence produced by cells of immature animals than by cells of mature animals in response to B. dermatitidis (Fig. 2). This difference correlates with the differences seen for killing of B. dermatitidis by these cells. In contrast, there were no significant differences in chemiluminescence between these cell populations after exposure to C. albicans (Fig. 1). This correlated with the lack of difference in killing of C. albicans between these groups.

**Peripheral blood PMN fungicidal activity.** Since the function of elicited PMN may be affected by the elicitant, we thought it important to also study a resting cell population, the peripheral blood PMN. Purified blood PMN from mature mice killed B. dermatitidis (41 ± 18)% (n = 5) to a significantly (P < 0.02) greater extent than did those from immature mice (10 ± 5)% (n = 5). The studies described above with caseinate-elicited cells showed greater variability in killing by the less active (immature) cell population, which may reflect the influence of the elicitant; thus, peripheral blood (unstimulated) cells may be a more appropriate cell population for study.

In contrast, purified blood PMN from both mature and immature mice were highly capable of killing C. albicans, with 98 and 97% killing, respectively. The potency of killing of C. albicans by peripheral blood PMN of mature mice has been previously reported (9).

The differences in killing B. dermatitidis were not due to differences in percent PMN in the preparations, as this was identical in preparations from mature and immature mice (82%, mean of two experiments each). The yield of peripheral blood leukocytes per gram of body weight was also not different, 1.5 × 10⁴ ± 0.4 × 10⁴ for mature mice and 1.1 × 10⁴ ± 0.8 × 10⁴ for immature mice.

Further evidence that the PMN are responsible for the fungicidal activity against B. dermatitidis described was provided by testing of the mononuclear cell fraction derived from peripheral blood leukocytes of mature animals. This fraction consisted of 85% mononuclear cells. No killing (0%) of B. dermatitidis was demonstrated by this fraction, in an experiment performed concurrently with the PMN (which killed 48%).

**Effect of macrophages on B. dermatitidis.** Peritoneal macrophages elicited with ConA from immature or mature mice had significant fungicidal activity against B. dermatitidis, whereas saline-elicited macrophages did not. ConA-elicited and saline-elicited macrophages from mature mice killed 34% ± 19% and
9% ± 12%, respectively ($P < 0.05$), and with these cells from immature mice, killing was 48% ± 12% and (7 ± 12)%, respectively ($P < 0.01$) ($n = 5$ for each of the four groups). The differences between killing by ConA-elicted macrophages from mature mice and killing by such macrophages from immature mice were not significantly different, nor were the differences with saline-elicted macrophages. Thus, neither “activated” nor resting peritoneal macrophages were different in their fungicidal activity against *B. dermatitidis*, in a comparison of mature and immature mice.

**DISCUSSION**

The present studies suggest that the enhanced susceptibility of immature mice may relate to the depressed capacity of PMN from immature mice to kill *B. dermatitidis*. This may be best illustrated in our study of peripheral blood PMN, although a disparity in effector function was also seen with PMN from an inflammatory exudate, induced by casein. These differences between PMN of mature and immature mice were not seen with respect to the killing of *C. albicans*, as PMN from both sources were highly effective killers of *C. albicans*. These differences and similarities in killing between mature and immature cells with respect to these two targets were mirrored in the chemiluminescence studies, where there were mature-immature differences with *B. dermatitidis* as the target, but not with *C. albicans*. In contrast, thioglyolate-induced PMN were impaired with respect to killing of *B. dermatitidis;* this finding in mature cells plus the superior killing of *B. dermatitidis* by ca-
ropophage anti-Listeria activity developed by 4 weeks (53). Blastogenesis or antibody formation to thymus-dependent antigens develops over 4 to 8 weeks (60), and lymphocyte-mediated natural cytotoxicity peaks by age 5 to 8 weeks (28). In contrast, other immune functions develop later, such as blastogenesis to staphyloccocal antigen (8 weeks) (59), and some reports of blastogenesis to phytohemagglutinin and ConA (8 weeks) (59), and the T-cell influence on antibody to pneumococcal antigen converts from the suppressor to helper mode by only 8 to 10 weeks of age (48). Some of the immune deficiencies in immature animals have been ascribed to suppressor mechanisms, though the suppressor activity is reversed before the period where mice remain susceptible to blastomycosis. For example, suppression of contact sensitivity has disappeared by age 5 days (67), suppression of primary and secondary antibody responses has disappeared by age 3 weeks (20, 23), non-T-cell suppressors of alloreactivity have disappeared by age 6 days (63), T-cell suppression of mixed lymphocyte cultures has disappeared by 3 weeks (4), and suppression of blastogenesis has disappeared by 4 weeks (37). Suppression of interleukin 2 (IL-2) production lasted until age 6 weeks (5).

Undoubtedly the best-studied immature animal, immunologically, is the human infant, and there are reports describing defects in almost every aspect of the immune system (30). In particular, PMN have previously described defects in adherence, aggregation, movement (30), phagocytosis or microbicidal activity in relation to some targets (of particular note, Candida [2, 30]), signal transduction, cell surface receptor up-regulation and mobility, specific granule content, cytoskeletal flexibility, microfilament contraction, chemiluminescence, oxygen metabolism (some studies), defense against auto-oxidative cell damage (30), chemiluminescence (74), storage pool size (58), chemotactic factor binding and hydroxyl radical generation (76), lactoferrin release (3), and myeloperoxidase (62). The susceptibility of neonates to candidiasis suggests the primary of a PMN defect. Other immune defects include humoral immunity: decreased B cells of the plasma cell type (50) and immunoglobulins (58) and the presence of plasma factors that may relate to cell movement (30), a problem which would be particularly relevant, since we previously have shown that IFN-γ up-regulates PMN (47) and macrophage (12) killing of Blastomyces, and a putative IFN-γ defect in immature mice might exaggerate the baseline PMN defect described in the present study. On the other hand, the fact that the responsiveness of naïve mature animals to blastomycosis occurs in <4 days (8), as previously mentioned, speaks against the involvement of cytokines dependent on the development of immunological memory. The therapeutic or prophylactic study of cytokines may provide an avenue of prospects for therapy (29) that might be applied in the future in this model.

The susceptibility of immature mice to Blastomyces is likely relevant to, in humans, not only the well-described problems of candidiasis in infancy (42) and the severity of acute pulmonary blastomycosis in children (57), but also the association of acute progressive histoplasmosis with childhood (21), increased dissemination of coccidioidomycosis in childhood (34), and association of the acute form of paracoccidioidomycosis with the young (61). The murine model of blastomycosis may thus prove useful for the study of the maturation of defenses against, in particular, extracellular targets, such as some fungi, some parasites, and tumor cells.

REFERENCES


