Mucosal and systemic candidiasis in IL-8Rh<sup>-/-</sup> BALB/c mice

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Abstract: Germ-free BALB/c mice, genetically engineered to be deficient for interleukin-8 (IL-8) receptor homolog (IL-8Rh<sup>-/-</sup>), were more susceptible to gastric candidiasis after oral challenge and to acute systemic candidiasis after intravenous challenge than IL-8Rh<sup>+/-</sup> controls. In comparison to IL-8Rh<sup>+/-</sup> mice, the IL-8Rh<sup>-/-</sup> mice had slower influx of polymorphonuclear neutrophils (PMN) into Candida albicans-infected tissues and a lower percentage of PMN in peritoneal exudate cells (PEC) elicited with heat-killed C. albicans. PEC from IL-8Rh<sup>-/-</sup> mice exhibited less luminol-dependent chemiluminescence in response to C. albicans and did not kill C. albicans hyphae as well as PEC from IL-8Rh<sup>+/-</sup> mice. C. albicans-colonized IL-8Rh<sup>-/-</sup> mice showed no histological evidence of systemic candidiasis. These results suggest a role for the IL-8Rh in murine resistance to gastric and acute systemic candidiasis, but not in resistance to systemic candidiasis of endogenous origin. J. Leukoc. Biol. 66: 144–150; 1999.

Key Words: chemokines · Candida albicans · neutrophils · chemotaxis

INTRODUCTION

Neutrophils play an important role in resistance to mucosal and systemic candidiasis: they predominate in Candida albicans-infected tissues and neutropenia is associated with increased susceptibility to systemic candidiasis [1-9]. Interleukin-8 (IL-8) is an alpha (CXC) chemokine that attracts human neutrophils and lamina propria lymphocytes to sites of infection [10-14]. IL-8 is a major neutrophil-activating agent [15-19] that also plays a role in delayed-type hypersensitivity [20]. C. albicans can stimulate the release of IL-8 from peripheral blood mononuclear cells [21].

Although mice apparently lack a homolog of IL-8, they do possess a chemokine receptor (CXC-R2b) that binds human IL-8, murine macrophage inflammatory protein (MIP-2), and mouse KC (a member of the c-x-c chemokine family) [22, 23]. The murine CXCR2b receptor is similar to human IL-8R-B (CXCR2) [23]. Mice genetically engineered with an inserted neomycin resistance gene to inactivate the IL-8 receptor homolog (IL-8Rh<sup>-/-</sup>) are unable to accumulate inflammatory neutrophils in vivo in response to CXC-dependent chemotactic stimuli [22, 23]. Conventional (normal flora) [22], but not germ-free (GF), IL-8Rh<sup>-/-</sup> mice are neutrophilic and their neutrophils apparently have normal bactericidal activity [22, 24, 25]. Neutrophils from IL-8Rh<sup>-/-</sup> mice are unable to migrate in response to human IL-8 or murine MIP-2 in vitro [22]. Conventional IL-8Rh<sup>-/-</sup> mice have been shown to develop lymphadenopathy, which is apparently caused by an increased number of B cells, and splenomegaly, which is attributed to an accumulation of leukocyte precursors and mature neutrophils [22].

Because of the important role that chemotaxis and neutrophils play in resistance to mucosal and systemic candidiasis [1-9] and because very little is known about chemotactic chemokines in resistance to candidiasis, we assessed the susceptibility of GF IL-8Rh<sup>-/-</sup> mice to mucosal and systemic candidiasis of acute (intravenous injection) or endogenous (alimentary tract) origin.

MATERIALS AND METHODS

Microorganism

Candida albicans B311 (type A) was maintained by monthly transfer on Sabouraud's dextrose agar (SDA; Difco, Detroit, MI) slants as previously described [26-28].

Mice

Breeding pairs of BALB/c IL-8Rh<sup>-/-</sup> mice were obtained from Dr. Mark Moore (Genentech, San Francisco, CA). A GF colony of IL-8Rh<sup>-/-</sup> mice was established by deriving IL-8Rh<sup>-/-</sup> pups into the GF state by cesarean section. The pups were raised to adulthood by GF BALB/c mothers. A breeding colony of the IL-8Rh<sup>-/-</sup> mice was established and maintained at the University of Wisconsin Gnotobiote Laboratory, Madison (http://www.biostat.wisc.edu/gnotolab/gnotolab.html).

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Colonization with *C. albicans*

Adult GF IL-8Rh⁻/⁻ and IL-8Rh⁺/⁺ (wild-type) mice were transferred into a separate GF isolator where they were colonized (alimentary tract) with a pure culture (monosassociated) of *C. albicans* (10⁶ viable *C. albicans*/mL culture was swabbed into the oral cavity and onto the ano-rectal area of each mouse). After one swabbing, both genotypes were quickly colonized with the fungus (24-h fecal samples had 10² to 10⁵ viable *C. albicans* CFU/g feces). Intestinal colonization was verified and quantified with gnotobiotic mice that were killed at different time intervals after colonization with *C. albicans* by plating dilutions of intestinal contents and tissues onto SDA (Diffco) and counting colonies that developed during a 24-h incubation at 37°C.

Evaluation of systemic candidiasis of endogenous origin

Internal organs from mice, killed at various times after colonization with *C. albicans*, were homogenized in Potter-Elvehjem tissue grinders (Fisher Scientific, Itasca, IL) in 5 mL sterile H₂O. The homogenized tissues were serially diluted and plated (100-µL aliquots) on SDA. The number of viable *C. albicans* in tissue homogenates was estimated by counting *C. albicans* colonies that formed on SDA plates during an incubation period of 24 h at 37°C. Data are reported as *C. albicans* CFU/g (dry weight) of tissue.

Resistance to systemic infection

IL-8Rh⁻/⁻ and IL-8Rh⁺/⁺ mice were injected intravenously with 10⁴ CFU of *C. albicans* as described [2, 5, 26]. Three mice from each group were killed at 1, 3, 7, and 14 days after intravenous challenge. The spleen, liver, kidneys, and brain from each mouse were individually homogenized (Potter-Elvehjem tissue grinders) in 5 mL of sterile distilled H₂O at 1, 3, 7, and 14 days after intravenous inoculation with *C. albicans*, and the number of CFU that developed on SDA plates were enumerated at 24–48 h of culture at 37°C. Data from two experiments are reported as CFU/g (dry weight) of tissue.

Histopathology

Groups of three IL-8Rh⁻/⁻ and IL-8Rh⁺/⁺ mice were removed from the gnotobiotic isolator at several time intervals after oral inoculation with *C. albicans* and killed. Tissue samples were aseptically collected and tongue, palate, esophagus, stomach, and the large and small intestines, spleen, vagina, mesenteric lymph node, liver, thymus, lung, and sternum (for bone marrow) were fixed in 10% formaldehyde buffered in phosphate-buffered saline (PBS). The tissues were processed in graded (100, 95, 80, and 70%) alcohol and xylene solutions and embedded in paraffin. Sections of tissues were cut (5 µm), placed on slides, and stained with hematoxylin and eosin and Grocott’s methenamine silver (GMS) stain for fungi. Histopathology of infected tissues was ranked by a pathologist (Dr. Thomas Warner) as follows: 0, no infected tissues observed; 1, 1–10 microorganisms (yeast and hyphae of *C. albicans*); high power field (HPF, ×400); 2, 10–50 microorganisms/HPF; 3, 50–100 microorganisms/HPF; and 4, confluent microorganisms (yeast and hyphae)/HPF.

Neutrophil isolation and candidacidal assay

Elicitation of peritoneal exudate cells (PEC) and isolation of neutrophils and assessment of their candidacidal activity was carried out as described previously [29].

Reactive oxygen intermediate (ROI) production

ROI synthesis by *C. albicans*-elicited neutrophils was estimated by the oxidation of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma) with a Los Alamos Diagnostics model 633 chemiluminometer as described previously [30, 31]. Neutrophils (7.5 × 10⁵) were incubated with live *C. albicans* blastoconidial at an effector/target ratio of 1:1 in a cuvette containing 100 µM luminol, and the chemiluminescence recorded in a computer interface.

Statistical analyses

Statistical analyses of these data were performed with Sigmastat software (SPSS Corp., Chicago, IL). Student’s t test was used to determine statistical significance between control and treatment groups; P < 0.05 was considered significant.

RESULTS

Colonization

After oral inoculation, *C. albicans* quickly colonized and persisted in the gastrointestinal tract of gnotobiotic IL-8Rh⁻/⁻ and IL-8Rh⁺/⁺ mice (Table 1). Similar counts of viable *C. albicans* from the alimentary tracts of monosassociated mice were recorded over the 12-week study. No significant differences were evident in the number of viable *C. albicans* isolated from the alimentary tracts of IL-8Rh⁻/⁻ and IL-8Rh⁺/⁺ mice.

Acute systemic candidiasis

*Figure 1* shows that the IL-8Rh⁻/⁻ mice were more susceptible to acute systemic candidiasis (intravenous injection), especially early after intravenous challenge, than IL-8Rh⁺/⁺ mice. Although both genotypes cleared *C. albicans* from the spleen, the IL-8Rh⁻/⁻ mice were unable to control the growth of *C. albicans* in the kidney, liver, and brain as well as IL-8Rh⁺/⁺ mice.

Recruitment of PMNs

A decreased percentage of neutrophils infiltrated into the peritoneal cavity (5–7 h) of IL-8Rh⁻/⁻ mice in comparison to IL-8Rh⁺/⁺ mice after 10⁷ heat-killed *C. albicans* yeast cells were injected into the peritoneal cavity (*Fig. 2*).

Candidacidal activity

*Table 2* shows that PEC from IL-8Rh⁻/⁻ mice, although able to kill *C. albicans* yeast cells as well as PEC from IL-8Rh⁺/⁺ mice, were less effective at killing *C. albicans* hyphae than PEC from IL-8Rh⁺/⁺ mice (*P < 0.05*).

Luminol chemiluminescence

*Figure 3* shows that PEC from IL-8Rh⁻/⁻ mice were not as efficient (*P < 0.05*) at producing luminol-dependent chemilu-

| Table 1. Colonization of GF IL-8Rh⁻/⁻ and IL-8Rh⁺/⁺ BALB/c Mice with *C. albicans* |
|-----------------------------------------------|----------------|----------------|----------------|----------------|
| Mouse genotype | Stomach Day 1 | Cecum Day 1 | Stomach Day 14 | Cecum Day 14 |
| IL-8Rh⁻/⁻ | 6.3 ± 0.5ᵃ | 9.3 ± 1.0 | 6.2 ± 0.1 | 8.2 ± 1.3 |
| IL-8Rh⁺/⁺ | 6.4 ± 1.0 | 9.1 ± 1.3 | 6.0 ± 0.4 | 8.3 ± 1.4 |

Values are mean ± SEM log₁₀ CFU/g (dry wt).

ᵃLog₁₀ number of viable *C. albicans* contents. Six mice per group. No significant difference (*P < 0.05*) was evident between CFU in IL-8Rh⁻/⁻ and IL-8Rh⁺/⁺ mice.

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minescence, which is associated with myeloperoxidase and other oxidative activity [30, 31], as IL-8Rh

Mucosal infections

Table 3 shows the histopathology results from 16 C. albicans-colonized IL-8Rh-/- and 17 IL-8Rh+/- mice killed at various time periods over the 12-week study. Two of the IL-8Rh-/- mice had lingual (one grade 2.5 and one grade 1) and one had gastric (grade 1) candidiasis at 1 and 7 days after colonization (Table 3). Conversely, of the 17 IL-8Rh+/- mice assayed over the 12-week study, two had esophageal (one grade 1 and one grade 2) and one had a palate (grade 1) infection at 14 days after oral challenge. Also shown in Table 3 are the histopathology results of four IL-8Rh-/- and four IL-8Rh+/- mice killed at 12 weeks. Four of the latter IL-8Rh-/- mice had gastric candidiasis (Table 3), three out of four IL-8Rh+/- mice had grade 4 infections of the stomach, and one had a grade 3 infection. Three of the four IL-8Rh+/- mice also had lingual candidiasis (grade 2). Conversely, only two of four IL-8Rh+/- mice, colonized for 12 weeks, had Candida infections (Table 3); one had a grade 2 infection of tongue and stomach and a second mouse had a grade 2 infection of the tongue only.

Histology

The keratinized gastric epithelium of the four IL-8Rh+/- mice that were colonized with C. albicans for 12 weeks was hyperplastic and several foci of PMN were present in the cornified layer. The numbers of PMN in three HPF (×400) in the grade 3-4 stomach lesions of the latter IL-8Rh+/- mice

| Table 2. Candidacidal Activity of PEC from IL-8Rh-/- and IL-8Rh+/- Mice |
|-----------------|-----------------|-----------------|
| PEC source      | Hyphal killing (%) | Blastoconidia killing (%) |
| IL-8Rh-/-        | 14 ± 4.3         | 46 ± 2.7         |
| IL-8Rh+/-        | 58 ± 2.0a        | 38 ± 6.3         |

* Significantly greater (P < 0.05) than IL-8Rh-/- mice.
ranged between 26 and 270 (Fig. 4). An IL-8Rh +/+ mouse colonized with Candida for 12 weeks, which had only grade 2 gastric lesions, contained 220 PMN/HPF. The lamina propria in the latter IL-8Rh +/+ mouse also contained scattered PMN.

There was a heavy lymphocytic infiltrate in the keratinized portion of the stomach and below the muscularis mucosae in IL-8Rh −/− mice that were colonized with C. albicans for 12 weeks. Although some lymphoid aggregates were present in the latter gastric lesions, germinal centers were absent but clusters of mature plasma cells were present. Perigastric lymphoid aggregates or enlarged perigastric lymph nodes were present in three of four of these IL-8Rh −/− mice. Aggregates of mature plasma cells were present in the sinuses of some lymph nodes and in mesenteric lymph nodes of two IL-8Rh −/− mice that were killed at 12 weeks. No lymphoid aggregates were noted in the gastric mucosae of C. albicans-monoassociated IL-8Rh +/+ mice.

**Table 3. Orogastric Candidiasis in Gnotobiotic IL-8Rh −/− and IL-8Rh +/+ Mice**

<table>
<thead>
<tr>
<th>Mucosal candidiasis</th>
<th>Days after alimentary tract colonization</th>
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<td>1</td>
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<td>IL-8Rh −/−</td>
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<td>Palate</td>
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<td>Gastric</td>
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<td>IL-8Rh +/+</td>
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* Total number infected/total number assayed. All mice were colonized with a pure culture of C. albicans for the indicated number of days. a Three of four mice had lingual and gastric candidiasis; one mouse had gastric candidiasis only. b Total number of mucosal infections/total number of mice assayed over the 84-day study. c Significant increase (P < 0.05) in gastric infections compared with IL-8Rh +/+ mice. d One mouse had gastric and lingual candidiasis; one mouse had lingual but no gastric candidiasis.

*Fig. 3.* Decreased (P < 0.05) chemiluminescence of PEC from IL-8Rh −/− compared with IL-8Rh +/+ mice in response to live C. albicans blastocondidia.

*Fig. 4.* Keratinized zone of stomach showing blastospores and hyphae (star) in keratin and focus of PMN (arrow). From an IL-8Rh −/− mouse colonized with C. albicans for 12 weeks (hematoxylin and eosin, original magnification ×100).

The intraepithelial PMN of grade 2 tongue lesions of three IL-8Rh −/− and two IL-8Rh +/+ mice that were colonized with C. albicans for 12 weeks were counted (magnification ×400); three IL-8Rh −/− mice had no PMN in the grade 2 tongue lesions (Fig. 5, A and B), whereas the grade 2 tongue lesions in two IL-8Rh +/+ mice contained 20–100 PMNs/HPF (Fig. 6, A and B).
The grade 2 tongue lesions were chosen for the latter comparison because all grade 4 lesions in the stomach of the IL-8Rh-/- mice contained many intraepithelial PMN and only grade 2 lesions were evident in IL-8Rh+/+ mice at 12 weeks. The esophagi of three of the IL-8Rh-/- mice that were colonized for 12 weeks showed subepithelial lymphocytes and basal intraepithelial lymphocytes despite the absence of demonstrable fungal elements (on GMS staining) (Fig. 7). Very few PMN were seen in the esophageal lamina propria of these three IL-8Rh-/- mice.

DISCUSSION

This study demonstrates that IL-8Rh h-/- mice were more susceptible to gastric (cardia-antrum) and systemic candidiasis (intravenous injection) than IL-8Rh h+/+ mice. These new data on the increased susceptibility of IL-8Rh h-/- mice to candidiasis supports the important role that PMNs play in controlling acute systemic candidiasis (i.e., intravenous inoculation) and gastric candidiasis [1-5, 7, 9, 12, 26]. Conversely, in C. albicans-colonized IL-8Rh h-/- mice we did not observe any disseminated candidiasis of endogenous origin or lethality (over a 12-week study) even though the gnotobiotic IL-8Rh h-/- mice were (chronically) colonized (Table 1) with large viable populations of a pure culture of C. albicans. In the absence of an inhibitory bacterial flora in the alimentary tract of the gnotobiotic mice, all mucosal sites of the IL-8Rh h-/- and IL-8Rh h+/+ mice were available for colonization and infection by C. albicans after oral challenge. We observed more extensive gastric (cardia-antrum) infections in IL-8Rh h-/- than in IL-8Rh h+/+ mice that were colonized with C. albicans for 12 weeks. The IL-8Rh h-/- mice, colonized for 12 weeks, had grade 3 and 4 infections of the stomach and three mice had a grade 2.0 infection of the tongue. In contrast, only two of four IL-8Rh h+/+ mice at 12 weeks had a grade 2 infection of the stomach (cardia-antrum section) and tongue.

We observed that IL-8Rh h-/- PMN did not infiltrate into the peritoneal cavity or into infected tissues as well as PMN in IL-8Rh h+/+ mice and the PEC from IL-8Rh h-/- mice did not kill C. albicans hyphae (in vitro) as well as PEC from IL-8Rh h+/+ mice. The PEC from IL-8Rh h-/- mice also had less chemiluminescence, which suggests less myeloperoxidase or other oxidative activity [30], than PEC from IL-8Rh h+/+ control mice. Peritoneal exudate cells from both genotypes were able to kill yeast cells of C. albicans equally well; however, we observed a diminished capacity of PEC from IL-8Rh h-/- mice to kill C. albicans hyphae. The diminished oxidative activity and deficient capacity of phagocytes from IL-8Rh h-/- mice to kill C. albicans hyphae could explain the more severe gastric candidiasis we observed at 12 weeks after colonization and their enhanced susceptibility to acute systemic candidiasis after intravenous challenge.

The impairment of PMN migration was also manifest on histological examination of Candida-infected tissues from IL-8Rh h-/- mice. The latter mice that were colonized with C. albicans showed no PMN in low-grade lesions (grade 2) of the tongue early after oral challenge; but at 12 weeks after challenge they showed a good PMN infiltrate into the keratinized layer and lamina propria of the stomach that was infected with grade 4 lesions. Therefore other chemoattractants for neutrophils such as leukotriene B4, complement components, or products of C. albicans likely played a late role in PMN recruitment in the IL-8Rh h-/- mice. Myeloid hyperplasia in the spleen was a constant finding in conventional IL-8Rh h-/- mice described by Calcano et al. [22, 24, 25]. We found splenic...
myeloid hyperplasia in only one of four C. albicans-colonized IL-8Rh−/− mice that had grade 4 Candida infection of the gastric mucosa.

In a comparison of GF and specific pathogen-free (SPF) IL-8Rh−/− mice [22], it was found [24, 25] that environmental microbes are necessary for the observed neutrophilia seen in the SPF IL-8Rh−/− mice. In the SPF mice, the bacterial flora apparently induce a chronic release of cytokines that can stimulate production of neutrophils [22, 24, 25]. The absence of splenic myeloid hyperplasia in GF and C. albicans-colonized IL-8Rh−/− mice in our study is very likely due to the absence of a viable bacterial flora in the gut. Apparently, the chronic, high-grade, C. albicans infection we observed in the IL-8Rh−/− mice at 12 weeks after C. albicans colonization of their alimentary tract was not sufficient to evoke an adequate myeloproliferative cytokine stimulus and subsequent myeloid hyperplasia response that is apparently induced by the bacterial flora in the gut of SPF IL-8Rh−/− mice [22, 24, 25]. Nevertheless, aggregates of PMN were present in intra-abdominal lymph nodes in two of four IL-8Rh−/− mice that were colonized for 12 weeks and infected with C. albicans. It is interesting that the nodal PMN in IL-8Rh−/− mice that were colonized for 12 weeks with C. albicans did not show evidence of degeneration or apoptosis. The sternal marrow sections appeared normal in all mice in this study but this central marrow source is not comparable to that in the distal long bones with respect to reactive hyperplasia [22, 24, 25].

Mucosal lymphoid infiltrates were restricted to the esophagus and keratinized gastric mucosa of the C. albicans-infected IL-8Rh−/− mice; however, they were not seen in the intestine, tongue, or palate. Peyer’s patches appeared normal in size and number in these gnotobiotic IL-8Rh−/− mice. The cervical lymph nodes were not sampled for histology but they were noticeably enlarged as reported previously by Calcano et al. [4] in IL-8−/− mice. The increased number of mature plasma cells in the stomach and perigastric lymphoid tissue with respect to reactive hyperplasia [22, 24, 25]. The absence of splenic myeloid hyperplasia in GF and C. albicans-colonized IL-8Rh−/− mice in our study is very likely due to the absence of a viable bacterial flora in the gut. Apparently, the chronic, high-grade, C. albicans infection we observed in the IL-8Rh−/− mice at 12 weeks after C. albicans colonization of their alimentary tract was not sufficient to evoke an adequate myeloproliferative cytokine stimulus and subsequent myeloid hyperplasia response that is apparently induced by the bacterial flora in the gut of SPF IL-8Rh−/− mice [22, 24, 25]. Nevertheless, aggregates of PMN were present in intra-abdominal lymph nodes in two of four IL-8Rh−/− mice that were colonized for 12 weeks and infected with C. albicans. It is interesting that the nodal PMN in IL-8Rh−/− mice that were colonized for 12 weeks with C. albicans did not show evidence of degeneration or apoptosis. The sternal marrow sections appeared normal in all mice in this study but this central marrow source is not comparable to that in the distal long bones with respect to reactive hyperplasia [22, 24, 25].

Thus, the IL-8 receptor homolog in mice appears to play a role in the resistance in gnotobiotic mice to gastric and acute systemic candidiasis; however, other innate and acquired immune mechanisms apparently can protect the mice from systemic candidiasis of endogenous origin.

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