Iron Overload Alters Innate and T Helper Cell Responses to *Candida albicans* in Mice

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The effect of iron overload on susceptibility of mice to *Candida albicans* infection and on the type of T helper (Th) immunity elicited was investigated. Iron overload greatly increased susceptibility to disseminated infection with low-virulence *C. albicans* cells of exogenous origin. The candidacidal activity and the ability to release nitric oxide and bioactive interleukin (IL)-12 were greatly impaired in neutrophils and macrophages from infected mice. CD4 T cells from spleens of iron-overloaded mice were found to produce high levels of IL-4 and IL-10 and low levels of interferon-γ. Treatment of iron-overloaded mice with the iron chelator, deferoxamine, resulted in the cure of mice from infection, restored the antifungal effector and immunomodulatory functions of the phagocytic cells, and allowed the occurrence of CD4 Th1 protective antifungal responses. These data indicate that iron overload may negatively affect CD4 Th1 development in mice with candidiasis, a function efficiently restored by therapy with deferoxamine.

Helper T (Th) cells play a central role in regulating immune responses to the fungus *Candida albicans* by secreting cytokines that modulate the development and activity of immune effectors. The dominance of either of the Th subsets (Th1 and Th2) correlates directly with the outcome and severity of infection [1, 2]. In experimental models of candidiasis, protection correlates with polarization toward the Th1 differentiation pathway, as observed in genetically resistant mice injected with a live vaccine strain of the yeast or in susceptible mice injected with virulent *C. albicans* cells, provided that the Th2 cytokines are neutralized [3–5]. In contrast, Th2 responses are associated with disease exacerbation and pathology [6–8].

A variety of factors control Th cell subset development and regulation in murine candidiasis [1, 2]. Among these, the initial handling of the yeast by cells of the innate immune system plays an essential role in shaping the subsequent adaptive immune response [5, 9]. The ability of macrophages to release nitric oxide (NO) and the early balance between opposing regulatory signals in this activity dictate the type of subsequent Th cell development, ultimately leading to disparate outcomes of *C. albicans* infection [5]. Likewise, depletion of polymorphonuclear neutrophils in genetically resistant mice at the time of infection with the live vaccine strain of the yeast leads to the onset of Th2 rather than Th1 cell-mediated responses, indicating that neutrophils could contribute to the appearance of Th1 responses in mice with candidiasis [1]. A defective innate immune response also contributes to the inability of interleukin (IL)-6–deficient mice to mount protective Th1 responses in infection with *C. albicans* [10]. These results indicate that phagocytic cells could act not only as effectors but also as regulators of cell-mediated immunity in mice with candidiasis. Such a finding, while evidencing the complexity of the interdependence among the different types of immunity [11], also suggests that factors affecting the efficiency of the innate immune system may have an impact on the subsequent anticandidal Th cell development.

Numerous studies have demonstrated that iron overload both increases susceptibility and aggravates the outcome of infections caused by a variety of microorganisms [12–14], probably including human immunodeficiency virus (HIV) [15]. Both the increased availability of iron, favoring microbial growth and virulence, and ineffective host iron-withholding strategies appear to be associated with increased risk of infection [14]. However, iron overload may act through additional mechanisms, such as impairment of both T and phagocytic cell functions [16, 17]. The availability of cellular iron has a distinct influence on the proliferation of Th1 and Th2 subsets [18] and regulates the expression of inducible NO synthase (iNOS) in a murine macrophage cell line [19]. In iron-overloaded patients, a reduced CD4:CD8 ratio and abnormalities in monocyte and neutrophil functions, including defective phagocytic and microbicidal capacity as well as chemotactic responsiveness, were observed [20, 21]. Exposure of neutrophils from iron-overloaded patients to the iron chelator deferoxamine (DFO) improved phagocytic activity, suggesting a critical role for iron excess in the generation of neutrophil phagocytosis abnormalities [21, 22].

In an experimental model of disseminated *C. albicans* infection, iron overload greatly exacerbated the course and pathol-

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ogy of infection [23]. Increased serum iron and iron saturation of transferrin, together with impaired phagocytic activity, accounted for the increased growth of the fungus.

To gain insight into the possible effects of iron overload on the immunoregulatory and effector functions of neutrophils and macrophages as well as on the type of Th cell involved in response to *C. albicans* infection, mice were overloaded with iron and injected intravenously or intragastrically with the fungus. The course and outcome of infection together with parameters of innate and acquired Th immunity were then assessed.

Materials and Methods

Mice. Hybrid (BALB/c × DBA/2)F1 (CD2F1) and inbred BALB/c mice were obtained from Charles River Breeding Laboratories (Calco, Milan, Italy). Female mice 2–4 months old were used.

Yeast, infections, and colony-forming unit (cfu) enumeration. The origin and characteristics of the highly virulent *C. albicans* strain (CA-6) and the live vaccine strain (PCA-2) used in this study have been described previously [3, 4, 6]. After a 24-h culture, yeast cells were harvested by low-speed centrifugation (1000 *g*), washed twice in saline, and diluted to the desired density to be injected into mice. For disseminated infection, yeast cells were injected intravenously into CD2F1 mice in a volume of 0.5 mL. Alimentary tracts of CD2F1 mice were colonized by oral intragastric inoculation of 10^8 CA-6 cells delivered via an 18-gauge 4-cm-long plastic catheter as described [24, 25]. Yeast cells in the organs of infected mice were enumerated by a plate-dilution method, by use of Sabouraud dextrose agar, and results (mean ± SE) were expressed as number of cfu per organ. For histology, tissues were fixed in formalin, sectioned, and stained with periodic acid–Schiff [24, 25] for yeasts or with Berlin blue (HematoGnost Fe; Merck, Darmstadt, Germany) reagents for iron detection [23].

In vivo treatments. Iron overload was established by administering iron dextran (Sigma, St. Louis) intraperitoneally every other day for 4 weeks before infection, at a dose of 40 or 60 mg of iron/kg, as described [23]. DFO (Desferal; Ciba Geigy, Basel, Switzerland) was administered intraperitoneally at a dose of 50 mg/kg every other day for 3 weeks, from the day of infection [26].

Growth of C. albicans in vitro. Fungal growth was evaluated in vitro in Sabouraud broth with a content of 0.41 to 0.45 ppm of iron, as measured by atomic absorption spectrometry. Iron citrate hydrate (Fe^{3+}, $[C_6H_8O_7Fe]_5/2H_2O$; Janssen Chimica, Beerse, Belgium; reference no. 2118539, 18%–20% Fe), was dissolved at 10 m*M* in distilled water, sterilized by filtration with a 0.22-μm filter, and distributed in the medium at a final concentration of 600 or 6000 μg/dL (corresponding to 0.0082 and 0.082 m*M*, respectively), as described [27]. Tubes containing 10 mL of medium were then added to 3×10^3 PCA-2 or CA-6 cells. After 24 h of incubation at 37°C, *Candida* cells were counted in a hemocytometer. Values (mean ± SE) were expressed as number of blastospores per milliliter [28].

Purification and culture of cells. Neutrophils were isolated from peritoneal exudate cells collected 18 h after intraperitoneal inoculation of aged, endotoxin-free 10% thioglycollate solution (Difco, Detroit), prepared according to manufacturer's instructions. Endotoxin was depleted from all solutions (Detoxi-gel; Pierce Chemical, Rockford, IL). To enrich for neutrophils (80%–85% of 18-h peritoneal exudate cells), cells were incubated with the anti-Ly6G monoclonal antibody (MAb) (gift of R. Coffman, DNAX Research Institute, Palo Alto, CA) for 30 min at 4°C and then mixed with magnetic polymer beads (Dynabeads M-450; Dynal, Oslo), at a bead-to-neutrophil ratio of 3:1, for 5 min at 4°C in a rotary mixer. Beads with attached neutrophils were removed by use of a magnet (Magnetic Particle Concentrator, MPC 1; Dynal), briefly vortexed, and incubated with 1% mouse serum albumin at 37°C for 1 h to allow beads to detach. This resulted in a 98% pure neutrophil population, referred to as neutrophils.

Monolayers of plastic-adherent macrophages (95% pure on esterase staining) were obtained by standard techniques from total spleen cells [29]. CD4 cells were positively selected from pools of spleen cells by means of a panning procedure using anti-murine CD4 MAb GK1.5, which resulted in a >95% pure population on cell cytometric analysis (FACS; Becton Dickinson, Mountain View, CA) [3, 4, 6]. Supernatants from mixed lymphocyte-*Candida* cultures were obtained by culturing CD4 lymphocytes from either uninfected or infected mice in the presence of yeast cells and accessory macrophages [3, 4, 6]. After a 48-h incubation, culture supernatants were collected for cytokine detection. Neutrophils and macrophages (5×10^6) were cultured in 1 mL of RPMI 1640 medium with 10% fetal calf serum and antibiotics in 24-well plates in the presence of either 400 U/mL interferon-γ (IFN-γ) or 40 ng/mL lipopolysaccharide for 24 h. Protease inhibitors (0.4 mg/mL aprotinin and 0.3 m*M* phenylmethylsulfonyl fluoride; Sigma) were added to the cultures to facilitate IL-12 protein detection in supernatants.

Candidacidal assay and NO production. For the candidacidal assay, 5×10^5 neutrophils or macrophages/0.1 mL/well were plated in 96-well flat-bottom microtiter plates (Costar, Cambridge, MA) and incubated with 10^5 PCA-2 cells for 1 h or 4 h, respectively, as described [29]. Triton X-100 (final concentration, 0.1%) was then added to the wells, and serial dilutions from each well were made in distilled water. Pour plates (4–6 replicate samples) were made by spreading each sample on Sabouraud glucose agar. The number of cfu was determined after 18 h of incubation at 37°C and the percentage of cfu inhibition (mean ± SE) was determined as follows: percentage of colony formation inhibition = 100 − (cfu experimental group/cfu control cultures) × 100. Control cultures consisted of *C. albicans* cells incubated without effector cells. Nitrite concentration, a measure of NO synthesis, was assayed in culture supernatants by a standard Griess reaction adapted to microplates, as described previously [5]. The Griess reagent was prepared by mixing equal volumes of sulfanilamide (1.5% in 1 *N* HCl) and naphthylethylene diamine dihydrochloride (0.15% in H_2O). A volume of 100 μL of reagent was mixed with 100 μL of supernatant and incubated for 30 min in the dark. Absorbance of the chromophore formed was measured at 540 nm in an automated microplate reader. Nitrite was quantitated using $NaNO_2$ as a standard. The data represent the means ± SE of quadruplicate determinations and are expressed as μM $NO_2^-/10^7$ cells.

Cytokine and IgE assays. The source and characteristics of the anti-cytokine MAb reagents used in IFN-γ, IL-4, and IL-10 ELISA have been described previously in detail [3–5, 30]. Briefly, culture supernatants were tested for their concentration of IFN-γ

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by using rat anti-murine IFN-γ MAb XMG1.2 as the primary antibody and biotinylated monoclonal AN-18.17.24 as the secondary antibody. In IL-4 and IL-10 measurements, two-site ELISA involved the use of MAb 11B11 in combination with biotinylated rat anti-murine IL-4 (PharMingen, San Diego) and MAb SXC-2 plus biotinylated SXC-1 (PharMingen), respectively. For IL-12p70 measurement in macrophages and neutrophils, an antibody-capture bioassay was used [30]. A micro-ELISA procedure was used to quantitate specific IgE in the sera of mice [3, 7]. The assay involved coating of the microtiter plate wells with *Candida* antigen, addition of appropriate dilutions of test antisera, and further reaction with biotinylated MAb anti-murine IgE (PharMingen) plus avidin-peroxidase (Sigma).

RNA preparation and polymerase chain reaction (PCR). CD4 lymphocytes, splenic macrophages, or peritoneal neutrophils (2×10^7) from 3 or 4 animals were subjected to RNA extraction by the guanidium thiocyanate–phenol–chloroform procedure [31]. Briefly, 3 μg of total RNA was incubated with 0.5 μg of oligo(dT) (Pharmacia, Uppsala, Sweden) for 3 min at 65°C and chilled on ice for 5 min. Each sample was then incubated for 2 h at 42°C after addition of 20 U of RNase inhibitors (Boehringer Mannheim, Milan, Italy), 1.5 m*M* dNTPs, 25 U of avian myeloblastosis virus reverse transcriptase (RT; Boehringer Mannheim), and RT buffer (50 m*M* TRIS-HCl, pH 8.3, 8 m*M* $MgCl_2$, 30 m*M* KCl, and 10 m*M* dithiothreitol, final concentrations) in a final volume of 20 μL. cDNA was diluted to a total volume of 500 μL with TE buffer (10 m*M* TRIS-HCl, 1 m*M* EDTA, pH 8.0) and frozen at −20°C until use. Amplification of synthesized cDNA from each sample was carried out as previously described [32]. Briefly, 5 μL of cDNA was added to a reaction mixture containing 50 m*M* KCl, 10 m*M* TRIS-HCl (pH 8.3), 3.0 m*M* $MgCl_2$, 0.01% gelatin, 0.2 m*M* dNTPs, 1 μM each primer, and 2.5 U of AmpliTaq polymerase (Perkin-Elmer, Hayward, CA). Each 100-μL sample was overlaid with 75 μL of mineral oil (Sigma) and incubated in a DNA thermal cycler (Perkin-Elmer) for a total of 30 cycles for each cytokine. For β-actin– and IFN-γ–specific primers, each cycle consisted of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C; for IL-4, 1 min at 94°C, 1 min at 67°C and 1 min at 72°C; and for IL-12p40, 1 min at 94°C, 1 min at 67°C, and 1 min at 72°C. For iNOS, the primers were synthesized using a 391 DNA synthesizer (PCR-MATE; Applied Biosystems, Foster City, CA). The sequences of 5′ sense primers and 3′ antisense primers for iNOS were as follows: 5′-AAGTCAAATCCTACCAAAGTGA; 3′-CCATAA-TACTGGTTGATGAACT.

For iNOS, each cycle consisted of 45 s at 94°C, 45 s at 60°C, and 1 min at 72°C. The positive controls were purchased from Clontech Laboratories (Palo Alto, CA). The primers and positive controls for β-actin, IFN-γ, IL-4, and IL-12p40 were as described elsewhere [30, 33]. The PCR fragments were analyzed by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. PCR-assisted mRNA amplification was repeated at least twice for at least 2 separately prepared cDNA samples for each experiment.

Statistical analysis. In all in vivo experiments, 8–10 animals per group for gastrointestinal infection and 6–8 mice per group for disseminated fungal infection were used. Mortality data were analyzed by Mann-Whitney *U* test. Student's *t* test was used to determine statistical significance between controls and treatment groups. Data are means ± SE of 3–5 separate experiments.

Results

Course of C. albicans infection in iron-overloaded mice. To examine the course and outcome of disseminated candidiasis in iron-overloaded mice, CD2F1 mice were treated intraperitoneally with iron dextran for 4 weeks before intravenous injection of either low-virulence PCA-2 (nonlethal infection) or highly virulent CA-6 (lethal infection). Mice were monitored for mortality, fungal load in visceral organs, and histology. The iron dextran–treated animals were clearly iron-overloaded, as indicated both by hematologic parameters (serum iron increasing from 167 ± 23 to 522 ± 27 μg/dL and unsaturated transferrin decreasing from 324 ± 12 to 44 ± 0.6 mg/dL) and by prominent hemosiderin deposition in the liver, as observed by Berlin blue staining (figure 1A, B). The iron content of circulating neutrophils was also increased, as revealed by the same staining procedure (data not shown). Iron treatment greatly increased the susceptibility to both types of infection, in that treated mice succumbed to PCA-2 infection whereas control, untreated mice survived long-term. Reduced survival was also observed on infection with CA-6 (table 1). Interestingly, increased susceptibility was also observed on infection with lower doses of both strains of *C. albicans* (data not shown). Studies on fungal growth in the kidneys and liver indicate that iron overloading was associated with an extensive fungal growth. This was also visible on histologic examination of the kidneys (figure 1C, D). An extensive inflammatory reaction in the cortex and medulla with numerous abscesses containing fungal and predominantly polymorphonuclear cells was observed in iron-overloaded mice, as opposed to the presence of few scattered healing inflammatory lesions in the cortex of control mice. Therefore, experimentally induced hemosiderosis is associated with an increased susceptibility to disseminated candidiasis.

To evaluate whether iron overload also modifies the course of gastrointestinal *C. albicans* infection, CA-6 cells were inoculated intragastrically into CD2F1 mice, in an experimental model of infection in which the course and outcome had been previously characterized [24, 25]. At 1, 2, and 3 weeks after infection, total viable yeast cells were enumerated in the esophagus, stomach, and small intestine. The results (figure 2) show that iron treatment was not associated with an increased fungal burden in the stomach and small intestine and did not cause dissemination to visceral organs, such as the esophagus. As expected [24, 25], both treated and untreated mice survived gastrointestinal infection (data not shown). These data indicate that iron overload differentially affects the susceptibility to disseminated and gastrointestinal candidiasis.

Effect of iron on C. albicans growth in vitro. Because inhibition of *Candida* growth in serum can be reversed by the addition of increasing concentrations of ferric nitrate to the growth medium [27], we assessed the growth capacity of both PCA-2 and CA-6 in vitro in the presence of serum from iron-overloaded mice or of different concentrations of ferric citrate.

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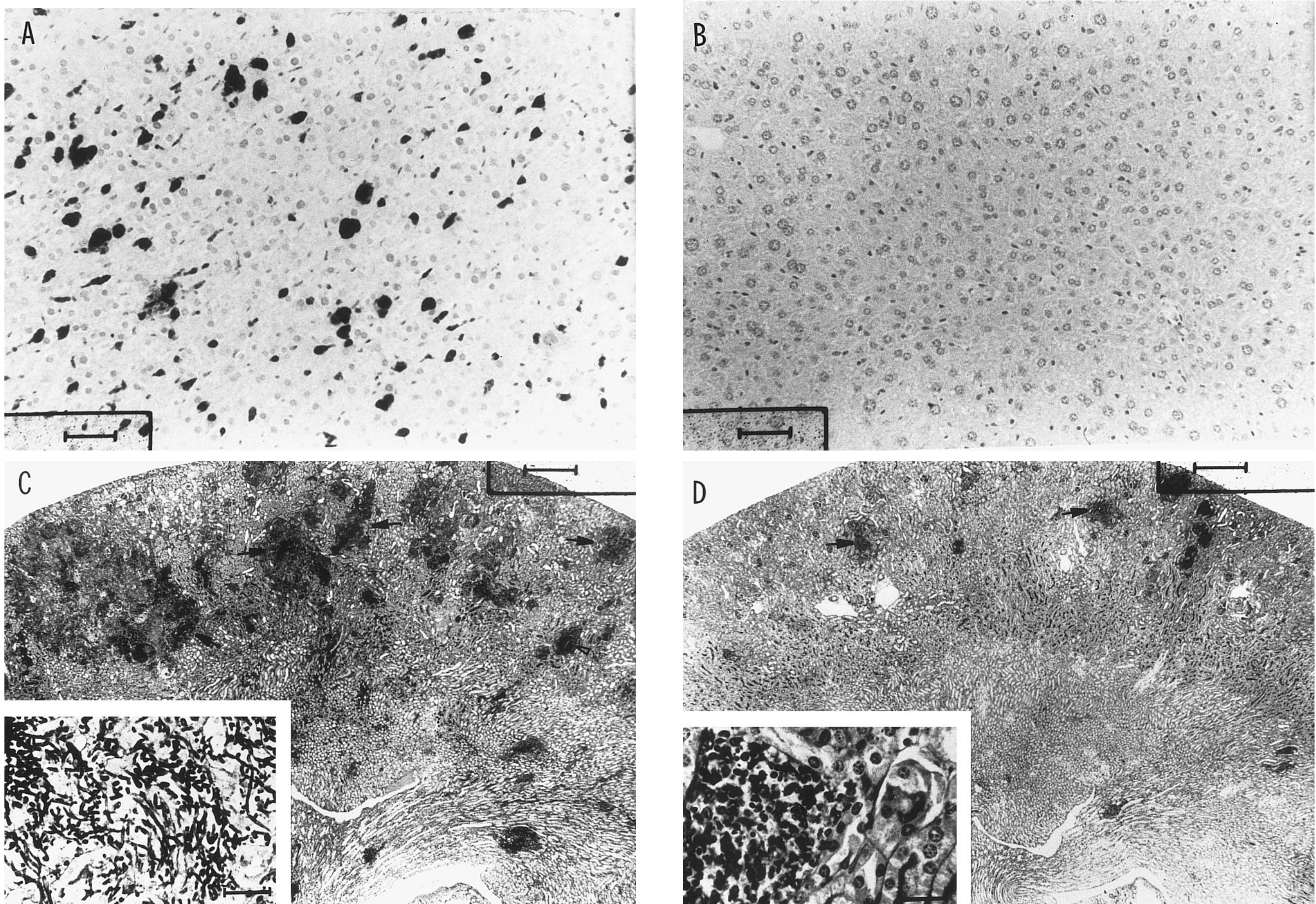

Figure 1. **A**, Prominent hemosiderosis in Kupffer cells of liver from mice on iron administration. **B**, Section of liver from control mouse (Berlin blue staining; bar = 50 μm). **C**, Pathologic analysis of renal candidiasis in iron-overloaded mice. Periodic acid–Schiff stained section from kidneys (largest transverse diameter) of mice given iron dextran (60 mg/kg) before intravenous infection with 10^6 *C. albicans* PCA-2 cells. Numerous abscesses (arrows) are present throughout section, with large aggregates of fungal yeasts and hyphae within the abscesses and tubules (bar = 400 μm). Inset: fungal yeasts and hyphae surrounded by few polymorphonuclear neutrophils in abscesses (bar = 25 μm). **D**, Healing inflammatory lesions (arrows) in cortex of iron-untreated mice infected with PCA-2, with no evidence of fungal growth (periodic acid–Schiff stain; bar = 400 μm). Inset: magnification of lesion showing predominant lymphomononuclear infiltrate (bar = 25 μm).

The concentration of iron in vitro (600 μg/dL) closely corresponded to the value found in the serum of iron-overloaded mice. After 24 h of incubation, yeast cells were counted in a hemocytometer. The results (figure 3) indicate that the growth of *Candida* organisms in vitro was not affected by the presence of 5% serum from iron-overloaded mice or by a concentration of iron that was close to that found in non-overloaded mice. In contrast, the addition of ferric citrate at a higher concentration to the growth medium significantly promoted the growth of both PCA-2 and CA-6 cells.

Anticandidal CD4 Th responses in iron-overloaded mice. We have previously shown that the dominance of either of the two Th subsets directly correlates with the outcome and severity of disseminated *C. albicans* infection [1, 2]. To verify whether the pattern of Th1/Th2 cytokine production by CD4 splenocytes and serum levels of IgE were modified in iron-treated mice, CD4 splenocytes and serum were obtained from iron-overloaded CD2F1 mice 6 days after PCA-2 infection, at a time when polarization of CD4 Th cells had already occurred [34]. Cytokine levels in the supernatants of antigen-stimulated CD4 cells and of *Candida*-specific IgE in serum were assessed (figure 4). In the absence of iron loading, the production of IFN-γ was elevated, whereas that of IL-4 and IL-10 was low in mice infected with PCA-2, which is consistent with the occurrence of a Th1-mediated, self-limiting infection [33]. Also, IgE levels were not detectable. However, with iron treatment, the production of IFN-γ decreased, while that of IL-4 and IL-10 and serum levels of IgE increased. In the absence of infection, iron treatment per se did not significantly modify either the pattern of cytokine production by CD4 cells or the serum levels of IgE. Therefore, a failure to mount protective Th1-mediated immunity and a predominant production of Th2 cytokines were observed in iron-overloaded mice on PCA-2 infection.

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Table 1. Course of systemic *C. albicans* infection in iron-overloaded CD2F1 mice.

Infecting strain, iron treatment (mg/kg)	Median survival time (days)	No. dead/ total	Organisms (cfu × 10^3) in	
			Kidneys	Liver
PCA-2				
—	>60	0/18	794 ± 65	24 ± 1
40	10	18/18	6860 ± 520*	71 ± 3†
60	7	18/18	11,260 ± 635*	182 ± 12*
CA-6				
—	12	16/16	1995 ± 82	19 ± 1
40	6	20/20	5011 ± 240†	79 ± 4†
60	3	20/20	10,350 ± 620†	213 ± 13*

NOTE. Iron was injected intraperitoneally as iron dextran every other day for 4 weeks before challenge. PCA-2 (10^6) or CA-6 (3 × 10^5) cells were injected intravenously. Parallel groups of mice were sacrificed 3 days after challenge for colony-forming unit enumeration in the kidneys and liver (mean ± SE).
* $P < .01$ (iron-treated vs. untreated mice).
† $P < .05$ (iron-treated vs. untreated mice).

Anticandidal innate immunity in iron-overloaded mice. Iron excess may impair neutrophil and monocyte/macrophage functions, such as phagocytic activity [20, 21] and NO release [19]. We have recently shown that IL-12 is both required and prognostic for CD4 Th1 development in mice with candidiasis [35]. Interestingly, both macrophages and neutrophils were found to release bioactive IL-12, either in vivo in infected mice or in vitro on exposure to fungal cells [33, 35, 36]. Therefore, we evaluated the antifungal effector and the immunomodulatory functions of macrophages and neutrophils in iron-overloaded mice either uninfected or at 3 days after *C. albicans* infection. To this purpose, freshly harvested splenic macrophages and peritoneal neutrophils were assessed for candidacidal activity after incubation with fungal cells (4 h for macrophages and 1 h for neutrophils) and expression of iNOS and IL-12p40 genes by RT-PCR. The results (figure 5) show that iron treatment greatly reduced the candidacidal activity in the two types of phagocytic cells from both uninfected and infected mice. In contrast, the effects of iron treatment on the expression of iNOS and IL-12p40 genes were dependent on the types of cells and on the presence of infection. Figure 6A indicates that iron loading re-

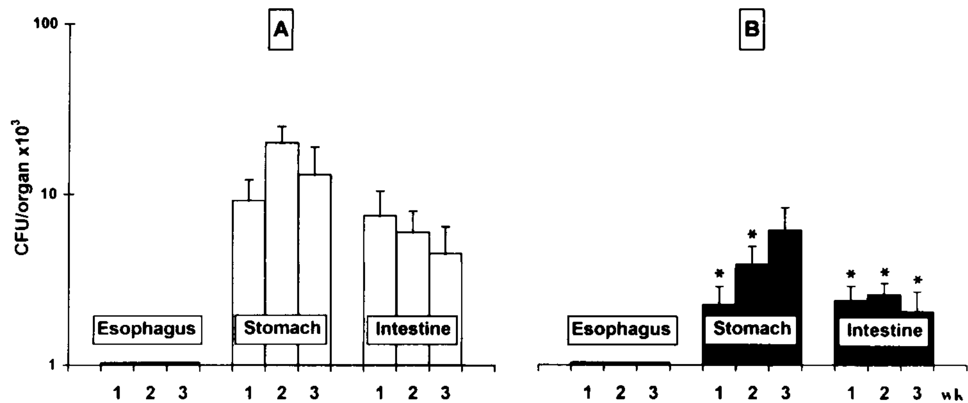

Figure 2. Total viable colony-forming units of *C. albicans* in esophagus, stomach, and small intestine at 1, 2, and 3 weeks after intragastric inoculation of control (**A**) or iron-overloaded (**B**) CD2F1 mice. Iron (60 mg/kg) was administered intraperitoneally as iron dextran every other day for 4 weeks before challenge. Values are mean ± SE. * $P < .01$ (iron-treated vs. untreated mice).

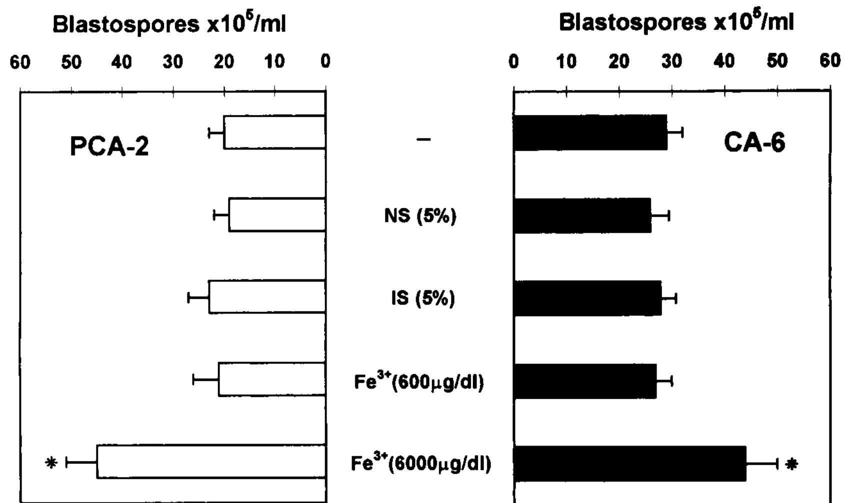

Figure 3. Effect of Fe^{3+} and serum from iron-treated (IS) or untreated mice (NS) on growth of *C. albicans* PCA-2 or CA-6 in vitro. PCA-2 or CA-6 cells (3 × 10^3) were incubated in 10 mL of Sabouraud broth at 37°C, supplemented with NS or IS or iron citrate hydrate (Fe^{3+}), at final concentration of 5%, or 600 and 6000 µg/dL, respectively. After 24 h, *Candida* blastospores were counted in hemocytometer. Values are mean ± SE. * $P < .05$ (tubes with iron vs. tubes with Sabouraud only).

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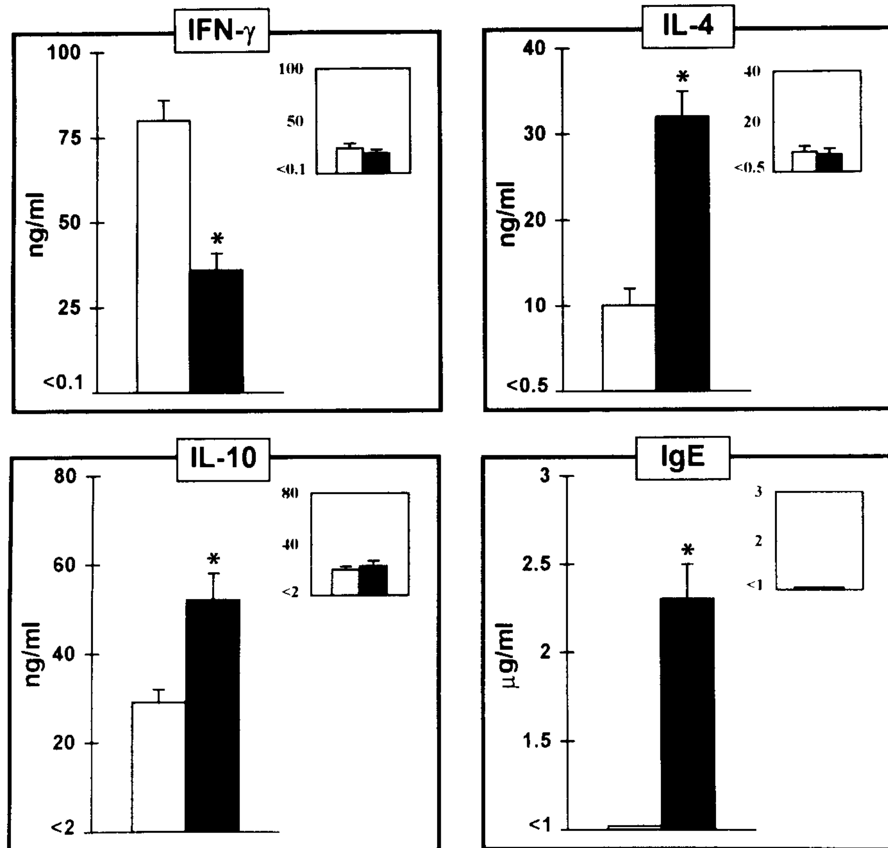

Figure 4. Cytokine production and serum IgE levels in untreated (open bars) or iron-treated (solid bars), *C. albicans* PCA-2–infected mice. At 6 days after infection, interferon-γ (IFN-γ), interleukin (IL)-4, and IL-10 were assayed in supernatants of mixed CD4 lymphocyte yeast cell cultures and antigen-specific IgE was measured in sera. Each value represents mean ± SE of 4 separate experiments. Inset: cytokine production and serum IgE levels in untreated (open bars) or iron-treated (solid bars), uninfected mice. * $P < .01$ (iron-treated vs. untreated mice). < indicates below detection limit of assay.

sulted in the disappearance of both types of messages in neutrophils from either uninfected or infected mice. In contrast, iron overload silenced the expression of both genes in macrophages from uninfected but not infected mice. Likewise, the in vitro production of NO and IL-12p70 was impaired in neutrophils but not in macrophages (figure 6B), indicating that iNOS and IL-12p40 gene expressions are differently regulated in these cells. The results also suggest the existence of redundant mechanisms of regulation of iNOS and IL-12p40 gene expressions in macrophages during *C. albicans* infection. Because the absolute numbers of circulating monocytes and neutrophils were apparently not affected by iron treatment (data not shown), these results indicate that qualitative defects in the antifungal effector and immunomodulatory functions are induced in macrophages and neutrophils by iron treatment.

Effect of DFO treatment on the course and outcome of C. albicans infection in iron-overloaded mice. To examine the effects of the iron chelator DFO on the course and outcome of *C. albicans* infection in the presence of iron overload, mice were administered iron for 4 weeks before infection and treated

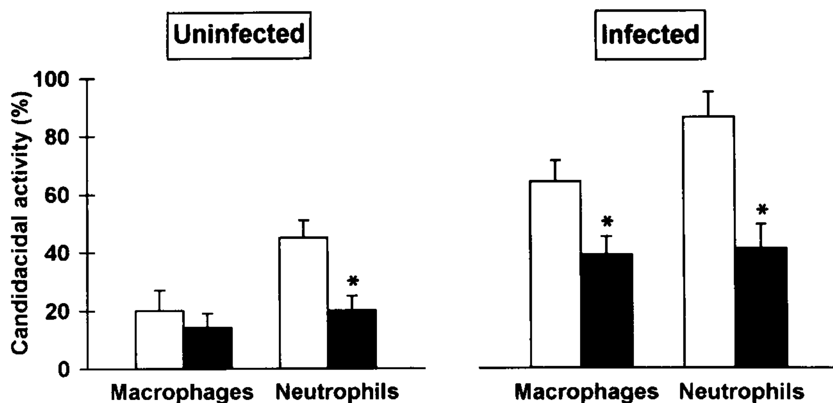

Figure 5. Candidacidal activity of splenic macrophages and peritoneal neutrophils from untreated (open bars) or iron-treated (solid bars) mice 3 days after *C. albicans* PCA-2 infection. Macrophages and neutrophils were incubated with PCA-2 at cell ratio of 5:1 for 4 h and 1 h, respectively, before assay. Values represent mean ± SE. * $P < .05$ (treated vs. untreated mice).

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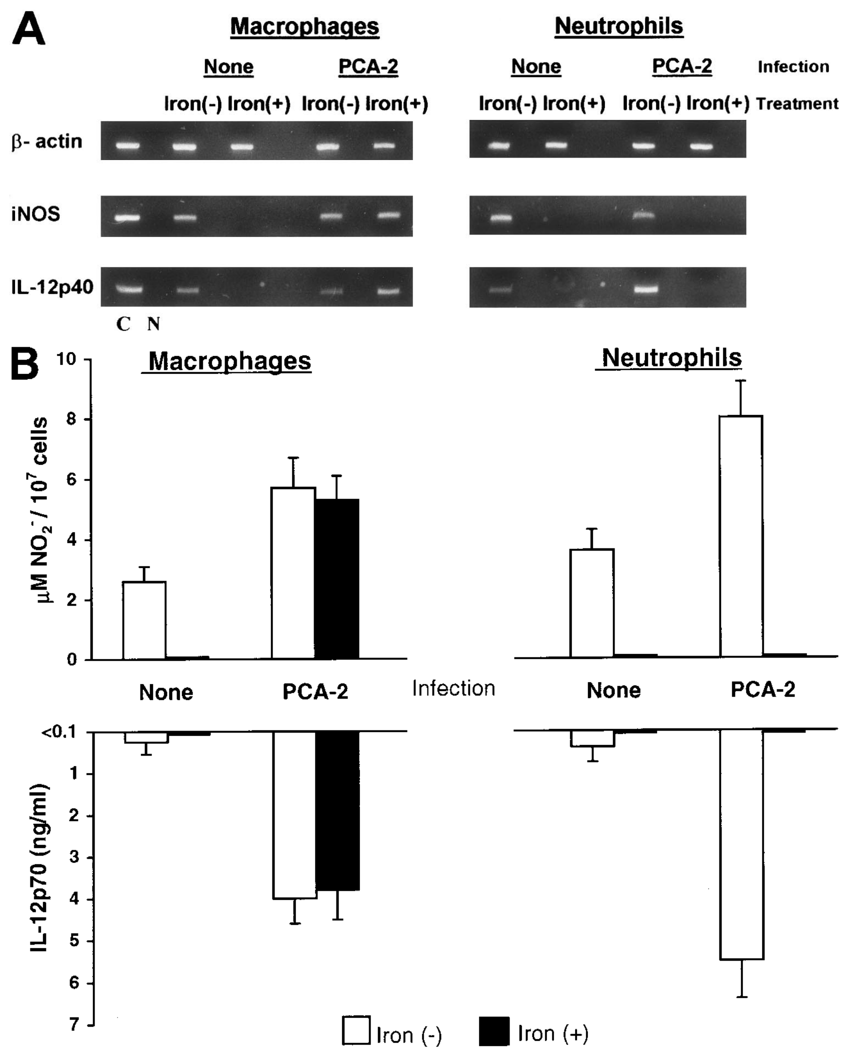

Figure 6. Analysis of inducible nitric oxide synthase (iNOS) and interleukin (IL)-12p40 gene expression (**A**) and nitric oxide (NO) and IL-12p70 production (**B**) in *C. albicans*–infected or control mice treated (+) or not (−) with iron dextran. At 3 days after infection, splenic macrophages and peritoneal neutrophils were assessed for iNOS and IL-12p40 gene expression by reverse transcriptase–polymerase chain reaction (PCR) and for production of NO and bioactive IL-12p70 on in vitro stimulation with interferon-γ and lipopolysaccharide. C, β-actin– or cytokine-specific control; N, no DNA added to amplification mix during PCR.

with DFO, starting the day of challenge with PCA-2 and continuing for 3 weeks after challenge. Mice were monitored for resistance to primary and secondary infections and fungal growth in visceral organs. The results (table 2) indicate that treatment with DFO completely restored the ability of mice to survive infection. Similar to PCA-2–infected but non–iron-overloaded mice, DFO-treated mice survived both primary and secondary infections and efficiently controlled yeast cell growth, as indicated by the reduced fungal load in the kidneys and liver. Interestingly, DFO treatment also reduced the iron content of neutrophils from infected iron-overloaded mice, as revealed by Berlin blue staining of peripheral neutrophils (data not shown). Treatment with DFO did not modify the course and outcome of infection in iron-untreated mice, thus confirming previous results obtained in guinea pigs [26].

Effect of DFO treatment on anticandidal Th development in iron-overloaded mice. To verify whether the beneficial effect of DFO treatment could be associated with the development of protective Th1-mediated anticandidal responses, the pattern

Table 2. Effect of deferoxamine on course and outcome of disseminated candidiasis in iron-overloaded CD2F1 mice.

Treatments		Median survival time (days)	No. dead/ total	Organisms (cfu × 10^3) in	
Iron	Deferoxamine			Kidneys	Liver
−	−	>60	0/18*	4510 ± 380	25 ± 1
+	−	7	18/18	41,100 ± 3800	184 ± 9
+	+	>60	4/20*	6200 ± 520†	39 ± 3†
−	+	>60	0/18	4130 ± 410	28 ± 2

NOTE. Mice were injected intravenously with 10^6 *C. albicans* PCA-2 cells. Iron was injected intraperitoneally as iron dextran every other day for 4 weeks before challenge. Deferoxamine (50 mg/kg) was injected intraperitoneally every other day for 3 weeks, from day of infection. Parallel groups of mice were sacrificed 6 days after infection for colony-forming unit enumeration in organs (mean ± SE).

* Parallel group of mice resisted intravenous reinfection with 10^6 *C. albicans* CA-6 cells, 14 days after primary infection. Median survival time of naive mice infected with 10^6 CA-6 cells was 3 days.

† $P < .01$ (deferoxamine-treated vs. untreated, iron-overloaded mice).

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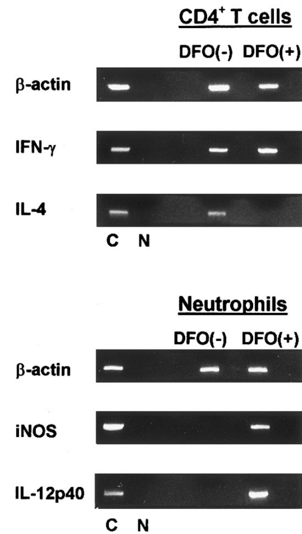

Figure 7. Analysis of cytokine and inducible nitric oxide synthase (iNOS) gene expression in iron-overloaded *C. albicans* PCA-2–infected mice treated (+) or not (−) with deferoxamine (DFO). At 6 days after infection, expression of interferon-γ (IFN-γ) and interleukin (IL)-4 in CD4 T splenocytes and IL-12p40 and iNOS in peritoneal neutrophils was assessed by reverse transcriptase–polymerase chain reaction (PCR). C, β-actin– or cytokine-specific control. N, no DNA added to amplification mix during PCR.

of Th1/Th2 cytokine gene expression was assessed in CD4 splenocytes from DFO-cured mice. In addition, IL-12p40 and iNOS expression was assessed in peritoneal neutrophils. We found (figure 7) that the IL-4 message disappeared while that of IFN-γ increased in CD4 splenocytes from iron-overloaded mice after therapy with DFO. This treatment also resulted in the appearance of iNOS and IL-12p40 messages in neutrophils from iron-overloaded mice. The candidacidal potential of neutrophils and macrophages was also increased after treatment with DFO (data not shown). Treatment of infected mice with DFO, in the absence of prior iron loading, did not modify the patterns of cytokine and iNOS gene expression in the cells (data not shown). These results indicate that cure of murine candidiasis with DFO is associated with the restoration of effector and immunomodulatory functions of cells of the innate immune system and with the occurrence of acquired Th1 protective anticandidal responses.

Discussion

C. albicans is a major cause of opportunistic fungal infection in immunocompromised hosts, especially neutropenic patients with hematologic malignancies [37] or those with HIV infection [38]. A number of clinical observations support the notion that iron overload may predispose to severe *C. albicans* infection [39–41]. In neutropenia, decreased lactoferrin availability has been proposed as one possible factor associated with impairment of the iron-withholding defense system and thus increased risk of infection [13]. Recently, increased iron accumulation in certain tissues was proposed to play an important role in the progression of HIV and its associated opportunistic infections [15]. The results of the present study may help to explain the increased risk of *C. albicans* infection noted in these clinical settings with an excessive iron burden. We found that iron overload greatly increased susceptibility of mice to *C. albicans* infection, which was associated with the detection of abnormal innate or acquired antifungal cell responses. Treatment with the iron chelator DFO, which is successfully used to remove iron from iron-overloaded patients [22], led to complete restoration of immune effector functions.

Iron is necessary for the growth of *C. albicans,* its growth being inhibited in an iron-restricted environment such as human serum [42]. A number of reports correlate serum inhibition with the presence of transferrin [42–45], a serum protein that binds iron with high affinity, thus restricting free iron to levels insufficient for microbial growth [12, 13]. Conditions in which the unbound iron–binding capacity of serum is decreased, such as those associated with increased serum iron, also predispose to fungal infections [12, 13]. In this regard, it has been reported that the high risk of disseminated fungal infections observed in leukemic patients is attributable to increased serum iron and decreased iron-binding capacity [39–41]. The proliferation of *Candida* organisms in vitro correlates positively with the iron saturation rate of transferrin and negatively with its unbound iron–binding capacity [46]. In the present study, we found that the growth of the fungus in vitro was promoted by iron only at concentrations higher than those found in iron-overloaded mice but not by serum from these mice. Therefore, the extensive growth of *C. albicans* observed in iron-overloaded mice may not be dependent on the serum availability of iron, although the possibility for *Candida* organisms to have access to iron at the site of infection cannot be excluded. In this regard, it is worth mentioning that *C. albicans* has evolved multiple strategies of iron acquisition [27, 47]. However, the finding that the growth of *C. albicans* was not promoted in the gastrointestinal tracts of orally infected mice argues against this possibility. The apparent improvement of the course of gastrointestinal candidiasis on iron treatment may underscore the importance of iron deficiency rather than iron overload in the pathogenesis of gastrointestinal candidiasis [48]. Moreover, relevant to this point may be the observation that down-regulation of Th1 responses could be beneficial to the gastrointestinally infected mice by limiting the gastrointestinal damage caused by *Candida* colonization and subsequent inflammatory stimuli [9].

It is well known that a link exists between cell-mediated immunity and iron metabolism [49]. Indeed, both iron deficiency and iron overload can exert subtle effects on the immune status by altering the proliferation of T and B cells [18, 49, 50]. In addition, iron plays a critical role in the antimicrobial activities of neutrophils and macrophages [17, 20], including the regulation of iNOS gene expression [19]. The results of the present study, while confirming the negative effect of excess iron on the antifungal effector functions of neutrophils and macrophages, also showed that iron overload negatively affected IL-12p40 gene expression, particularly in neutrophils from infected mice. Excess iron down-regulated the expression of the IL-12p40 gene in macrophages from noninfected but not from infected mice. In contrast, iron overload silenced this cytokine gene in neutrophils from both

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uninfected and infected mice. Therefore, these results identify a novel mechanism by which iron may regulate the overall antifungal effector functions of phagocytic cells, through the release of IL-12. The recent finding that IL-12 gene expression is regulated in a macrophage cell line by NO [51] adds further support to our observation. This finding is particularly relevant in the case of *C. albicans* infection, in which production of IL-12 is required for Th1 development [33, 35]. We have recently reported that production of IL-12 by neutrophils is important for the initiation of protective cell-mediated immunity in murine candidiasis [36, 51a]. Therefore, it appears that one possible mechanism by which iron overload increases the susceptibility to infection is through the inhibition of a directive cytokine, such as IL-12. Whether this event is directly responsible for the induction of nonprotective Th2 cell responses by a default pathway remains to be determined. Alternatively, the growth-promoting effect of iron on *Candida* organisms may result in an increased fungal load, which could be responsible per se for the selective activation of Th2 cell responses [1, 52]. Moreover, since the availability of cellular iron has a distinct influence on the proliferation of Th1 and Th2 subsets [18], it is possible that iron may have a direct influence on Th cell expression.

Whatever the mechanisms through which iron regulates Th selection in mice with candidiasis, Th2 cell responses predominated in iron-overloaded mice. The production of IFN-γ decreased while that of IL-4 and IL-10 increased, thus further contributing to the impairment of antifungal effector functions of phagocytic cells [29]. Treatment with DFO completely restored the animals' ability to resist the infection, which was associated with recovery of protective Th1 cell functions, such as detection of IFN-γ and IL-12 but not IL-4. Both macrophages (data not shown) and neutrophils expressed detectable levels of iNOS on DFO treatment, suggesting a near complete recovery of antifungal effector functions.

It is known that DFO may have complex immunomodulatory effects on the microbicidal potential of phagocytic cells [53] as well as T cell–mediated immunity [54]. However, because DFO per se was found to decrease rather than increase IL-12 production of bone marrow macrophages infected with *Yersinia enterocolitica* [55], while DFO treatment decreased the iron content of neutrophils in iron overload (this study), it is likely that the effect of DFO observed in iron-overloaded *C. albicans*–infected mice is due to iron deprivation. Recent data provide evidence that iron chelation therapy strengthens Th1-mediated immune effector functions in children with cerebral malaria [56].

In conclusion, the present study shows that disturbances of iron metabolism may profoundly affect Th cell development in mice with candidiasis, thus altering the course and outcome of infection. Iron chelation leads to a complete recovery of protective anticandidal acquired immunity, thus offering the potential for new strategies of immunointervention in candidiasis.

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