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Gamma Interferon Is Not Essential in Host Defense against Disseminated Candidiasis in Mice

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In vitro studies have suggested a role for interferon gamma (IFN-γ) in host defense against disseminated candidiasis, but in vivo studies are inconclusive. We utilized homozygous IFN-γ knockout (GKO) mice to determine if the cytokine is essential in host defense against this disease. Genotypes of mice were determined by PCR with specific primers for the normal or disrupted IFN-γ gene. The GKO status of the mice was confirmed by an enzyme-linked immunosorbent assay, which showed no detectable IFN-γ produced by their splenocytes stimulated by concanavalin A. To test the susceptibility of GKO mice to candidiasis, the animals were infected either intravenously (i.v.) or intragastrically (i.g.) with *Candida albicans*. GKO mice infected i.v. survived as long as wild-type (WT) mice and showed no difference in *Candida* CFU counts in liver, spleen, or kidneys compared to those for WT mice. When animals were given *Candida* i.g., at 3 h or at 10 or 21 days after infection, there was no dissemination of *Candida* to the lung, liver, spleen, or kidneys for either GKO or WT mice. There was no difference in *Candida* CFU counts recovered from the stomach or intestines between GKO and WT mice. Histological examination of the stomach cardial-atrium fold, where the fungus was located, showed that GKO mice did not have evidence of more tissue damage or fungal invasion than WT mice. Finally, the jejunum for both types of mice showed no evidence of tissue damage or fungal invasion. These studies indicate that IFN-γ is not essential in host defense against *C. albicans* that originates from a mucosal site or that is given directly into the bloodstream in a mouse model.

Candida albicans is an increasingly important opportunistic fungal pathogen in immunocompromised patients. It is now the fourth leading cause of nosocomial bloodstream infections (26). Hematogenous disseminated candidiasis has increased dramatically as a result of widespread use of chemotherapeutic drugs, indwelling catheters, and certain kinds of surgical and other medical procedures (15). This serious disease often leads to death even with treatment (33). Reasons for treatment failures are complex and may include problems with fungal resistance and/or the toxicity of these drugs to the host (22, 43). Clarification of host defense mechanisms against *C. albicans* may well lead to improved methods of prevention and treatment of this disease through immune therapy.

Components of both innate nonspecific immune responses and acquired specific immunity have been shown to be important in host defense against disseminated candidiasis. Studies on neutrophils in vitro (35), in animal models (2), and in patients who develop disseminated disease (54) indicate that neutrophils are critical innate host defense mechanisms against this disease. Results from in vitro studies (24) and from experimental animals (5, 28, 42, 53) also show that macrophages are important in host defense against candidiasis. With regard to specific immune defense against the disease, specific antibodies have been shown to protect mice against disseminated candidiasis (23, 36, 38, 51). T-cell-deficient patients, such as those with AIDS, usually do not develop this disease. Furthermore, T-cell-deficient mice, such as SCID (3) and nude (12, 30) mice, are not more susceptible to acute-phase disseminated candidiasis. Studies in vitro and in animal models, however, show evidence that T cells may protect the host against this disease. For example, $CD8^+$ T cells can inhibit the growth of *C. albicans* hyphae in vitro (4), and a *C. albicans*-specific T-cell line (47) and Th1-predominant immune responses (46) protect mice against disseminated candidiasis.

Interferon gamma (IFN-γ), which is mostly produced by T cells and NK cells (52), induces activation of macrophages and enhances both macrophage and neutrophil anti-*Candida* activity in vitro. For example, IFN-γ augments intracellular killing of *Candida* yeast cells by macrophages (34) and neutrophils (49), and hyphal killing by neutrophils (16). While these investigations suggest a role for IFN-γ in host defense against disseminated candidiasis, in vivo studies are inconclusive. Administration of an interferon stimulator, poly(I · C), increased the severity of experimental candidiasis (27, 56). Treatment of mice with neutralizing anti-IFN-γ monoclonal antibodies caused increased susceptibility to disseminated disease in one study (8), but others found that this treatment resulted in increased resistance (27). In another investigation, administration of recombinant IFN-γ protected mice against the disease (29), whereas others showed that mice who received the cytokine had increased susceptibility to this form of candidiasis (20).

Mice with a targeted disruption of the IFN-γ gene have been described (14). These IFN-γ gene knockout (GKO) mice appear normal, fertile, and healthy in the absence of pathogens. Furthermore, they appear not to have alterations in splenic or thymic cell populations (14). In the present study, we examined the susceptibility of GKO mice to disseminated candidiasis with the aim of determining whether IFN-γ is essential in host defense against this disease.

MATERIALS AND METHODS

Microorganisms and culture conditions. *C. albicans* strains Ca-1 (strain 1, serotype A) (7, 31, 32) and Ca-222 (serotype B) (13) have been described previously. They were stored as yeast cells in 50% glycerol at −20°C and were prepared as washed hydrophilic stationary-phase yeast-form cells in sterile Dulbecco's phosphate-buffered saline (DPBS) as previously described (25). Strain Ca-1 was used in all studies unless indicated otherwise.

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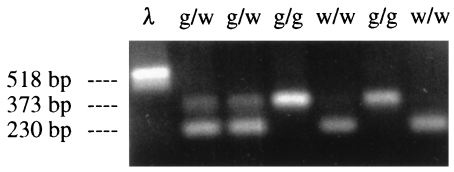

FIG. 1. PCR confirmation of genotypes of GKO, heterozygote, and WT control mice. The WT IFN-γ genomic band (w) is about 230 bp, the GKO disrupted IFN-γ genomic band (g) is about 373 bp, and the bacteriophage λ DNA band is about 518 bp. Samples from homozygote GKO mice showed only a g band (g/g); heterozygote mice had both g and w bands (g/w); and WT mice had only a w band (w/w). The control bacteriophage lambda DNA band (λ) is also shown.

Mice. Mating pairs of heterozygote BALB/c mice were kindly provided by Genentech, Inc. (San Francisco, Calif.), and were bred for GKO (homozygous recessive, g/g), wild-type (WT) (homozygous normal dominant, w/w), and heterozygous (w/g) mice in an animal resource center at our university that is certified by the American Association for Accreditation of Laboratory Animal Care. The genetic designations are the same as those used in the original description of these mice (14).

PCR genotyping of mice. F_1 offsprings of the original heterozygote mating pairs were screened for GKO and WT genotypes by PCR amplification of DNA primed specifically for either the normal (5′-AGAAGTAAGTGGAAGGGCC CAGAAG-3′ and 5′-AGGGAAACTGGGAGAGGAGAAATAT-3′) or the disrupted (5′-TCAGCGCAGGGGCGCCCGGTTCTTT-3′ and 5′-ATCGACAAG ACCGGCTTCCATCCGA-3′) IFN-γ gene. The specific primers were obtained from Microcellular Resources, Fort Collins, Colo. (14). Bacteriophage λ DNA-specific primers (5′-GATGAGTTCGTGTCCGTACAACTGG-3′ and 5′-GGT TATCGAAATCAGCCACAGCGCC-3′) (PCR control) and other reagents used for PCR were from the GeneAmp PCR reagent kit with Ampli*Taq* DNA polymerase (Perkin-Elmer, Norwalk, Conn.). Briefly, about 4 mm of mouse tail was cut from each animal and digested in 0.04% proteinase K (Boehringer Mannheim GmbH, Mannheim, Germany) containing 5% sodium dodecyl sulfate, 0.1 M NaCl, 50 mM Tris-HCl, and 7.5 mM EDTA (pH 7.2) at 60°C for 16 h, and DNA was purified by chloroform extraction and precipitation by ethanol. DNA preparations were suspended in Tris-EDTA buffer (10 mM Tris-HCl–1 mM EDTA [pH 8.0]), and stored at 4°C until use. The DNA samples were amplified in the presence of the specific primers (PTC-100 Programmable Thermal Controller; MJ Research, Inc., Watertown, Mass.). The amplified DNA was electrophoretically run on a 1.7% agarose gel and stained with ethidium bromide.

Phenotype of mice as determined by ELISA. In addition to genetic confirmation, we used an IFN-γ-specific enzyme-linked immunosorbent assay (ELISA) (PharMingen, San Diego, Calif.) to measure IFN-γ protein produced in the supernatant fluid of mouse splenocytes stimulated by concanavalin A (ConA) (Sigma Chemical Co. St. Louis, Mo.). In brief, spleens were obtained from 6- to 8-week-old mice. A syringe and needle were used to gently flush each spleen with 8 ml of RPMI 1640 complete medium (CM) (Sigma Chemical Co.), which contained 10% fetal bovine serum, 20 mM HEPES (Sigma Chemical Co.), 50 U of penicillin/ml, 50 μg of streptomycin/ml (Microbiological Associates Co., Walkersville, Md.), and 2 mM L-glutamine (Sigma Chemical Co.). The erythrocytes in the resulting splenocyte preparation were lysed by treatment with distilled water for 10 s at room temperature, and the remaining cells were washed with CM and suspended at 5×10^6 or 1×10^7 cells/ml of CM containing 5 μg of ConA/ml. The suspension of splenocytes was incubated in wells (1 ml/well) of a 24-well polystyrene plate (Corning Glass Works, Corning, N.Y.) for 72 h at 37°C under a 5% CO_2 atmosphere. The supernatant material was collected, aliquoted, and stored at −80°C. Aliquots were thawed and tested for IFN-γ by ELISA (19).

Determination of *C. albicans* CFU in mouse organs. For intravenous (i.v.) infections, 6- to 8-week-old GKO and WT mice were given i.v. 5×10^5 or 2.5×10^5 *C. albicans* yeast cells in DPBS. Forty-eight or 72 h later, mice were sacrificed by cervical dislocation, and the liver, spleen, and kidneys from each mouse were removed and weighed. Each liver or both kidneys were homogenized in 5 ml of sterile saline, and each spleen was homogenized in 1.5 ml of sterile saline. Appropriate dilutions in saline of each homogenate were made, plated onto glucose (2%)-yeast extract (0.3%)-peptone (1%) agar plates containing 100 U of penicillin/ml and 100 μg of streptomycin/ml (Microbiological Associates Co.), and incubated for 48 h at 37°C, and numbers of *Candida* CFU per gram of tissue were determined.

Five- to six-day-old infant mice were used for intragastric (i.g.) inoculations with *C. albicans*. The GKO or WT infant mice were isolated from their mothers and kept at 35°C for 3 h before i.g. inoculation with 2×10^8 or 5×10^8 *C. albicans* strain Ca-1 or Ca-222 yeast cells in 50 μl of DPBS. The mice were returned to their respective mothers after inoculation. Quantitative cultures of lung or gastric contents performed immediately after the i.g. inoculation showed that the entire inoculum reached the stomach. Three hours, 10 days, and 21 days after i.g. inoculation, the mice were sacrificed by decapitation, and lung, liver, spleen, kidneys, stomach, and intestines were removed and weighed. Before the mice were sacrificed, they were isolated from their mothers for 1 to 2 h to allow their stomachs to empty. Each lung, liver, and spleen or both kidneys were homogenized in 2 ml of sterile saline, and each stomach or whole intestine was homogenized in 5 ml of sterile saline. Appropriate dilutions in saline of each homogenate were made and plated onto Mycobiotic agar plates (Difco Laboratories, Detroit, Mich.). The plates were incubated for 72 h at 37°C, and numbers of CFU per gram of tissue were determined.

TABLE 1. Distribution of *C. albicans* in different organs of GKO and WT mice after i.v. infection[a]

Expt and subject	Level of *C. albicans* in:					
	Liver (10^3 CFU/g)		Spleen (10^3 CFU/g)		Kidneys (10^3 CFU/g)	
	GKO	WT	GKO	WT	GKO	WT
Expt 1						
1[b]	0.1	0.1	1.0	1.6	4	12
2	0.2	0.2	1.0	0.4	26	89
3	0.3	ND[c]	0.2	0.3	185	18
Expt 2						
1	5	4	17	11	32,270	7,701
2	18	3	78	17	160	3,362
3	8	3	7	11	79	3,255
4	24	4	57	30	534	49,716
5	6	6	10	20	2,480	47,504

[a] GKO and WT mice were given i.v. 2.5×10^5 (experiment 1) or 5×10^5 (experiment 2) viable *C. albicans* yeast cells. Three days (experiment 1) or 48 h (experiment 2) later, various organs were removed, homogenized, and plated for determination of CFU per gram of tissue. Values are from individual mice. There was no difference between GKO and WT mice in each set of data ($P > 0.05$).

[b] Individual mice.

[c] ND, not determined.

Survival of mice with experimental disseminated candidiasis. Six- to eight-week-old GKO and WT control mice (five mice per group) were given i.v. 0.1 ml of a suspension containing 1×10^6, 5×10^5, or 2.5×10^5 *C. albicans* yeast cells in DPBS. The mice were observed daily for survivors for up to 40 days.

Histological studies of stomach and jejunum for mice infected i.g. with *C. albicans*. Five- to six-day-old infant GKO or WT mice were given i.g. 2×10^8 *C. albicans* yeast cells. Twenty-one days later, mice were sacrificed, and the stomach and jejunum were removed. For each mouse, the stomach cardial-atrium fold (CAF) regions were cut into two pieces, and four pieces from the middle part of the jejunum were collected and fixed in 10% formalin. The two pieces of stomach were embedded in one paraffin wax block, and the four pieces of jejunum were embedded in one paraffin wax block. The tissues were cut in 5-μm-thick sections at five different levels; five sections of each level were stained with Grocott methenamine silver (GMS), and five sections were stained with hematoxylin and eosin (H & E). The tissues were evaluated by the following criteria: (i) observation for evidence of hyphal and yeast invasion beyond the keratinized layer and for hyphal extension into the epithelium; (ii) making these observations at the five different levels of the stomach from each mouse; (iii) observation of the two groups of mice for fungal load in the tissue; and (iv) observation of the extent of infiltration of inflammatory cells associated with the presence of fungi.

Statistical analysis. The Mann-Whitney test was used for comparisons of CFU from cultures of tissues and for analysis of survival experiments. The Student *t* test was used as a measure of significance for comparing levels of IFN-γ produced by stimulated splenocytes from the various kinds of animals.

RESULTS

Confirmation of the genotype of GKO mice. As shown in Fig. 1, the PCR-amplified WT IFN-γ genomic band (w) was about 230 bp, and the GKO band (g) containing the IFN-γ disruption (neomycin band) was about 373 bp. The bacteriophage λ DNA PCR control band was about 518 bp. The DNA samples showing only a normal IFN-γ band were from WT mice (w/w), those with only a neomycin band were from homozygote GKO mice (g/g), and those showing both bands were from heterozygote mice (g/w).

GKO mice were also confirmed by the ELISA test for

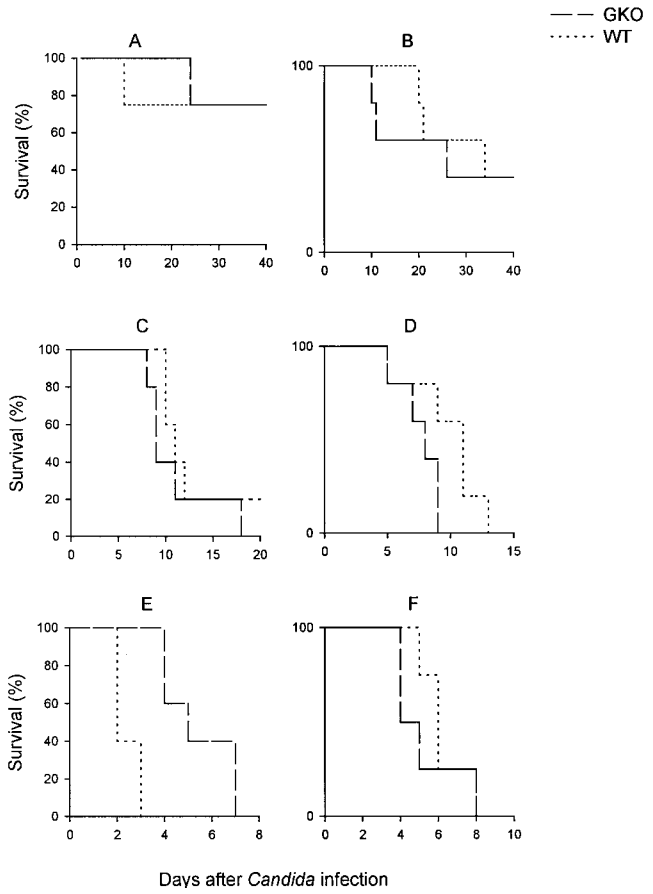

FIG. 2. Effect of IFN-γ gene knockout on hematogenous disseminated candidiasis in mice. GKO and WT mice were infected i.v. with 2.5×10^5 (A and B), 5×10^5 (C and D), or 1×10^6 (E and F) *C. albicans* yeast cells, and were observed for survivors for up to 40 days. There was no significant difference between GKO and WT mice in the experiments for which results are shown in panels A through D and F ($P > 0.05$), but GKO mice survived longer than WT mice in the experiments for which results are shown in panel E ($P < 0.05$).

TABLE 2. Effect of IFN-γ gene knockout on colonization and dissemination of *C. albicans* in mice by i.g. inoculation[a]

Time and mouse	*C. albicans* CFU (10^4)/g of tissue					
	Lung	Liver	Spleen	Kidneys	Stomach	Intestines
3 h						
GKO						
1[b]	0	0	0	0	2,216	26,557
2	0	0	0	0	980	32,754
3	0	0	0	0	3,667	41,148
WT						
4	0	0	0	0	818	35,000
5	0	0	0	0	7,690	42,333
6	0	0	0	0	1,281	33,684
10 d[c]						
GKO						
1	0	0	0	0	6	2
2	0	0	0	0	0.4	0.4
3	0	0	0	0	15	1
4	0	0	0	0	6	1
5	0	0	0	0	5	1
WT						
6	0	0	0	0	18	2
7	0	0	0	0	12	1
8	0	0	0	0	11	2
21 d						
GKO						
1	0	0	0	0	17	1
2	0	0	0	0	81	13
3	0	0	0	0.016	147	5
4	0	0	0	0	23	2
WT						
5	0	0	0	0	35	2
6	0	0	0	0	26	2
7	0	0	0	0	9	1
8	0	0	0	0	10	2

[a] GKO and WT mice were given i.g. 2×10^8 viable *C. albicans* yeast cells. Three hours, 10 days, or 21 days later, various organs were removed, homogenized, and plated for determination of CFU per gram of tissue. Values are from individual mice.
[b] Individual mice.
[c] d, days.

IFN-γ. The splenocytes of the three GKO mice stimulated with ConA did not produce detectable IFN-γ, while the splenocytes of the three WT control mice produced 1.38, 7.37, and 1.58 ng of IFN-γ/ml of splenocyte culture supernatant, respectively. The minimum detection of this test is 79 pg of IFN-γ/ml. These results indicate that GKO mice do not have a normal IFN-γ gene and do not produce detectable levels of IFN-γ.

Effect of IFN-γ gene knockout on the distribution of *C. albicans* in liver, spleen, and kidneys for mice infected intravenously. There was no significant difference in *Candida* CFU recovered from livers, spleens, or kidneys between GKO and WT mice in the two experiments (Table 1). *P* values were calculated for each set of data, and none was less than 0.05. Surprisingly, the numbers of CFU in the kidneys of GKO mice tended to be less than those in WT mice.

Effect of IFN-γ gene knockout on hematogenous disseminated candidiasis of mice. GKO mice survived as long as WT mice at different infection doses (Fig. 2A through D and F). For each comparison set of data, the *P* value is >0.05. Yet in one experiment (Fig. 2E), GKO mice survived longer than control mice ($P < 0.05$). These results indicate that GKO mice were not more susceptible than WT mice to death by i.v. infection.

Effect of IFN-γ gene knockout on colonization and dissemination of *C. albicans* in mice infected by the i.g. route. Three hours, 10 days, and 21 days after i.g. inoculation of *C. albicans*, no dissemination of *Candida* was detectable in the lung, liver, spleen, or kidneys of either GKO or WT mice. The CFU count for the kidneys of one mouse was due to only 1 CFU on the plate of undiluted homogenate. There was also no significant difference in the CFU in the stomach or intestines between GKO and WT mice at different time points (Table 2). For each comparison set of data, the *P* value was >0.05. GKO mice inoculated i.g. with *C. albicans* strain Ca-222 produced essentially identical results 3 h after inoculation (data not shown).

As a control for the neonate mouse model, splenocytes from infant mice were evaluated for their ability to produce IFN-γ. Splenocytes from 6-, 16-, and 27-day-old mice were compared with splenocytes from 6- to 8-week-old mice (three mice/group). Following stimulation with ConA, the amount of IFN-γ (in nanograms per milliliter of splenocyte culture supernatant, ± standard deviation) was 4.0 ± 2.2, 6.0 ± 0.10, and 5.84 ± 0.16 for 6-, 16-, and 27-day-old mice, respectively, and 5.75 ± 0.16 for 6- to 8-week-old mice. None of the averages differed significantly ($P > 0.05$) when tested by the Student *t* test. These results indicate that infant mice used in our studies

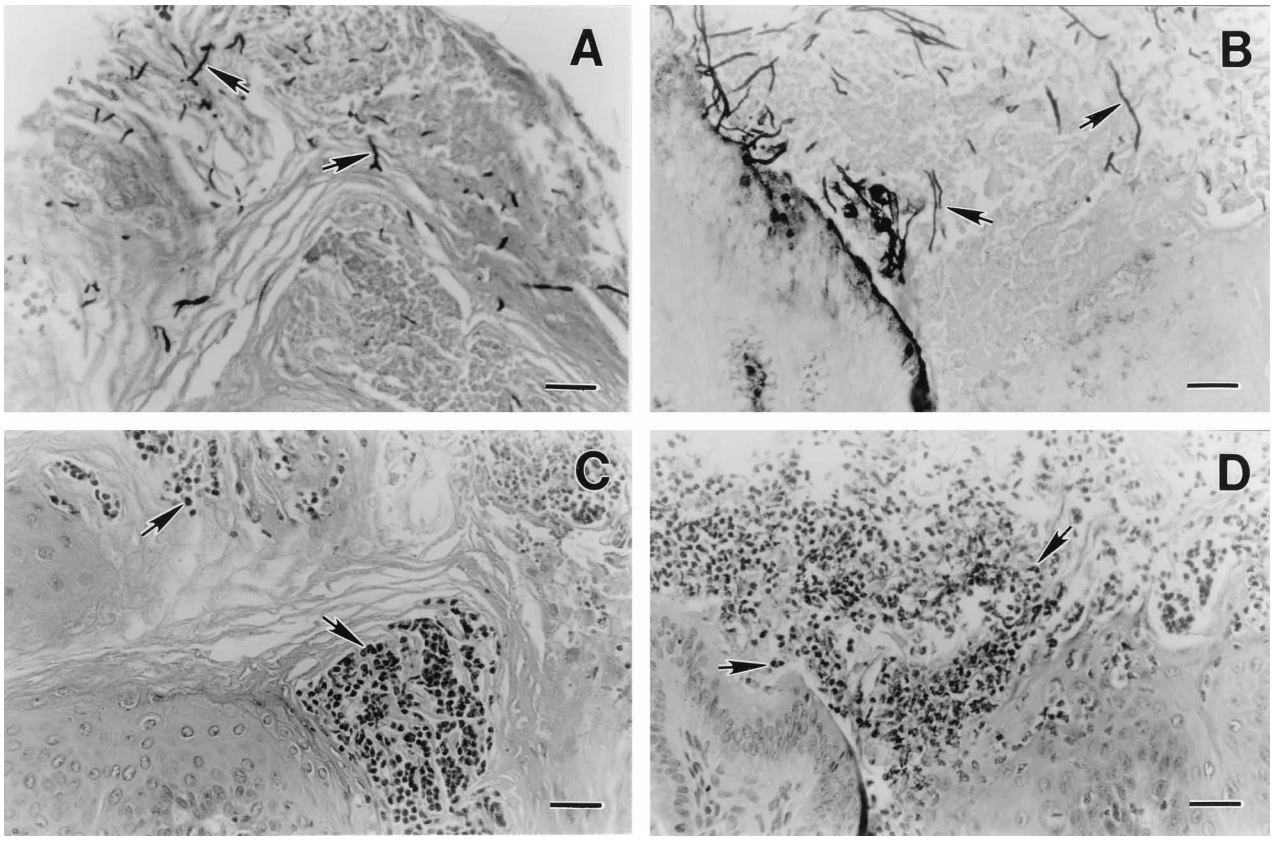

FIG. 3. Histology studies of the stomach CAF regions of GKO and WT mice infected with *C. albicans* i.g. GKO and WT mice were infected i.g. with 2×10^8 *C. albicans* yeast cells, and 21 days later, mice were sacrificed and their stomachs were removed, fixed in buffered formalin, and processed for GMS (A and B) and H & E (C and D) staining. Tissues from GKO (A and C) and WT (B and D) mice are shown. Arrows in panels A and B show hyphae in the tissue, and arrows in panels C and D show inflammatory cells. Bars, 100 μm.

had the ability to produce IFN-γ to a similar extent as adult mice.

Effect of IFN-γ gene knockout on fungal invasion and tissue damage of mice by i.g. inoculation of *C. albicans*. GMS-stained mouse stomach CAF regions of both GKO and WT mice, which had been inoculated i.g. with *C. albicans* 21 days earlier, showed hyphal and yeast (data not shown) forms of *C. albicans* in the keratinized layer, but there was little evidence of fungal invasion in either the GKO or the WT mice (Fig. 3A and B). Examination of H & E stains of the same areas showed inflammatory cell infiltration primarily at the keratinized layer, where the fungi were located (Fig. 3C and D). For both types of mice, the infiltration was similar and consisted of 75 to 95% neutrophils, depending on the high-power field that was examined. Inflammatory cells were also noted, but to a lesser extent, in the lamina propria and submucosal areas immediately below the fungus-associated keratinized layer in both kinds of mice (data not shown). There was no apparent difference in tissue damage and inflammatory cell infiltration between GKO and WT mice in any of the sections examined. There was no evidence of fungal invasion or tissue damage in the jejunum for either GKO or WT mice (data not shown).

DISCUSSION

Results from in vitro studies have suggested that IFN-γ may be important in host defense against disseminated candidiasis, but in vivo studies have yielded conflicting conclusions. Differences in experimental design may partly explain the controversy. For example, Kullberg et al. (29) gave mice 10^5 U of IFN-γ by an i.v. route, while Garner et al. (20) gave mice 1×10^2 to 5×10^5 U of IFN-γ by an intraperitoneal (i.p.) route. Mice in the former experiment showed enhancement of protection against the disease, but the latter experiment resulted in increased susceptibility. Similarly, mice had increased susceptibility to the disease after they were given i.v. an anti-IFN-γ monoclonal antibody (8), while mice given the antibody i.p. showed increased resistance (27).

GKO mice provide a good tool for assessing the essential role of IFN-γ in host defense against infectious diseases. Previous studies demonstrated the utility of the GKO mouse in showing the importance of IFN-γ in host defense against *Mycobacterium tuberculosis* (19) but not against *Schistosoma mansoni* (1). Our data show that GKO mice are not more susceptible to a direct i.v. presentation of *C. albicans* than WT mice. The numbers of CFU recovered from the kidneys of GKO mice were not greater than those for WT mice, and GKO mice survived as long as, or longer than, control mice.

In humans who develop the disease, the *C. albicans* is believed to be mostly from mucosal surfaces of the gastrointestinal (GI) tract (50, 55), where *C. albicans* is normal flora. Neutrophils and macrophages are important in host defense against disseminated candidiasis, and neutrophils accumulate at the mucosal infection sites where fungal yeast and hyphal forms are located (9, 42a). IFN-γ increases neutrophil and macrophage anti-*Candida* activity in vitro (16, 34, 49). To test if IFN-γ may be essential to the host in limiting *Candida*

spread from mucosal sites, we used infant mice to establish mucosal colonization of *C. albicans* (41). This model of mucosal colonization was chosen because *C. albicans* does not colonize the GI tract of normal adult mice. Pope et al. (41) reported that 5- or 6-day-old CFW mice showed evidence of disseminated disease 30 min to 3 h after an i.g. dose of the fungus. In our studies, no dissemination was detected in GKO or WT mice (BALB/c background) at 30 min (data not shown), 3 h, 10 days, and 21 days after i.g. inoculation. The extent of fungal colonization and of tissue damage in the stomach and jejunum was similar to that for CFW mice (9). The BALB/c strain was chosen in our studies because this is the strain of mouse used by others who showed protection or no protection by IFN-γ (8, 20, 45). *C. albicans*-colonized GKO mice were just as resistant as normal mice colonized with the fungus to subsequent development of disseminated disease. Both kinds of mice were colonized to a similar extent following an i.g. dose of yeast cells, and both resisted dissemination from the GI tract equally well. This conclusion is based on our observation of organ counts and histologic studies of tissue areas that, according to others (41), should be the areas of interest. The results of our experiments, therefore, show that IFN-γ is not essential in host resistance against disseminated candidiasis.

Our experiments do not rule out a role for IFN-γ in normal mice. A complex network of endogenous mediators, such as cytokines and chemokines, orchestrates the host immune response. In a normal animal, there appears to be redundancy in the production of cytokines with overlapping functions, and these pleiotropic effects may have a protective advantage for the host. For example, not only IFN-γ but also interleukin-1 (IL-1), tumor necrosis factor alpha (TNF-α), and IL-4 up regulate macrophage functions (10, 11, 40). It has been shown that IL-1 and TNF-α augment neutrophil and macrophage anti-*Candida* activity in vitro (6, 18), and IL-1 had a protective effect against disseminated candidiasis in vivo (37). IFN-γ may protect the host by enhancing IL-1 or TNF-α production (21, 44), and this could be compensated by other cytokines, for example, IL-2 (17, 39), which induces IL-1 or TNF-α production. IFN-γ may also protect the host by direct augmentation of phagocyte *anti-Candida* functions (34). Perhaps in the absence of IFN-γ, phagocyte antifungal activity is increased by other cytokines, such as granulocyte-macrophage colony-stimulating factor (48), IL-1, and TNF-α (6, 18).

In conclusion, our data show that IFN-γ is not essential in host defense against *C. albicans* that originates from a mucosal site or that is given directly into the bloodstream.

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