Cytokine Treatment of Central Nervous System Infection: Efficacy of Interleukin-12 Alone and Synergy with Conventional Antifungal Therapy in Experimental Cryptococcosis

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Received 30 August 1993/Returned for modification 8 October 1993/Accepted 16 December 1993

Cell-mediated immune responses appear to be critical in the outcome of cryptococcosis. Interleukin-12 (IL-12) was studied for its potential use as a therapeutic agent because of its stimulation of natural killer cells and gamma interferon production by stimulated T cells and natural killer cells. Gamma interferon-activated macrophages are important in host resistance against cryptococcosis. In two separate studies, male BALB/c mice were infected intravenously with Cryptococcus neoformans. In the first study, mice received either no treatment, 5.0 mg of fluconazole alone per kg of body weight per day (by gavage twice daily), or IL-12 subcutaneously at 0.01, 0.1, or 1.0 μg/day once daily (low-dose study) alone or in combination with 5.0 mg of fluconazole per kg/day. In a second study (high dose), the dosages of IL-12 used were 1.0, 2.5, or 5.0 μg/day. Therapy was given for 10 consecutive days, and the number of CFU of C. neoformans remaining in various organs was quantitated 1 or 2 days after administration of the last dose. In the low-dose study, IL-12 at 0.1 or 1.0 μg reduced the level of brain infection by 10-fold (P < 0.05) and IL-12 at 1.0 or 0.1 μg/day enhanced the efficacy of fluconazole. In liver, both the efficacy of IL-12 alone (0.01 or 0.1 μg; P < 0.05) and enhancement of the efficacy of fluconazole (P < 0.05) were seen. No efficacy of IL-12 was seen in spleens or lungs, although spleen weights increased fourfold in mice given 1.0 μg of IL-12 per day. In the high-dose study, all IL-12 doses alone again reduced the levels of brain infection (5- to 8-fold; P < 0.05) and significantly enhanced (2.4- to 3.2-fold; P < 0.05) the already significant effect of fluconazole (P < 0.05) when the two were given in combination. No overt toxicities were observed at any dose, and overall, 1.0 μg of IL-12 per day was found to be the optimal dosage for reducing infection in the brain. To our knowledge, this is the first demonstration of the efficacy of cytokine therapy in systemic, and particularly brain, infections with C. neoformans. The stimulation of cell-mediated immunity represents a new approach to therapy and can enhance suboptimal antifungal chemotherapy. IL-12 should be considered for further study and for clinical trials. These studies suggest that other opportunistic central nervous system pathogens should also be investigated.

The ubiquitous fungal pathogen Cryptococcus neoformans is an opportunist that causes serious life-threatening disease in both healthy and immunocompromised persons. In particular, patients with AIDS as well as other immunosuppressed patients (e.g., patients with leukemia and those undergoing cancer therapy) are at risk of C. neoformans infection (6, 24). Although current antifungal treatments have been demonstrated to be somewhat efficacious in the treatment of this disease, relapses and clinical failures while on therapy have been reported (6, 24), thus demonstrating the need for improved and curative treatment modalities. Because many of the patients with this disease are in some state of immunosuppression and because cell-mediated immunity plays a significant part in host resistance to cryptococcosis (9, 11, 19), stimulation of the cell-mediated immune response to enhance resistance would be very desirable.

The therapeutic administration of cytokines is one potentially useful way to stimulate the host immune response. Because gamma interferon (IFN-γ)-activated macrophages and natural killer (NK) cells have been shown to be important in host resistance to cryptococcosis (9, 11, 19), cytokine stimulation of these cells therapeutically might prove to be beneficial in the treatment of this disease. Interleukin-12 (IL-12) is a heterodimeric cytokine produced by monocytes and B cells (4, 7, 8, 16). Various biological activities that affect NK and T cells, including the enhancement of proliferation of CD4⁺ and CD8⁺ lymphocyte subsets, induction of IFN-γ production, and enhanced cytolytic activity by NK and T cells, have been demonstrated for IL-12 (4, 7, 8, 14, 16, 22). IL-12-induced stimulation of IFN-γ production by T cells and NK cells could potentially enhance host resistance to C. neoformans by stimulation of the cytolytic properties of NK cells and/or IFN-γ activation of macrophages.

In the studies described here, we examined the potential of immunomodulation of the host immune response by IL-12 as a treatment regimen in a murine model of systemic cryptococcosis. In addition, because conventional fluconazole therapy does not appear to be curative in the treatment of human cryptococcosis (6), IL-12 also was given in conjunction with a suboptimal, noncurative dosage of fluconazole to examine the possibility of its utility as an adjunctive therapy. Our results demonstrate that IL-12 alone or in combination with fluconazole proved to be a useful treatment for controlling cryptococcosis.
MATERIALS AND METHODS

Therapeutic agents. Recombinant murine IL-12 was provided by Genetics Institute, Inc. (Cambridge, Mass.), and was diluted in sterile phosphate-buffered saline containing 1% normal mouse serum prior to injection into mice. All solutions of IL-12 were stored at −80°C. Fluconazole powder was obtained from Pfizer, Inc. (Groton, Conn.), was solubilized in polyethylene glycol 200, and was stored at 4°C.

Experimental infections. Six- to eight-week-old male BALB/cAnNSim mice (Simonsen Laboratories, Gilroy, Calif.) were used in the studies. On day 1, mice were infected intravenously with C. neoformans 9-759 yeast cells given in 0.1 ml of saline and prepared as described previously (13). In the first study, mice were infected with 3.2 × 10⁹ viable C. neoformans yeast cells, and in the second study, mice were infected with 3.8 × 10⁶ viable C. neoformans yeast cells. Mice were provided food and acidified water ad libitum.

Experimental design. Infected mice were randomly chosen for treatment and were placed into groups of 10 mice each. Various dosages of IL-12 were given in 0.1-ml volumes subcutaneously in the nape of the neck. Fluconazole was given by gavage twice daily in 0.1 ml at 2.5 mg/kg of body weight per dose (i.e., 5.0 mg/kg/day). The treatment groups for the first study were as follows: untreated controls, 5.0 mg of fluconazole alone per kg, or IL-12 at 1.0, 0.1, or 0.01 µg per dose alone or in combination with 5.0 mg of fluconazole per kg/day. Treatments were initiated on day 2 and were continued through day 11 of infection. The treatment groups for the second study were the same as those for the first study except that the doses of IL-12 given were 1.0, 2.5, or 5.0 µg per dose. Therapy in the second study began on day 3 and continued through day 12 of infection.

On days 12 and 13 (first study) or day 13 (second study), all mice were killed by CO₂ asphyxiation. Various organs of each mouse were removed aseptically, and the number of viable C. neoformans was determined by quantitative plating of serially diluted homogenates onto Sabouraud dextrose agar plates with chloramphenicol (50 mg/liter). Plates were incubated at 35°C for 3 days, and the number of colonies was enumerated (13).

Evaluation of the efficacy of treatment was based on the comparative numbers of CFU remaining in the organs. The number of CFU was expressed as the log₁₀ CFU per entire organ and the log₁₀ geometric mean of each group (23). The 95% confidence intervals for the geometric means were determined by using GB-STAT version 4.0 (Dynamic Microsystems, Inc., Silver Spring, Md.). Analyses of variance (23) were performed on the geometric mean organ CFU by using SAS version 6.08 (SAS Institute, Inc., Cary, N.C.) to determine the treatment effects on each organ. Those organs with significant treatment effects, as determined by analysis of variance, were further analyzed by a Student-Newman-Keuls (SNK) test to determine the comparative effects of the treatment regimens (23).

RESULTS

Various dosages of IL-12 were administered alone or in combination with suboptimal fluconazole therapy to assess the potential therapeutic utility of IL-12 in the treatment of systemic cryptococcosis. The results of the first study with doses of 0.01 to 1.0 µg of IL-12 per day are presented in Fig. 1. Efficacy was assessed on the basis of the comparative numbers of CFU recovered from brain, spleen, liver, and lung tissues. By analysis of variance, significant treatment effects were observed in the brain (P < 0.0001), liver (P < 0.0001), and spleen (P < 0.003), but not in the lung (P > 0.5). Upon further analyses, SNK rankings showed that all therapeutic regimens except IL-12 at 0.01 µg resulted in significantly lower burdens of C. neoformans in the brains of treated mice than in the brains of untreated controls (P < 0.05; Fig. 1). Although mice that were treated with the combination regimen of 5.0 mg of fluconazole per kg/day plus 0.1 or 1.0 µg of IL-12 carried mean burdens of C. neoformans in the brain that were lower than the burdens in the brains of mice given 5.0 mg of fluconazole alone per kg/day, they were not significantly lower (P > 0.05).

Similar to the results for the brains, the burdens of C. neoformans recovered from the livers of treated mice demonstrated that some regimens were efficacious (Fig. 1). By the SNK test, mice that were given 5.0 mg of fluconazole alone per kg/day or 1.0 µg of IL-12 alone per day had burdens equivalent to those in the livers of untreated controls (P > 0.05). In contrast, combination regimens of fluconazole and IL-12 at 1.0 or 0.1 µg as well as the 0.1- or 0.01-µg doses of IL-12 alone caused a significant reduction in the infectious burden below those in untreated controls or those treated with 5.0 mg of fluconazole alone per kg/day (P < 0.05; Fig. 1). However, mice treated with any combination regimen carried burdens of C. neoformans equivalent to those in their comparison group given IL-12 alone (P > 0.05). Equivalent numbers of C. neoformans were recovered from the lungs of mice regardless of the treatment that they received (P > 0.05); no therapeutic regimen was effective in reducing the burdens of C. neoformans yeasts in the lungs below the burdens recovered from mice that received no treatment (P > 0.05) (Fig. 1). By the SNK rankings, the spleens of mice treated with IL-12 at 0.01 or 1.0 µg, fluconazole at 5.0 mg/kg/day, and fluconazole plus IL-12 at 1.0 µg carried equivalent burdens, which were higher than those recovered from untreated controls or those recovered from mice treated with other regimens (P < 0.05). Among those treated with other regimens, none of the treatment regimens resulted in a lower mean burden of C. neoformans than that in untreated controls (P > 0.05). However, as presented in Table 1, IL-12 treatment caused a dose-responsive increase in the average weight of the spleens of treated mice, whereas fluconazole treatment did not. The average weight of the spleens of mice given 1.0 µg of IL-12 per day alone or in combination with fluconazole was fourfold higher than the average weight of the spleens of mice given no treatment (P < 0.0001) (Table 1). The weights of the spleens of mice treated with IL-12 alone at 0.01 or 0.1 µg per day was also significantly increased in comparison with the weights of the spleens of untreated controls (P < 0.01 and P < 0.001, respectively). In combination with fluconazole, the weights of the spleens of mice treated with IL-12 at 0.1 µg/day but not 0.01 µg/day were also significantly increased in comparison with the weights of the spleens of untreated controls (P < 0.001) (Table 1).

Because in the first study IL-12 alone exhibited efficacy and a trend toward synergistic activity when given in combination with fluconazole in reducing C. neoformans infection in the brain, a second study was done to better define the optimal dosage of IL-12. The results of the second study are presented in Fig. 2 and confirmed those of the first study that IL-12 alone or in combination with fluconazole has therapeutic efficacy against C. neoformans infection in the brain. Significant treatment effects were demonstrated by analysis of variance (P < 0.0001). Upon further SNK analyses, treatment with IL-12 alone at all doses (1.0, 2.5, or 5.0 µg/day) resulted in significantly fewer CFU in the brain in comparison with the burdens in the brains of untreated controls (P < 0.05) (Fig. 2). The CFU was...
reduced by factors of 8.1-, 5.9-, and 4.8-fold in mice treated with IL-12 at 1.0, 2.5, and 5.0 μg/kg/day, respectively. Although 1.0 μg of IL-12 per day caused a greater reduction in the CFU of *C. neoformans* than did higher doses, the mean CFU burdens were not statistically significantly different (*P* > 0.05). Fluconazole given alone at 5.0 mg/kg/day caused a 32.4-fold reduction in CFU over that in controls and was superior to any dosage of IL-12 alone or no treatment (*P* < 0.05) (Fig. 2). In groups that received IL-12 and fluconazole in combination, the number of CFU recovered was significantly lower than that recovered from mice given either drug alone or no treatment (*P* < 0.05) (Fig. 2). The reductions in CFU achieved by the combination regimens over those achieved by 5.0 mg of fluconazole alone per kg/day were 2.4-, 3.2-, and 2.7-fold after treatment with IL-12 at 1.0, 2.5, and 5.0 μg/kg/day in combination with 5 mg of fluconazole per kg/day, respectively. Furthermore, the combination regimens reduced the CFU burdens over those recovered from untreated controls by 77.6-fold (IL-12 at 1.0 μg/day), 104.7-fold (IL-12 at 2.5 μg/day), and 87.1-fold (IL-12 at 5.0 μg/day). However, mice treated with all combination regimens carried equivalent burdens of *C. neoformans* (*P* > 0.05).

**DISCUSSION**

Our studies were done to assess the potential efficacy of IL-12 as a treatment for systemic cryptococcosis, and more specifically its effects on meningeal disease. We found that IL-12 had a profound effect on the resistance of the brain to infection with *C. neoformans*. This effect could be demonstrated whether it was administered alone or in combination with a suboptimal dose of fluconazole. Our choice of a suboptimal noncurative dose of fluconazole was by design, to avoid the use of a fluconazole dosage that could not be improved upon and thus that would prevent the demonstration of synergy between fluconazole and IL-12. Since relapse and recrudescence is seen in patients on therapy, the dose of fluconazole is relevant, in that meningeal cryptococcosis is not cured by fluconazole therapy (6, 24). Thus, these results are of particular interest because the brain is the major target organ for this pathogen.

It is of interest that resistance was also conferred by IL-12 alone in the liver, but not by fluconazole alone; mice given fluconazole alone carried higher mean burdens than untreated controls. Although mice given the lower IL-12 dose combina-
TABLE 1. Spleen weights of mice treated with IL-12 and fluconazole alone or in combination

<table>
<thead>
<tr>
<th>Therapy group</th>
<th>Mean ± SD spleen wt (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>0.094 ± 0.015</td>
</tr>
<tr>
<td>Fluconazole at 5 mg/kg/day</td>
<td>0.068 ± 0.008</td>
</tr>
<tr>
<td>IL-12 at (µg/day)</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>0.415 ± 0.136</td>
</tr>
<tr>
<td>0.1</td>
<td>0.236 ± 0.036</td>
</tr>
<tr>
<td>0.01</td>
<td>0.118 ± 0.021</td>
</tr>
<tr>
<td>Fluconazole at 5 mg/kg/day plus IL-12 at (µg/day)</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>0.396 ± 0.073</td>
</tr>
<tr>
<td>0.1</td>
<td>0.239 ± 0.034</td>
</tr>
<tr>
<td>0.01</td>
<td>0.106 ± 0.017</td>
</tr>
</tbody>
</table>

* n = 10 mice per group.

** P < 0.01 compared with untreated controls.

*** P < 0.01 compared with untreated controls.

...tion therapies carried somewhat higher mean burdens than those given only IL-12, these were not statistically different. However, IL-12 at 0.1 or 1.0 µg/day in combination with fluconazole was efficacious in comparison with no treatment (controls). Thus, we feel that there was neither synergy nor antagonism by the combination of IL-12 and fluconazole; IL-12 was the sole efficacious agent in the combination treatment in the liver. While no treatment efficacy was observed in the lungs or spleens (the least infected organs), the spleens of mice given IL-12 at 1.0 µg had significantly higher burdens of C. neoformans than the spleens of untreated controls. This may not have been due to the exacerbation of disease by IL-12 at this dose but, rather, may have been due to the significant increase in spleen weight induced by IL-12 when it was given at 1.0 µg, since calculation of the number of CFU per gram of spleen indicated no differences in the numbers of CFU (data not shown).

The dosages of IL-12 used in our studies covered a 500-fold range, with no overt toxicities observed with any treatment regimen. All doses but 0.01 µg of IL-12 proved to have efficacy, but IL-12 showed only a minimal dose-responsiveness when it was given alone or in combination with fluconazole. Although efficacy in the brain was observed with IL-12 when IL-12 treatment alone and no treatment were compared (up to 8.1-fold reduction in mean infectious burden), a combination regimen of IL-12 at 0.1 µg and fluconazole produced results superior to those obtained with either agent alone; the results with the combinations were the best results recorded.

With respect to the optimal dose of IL-12 necessary to cause the greatest reduction in CFU, we found that 1.0 µg of IL-12 per day was as effective as a fivefold higher dose and concluded that a dose of approximately 1.0 µg/day is optimal in our dosing schedule. This dose can now be used in subsequent studies to assess the number of doses and the IL-12 dosing schedule needed to induce an optimal and possibly curative response in animals infected with C. neoformans.

Stimulation of the host immune response by administration of a cytokine or other immunomodulatory compound has been pursued as a mechanism of improving treatments for a variety of diseases. Various biologic activities have been attributed to the heterodimeric cytokine IL-12. These include enhanced proliferation of NK and T cells, facilitation of the generation of cytolytic lymphocyte responses, induction of IFN-γ production, suppression of IL-4-stimulated immunoglobulin E synthesis, and a role in the initiation and development of a Th1 helper cell response (4, 7, 8, 15, 16, 22). There is evidence that a Th1 helper cell response to fungal infections correlates with successful host defenses, whereas a Th2 helper cell response correlates with progression of disease (3, 12, 20), and that a shift to a Th1 response results in a better outcome (12). IL-12 appears to drive the lineage of the T-helper cell response toward Th1 cells (15, 22). Administration of IL-12 to mice initiates a protective Th1 cellular immune response, causing the resolution of cutaneous Leishmania major infection and the prevention of visceral dissemination (10, 25).

Similarly, cell-mediated immunity has been demonstrated to play an important role in resistance to cryptococcosis (9, 11, 19). In vivo, nonspecific activation of macrophages by administration of Mycobacterium bovis BCG increased the resistance of mice to cryptococcosis, while abrogation of macrophage function by silica administration increased their susceptibilities to infection (18). In vitro, both IFN-γ-activated murine macrophages as well as NK cells were shown to have anticytotoxic activities (17). The important role of IFN-γ in mediating resistance in mice with cryptococcosis has been addressed by treatment of nu/nu and nu/+ mice with anti-interferon antibody and the subsequent abrogation of the increased splenic NK cell activity found during infection, as well as the increased susceptibility of treated nu/+ mice to cryptococcosis after treatment with the anti-interferon antibody (21). Thus, the use of a cytokine such as IL-12 to stimulate cell-mediated immune responses, especially in patients with suppressed cell-mediated immunity, might improve their resistance to infection.

We conclude that IL-12 has potential utility as either a sole therapy or as an adjunct to conventional antifungal therapy against cryptococcosis. The mechanisms by which IL-12 confers enhanced resistance to C. neoformans in the brain remain to be defined, but they might be a result of activation of microglial cells for phagocytosis and killing or inhibition of proliferation of C. neoformans yeasts. IFN-γ plus lipopolysaccharide has been demonstrated to activate microglial cells to suppress the intracellular growth of Toxoplasma gondii, and antibody to either tumor necrosis factor alpha or transforming growth factor beta blocked the antitoxoplasma activities of the activated microglial cells (5). Thus, these cells appear to be
involved in the host defense against this organism. Microglial cells have recently been demonstrated to be anticytotoxic effectors cells in vitro and have been shown to be capable of phagocytosing opsonized Candida albicans and inhibiting its proliferation (1, 2). Furthermore, treatment of mice with chloroquine or colchicine to block, for example, phagocytosis increased their susceptibilities to intracerebrally induced infection (2). In other studies, enhancement of macrophage-mediated functions by treatment of mice with picolinic acid caused significant reductions in the number of recoverable Candida albicans or C. neoformans from the brains of intracranially infected mice (1). Although picolinic acid provided greater protection against candidal infection than against cryptococcal infection, these data provide additional evidence of the immunologic defense against this organism (3).

ACKNOWLEDGMENTS

We thank Margit Homola and Martha Martinez for excellent assistance during the course of the studies. We also thank Gupta and Mark Knowles of ALZA Corp. for advice and help with the statistical analyses of some of these data.

These studies were funded in part by a grant from Genetics Institute, Inc., Cambridge, Mass.

REFERENCES


