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Role of Tumor Necrosis Factor and Gamma Interferon in Acquired Resistance to *Cryptococcus neoformans* in the Central Nervous System of Mice

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Although naive C.B-17 and BALB/cBy mice die of meningoencephalitis within 5 weeks of intravenous infection with an opportunistic strain of *Cryptococcus neoformans*, immunized mice express an acquired, CD4$^+$ T-cell-dependent immunity and survive an intravenous infection. Infusion of lymphocytes from immune mice into severe combined immunodeficiency (SCID) mice renders these mice more resistant to cryptococcal brain infection than uninfused controls. We have investigated the role of gamma interferon (IFN-γ) and tumor necrosis factor (TNF) in acquired resistance to *C. neoformans*. Neutralization of either IFN-γ or TNF impaired resistance of immune BALB/cBy or C.B-17 mice to cryptococci. At 10 days postinfection, there were approximately 10 times as many yeast cells in the brains of mice treated with either anticytokine antibody as in the brains of mice treated with control antibody. Simultaneous neutralization of IFN-γ and TNF further exacerbated infection. Neutralization of IFN-γ or TNF also impaired resistance in immune lymphocyte-infused SCID mice, resulting in significantly higher yeast burdens in brains of cytokine-neutralized mice than in brains of controls. Concurrent neutralization of IFN-γ and TNF rendered SCID recipients of immune cells equivalent to uninfused SCID mice with respect both to brain yeast burdens at 10 days and to survival. Anti-TNF treatment alone also curtailed survival. Histological examination of the brains of cytokine-neutralized mice revealed deficiencies in ability to focus inflammatory cells at brain lesions. These data demonstrate that both IFN-γ and TNF are important mediators of acquired resistance to cryptococcal meningoencephalitis.

Cryptococcus neoformans is an opportunistic fungal pathogen that is the third most frequent central nervous system (CNS) infection in AIDS patients. The majority of those diagnosed with pulmonary cryptococcosis are found to have brain infections as well (23). Previously, it has been proposed that the paramount defect rendering AIDS patients peculiarly at risk for lethal cryptococcal meningoencephalitis is failure of a CD4$^+$ T-cell-dependent mechanism that serves to sequester yeast cells in the lungs (13, 15), where they are cleared by a process that requires both CD8$^+$ and CD4$^+$ cells in immunocompetent individuals (15, 16, 22). Yet, according to our observations and those of others (16, 17), yeast cells can be recovered from the brains of greater than 50% of fully immunocompetent mice at some point after intratracheal instillation of 10^6 organisms.

Although yeast cells are detectable in the brains of more than half of mice within 1 to 4 weeks after intratracheal instillation, fewer than 5% of them die by 6 to 8 weeks in our experience. Indeed, few if any yeast cells can be found in brains of the 95% or so of the mice that survive this long. Yet if brains are colonized by only a few yeast cells immediately after intravenous (i.v.) inoculation, death occurs from 4 to 5 weeks later, with greater than 10^8 yeast cells per brain. We therefore propose that hematogenous dissemination of yeast cells and establishment of foci within the CNS after pulmonary infection is a not infrequent event even in immunocompetent individuals, but that such individuals possess potent inducible mechanisms that serve to eradicate or limit proliferation of yeast cells in the brain. This capability is not available to individuals lacking CD4$^+$ cells or manifest to any great extent in mice inoculated i.v.

Recently, a report from this laboratory described a CD4$^+$ T-cell-dependent mechanism of resistance that controls yeast proliferation at established foci of infection in the brain (14). This resistance is acquired by mice that previously experienced a sublethal pulmonary infection with *C. neoformans* and can be conferred upon severe combined immunodeficiency (SCID) mice by passive transfer of lymphocytes obtained from immune donors. Immunized mice are protected and survive doses of i.v.-delivered cryptococci that kill SCID mice in approximately 14 days and nonimmune immunocompetent mice at 28 to 35 days.

In this report, an investigation of a putative role for the cytokines tumor necrosis factor (TNF) and gamma interferon (IFN-γ) in acquired resistance to *C. neoformans* within the brain was prompted by the following published findings. IFN-γ-induced murine macrophages can inhibit yeast proliferation in vitro (1, 6, 19). It has also been shown that human astrocytes activated with IFN-γ and interleukin-1β (IL-1β) kill *C. neoformans* via a nitric oxide-mediated mechanism (18). Another study implicated TNF in activation of murine peritoneal macrophages to ingest encapsulated opsonized yeast cells (2). Furthermore, neutralization of TNF in vivo resulted in more severe infection and reduced survival time in mice infected intraperitoneally (i.p.). More recently, it has been shown that TNF is produced by peripheral blood monocytes, polymorphonuclear leukocytes, and macrophages if they are activated by opsonized *C. neoformans* (20). Following culture in vitro, the capacity to release TNF after cryptococcal stimulation decreased in both monocytes and bronchoalveolar macrophages,

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but this trend could be reversed by addition of IFN-γ to cultures.

We sought to determine the role of IFN-γ and TNF in acquired anticryptococcus resistance expressed in the brains of mice. Either or both cytokines were neutralized in vivo, and the course of infection was monitored by killing mice and determining brain burdens of yeast cells at 10 days after i.v. infection, by allowing progression to morbidity/mortality and comparing survival of cytokine-neutralized and control mice, and by histological comparison of brain tissues from these mice.

MATERIALS AND METHODS

Mice. BALB/cBy and C.B-17 mice were purchased from the Animal Breeding Facility of the Trudeau Institute. Immunocompetent mice were maintained under conventional husbandry conditions and were free of common pathogens as evidenced by serological testing performed by the Research Animal Diagnostic and Investigative Laboratory, University of Missouri, Columbia. Mice received commercially prepared chow and acidified water ad libitum. C.B-17 SCID mice were bred at the Trudeau Institute and were kept in autoclaved microisolator cages provided with HEPA-filtered air. Mice were fed sterile food and water.

Mice were routinely immunized at 6 to 8 weeks of age, making them 3 to 4 months old at the time of i.v. challenge.

C. neoformans. Two strains of *C. neoformans* were used in this study. Serotype A strain 184 (14) was the gift of J. Murphy. This strain grows on Sabouraud-dextrose agar and yeast synthetic minimal medium (YSM) agar. This strain is mildly virulent for mice. Intravenous infection of 2×10^4 strain 184 yeast cells results in death of immunocompetent C.B-17 or BALB/cBy mice at 3 to 4 weeks postinjection. The serotype D *ura5* mutant strain (4, 25) was the gift of K. J. Kwon-Chung. *ura5* mutants require exogenous uracil and although able to grow on Sabouraud-dextrose agar, which contains a source of uracil, are unable to grow on YSM agar plates. *ura5* yeast cells are avirulent for the doses and immunocompetent mouse strains used in this study.

Working stocks of both yeast strains were maintained on Sabouraud-dextrose agar slants at 26°C. A fresh batch of slants was prepared from one of these slants every 2 weeks. At approximately 6-month intervals, fresh working stocks were initiated from seed stocks maintained in long-term storage at 4°C in distilled water.

Preparation and delivery of *C. neoformans* inocula. Inocula were prepared by seeding yeast cells of either strain onto fresh Sabouraud-dextrose agar slants and allowing them to grow for 24 h. Yeast cells from these 24-h slants were inoculated into Sabouraud-dextrose broth and allowed to grow for 24 h at 37°C. Log-phase suspensions were centrifuged to pellet yeast cells, washed once in phosphate-buffered saline (PBS), then resuspended in PBS, and counted. Suspensions were adjusted to the desired concentration. Mice were infected intratracheally with 10^6 yeast cells in 0.1 ml of PBS as previously described (15) and i.v. via the retro-orbital sinus with 2×10^4 yeast cells in 0.2 ml of PBS.

Harvest and enumeration of *C. neoformans* from brains and lungs of mice. Mice were killed by CO_2 asphyxiation, except in cases where serum or cerebrospinal fluid (CSF) was harvested, in which case they were killed by halothane gas overdose. The lungs and/or brain were then removed, homogenized, and plated on Sabouraud-dextrose agar. When the *ura5* strain was used, organ homogenates were plated on both Sabouraud agar and YSM agar plates. Only strain 184 organisms grow on YSM agar plates. In the few instances when significant numbers of strain 184 organisms from the immunizing inocula were detected in the brain homogenates, the animal was excluded from analysis. The numbers ($\log_{10}$) of viable *C. neoformans* are expressed as the mean ± standard deviation. These data were statistically analyzed with Student's *t* test. Significance was defined by a P value of <0.05.

Antibody treatments. The following monoclonal antibodies (MAbs) were used: anti-IFN-γ (R46A2, rat immunoglobulin G1 [IgG1], ATCC HB170) and anti-TNF (XT3.11, rat IgG1; DNAX Corp.). A rat IgG1 MAb (HRPN) of irrelevant specificity was used as a control for these MAbs.

In several experiments, polyclonal rabbit anti-TNF IgG was used. Polyvalent, monospecific rabbit anti-recombinant TNF IgG was generated and purified from serum as described previously (11). The neutralizing titer (neutralizing units [NU] per milliliter) is defined as the reciprocal of the highest dilution of anti-recombinant TNF that when mixed with an equal volume of a preparation of TNF having 20 U of cytotoxic activity per ml neutralizes 50% of the cytotoxic activity as determined in vitro on actinomycin D-treated L929B cells.

All antibodies and sera were diluted in PBS and injected into the peritoneal cavities of mice. Dosages were as stated in the text.

Quantitation of anti-IFN-γ MAb in mouse sera. Sera were harvested by cardiac puncture from mice at time of sacrifice and frozen at −70°C until assayed for content of anti-IFN-γ. These assays were performed to establish whether the amount of anti-IFN-γ MAb administered to mice was in excess of that required to neutralize all endogenously produced IFN-γ. The IFN-γ neutralization assay has been described elsewhere (9). Briefly, serial twofold dilutions of serum (50 μl) were incubated with an equal volume of IFN-γ (20 U/ml) in a 96-well plate for 1 h at 37°C. At the end of this time, 2×10^4 L929B mouse fibroblasts in 100 μl were added to each well, and the plates were incubated at 37°C. Eighteen hours later, 10^3 PFU of vesicular stomatitis virus was added to each well. The plates were then incubated for 48 h, after which viral cytopathic effect was scored. The neutralizing titer of a serum sample is defined as the reciprocal of the highest dilution of the sample that when mixed with an equal volume of IFN (final IFN concentration, 10 U/ml) neutralizes 50% of the antiviral activity as judged by the development of viral cytopathic effect.

The presence of anti-TNF IgG in sera was determined in a procedure analogous to that used for quantitation of anti-IFN-γ antibody. Anti-TNF IgG administration has been shown to neutralize the cytotoxic activity of endogenously produced TNF, with the presence of anti-TNF IgG in sera correlating with ablation of cytotoxic activity in infected organ homogenates (10). Serial twofold dilutions of sera were added to an equal volume of a mixture containing Eagle's minimal essential medium (Gibco, Grand Island, N.Y.), 10% (vol/vol) fetal calf serum, actinomycin D (1 μg/ml), and TNF (20 U/ml) and incubated for 1 h at 37°C in a 96-well plate; one set of wells contained serial dilutions of a known concentration of MAb against TNF, for use as a standard. These mixtures were then transferred to a 96-well plate containing an equivalent volume of nearly confluent L929B cells and incubated for 24 h more, at 37°C, in a humidified 5% CO_2 atmosphere. TNF-mediated cytotoxicity was scored at 24 h. The neutralizing titer of the sample is defined as the reciprocal of the highest dilution of sample that when mixed with an equal volume of TNF (final TNF concentration, 10 U/ml) neutralizes 50% of the cytotoxic activity as judged by the destruction of the fibroblast monolayer.

An enzyme-linked immunosorbent assay (ELISA) was used to quantify rat IgG1 control antibody in the CSF of mice. Five mice infected 7 days previously with 2×10^4 strain 184 yeast cells and five untreated mice were killed, and the foramen magnum was exposed. The membrane was nicked with a 16-gauge needle, and then a P20 pipettor and tip were used to quickly extract approximately 5 μl of CSF per mouse. CSF contaminated with erythrocytes was excluded from analysis. Pools from three to four mice were used for the ELISA. These were diluted 1:5 in PBS and 10% bovine serum albumin (BSA)–Tween–PBS, and 1:2 serial dilutions were added to a polyvinyl chloride (PVC) plate, the wells of which had been coated 24 h previously with sheep anti-rat $F(ab')_2$, and blocked with 3% BSA–Tween–PBS for 2 h at 37°C. One set of wells contained dilutions of a known quantity of rat IgG1. These wells were incubated for 1.5 h at room temperature. Plates were washed three times with 3% BSA–Tween–PBS and then incubated with alkaline phosphatase-labelled goat anti-rat IgG (Bethesda Research Laboratories, Gaithersburg, Md.) at room temperature for 1 h. *p*-Nitrophenyl phosphate was then added to each well as instructed by the manufacturer. Absorbance was read at 405 nm on a Titertek Multiskan Plus (Flow Laboratories, Inc., McLean, Va.) and compared against a standard curve of known concentrations of rat IgG.

Histopathology. After the brain was removed, it was sagitally bisected, and half of the organ was plated to enumerate yeast cells. The other half was fixed in 10% neutral buffered formalin. Tissues were dehydrated and embedded in paraffin. Sections were stained with hematoxylin and eosin and by the Fontana-Masson alcian blue method, which stains yeast cell wall, capsule, and cytoplasm (21), with the omission of the silver-staining step.

For the histopathologic observations, four to five serial sagittal sections of brain taken adjacent to the midline were examined for each animal. The number of yeast organisms, the intensity of the mononuclear cell response, and the intensity of the neutrophil/eosinophil response were assessed for brain parenchyma and meninges. Lesions were scored subjectively on a five-point scale where 0 indicated complete absence of a finding and 1 to 4 indicated slight, mild, moderate, or severe findings. For inflammatory lesions, individual inflammatory cells or small groups of cells migrating from the perivascular space or diffusely dispersed in the meninges were graded 1 or 2. In these cases, inflammatory cells were absent from at least some of the foci of cryptococcal organisms. Scores of 3 and 4 were used for robust inflammatory responses which included dense accumulations of inflammatory cells surrounding organisms. The severity of cryptococcal infection was scored on a similar scale according to the number of foci of yeast cells and the number of visible organisms within foci. The treatment accorded to each group under the experimental protocol was unknown to the scorer.

RESULTS

Neutralization of IFN-γ decreases the resistance of immune mice to systemic cryptococcal infection. Three groups of mice were infected i.v. with *C. neoformans* 184 at 2×10^4 organisms per mouse. A first group consisted of five nonimmune mice. Three other groups of mice were immunized by the intratracheal instillation of 10^6 strain 184 yeast cells 6 weeks prior to the i.v. infection. The positive control group of immunized mice received an irrelevant antibody of the same isotype and at the same concentration as the anti-IFN-γ MAb which was administered to a third group. These mice received 2×10^4

TABLE 1. Effect of neutralization of TNF on brains and lungs of mice challenged with *C. neoformans* i.v.[a]

Group	Treatment	Log_{10} *C. neoformans*	
		Brain	Lungs
A	Nonimmune, control IgG	6.67 ± 0.21[b]	2.86 ± 0.54
B	Nonimmune, anti-TNF IgG	6.79 ± 0.12	2.73 ± 0.75
C	Immune, control IgG	5.01 ± 1.18	2.69 ± 0.42
D	Immune, anti-TNF IgG	6.15 ± 0.79	5.09 ± 2.37

[a] Groups of 5 C.B-17 female mice were immunized by intratracheal instillation of 10^6 strain 184 yeast cells. After 6 weeks, they received 2×10^4 NU of polyclonal anti-TNF or appropriate control i.p. at 1 h prior to i.v. challenge and again 5 days later. Mice were challenged with 2×10^4 184 i.v. Mice were killed at 10 days. Results shown are the means for five mice ± standard deviations.
[b] $P < 0.05$ compared with group C.

TABLE 2. Effect of neutralization of IFN-γ and TNF on brains of immune mice challenged with *ura5* mutant *C. neoformans* i.v.[a]

Group	Treatment	Log_{10} *C. neoformans*/brain
A	Nonimmune, control antibody	5.21 ± 0.32[b]
B	Nonimmune, saline	5.23 ± 0.15[b]
C	Immune, control antibody	2.46 ± 0.32[c]
D	Immune, 4×10^4 NU of anti-IFN-γ MAb	3.63 ± 0.15[b,c]
E	Immune, 4×10^4 NU of polyclonal anti-TNF IgG	3.50 ± 0.12[b,c]
F	Immune, anti-IFN-γ MAb + polyclonal anti-TNF IgG at 4×10^4 NU of each	4.14 ± 0.29[b,c]

[a] Groups of five BALB/cBy female mice were immunized by intratracheal instillation of 10^6 strain 184 yeast cells. After 6 weeks, they were treated i.p. with 4×10^4 NU of MAb against IFN-γ or rabbit polyclonal antibody against TNF. One hour later, mice were challenged with 2×10^4 *ura5* yeast cells. Ten days later, animals were killed. *ura5* yeast cells in the brain were distinguished from strain 184 cells by differential plating (see Materials and Methods). Results shown are means for five mice ± standard deviations.
[b] $P < 0.05$ compared with group C.
[c] $P < 0.05$ compared with group A.

NU of anti-IFN-γ MAb. A fourth group of immunized mice received 2×10^4 NU of anti-IFN-γ MAb but was left unchallenged by i.v. infection. Antibodies were administered i.p. 1 h prior to i.v. challenge and at 5 days after initiation of infection. At 10 days postinfection, mice in all four groups were killed and viable yeast cells in their brains were enumerated. Immune control mice had almost 10,000-fold fewer yeast cells than did the nonimmune mice, corresponding to a log_{10} resistance of 3.8 (data not shown). Although immune mice receiving anti-IFN-γ MAb had more than 100-fold fewer yeast cells in their brains than did nonimmune mice, they nonetheless exhibited significantly less resistance than immune mice given a control antibody. Yeast cells were not detectable in the unchallenged group, demonstrating that neutralization of IFN-γ did not cause significant seeding of the brain by residual yeast cells remaining in the lung from the immunizing pulmonary infection. We conclude that IFN-γ plays a role in acquired resistance to *C. neoformans* in the brains of immunized mice. Neutralization of IFN-γ did not exacerbate infection in the brains of nonimmune mice inoculated i.v. with *C. neoformans* (data not shown).

Effects of neutralization of TNF on resistance to *C. neoformans* 184 in the brains and lungs of immunized animals. The effect of neutralization of TNF in immune and naive animals was also examined. Animals were immunized as described above and, along with naive control groups, treated with 2×10^4 NU of polyclonal rabbit anti-TNF IgG i.p. at 1 h prior to infection with strain 184 and again on day 5 postinfection. Controls were treated with concentration-matched rabbit IgG. Infections were evaluated at day 10. Table 1 shows that neutralization of endogenous TNF did not affect brain burdens of yeast cells in nonimmune mice. While a slightly decreased resistance is apparent in immune animals receiving anti-TNF IgG compared with immune animals receiving control IgG, this difference was not statistically significant, owing to considerable intragroup variability. It should be noted that the immune control mice in this experiment exhibited only 1.5 to 2 logs of resistance. That is to say, the immunization did not serve to protect even the control antibody-treated animals in this experiment as well as is usual in this system, possibly because TNF neutralization reduced the capacity of immune mice to control proliferation of yeast cells remaining in the lungs from the primary infection. Again, extreme variability in lung burdens was observed.

In light of concern regarding the possibility that residual lung-derived yeast cells may have contributed extensively to observed brain burdens, and the general systemic debilitation of the anti-TNF-treated animals as a result of the exacerbation of pulmonary disease, this experiment was performed with mice at 18 weeks postimmunization. At that time, immune mice have few if any yeast cells in their lungs but continue to express robust acquired resistance (data not shown). Although better protection was achieved (3 to 4 logs fewer brain yeast cells in immune mice receiving control antibodies than in nonimmune mice), only a statistically insignificant decrease in resistance was observed if TNF was neutralized. While these experiments suggest that TNF does not play an important role in either innate or acquired resistance to cryptococcal brain infection, it is difficult to draw conclusions confidently because of exacerbation of pulmonary infections.

Neutralization of IFN-γ or TNF results in reduction in the capability of immune mice to resist *ura5* i.v. challenge. Mice were immunized by instillation of 10^6 strain 184 cells intratracheally and held for 6 weeks. At that time, animals received concentration- and isotype-matched control antibody or 4×10^4 NU of MAb against IFN-γ or polyclonal anti-TNF IgG, or both, i.p. 1 h prior to infection. A saline control was introduced to verify that the rabbit polyclonal IgG contained no anticryptococcal activity. All four groups, as well as a group of nonimmune mice, received 2×10^4 *ura5* mutant cryptococci i.v. Mice were then killed at 10 days postinfection, and brains were plated on Sabouraud-dextrose agar and YSM agar plates, to differentiate between yeast cells derived from the immunizing and challenge inocula, as explained in Materials and Methods. The results are shown in Table 2. There was no difference between nonimmune mice treated with control antibodies (group A) and nonimmune mice given saline (group B), implying that the rabbit IgG had no anticryptococcal activity. Immune mice treated with control antibodies (group C) had between 100- and 1,000-fold fewer yeast cells in their brains than did the nonimmune groups. Immune mice receiving anti-IFN-γ MAb (group D) had lesser resistance capabilities, with about 10-fold more yeast cells than in immune control mice. Immune mice receiving polyclonal anti-TNF IgG also showed less resistance capability than immune control mice. Combined antibody treatment further weakened the resistance capabilities of immune mice in group E. These mice had between 10- and 100-fold more yeast cells than did immune controls but still about 10-fold fewer than nonimmune controls. These results imply that both IFN-γ and TNF play a role in mediating the acquired resistance to *C. neoformans* that is observed in immune mice but that neither treatment completely abolishes their potentiated resistance capability.

Neutralization of IFN-γ and TNF in immune mice does not affect their ability to clear yeast cells from the blood or the level of seeding of yeast cells to the brain. We wished to determine if the increased brain burden of yeast cells seen in anti-IFN-γ- and anti-TNF-treated immunized mice was due to impaired clearance of yeast cells from the blood or increased seeding of yeast cells to the brain. We therefore performed the following experiment. A group of 10 naive mice and four additional immunized groups of 10 mice each were challenged with 2×10^4 yeast cells. The four additional groups were treated as follows. The positive control group received a concentration- and isotype-matched irrelevant antibody. A second immunized group received 2×10^4 NU of anti-IFN-γ MAb, and a third received 2×10^4 NU of anti-TNF MAb. A fourth group received a combined treatment of 2×10^4 NU of each of the anti-cytokine MAbs. At 24 h postinfection, all mice were exsanguinated, organs were harvested, and brain yeast burdens were enumerated. No yeast cells were detectable in the blood drawn from any mouse in any group. The limit of detection was 100 yeast cells per ml of blood. There was no significant difference in number of yeast cells recovered from brains of any group (data not shown). Therefore, we find no evidence that treatment of mice with MAbs against IFN-γ or TNF, or both, impairs their ability to clear yeast from the vasculature or increases their susceptibility to colonization of the brain.

Neutralization of IFN-γ and TNF renders SCID mice infused with lymphocytes from immune donors less resistant to cryptococcal brain infection. As discussed above, we wished to confirm these results in SCID mice rendered more resistant to *C. neoformans* by infusion of lymphocytes from immunized donors. This allows us to avoid the difficulties in analysis arising from underlying lung burdens remaining from an immunizing inoculum in immunocompetent hosts and from possible neutralization of antibodies due to murine response to the rat or rabbit Ig used for neutralization. The shorter survival of SCID mice facilitates determination of the effects of cytokine neutralization on survival time of infected animals. SCID mice survive approximately 2 weeks after i.v. infection with *C. neoformans* 184, and SCID mice protected by infusion of lymphocytes from immune donor animals survive about twice as long.

Lymphocytes obtained from spleens and lung-draining lymph nodes of donor animals at 6 weeks after intratracheal infection with 10^6 strain 184 yeast cells were infused into SCID recipients; 24 h later, reconstituted SCID mice and SCID controls received either monoclonal anti-IFN-γ, anti-TNF IgG, or both at 4×10^4 NU i.p. or concentration- and isotype-matched antibodies. Intravenous infection followed 2 h later. Mice were killed at 10 days, and one half of each brain was fixed and set aside for histology. The remaining half brains were plated to assess organ burdens. The results are shown in Table 3. Reconstituted mice receiving anti-IFN-γ MAb (group D) showed a modest but significant loss of resistance capability, and anti-TNF treatment of group E mice also weakened resistance conferred by the infusion of immune lymphocytes. Neutralization of both IFN-γ and TNF in reconstituted mice (group F) rendered them not significantly different from control uninfused SCID mice; i.e., the protective effect of infusion of lymphocytes from immune donors was ablated. These results confirm the data and conclusions drawn from the experiment in immunocompetent animals shown in Table 2.

Neutralization of IFN-γ and TNF decreases the survival time of SCID mice infused with lymphocytes from immune donors. The $\log_{10}$ resistance value of 0.9 seen at day 10 when we compared reconstituted with control SCID mice in the previous experiment represents a reduction in yeast burdens shown in a previous study to result in approximately a twofold life extension for reconstituted SCID mice (14). To determine the effects of anticytokine treatment on survival time after i.v. infection, we performed an experiment similar in protocol to the one described above. The results are summarized in Fig. 1. No effect of anticytokine treatment on innate resistance of

TABLE 3. Effect of neutralization of IFN-γ and TNF on brains of SCID mice challenged with *C. neoformans* 184 i.v.[a]

Group	Treatment	$\log_{10}$ *C. neoformans*/brain
A	Noninfused, control antibody	7.56 ± 0.26^b
B	Noninfused, saline	7.54 ± 0.29^b
C	Reconstituted, control antibody	6.66 ± 0.07^c
D	Reconstituted, 4×10^4 NU of anti-IFN-γ	$6.95 \pm 0.14^{b,c}$
E	Reconstituted, 4×10^4 NU of polyclonal anti-TNF IgG	$7.15 \pm 0.21^{b,c}$
F	Reconstituted, anti IFN-γ + polyclonal anti-TNF IgG at 4×10^4 NU of each	7.31 ± 0.17^b

[a] Twenty-five C.B-17 male mice were immunized by intratracheal instillation of 10^6 strain 184 yeast cells. After 6 weeks, they were killed and lymphocytes were harvested from the spleens and lung-draining lymph nodes of donor mice. Groups of five C.B-17 male SCID recipients were infused i.v. with 4×10^7 lymphocytes each. Two hours later, mice received antibody or control treatments i.p. One hour after antibody treatment, mice were infected i.v. with 2×10^4 strain 184 cells. Mice were killed at 10 days. Results shown are means for five mice ± standard deviations.

[b] $P < 0.05$ compared with group C.

[c] $P < 0.05$ compared with group A.

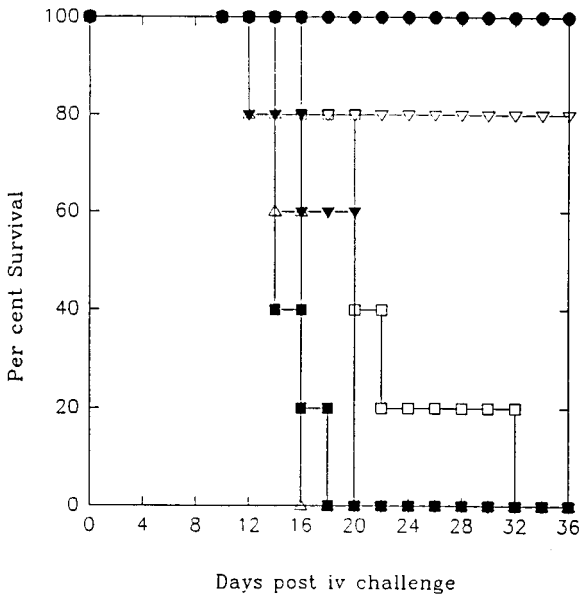

FIG. 1. Effect of neutralization of IFN-γ and TNF on survival of SCID mice after i.v. infections with *C. neoformans*. Male C.B-17 SCID mice were reconstituted with 4×10^7 lymphocytes harvested from the spleens and lung-draining lymph nodes of congeneic immune mice. Two groups of unreconstituted SCID mice were also set aside for this experiment. Twenty-four hours later, mice received 4×10^4 NU of antibody or control i.p. Two hours later, all mice received 2×10^4 strain 184 cells i.v. Mice were allowed to progress to morbidity/mortality. Groups of five mice each were treated as follows: uninfused mice, control antibodies (filled boxes); uninfused mice, anti-IFNγ plus anti-TNF (open triangles); reconstituted mice, control antibodies (filled circles); reconstituted mice, anti-IFN-γ (inverted open triangles); reconstituted mice, anti-TNF (open boxes); reconstituted mice, anti-IFN-γ plus anti-TNF (filled triangles). Reconstituted mice in the anti-IFN-γ-treated group received a second dose of 4×10^4 NU of antibody i.p. at 20 days after i.v. infection. At the same time, reconstituted mice receiving control antibody received a concentration-matched dose of control rat IgG1.

TABLE 4. Histological comparison of brains of SCID mice and lymphocyte-reconstituted SCID mice after cytokine neutralization[a]

Group	Treatment	Severity of infection	Inflammation			
			Meningeal		Parenchymal	
			M	NE	M	NE
A	Noninfused, control antibody	2	0	0	0	0
B	Noninfused, saline	2	0	0	0	0
C	Reconstituted, control antibody	1	3	2	1/0	1/0
D	Reconstituted, anti-IFN-γ	2	2	2	0	2
E	Reconstituted, anti-TNF	3	3	3	0	0
F	Reconstituted, anti-IFNγ + anti-TNF	2	1	1	0	0

[a] The experimental protocol is described in footnote *a* to Table 3. Slides were scored for severity of infection and presence and extent of inflammatory response as detailed in Materials and Methods. Scores are medians for three mice per group except for group C, in which case only two samples were evaluated. M, monocytes; NE, neutrophils or eosinophils.

SCID mice to cryptococcal proliferation in the brain was detected. Four of five reconstituted mice who received only anti-IFN-γ treatment survived to day 35, when the experiment was concluded (despite an antibody boost of 4×10^4 additional NU and concomitant boost of the control group animals at 20 days after i.v. infection). These animals thus behaved similarly to the reconstituted group receiving only irrelevant control antibodies, which enjoyed greater than twofold extension of survival time. Neutralization of TNF alone in reconstituted SCID mice significantly hastened their deaths compared with reconstituted controls. Mortality times in all groups were analyzed by the Mann-Whitney U test. Time to death of reconstituted SCID mice treated with a combination of anti-IFN-γ and anti-TNF was reduced to the extent that it was not significantly different from that of control uninfused SCID mice.

These data demonstrate that TNF mediates resistance to proliferation of yeast cells in the brain. They also imply that neutralization of IFN-γ has but a transient effect on the resistance mechanism studied here, causing higher organ burdens of yeast cells at early time points but not wholly preventing the animals from mounting a defense against the infection. An additional test of IFN-γ neutralization in this system also failed to produce a statistically significant reduction in survival time.

Comparative histology of cryptococcal lesions in the meninges and parenchyma in brains of SCID mice. Brains were taken from mice at day 10 of the experiment referred to in Table 4 and stained with combined hematoxylin and eosin and Fontana-Masson alcian blue stain. This combined technique allows us to visualize cryptococci readily against a background where familiar brain structures are recognizable and the presence or absence of inflammatory infiltrate is easily assessed. Brains were scored for inflammatory response, with ascending scores indicating increased number of inflammatory cells, as described in Materials and Methods. Although the severity of yeast infestation was comparable in the meninges and parenchyma (Table 4), the meningeal inflammatory response was always more developed. All groups of infused mice had detectable mononuclear cells, eosinophils, and neutrophils at meningeal foci of infection. However, the number of inflammatory cells in the mice that received combined anti-IFN-γ and anti-TNF treatment was considerably lower and therefore more similar to the number in uninfused SCID controls, which were characterized by complete absence of inflammatory cells in the meninges and in lesions abutting the meningeal or perivascular space.

In the parenchyma of reconstituted mice given either anti-TNF or given anti-TNF plus anti-IFN-γ, despite a moderate to marked severity of infection, there were few inflammatory cells, similar to findings for uninfused SCID mice. In contrast, one of two control reconstituted mice was able to recruit modest numbers of monocytes and eosinophils and an intermediate number of neutrophils from the vasculature into the parenchyma. The second control reconstituted mouse had very few parenchymal yeast cells, and so no host defense cells were identified despite a robust response in the meninges of this same animal. Two of three IFN-γ-neutralized reconstituted mice were similarly capable of recruiting eosinophils and neutrophils to the parenchyma, but monocytes were absent. The third showed no host response despite considerable yeast infestation. It should be noted that when brains of normal immunocompetent immune mice challenged with cryptococcal brain infection are examined, lesions are reliably surrounded by such an inflammatory infiltrate. The brains of the mice in groups C and D did not have a detectable infiltrate at every lesion, and in general, their inflammatory responses were less robust than those seen in the meninges. But since some lesions were attended by host inflammatory cells, particularly neutrophils and eosinophils, these animals were to some extent capable of mediating extravasation of host defense cells and the mobilization of these cells to the brain parenchyma.

These histological observations suggest that IFN-γ and TNF are involved in recruitment of inflammatory cells into the CNS, with TNF playing an important role in mediating extravasation of host defense cells and their entry to the deep parenchyma in *Cryptococcus*-infected mouse brains.

DISCUSSION

A previous report from our laboratory demonstrated an acquired resistance to *C. neoformans* expressed in the brains of mice sensitized by a sublethal pulmonary infection and challenged with i.v. infection (14). The resistance is $CD4^+$ T-cell dependent, serves to inhibit proliferation of yeast cells at already established foci within the CNS, and can be conferred on SCID mice by passive transfer of lymphocytes from spleens and lung-draining lymph nodes of immune donors. In the present study, we have extended our characterization of this acquired resistance, demonstrating that neutralization of IFN-γ and/or TNF impairs this resistance mechanism in intact immune mice or reconstituted SCID mice. In reconstituted SCID mice, treatment with anti-TNF or anti-TNF plus anti-IFN-γ concurrently completely ablates the protective effects of the infused lymphocytes, exacerbating yeast burdens at early time points, blocking the ability to focus inflammatory defense cells at foci of infection within the CNS, and ultimately decreasing the survival time of infected mice. Treatment with anti-IFN-γ MAb produces less dramatic results. Brain yeast burdens are higher at day 10 after i.v. infection in reconstituted SCID mice receiving the neutralizing antibody compared with reconstituted control SCID mice, but ability to mount an inflammatory response within the CNS is only partially impaired, and the animals' survival time after infection is not significantly reduced.

From these observations, and established effects of IFN-γ or TNF which have been reported in the literature, it is possible to propose a number of models of action and possibly interaction of the two cytokines in resistance to cryptococcal meningoencephalitis. The infected mouse must first recruit T cells from extra-CNS circulation across the blood-brain barrier and

into the brain parenchyma. It is known that both IFN-γ and TNF promote lymphocyte-endothelial cell binding, via interaction of the LFA1-ICAM receptor-ligand pair (8). Systemic neutralization of either or both cytokines might therefore prevent access of T cells to sites of infection within the CNS. However, it should be recalled that some T cells in our model system have already been activated by prior exposure to cryptococci. Indeed, infusion of lymphocytes from naive intact donors is not helpful to SCID animals infected i.v. with *C. neoformans* (14), either because the nonimmune population of lymphocytes simply contains too few cryptococcus-specific T cells or because nonimmune T cells lack certain cell surface activation markers which would allow the lymphocytes to productively interact with the vascular endothelia of the brain (12). The presence of the invading organisms themselves may set off a chain of events including increase of vascular permeability and culminating in egress of T cells and other effector cells from the blood. It is therefore not at all certain that neutralization of either cytokine can block this first step of immune T-cell entry to the CNS in this system.

Once within the CNS, T cells might mediate immunity in a number of ways. IFN-γ-secreting $CD4^+$ cells might interact with antigen-presenting cells (APCs) in the CNS in a major histocompatibility complex (MHC)-restricted fashion. Microglial cells, astrocytes, and pericytes are all capable of expression of MHC II expression under appropriate conditions, for example, stimulation by IFN-γ (5, 7). Upregulation of MHC II would promote efficient presentation of *C. neoformans* epitopes in proper context for recognition by $CD4^+$ T cells. Interaction of the $CD4^+$ T cells with antigen of proper specificity displayed on the surface of microglial cells or astrocytes would provide cues for T-cell proliferation and/or cyclical reentry of *C. neoformans*-reactive T cells into the CNS parenchyma (12). Egress of a number of activated T cells into the CNS, stimulation by IFN-γ derived from those T cells of APCs in the CNS to express MHC II molecules on their surfaces, and presentation of processed antigen to a small pool of *C. neoformans*-reactive T cells would ultimately result in a relatively large pool of antigen-specific T-cell clones at foci of infection. It has recently been demonstrated that *C. neoformans*-laden human alveolar macrophages efficiently stimulate proliferation of T lymphocytes through an MHC II-restricted mechanism (26). But the APC–T-cell interaction presumably has reciprocal effects. Not only do the microglia or other APCs stimulate T cells, but engagement by the T-cell receptor of the antigen in the context of the MHC II molecule, or cytokine stimulation by the cognate T cell, may stimulate these semiprofessional phagocytes to ingest more yeast cells. For example, IFN-γ has been reported to induce macrophages to increased fungistatic capability (1, 6, 19) and to stimulate control of cryptococcal proliferation by astrocytes via a nitric oxide-mediated mechanism (18). Finally, IFN-γ may play an important role through its capacity to prime T cells and macrophages to produce TNF (26).

TNF has been shown to activate macrophages to increased phagocytosis of opsonized *C. neoformans* (2), and its role in recruitment of nonspecific host inflammatory cells to tissue lesions by increasing adhesiveness of endothelial cells for neutrophils, eosinophils, and basophils is well known (3). Indeed, neutralization of TNF and IL-1β has been shown to completely suppress the CSF inflammatory response, and administration of recombinant TNF and recombinant IL-1β results in rapid influx of leukocytes into the CSF during infection with *Haemophilus influenzae*, a model for CNS infection in rodents (24).

Neutralization of IFN-γ, then, could short-circuit expression of acquired immunity to *C. neoformans* by interfering with entry, proliferation, or accumulation of T cells at sites of infection and possibly with activation of resident and recruited phagocytes in potentiated yeast ingestion. Neutralization of TNF could block the capacity of T cells, even if present in sufficient numbers at lesions, to recruit inflammatory mediators like neutrophils, eosinophils, and monocytes from the extra-CNS circulation or to focus such cells at lesions within the brain parenchyma. Just such a role has been reported for TNF in recruitment of mononuclear cells to *Listeria monocytogenes* lesions in the murine liver (10). Indeed, upon examination, TNF-neutralized animals in this study showed large, in some cases even macroscopic, lesions in the deep brain, to which the animals were apparently incapable of responding, although host response to meningeal yeast cells was intact.

In fact, the meningeal response was more developed than parenchymal response in all cases examined, although the severities of infection were similar in the two compartments. Even animals with detectable parenchymal host response had less robust inflammatory responses at foci that were distant from the meningeal or perivascular space. In our experience, when naive normal mice and immunized normal mice are simultaneously challenged with i.v. cryptococcal infection, immune mice are quite efficient at recruitment and focusing of inflammatory cells at brain lesions from early on. In naive animals, however, appearance of any inflammatory infiltrate is delayed until later time points and even then is not apparent at all lesions. Immune mice generally control the proliferation of yeast cells and eventually sterilize their brains, while naive mice eventually die of meningoencephalitis. It would seem that, given the accelerated proliferation of cryptococci in the CNS, even in immunocompetent animals, time is the limiting factor in fighting the infection. Therefore, cytokine-mediated amplification of response may well become crucial in fighting cryptococcal brain infection. By all measures detailed in this study, concurrent neutralization of TNF and IFN-γ effectively paralyzes the host resistance mechanisms.

Examination of infected brain tissue afforded some insight into the resistance mechanisms impaired by cytokine neutralization, particularly for TNF. Infected mice that received anti-TNF treatment had large lesions in the deep brain, to which the mice appeared incapable of responding.

The observation that treatment with anti-IFN-γ MAbs decreased but did not ablate T-cell protection may be rationalized in several ways, although the possibility that neutralization of IFN-γ was incomplete is unlikely. Excess anti-IFN-γ antibody was detected in serum at the time of death, suggesting that all endogenously produced IFN-γ was neutralized. Furthermore, introduction of isotype-matched control antibody i.p. resulted in detectable levels of antibody in the CSF of cryptococcus-infected but not sham-infected controls. It is possible, however, that the presence of excess unbound anti-IFN-γ MAb in sera is not an adequate index of total IFN-γ neutralization at foci of infection within the CNS. Some portion of the IFN-γ activity may be autocrine, or the cytokine could be secreted by one member and act upon the other member of closely apposed T-cell–APC couples. Elevated local concentrations of target IFN-γ and short-range interactions of IFN-γ with its ligands may preclude complete neutralization of the cytokine in this situation, causing the decrease, but not absolute loss, of potentiated resistance capability that was observed. It is possible that a total depletion of IFN-γ, if achieved, would indeed result in total ablation of the protective effects of immune lymphocytes.

Another explanation for the relatively mild effect of anti-IFN-γ treatment involves the possibility that the capacity of IFN-γ to activate T cells to produce TNF is of paramount

importance in this system. Neutralization of IFN-γ, while blocking a pathway to T-cell production of TNF, would not eradicate all production of TNF if redundant mechanisms exist for stimulation of its production by T cells and/or other cells. Recently, it has been reported that in the presence of a source of complement, *C. neoformans* itself stimulated monocytes and macrophages to secrete TNF (20). Finally, if an important role of IFN-γ in these brain infections is to potentiate the fungicidal or fungistatic activity of phagocytic cells active at the sites of infection, neutralization of this cytokine stimulus, while weakening the response, would not necessarily eradicate the innate defense capabilities of these cells.

It appears clear from the data presented here that the presence of TNF is crucial to the infected mouse if inflammatory effector cells are to be recruited to the deep brain and an efficacious defense is to be made against cryptococcal proliferation in the brain.

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