A Combination of Thalidomide plus Antibiotics Protects Rabbits from Mycobacterial Meningitis—Associated Death

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Tuberculous meningitis (TBM) is a devastating form of tuberculosis that occurs predominantly in children and in immunocompromised adults. It is predominantly a disease of children in the developing world and of the elderly or immunocompromised in the United States [1, 2]. The mortality rate of TBM is ~50%, and most of the survivors of this infection suffer permanent neurologic sequelae [1, 3]. The underlying mechanisms that determine the development of the immune response and clinical manifestations of TBM are poorly understood. Insights into its pathophysiology still rest on anatomic and histologic descriptions of 50 or 60 years ago [4–6].

It has long been known that infections in the central nervous system (CNS) are extremely dangerous because of the inflammation resulting from the mobilization of the host defense. Macrophages exposed to invading mycobacteria or their products release proinflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) [7–11]. TNF-α plays a definitive role in granuloma formation and the containment of mycobacterial infection [12, 13]. However, in addition to the protective role of TNF-α, it has been shown that the local production of inflammatory cytokines (including TNF-α) in the brain in response to experimental bacterial meningitis leads to altered blood-brain barrier (BBB) permeability, cerebrospinal fluid (CSF) leukocytosis, increased protein influx, and lactate accumulation [14–16]. In humans with bacterial meningitis, CSF levels of TNF-α and IL-1β correlate with the severity of disease and clinical outcome [17, 18]. This inflammatory process ultimately leads to brain edema, vascular infarcts, necrosis, and irreversible brain damage. Hence, control of inflammation even during antibiotic treatment of CNS infections is of vital importance.

We describe here a rabbit model of acute mycobacterial infection in the CNS, which resembles human TBM immunologically, clinically, and pathologically. We used this model to study the inflammatory cascade of mycobacterial CNS infection and then intervened with a combination of antimycobacterial drugs and thalidomide (α-N-phthalimido glutarimide), a drug recently shown to inhibit production of TNF-α [11, 19–23]. The effects of this combination therapy on brain pathology and on survival of mycobacteria-infected rabbits were evaluated.

Materials and Methods

Infecting organism. Mycobacterium bovis Ravenel (Trudeau Mycobacterial Culture Collection, TMC no. 401), known to be highly virulent for rabbits, was selected for this study. Mycobacteria were grown initially in Middlebrook 7H9 broth (Difco Laboratories, Detroit) for ~2 weeks and then cultured in Proskauer and Beck medium [24] containing 0.01% Tween 80. Mycobacteria (1.7 × 10^8 cfu/mL) were frozen and stored at −70°C and were used as stock cultures for experiments. Before each experiment, a vial was thawed and subjected to brief ultrasonication to break up aggregates. Final suspensions were prepared to achieve an inoculum of 2 × 10^5 cfu/0.12 mL.

Induction of meningitis. New Zealand White rabbits weighing 2–3 kg (Hare Marland, Nutley, NJ) were used as previously described [23]. After arrival at the animal facility, all animals underwent a 1-week period of adaptation. Thereafter, a helmet of dental acrylic was attached to the skulls of the rabbits. On the next day,
the animals were anesthetized with a combination of ketamine (0.5 mL/kg) and xylazine (3.5 mL/kg) administered intramuscularly. The rabbits were then placed in a stereotaxic frame. A spinal needle was introduced into the cisterna magna, and 0.3 mL of CSF was withdrawn. Next, 0.12 mL of live mycobacteria was injected intracisternally. For each experiment, 4–6 animals were infected. Control animals were injected intracisternally with 0.12 mL of pyrogen-free saline. In some of the experiments, CSF samples were withdrawn at 0, 2, 4, 6, and 24 h after inoculation. In other experiments, meningitis was allowed to progress for 8 days, and the samples were obtained every 24 h. After infection, animals were housed separately, each in an individual cage. During the experiments, rabbits were rehydrated daily with 60 mL of pyrogen-free saline subcutaneously. At the end of each experiment, rabbits were euthanized by an overdose of pentobarbital. The brains were removed and fixed in 10% buffered formalin (vol/vol) (Fisher Chemical, Fairlawn, NJ).

CSF. Immediately after collection, 100 μL of CSF was removed for determination of the bacterial load. Ten-fold serial dilutions were plated onto Middlebrook 7H10 agar (Difco). Plates were incubated at 37°C for 2–3 weeks. Organisms were counted as colony-forming units (cfu). The remainder of the CSF was centrifuged at 10,000 g for 5 min, and the supernatant was stored at −70°C until tested for cytokines and protein. CSF samples were analyzed for numbers of white blood cells (Coulter Electronics, Hialeah, FL). The cellular sediment was used for differential cell counts (Diff-Quick; Baxter, Miami). CSF supernatant protein concentration was determined using the bicinchoninic acid method (BCA Kit; Pierce Chemical, Rockford, IL), as described by the manufacturer.

Blood. Blood was collected from the auricular artery using a heparinized syringe at different time points and centrifuged at 10,000 g. Plasma was separated and frozen at −70°C for cytokine analysis.

TNF-α assay. TNF-α was measured in CSF supernatants and in plasma, using a modification of a cytotoxicity assay with the murine L929 cell line, as previously described [23]. The minimal detection level was 16 U/mL.

IL-1α and IL-1β assays. Both cytokines were assayed in CSF supernatants as described by the manufacturer, using commercial RIA kits designed for the rabbit (Cytokine Sciences, Boston). Duplicate 100-μL CSF samples were assayed per tube. The minimal detection level of IL-1β was 30 pg/mL and of IL-1α was 40 pg/mL.

Histopathology. After fixation in 10% formalin (Fisher Chemical), the brains were cut transversely in serial 2- to 3-mm sections from rostral to caudal. Four sections were selected representing the fore-, mid-, and hindbrains. The tissues were processed routinely, embedded in paraffin (Tissue Prep-2; Fisher Scientific, Fairlawn, NJ), cut into 4-μm sections, and stained with hematoxylin-eosin and acid-fast (Kinyoun’s) stains.

Thalidomide treatment. Thalidomide (Celgene, Warren, NJ) was prepared as a suspension in sterile saline and administered intragastrically via a flexible rubber tube. In order to obtain sufficient plasma and CSF thalidomide concentrations, the animals were pretreated with the drug (at −24 h, −17 h, and −2 h) before infection with mycobacteria. Initially, 100 mg/day thalidomide was used; in the second group of experiments, the dose was 200 mg/day.

Antituberculosis therapy. Isoniazid (Nydrazid Injection, Apothecon; Bristol-Myers Squibb, Princeton, NJ) was administered at a dose of 30 mg/day as an intramuscular injection. Rifampicin (Rifadin; Merrell Dow Pharmaceuticals, Kansas City, MO) was administered at a dose of 30 mg/day in a suspension of saline via a rubber intragastric tube. Thus, the dose of the antibiotics was ~15 mg/kg per day, which is the standard human antitubercular treatment dosage.

Data. Results are presented as the means of values obtained for all animals evaluated at each time point. SDs are shown for all data points unless the number of animals remaining for evaluation was <3. Data were analyzed using an independent t test to compare controls with treated animals. For the survival data, the Kaplan-Meier survival analysis was used. P < .05 was considered significant. Extreme outlying values for TNF-α levels and CSF leukocyte counts were found to be indicators of imminent death of the animals. Thus, when an individual value for TNF-α or leukocyte number was >3-fold higher than the mean, that value was not used in the calculation of the SD.

Results

Mycobacterial infection of the CNS in rabbits. New Zealand White rabbits were infected by intracisternal inoculation of M. bovis Ravenel, which is highly virulent for these animals [25]. Rabbits were monitored for immunologic, clinical, and pathologic responses. Samples of CSF and blood were obtained at different time points following infection. Our preliminary experiments showed that 2 × 10⁷ cfu of M. bovis Ravenel induced a highly reproducible acute inflammatory response in the CSF. Lower inocula produced a chronic infection, which resulted in delayed onset (>14 days) of inflammation.

In the present experiments, following intracisternal inoculation of 2 × 10⁷ cfu M. bovis Ravenel into rabbits, viable mycobacteria were isolated and could be cultured from the CSF throughout the course of the experimental infection (figure 1A). Examination of cytospin preparations of the CSF showed that the acid-fast bacilli were usually associated with mononuclear phagocytes that had entered into the subarachnoid space (not shown). Leukocyte accumulation in the CSF increased from saline control levels of ≤300 cells/mL to ≥1000 cells/mL by day 1 after mycobacterial infection, indicating that the BBB had been breached (figure 1B). Leukocyte numbers continued to increase slowly. When infected animals were moribund at the time of CSF sampling, a >5-fold increase in the number of leukocytes compared with day 1 after infection was observed. The differential white blood cell count revealed an acute polymorphonuclear leukocyte response for the first 24 h after infection, followed by a shift to mononuclear cells. By 48 h after infection, 75% of the cells in the CSF were monocytes and lymphocytes. At day 6, >90% of the leukocytes were mononuclear.

One of the hallmarks of BBB injury is protein influx into the CSF. In this model, the animals had an increased amount of CSF protein from baseline control levels of 0.5 mg/mL to >1 mg/mL by 2 h after infection. Protein levels continued to
Figure 1. Inflammatory response of rabbits infected intracisternally with $2 \times 10^7$ cfu of *M. bovis* Ravenel and monitored for 8 days after infection. A, Growth (cfu/mL) of *M. bovis* Ravenel in cerebrospinal fluid (CSF) of infected rabbits ($n = 6$). B, Leukocyte density (no./mm$^3$) in CSF of infected rabbits ($n = 10$). C, Protein levels (mg/mL) in CSF of infected rabbits ($n = 8$). D, Tumor necrosis factor-α (TNF-α) concentration (U/mL) in CSF (□) and in plasma (△) of infected rabbits ($n = 10$). Nos. in parentheses pertain to no. of animals included in each study group at start of study. All values are means ± SD for no. of animals studied at each time point. Following death of animals, if $n < 3$, SD was not included.

increase during the period of observation (figure 1C). In addition to protein accumulation in the CSF, cytokines were also induced. TNF-α in the CSF, which at baseline is 0, peaked 2 h after mycobacterial inoculation and was maintained at measurable levels throughout the experiment (figure 1D). Secondary peaks of TNF-α preceded the death of infected animals. In the plasma of the rabbits, TNF-α was detected 1 day after the infection and remained elevated throughout the experiment (figure 1D). Low levels of IL-1β (199 ± 102 pg/mL at 2 h) and IL-1α (173 ± 111 pg/mL at 2 h) were detected in CSF of infected animals (data not shown).

The inflammatory process described above correlated with the clinical course of experimental mycobacterial meningitis. All infected rabbits showed severe clinical signs, including somnolence or irritability, loss of coordination, lateral recumbency, ophthalmitis, hemiparesis, or hemiplegia. Infected animals survived 2–8 days after intracisternal inoculation of mycobacteria (see below).

Histopathologic examination of brains of rabbits infected with *M. bovis* Ravenel demonstrated a granulomatous meningitis, which progressed in severity over time. By 24 h, there was marked thickening of the leptomeninges and distension of the arachnoid space with large numbers of inflammatory cells, which consisted primarily of mononuclear cells (macrophages and lymphocytes) and fewer polymorphonuclear leukocytes (figure 2B). Vasculitis of blood vessels within the meninges was frequently observed and was characterized by infiltration of the adventitia (figure 2C). This process progressed in the later stages of infection (by 7 days) to extension of the inflammation transmurally with fibrinoid necrosis of the smooth muscle of the vessel walls (figure 2D). In some cases, there was also extension of the inflammation to the deeper perivascular areas within the neuropil (encephalitis) (figure 2E).

Acid-fast staining of sections of tissue sampled 4–8 days after infection showed abundant intracytoplasmic bacilli within macrophages in the meninges as well as perivascularly in the neuropil. In addition, scattered acid-fast bacilli were seen in the macrophages within histologic sections of the lung and spleen, and viable bacilli could be cultured from these organs (not shown). The presence of viable bacilli in organs other than the CNS indicated that once the BBB was breached, mycobacteria-infected cells probably crossed back from the subarachnoid space into the blood. The systemic infection may have contributed to the presence of TNF-α in the plasma (figure 1D).
Effect of thalidomide on CSF cytokine levels and survival of rabbits. The immunomodulatory drug thalidomide has been previously shown to reduce TNF-α production in vitro and in vivo in rodents [26–29]. To determine whether such immunomodulation would affect the pathophysiology of CNS mycobacterial infection, experimentally infected rabbits were treated with thalidomide. Infected rabbits received either 100 or 200 mg/day of thalidomide orally (by gavage) starting 24 h before infection. A group of untreated, infected animals served as controls. Thalidomide treatment was continued for 6 days. Animals were monitored for 8 days after infection.

Administration of 200 mg/day thalidomide to rabbits resulted in plasma drug levels ranging from 10–20 μg/mL at 2 h to 20–25 μg/mL at the 8-h drug delivery. By 24 h (the time of the next dose delivery), plasma levels of thalidomide had dropped to <0.2 μg/mL. CSF drug levels reached 10–13 μg/mL by 2 h after delivery of thalidomide. Treatment of rabbits with this dose of thalidomide (200 mg/day) significantly (P = .01) reduced peak TNF-α levels in the CSF (figure 3A). Similar inhibition was observed in the plasma, where TNF-α concentrations were reduced in a dose-dependent manner (figure 3B). Furthermore, a delay in TNF-α production was seen in animals treated with 200 mg/day thalidomide. Administration of a lower dose (100 mg/day) of thalidomide did not result in significant changes in peak CSF TNF-α levels at 2 h after infection nor were plasma TNF-α levels fully reduced compared with controls (figure 3A, B). In control animals, TNF-α was detected in the plasma by 1 day after infection, whereas in rabbits treated with thalidomide at either 100 or 200 mg/day, TNF-α was detected in the plasma only on day 2.

Thalidomide treatment was associated with prolonged survival of the infected rabbits (figure 3C). However, when thalidomide treatment was discontinued at day 6, the rabbits became moribund, and by day 8 after infection, no significant residual effect on survival of the infected rabbits was observed. Thus, thalidomide protected rabbits from death only during the time infected animals were receiving the drug. A side effect of thalidomide treatment was sedation, which was dose-dependent.
that the antituberculous drugs alone cannot protect the rabbits cause of cranial nerve palsies, hemiparesis, paraplegia, and infected rabbits treated with antibiotics had less severe symp-toms, and 67% died within a few days (figure 4).

Clinical signs (figure 4). It is of interest to note that in rabbits Ravenel induced a meningitis characterized by release of TNF- in the CSF concentrations of thalidomide, treatment with this drug administered 30 mg/day isoniazid intramuscularly and 30 mg/ day rifampicin orally. The strain of M. bovis used in the experi-ments was completely sensitive to these drugs. A third group of infected rabbits received 200 mg/day thalidomide by gavage, in addition to the antituberculous drugs. To achieve efficient CSF concentrations of thalidomide, treatment with this drug was started 24 h before antibiotics. All rabbits were treated with the combination of drugs daily for 8 days.

Control infected animals developed acute, rapidly progres-sive meningitis, and 67% died within a few days (figure 4). Infected rabbits treated with antibiotics had less severe symp-toms, and 50% survived until day 8. These data clearly indicate that the antituberculous drugs alone cannot protect the rabbits from death due to mycobacterial meningitis. In contrast, all infected rabbits treated with both antibiotics and thalidomide survived until the end of the observation period with no obvious clinical signs (figure 4). It is of interest to note that in rabbits treated with antibiotics and thalidomide, interruption of thalido-mide treatment after 6 days while maintaining antibiotic ther-apy resulted in continued survival of 100% of the animals (not shown).

Histopathologic evaluation of the brains showed significant attenuation of the inflammatory response in the combined anti-biotic and thalidomide therapy group. In these rabbits, there were only small numbers of inflammatory cells distending the subarachnoid space in the meninges, as well as an absence of extension into the deeper perivascular areas of the neuropil (figure 5C). Treatment with antibiotics alone did not signifi-cantly alter the morphologic severity of the meningitis (figure 5B), although the inflammation appeared to occur more focally relative to the control infected animals. Some acid-fast organisms could still be demonstrated within phagocytic cells in the meninges in response to antibiotic therapy with or without thalidomide but in decreased numbers compared with the untreated, infected animals.

To measure the efficacy of antituberculous treatment, myco-bacteria were cultured from the CSF and quantitated by the cfu assay. By day 2 after the infection and initiation of antitu-berculous treatment, significant reductions in cfu were observed in the antibiotic-treated animals compared with the cfu in the control group (P < .04) (figure 6A). The addition of thalido-mide to the antibiotic treatment did not interfere with bacillary clearance.

Thalidomide treatment reduced TNF-α concentrations in the CSF and plasma of infected animals receiving concomitant antituberculous therapy (figure 6B, C). Thalidomide prevented the peaks of TNF-α detected in CSF of untreated and antibiotic-treate rabbits at 2 h after infection as well as at later time points (4–8 days). In rabbits receiving antibiotics combined with thalidomide, TNF-α production in plasma was fully blocked for the first 4 days. In these animals, TNF-α was first detected in the plasma 5 days after the infection at reduced levels (figure 6C). Leukocyte counts in the CSF of animals treated with antibiotics were lower than the levels in the CSF of control untreated rabbits at the later time points (days 6–8) (figure 6D). In animals treated with antibiotics and thalidomide, leukocyte numbers were much lower than in the CSF of either antibiotic alone or untreated infected rabbits (figure 6D).

Discussion

The present study describes a rabbit model of acute myco-bacterial meningitis and demonstrates the efficacy of an immu-nomodulatory approach that improves neurologic outcome and survival. M. bovis infection in rabbits is similar to M. tuberculo-sis infection in humans [30]. In human TBM, occlusions of large or small vessels leading to infarcts are the most common cause of cranial nerve palsies, hemiparesis, paraplegia, and death [31]. In our animal infection model, we observed all of these neurologic signs following mycobacterial infection of the CNS. Intracisternal inoculation of high doses of M. bovis Ravenel induced a meningitis characterized by release of TNF-α and IL-1β, high protein influx, and a massive cellular re-sponse with predominance of mononuclear cells in the CSF. In our model, CSF TNF-α levels correlated with the clinical course of meningitis. The peaks of individual TNF-α levels paralleled the exacerbation of the infection and, in many ani-mals, preceded death. These observations are analogous to those in clinical studies of bacterial meningitis, in which rela-tively higher concentrations of TNF-α in the CSF were associ-ated with more severe disease [15, 17].
Figure 5. Histologic sections of brain and meninges of rabbits infected with *M. bovis* Ravenel and followed for 8 days after infection. A, Control rabbit (infected, untreated) with severe meningitis, with marked thickening of meninges (arrows) and extension of perivascular inflammation to neuropil (arrowheads) (hematoxylin-eosin; photographed at ×25). a, High magnification of A. Note presence of primarily mononuclear cells (macrophages and lymphocytes) (arrows) and acid-fast bacilli (encircled) (acid-fast stain; photographed at ×100). B, Rabbit infected and treated with antibiotics only. Severity of meningitis is not significantly reduced on morphologic evaluation compared with control animal (hematoxylin-eosin; photographed at ×25). b, High magnification of B. Note similar appearance of mononuclear inflammatory infiltrates compared with controls and presence of acid-fast bacilli (encircled) (acid-fast stain; photographed at ×100). C, Rabbit infected and treated with antibiotics and thalidomide (200 mg/day). Note attenuation of inflammatory response within meninges compared with control animal (arrows) with no evidence of perivascular infiltration within neuropil (arrowhead) (acid-fast stain; photographed at ×25). c, High magnification of C. There are reduced nos. of inflammatory cells in arachnoid space compared with control animal (arrows) (acid-fast stain; photographed at ×100).

The meningitis induced in the experimental model studied here is more acute than the naturally occurring infection in humans. Because the infection is so acute and animals start dying so soon after infection (2 days), intervention with thalidomide had to be started before induction of CNS inflammation, which is observed as soon as 2 h after infection (TNF-α peak). Thus, the therapeutic window used here did not mimic that in human disease, in which interventions are always initiated after symptoms are manifest. To better mimic human disease, we are now developing a more chronic mycobacterial infection...
Figure 6. Effect of thalidomide as adjunct to antituberculous treatment. All rabbits were infected intracisternally with M. bovis Ravenel. Control animals received no treatment (□) (n = 6); 1 group of rabbits was treated with antibiotics only (●) (n = 6); third group received 200 mg/day thalidomide in addition to antituberculous drugs (▲) (n = 7). Treatment continued 8 days. A, Growth (cfu/mL) of M. bovis Ravenel in CSF. Treatment with antibiotics resulted in statistically significant reduction in growth by 2 days (t test, P < .04). B, Tumor necrosis factor-α (TNF-α) concentration (U/mL) in CSF. C, Plasma TNF-α levels (U/mL). D, Leukocyte density (no./mm³) CSF. All values are expressed as means ± SD for group. SD not shown when n < 3.

model, which will enable us to initiate treatment with antibiotics and/or thalidomide 10-14 days after infection of the animals.

The inflammatory response in human TBM meningitis is complex and causes much of the damage to the CNS. TNF-α and IL-1β affect vascular endothelium, inducing endarteritis [32]. Even very small amounts of TNF-α can exert deleterious effects on capillaries already sensitized by exposure to mycobacterial products [33]. The impairment of the vasculature, coupled with edema and hydrodynamic alterations, can lead to a rise in intracranial pressure and vascular change. Thus, in addition to bacteriologic cure, the major therapeutic goal in TBM is to obviate the vascular impairment induced by inflammatory cytokines and thus prevent permanent neurologic damage.

Immune modulatory therapy in human TBM is surrounded by controversy. It is widely believed that the use of corticosteroids soon after the start of antituberculous treatment can be life-saving, especially during clearance of bacilli [34]. Experimental studies suggest that corticosteroids depress inflammatory processes and thereby reduce cerebral edema and connective tissue formation, thus preventing arteritis and meningeal adhesions [35–37]. Patient trials have also shown that dexamethasone administration before the start of antibiotic treatment reduces the inflammatory response and improves outcome [15]. On the other hand, corticosteroids have been reported to increase tuberculous inflammation, leading to massive caseation [37, 38]. In addition, a recent clinical study in children with TBM suggests that the use of large doses of prednisone does not have any significant effect on cytokine levels in the CSF. Other inflammatory parameters also did not show improvement with prednisone treatment [39]. To further complicate matters, it appears that antituberculous drugs such as rifampicin and ethambutol penetrate the BBB only when the meninges are inflamed, while streptomycin penetrates poorly even into inflamed meninges [1, 40]. Thus, it appears that the corticosteroids used to reduce the inflammatory damage to the brain in TBM can impair the delivery of antituberculous antibiotics into the subarachnoid space. An alternative adjunctive antinflammatory therapy for TBM is clearly needed. We therefore evaluated the effect of thalidomide, a known TNF-α inhibitor, on antibiotic activity in the CSF and on CSF inflammation and disease outcome in mycobacteria-infected rabbits.

In our experimental model, thalidomide reduced TNF-α production in the CSF and in the blood in a dose-dependent manner. Leukocytosis was also reduced throughout the whole pe-
period of observation. The effect of the drug on meningeal inflammation indicates that thalidomide crosses the BBB and enters the CSF in effective concentrations. We have found that at the doses used here, thalidomide accumulates in the CSF of rabbits at concentrations of >10 µg/mL, which are higher than those concentrations shown to have clinical efficacy in patients with graft-versus-host disease [41]. In animals treated with thalidomide, the antituberculous activity of isoniazid and rifampin is maintained, suggesting that the antiinflammatory effects of thalidomide treatment do not interfere with antibiotic penetration and/or antimycobacterial activity in the CSF.

In summary, we have described a rabbit model of TBM that can be used to study the course and sequelae of mycobacterial infection of the CNS. In addition, our experiments indicated that a combination of antituberculous drugs plus thalidomide dramatically improves outcome and survival of infected rabbits. These results suggest a potential role for thalidomide as an adjunctive immune modulator in TBM to diminish neurologic consequences. Investigations are ongoing to determine the efficacy of thalidomide administered with antituberculous drugs in children with TBM.

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References

27. Ching LM, Xu ZF, Glummer BH, Palmer BD, Joseph WR, Baguley BC. Thalidomide in Mycobacterial Meningitis