Effect of Thalidomide on Chemokine Production by Human Microglia

James R. Lokensgard,1,2 Shuxian Hu,1,2
Esther M. van Fenema,1,3 Wen S. Sheng,1,2
and Phillip K. Peterson,1,2

1Institute for Brain and Immune Disorders, Minneapolis Medical Research Foundation, and 2University of Minnesota Medical School, Minneapolis; 3Eijkman Winkler Institute, Utrecht University Medical School, Utrecht, The Netherlands

Thalidomide, a psychoactive drug that readily crosses the blood-brain barrier, has been shown to possess immunomodulatory attributes, including the inhibition of cytokine production by monocytes and microglia. In this study, we investigated the effect of thalidomide on chemokine production by human microglial cells. Microglial cells were stimulated with lipopolysaccharide, a key cell-wall component of gram-negative bacteria responsible for meningitis, and production of chemokines (regulated upon activation normally T cell expressed and secreted [RANTES], monocyte chemoattractant protein [MCP]-1, macrophage inflammatory protein [MIP]-1α, and interleukin [IL]-8) was examined by ELISA. Thalidomide treatment was found to cause potent and selective inhibition of IL-8 production in a dose-responsive manner. This inhibition was associated with decreased intracellular IL-8 staining as well as reduced transcription of IL-8 mRNA. In addition, thalidomide treatment of lipopolysaccharide-stimulated microglia inhibited the activation of protein NF-kB, a transcription factor known to be important for IL-8 production. These results suggest thalidomide could have a therapeutic role in acute bacterial meningitis through inhibition of IL-8–mediated neutrophil chemotaxis.

Successful approaches to the treatment of bacterial meningitis must control meningeal inflammation in addition to eradicating bacterial pathogens. The development of more potent antimicrobial agents has not led to appreciably decreased fatality rates, and new therapeutic modalities are desperately needed for this life-threatening disease [1]. Inflammatory cytokines and chemokines are known to play an important role in acute bacterial meningitis leading to the accumulation of large numbers of polymorphonuclear leukocytes (PMNL) in the cerebrospinal fluid [2].

Chemokines are low–molecular-weight chemotactic peptides that are important for the transendothelial migration of leukocytes into infected tissues. PMNL are the first leukocytes found at the onset of bacterial meningitis. Interleukin (IL)–8 is a CXC chemokine that is produced by many cell types, including microglia [3], and is known to be involved in the activation and chemotaxis of PMNL. This chemokine regulates cell adhesion to activated receptors on endothelial cells, facilitating the transendothelial migration of PMNL into infected tissues [4]. When a host is infected with bacterial meningitis, high levels of IL-8 are present in the cerebrospinal fluid [5], and this molecule may induce PMNL migration into the central nervous system. Although the source of chemokines in the central nervous system remains unclear, it seems likely they are produced locally by endogenous glial cells.

Thalidomide, a psychoactive drug that readily crosses the blood-brain barrier, has been shown to possess immunomodulatory attributes, including the alteration of adhesion molecule expression and the suppression of tumor necrosis factor alpha (TNF–α) production by monocytes and microglia [6, 7]. It is possible that the inflammatory response during meningitis could be modulated by drugs such as thalidomide, which inhibit the production of proinflammatory cytokines or inhibit the migration of leukocytes into the central nervous system. In this study, we examined the effect of thalidomide on chemokine production by human microglial cells, which were stimulated by the gram-negative bacterial cell-wall component lipopolysaccharide (LPS).

Materials and Methods

Preparation of cell cultures and thalidomide. Primary human microglial cell cultures were prepared as previously described [3]. Briefly, brain tissues from 16–22-week-old aborted fetuses were dissociated and plated in 75-cm² Falcon culture flasks in Dulbecco’s
modified Eagle medium containing 10% fetal bovine serum. The medium was replenished 4 days after plating, and microglia were harvested after 10–14 days and replated into 24-well (5 × 10⁴ cells per well) or 96-well (2 × 10⁴ cells per well) plates. Purified microglial cell cultures were composed of a cell population of which > 99% stained with antibodies to CD68. Stock solutions of thalidomide (Sigma Chemical, St. Louis) were prepared in 100% dimethyl sulfoxide and diluted in tissue culture medium immediately before use. The final concentration of dimethyl sulfoxide was kept at <0.25%.

**ELISA.** Chemokine levels in microglial cell culture supernatants were measured with a modified sandwich ELISA. Purified mouse anti-human chemokine antibodies were coated overnight onto microtiter plates at a concentration of 1–2 µg/mL. Culture supernatants (50 µL) were added in triplicate for 2 h at 37°C. The wells were then incubated with goat anti-human chemokine antibodies (1–4 µg/mL) followed by donkey anti-goat IgG–horseradish peroxidase conjugate. Substrate buffer K-blue (ELISA Technology, Lexington, KY) was used for color development. Sensitivities of the ELISA for IL-8, regulated upon activation normally and transiently expressed and secreted (RANTES), monocyte chemoattractant protein (MCP)-1, and macrophage inflammatory protein (MIP)-1β were determined to be 10, 20, 7.8, and 30 pg/mL, respectively.

**Reverse transcriptase (RT)–polymerase chain reaction (PCR).** Reverse transcription of 1.5 µg of RNA was performed with 50 µM oligo(dT)12-18, first-strand buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM MgCl₂, 0.1 M dithiothreitol), 10 mM dNTPs, and SuperScript II reverse transcriptase. Amplification of IL-8 or glyceraldehyde-3-phosphate dehydrogenase (GADPH) cDNA was performed in a reaction mixture containing polymerase chain reaction (PCR) buffer (500 mM KCl, 100 mM Tris, 25 mM MgCl₂), 10 mM deoxynucleoside triphosphate mixture, 5 U Taq DNA polymerase with 0.22 µg/mL TaqStart antibody, and 25 µM primer and cDNA. Amplification parameters were 94°C for 45 s, 65°C for 45 s, and 72°C for 90 s, followed by a final 10-min extension at 72°C. The IL-8 PCR product (298 bp) was amplified for 17–19 cycles, and the GADPH (600 bp) was amplified for 22 cycles. The IL-8 primer sets were 5'-ATGACTTCCAAGCTGGCCGTGGCT-3' and 5'-TCTCAGCCCTCTTCAAAAAACTTCTC-3'. The GADPH primer sets were 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCTGTGTTGCTGA-3'.

**Northern blot tests.** Total microglial cell RNA (8 µg, 6 h after stimulation) was separated on denaturing formaldehyde gels and transferred to nylon membranes (Magna Graph; Micron Separation, Westborough, MA). The blots were probed for IL-8-specific mRNA with a 32P-labeled PCR amplification product of the human IL-8 gene as a probe. Blots were hybridized overnight in 50% formamide at 42°C, washed under high stringency conditions, developed on a phosphoimager (Molecular Dynamics, Sunnyvale, CA), and analyzed with ImageQuant software (Molecular Dynamics). Ratios of GADPH to IL-8 band density were calculated for each treatment to determine the decrease in mRNA expression after thalidomide treatment.

**Results**

**Effect of thalidomide on microglial cell chemokine production.** We first examined the effect of thalidomide on the production of chemokines by LPS-stimulated primary human microglial cells with ELISA. Microglial cells were pretreated with various doses (1–30 µg/mL) of thalidomide for 30 min and then stimulated with LPS (1 µg/mL). When culture supernatants from thalidomide-treated, LPS-stimulated microglial cells were assayed for production of the β chemokines RANTES, MCP-1, and MIP-1β, thalidomide was found to have no effect on β-chemokine production (figure 1A–1C). In contrast, thalidomide treatment was found to potently inhibit microglial cell production of IL-8 in a dose-responsive manner, at levels of 65.6% ± 6.7% inhibition with maximal doses (figure 1D).

LPS-induced production of the cytokine TNF-α was included as a positive control for inhibition by thalidomide (figure 1E).

We next examined whether the thalidomide-mediated decrease in IL-8 levels, as detected by ELISA, was the result of an inhibition of chemokine production or whether it was caused by diminished chemokine release into the microglial culture supernatants. Microglial cell cultures were pretreated with thalidomide for 30 min before stimulation with LPS; the cells were fixed 16 h after LPS stimulation and stained for IL-8 by immunohistochemical methods. A marked decrease in intracellular IL-8 staining was observed after thalidomide treatment (data not shown), indicating that thalidomide does not inhibit chemokine release from microglia but inhibits the production of IL-8.

**Effect of thalidomide on IL-8 mRNA expression.** We then examined the effect of thalidomide on IL-8 mRNA expression with RT-PCR. Microglial cells were pretreated with thalidomide for 30 min before stimulation with LPS. Total RNA was harvested 6 h after LPS stimulation and analyzed for IL-8 mRNA. The 298-bp IL-8–specific PCR product was observed in all microglial RNA samples tested (figure 2A). Qualitative analysis of the PCR gels revealed that thalidomide suppressed IL-8 mRNA levels higher than baseline values (figure 2A, lane 3) with a corresponding return to constitutive levels after thalidomide pretreatment, before LPS stimulation (figure 2A, lane 4). Analysis of these data suggests that thalidomide treatment inhibits microglial cell IL-8 production at the transcriptional level. Finally, we found thalidomide treatment to have no effect on GADPH mRNA levels (figure 2, lanes 5–8).

The PCR results were confirmed, and the amount of thalidomide-mediated inhibition of IL-8 expression was quantified, by Northern blot analysis. Decreased levels of IL-8 transcripts were detected in thalidomide-treated, LPS-stimulated microglial cells when compared with LPS stimulation without thalidomide pretreatment (figure 2B). Analysis of these results with ImageQuant software (Molecular Dynamics) showed that thalidomide treatment markedly inhibited microglial cell IL-8 mRNA expression in response to LPS stimulation when compared with untreated LPS-stimulated cells (figure 2C).

The transcriptional regulatory protein NF-κB is important for IL-8 expression, and treatment of human microglia with IL-10 has been shown to down-regulate IL-8 production...
Figure 1. Effect of thalidomide treatment on lipopolysaccharide (LPS)-induced chemokine production by primary human microglial cells. Microglial cells were pretreated with the indicated concentrations of thalidomide for 30 min and subsequently stimulated with LPS (1 \( \mu g/mL \)). Culture supernatants were collected and assayed by ELISA for production of chemokines: A, RANTES; B, MCP-1; C, MIP-1\( \beta \); D, interleukin (IL)-8; E, tumor necrosis factor (TNF)-\( \alpha \). Thalidomide-mediated inhibition of the cytokine TNF-\( \alpha \) was included as a positive control. Background chemokine levels (control) were measured in supernatants from microglial cells without LPS stimulation. Data are presented as the mean \( \pm \) SE of triplicate determinations and are representative of 3 separate experiments performed using microglial cells isolated from different brain tissue specimens.

through inhibition of NF-\( \kappa \)B activation [8]. For these reasons, we next examined the effect of thalidomide on the nuclear translocation of NF-\( \kappa \)B. Microglial cells that were incubated in culture medium alone or with thalidomide displayed diffuse staining throughout the cytoplasm when probed with a monoclonal antibody specific to the p65 subunit of NF-\( \kappa \)B. In contrast, intense nuclear p65 staining was observed in microglia that were stimulated with LPS. Thalidomide pretreatment for 30 min before stimulation with LPS resulted in decreased translocation of NF-\( \kappa \)B into the nucleus (data not shown), and the staining pattern observed in these thalidomide-treated cells was comparable to that seen in cells without LPS. Thus, the mechanism of thalidomide-mediated suppression of IL-8 production appears to involve the inhibition of NF-\( \kappa \)B.

Discussion
Inflammation within the central nervous system is regulated by the local production of soluble chemotactic mediators produced by glial cells and infiltrating immunocytes in response to microbial products or tissue injury. Human microglial cells have been shown to produce the CC chemokines MCP-1, MIP-
Effect of thalidomide on interleukin (IL)-8 mRNA expression. Microglial cells were pretreated with thalidomide for 30 min before stimulation with lipopolysaccharide (LPS). Total RNA was harvested 6 h after LPS stimulation and analyzed for IL-8 mRNA. 

A, Reverse transcriptase–polymerase chain reaction analysis: lane M, marker; lanes 1 and 5, untreated microglia; lanes 2 and 6, thalidomide alone; lanes 3 and 7, LPS alone; lanes 4 and 8, thalidomide treatment followed by LPS stimulation. Data are representative of 2 separate experiments. B, Northern blot test: lane 1, untreated microglia; lane 2, thalidomide alone; lane 3, LPS alone; lane 4, thalidomide treatment followed by LPS stimulation. Data are representative of 2 separate experiments. C, Ratio of IL-8 to glyceraldehyde-3-phosphate dehydrogenase (GADPH) mRNA expression levels. Data were quantified and standardized with GADPH mRNA expression levels, and they are expressed relative to the control value. Control, culture medium; Thd, thalidomide treatment alone (30 μg/mL); LPS, LPS alone (1 μg/mL); Thd + LPS, thalidomide treatment (30 μg/mL) followed by LPS stimulation (1 μg/mL).

Bacterial meningitis is characterized by the rapid accumulation of PMNL into the subarachnoid space. IL-8 is known to be a potent inducer of neutrophil chemotaxis, and it may contribute to the influx of these cells during acute bacterial meningitis. Excess numbers of activated neutrophils may have a detrimental effect. Most of the damage produced during bacterial meningitis appears to result from a cascade of pathogenic events precipitated by the influx of activated PMNL rather than by the invading microorganism itself. Although there are few data to support the association between reduced influx of PMNL and improved outcomes of bacterial meningitis, the use of inhibitors that regulate IL-8 production and its corresponding neutrophil activation may present new therapeutic approaches to the treatment of acute bacterial meningitis.

Despite its checkered past, thalidomide has recently been approved by the Food and Drug Administration for the treatment of erythema nodosum leprosum and esophageal aphthous stomatitis during infection with the human immunodeficiency virus. Potential therapeutic options for this drug exist in many other diseases. The reemergence of thalidomide in clinical practice is primarily due to the broad spectrum of pharmacologic and immunologic effects it possesses, and the immunomodulatory properties of thalidomide are becoming increasingly apparent. In vitro, thalidomide inhibits production of TNF-α, IL-6, and IL-12 at the transcriptional level [6, 7]. It also decreases β2 adhesion molecule expression on lymphocytes, monocytes, and granulocytes and induces a switch from Th1 to Th2 type cytokine production by human peripheral blood mononuclear cells [10]. With respect to IL-8, thalidomide has been found to induce a 2–4-fold increase in keratinocyte-derived IL-8 production [11], but it has also been shown to inhibit LPS-induced production of IL-8 by endothelial cells [12].

The mechanisms by which thalidomide imparts these immunoregulatory functions are still unclear, although it seems likely that the inhibition of NF-κB and the translocation of NF-κB to the nucleus are involved [13]. In the present study, thalidomide treatment resulted in decreased translocation of NF-κB into the microglial cell nucleus. This decrease could explain the effect of thalidomide on IL-8 production, where NF-κB plays an important role [8]. Thalidomide has been reported elsewhere to have a limited effect on cytokines associated with bacterial meningitis in vivo [14]. Thalidomide treatment was found to reduce TNF-α levels in the cerebrospinal fluid of rabbits by >50% and 29% in response to lysates of gram-positive or gram-negative bacteria,
respectively. In vivo evidence supporting a therapeutic role for thalidomide during tuberculous meningitis has also recently been found. In humans, antibiotic treatment alone is insufficient to fully diminish the pathologic manifestations of tuberculous meningitis. By use of a rabbit model of tuberculous meningitis, it was found that animals treated with thalidomide along with antituberculosis drugs displayed significantly decreased inflammation and had dramatically higher rates of survival than did those given antituberculosis drugs alone [15]. Because of these anti-inflammatory properties, thalidomide may have analogous effects during acute bacterial meningitis.

The results of our studies show that thalidomide potently inhibits IL-8 production from LPS-stimulated human microglial cells and that this inhibition is mediated at the transcriptional level. Analysis of these data suggests that thalidomide could play a therapeutic role in acute bacterial meningitis through the inhibition of IL-8-mediated neutrophil chemotaxis.

References