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Intracisternally Localized Bacterial DNA Containing CpG Motifs Induces Meningitis

Guo-Min Deng, Zai-Qing Liu, and Andrej Tarkowski

Unmethylated CpG motifs are frequently found in bacterial DNA, and have recently been shown to exert immunostimulatory effects on leukocytes. Since bacterial infections in the CNS will lead to local release of prokaryotic DNA, we wanted to investigate whether such an event might trigger meningitis. To that end, we have intracisternally injected mice and rats with bacterial DNA and oligonucleotides containing CpG motifs. Histopathological signs of meningitis were evident within 12 h and lasted for at least 14 days, and were characterized by an influx of mononuclear, Mac-3+ cells and by a lack of T lymphocytes. To study the mechanisms whereby unmethylated CpG DNA gives rise to meningitis, we deleted the monocyte/macrophage population leading to abrogation of brain inflammation. Also, interaction with NF-κB using antisense technology led to down-regulation of proinflammatory cytokine production and frequency of meningitis. Furthermore, specific interactions with vascular selectin expression and inhibition of NO synthase led to a significant amelioration of meningitis, altogether indicating that this condition is dependent on macrophages and their products. In contrast, neutrophils, NK cells, T/B lymphocytes, IL-12, and complement system were not instrumental in meningitis triggered by bacterial DNA containing CpG motifs. This study proves that bacterial DNA containing unmethylated CpG motifs induces meningitis, and indicates that this condition is mediated in vivo by activated macrophages. The Journal of Immunology, 2001, 167: 4616–4626.

Bacterial infections can be localized to the subarachnoid space, causing bacterial meningitis, a relatively common and devastating disease despite adequate use of antibiotics. Fever, headache, meningismus, and signs of cerebral dysfunction are found in ~85% of patients who present with acute bacterial meningitis (1). Despite early and adequate antibiotic treatment, bacterial meningitis remains an infection with a high mortality rate, particularly for very young and elderly patients. In addition, survivors may have permanent neurological damage, e.g., learning deficits, hearing loss, seizures, motor handicaps, and other sequelae (2). To study the pathogenesis of bacterial meningitis, animal models of this disease have been developed. It has been shown that certain bacterial virulence factors as well as host immune responses are important in the induction phase and progression of this disease (3). It is believed that exacerbation of meningitis is due to increased levels of proinflammatory cytokines, partly a consequence of antibiotic therapy leading to disruption of bacterial cell walls, resulting in the local release of biologically active cell wall products such as LPS. The release of bacterial cell wall fragments giving rise to the brain production of IL-1, IL-6, and TNF-α will not only exacerbate inflammation, but also further damage the blood-brain barrier (4–7). However, in recent studies of experimental Escherichia coli meningitis, the amount of bacterial endotoxin ultimately released as a consequence of bacteriolysis was much lower than that released by bacteria not being exposed to antibiotics (8). This finding led us to hypothesize whether there might be another virulence factor for exacerbating inflammation as a consequence of antibiotic treatment. Recently, bacterial DNA was reported to have an immunostimulatory effect on leukocytes (9–12). Indeed, intraarticularly deposited bacterial DNA induces arthritis (13, 14). In the case of human bacterial meningitis, the PCR has shown the occurrence of bacterial DNA in cerebrospinal fluid (CSF) (15).

We propose that bacterial DNA triggers macrophage accumulation that in turn contributes to meningeal inflammation and blood-brain barrier permeability. In addition, we suggest that adhesion molecules responsible for macrophage recruitment, and macrophage-derived inflammatory cytokines/chemokines are likely to contribute to the pathogenesis of the condition.

Materials and Methods

Animals and reagents

BALB/c, C57BL/6, and NMRI mice were purchased from ALAB (Stockholm, Sweden). C3H/HeJ, C3H/HeN mice, SCID mice, and their congenic strain C.B.17, Sprague Dawley (SD) rat were purchased from M&B (Bomholtvej, Denmark). IL-12 p40 knockout mice were kindly provided by J. Magram (Nutley, NJ). All mice were housed in the animal facility of the Department of Rheumatology, Göteborg University (Göteborg, Sweden). Male mice and rats of 6–8 wk of age were used in all the experiments. The hybridoma cells secreting RB6-8C5 were a gift from R. Coffman (DAX Research Institute, Palo Alto, CA). PK136 Ab was obtained from Sjögren-Jansson (Göteborg, Sweden). Etoposide was supplied by Bristol-Myers Squibb (Bromma, Sweden). α-Melanocyte-stimulating hormone (α-MSH), fucoidin, and cobra venom factor (CVF) were bought from Sigma. N4′-Monomethyl-L-arginine monoacetate salt (L-NMMA) and Nω-nitro-L-arginine methyl ester (L-NAME) were purchased from Kelab (Göteborg, Sweden). CR1Abs (SC12) were kindly supplied by T. Kinoshita

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3 Abbreviations used in this paper: CSF, cerebrospinal fluid; α-MSH, α-melanocyte-stimulating hormone; CR, complement receptor; CVF, cobra venom factor; NOS, NO synthase; iNOS, inducible NOS; L-NAME, Nω-nitro-L-arginine methyl ester; L-NMMA, Nω-nitro-L-arginine methyl ester; ODN, oligodeoxynucleotide; PGN, peptidoglycan.

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FIGURE 1. The histopathological appearance of bacterial DNA-induced meningitis. A, Histopathology of brain of a C57BL/6 mouse killed 3 days after intracisternal inoculation of 6 µg ODN 1668, showing meningeal and submeningeal deposits of leukocytes. B–D, Histopathological appearance of a SD rat killed 3 days after intracisternal inoculation of 6 µg ODN 1668 (B) or 60 µg ODN 1668 (C, D). Infiltration of mononuclear cells in meninges and around blood vessels is apparent. E–G, Normal histopathological appearance of a mouse subarachnoid space after intracisternal inoculation of calf thymus DNA (30 µg), and a SD rat subarachnoid space after intracisternal inoculation of 6 µg ODN 1668 m (F) and 6 µg ODN 1720 (G). Magnification ×20.
(Uppsala, Sweden). Peptidoglycan (PGN) from *Staphylococcus aureus* was kindly provided by S. Foster (Sheffield, U.K.).

**Genomic DNA and oligonucleotides**

*E. coli* (strain B) DNA and calf thymus DNA were purchased from Sigma (Stockholm, Sweden) and further purified by extraction with phenol-chloroform-isooamyl alcohol (25:24:1), followed by ethanol precipitation. Genomic DNA from *S. aureus* strain LS-1 was prepared by lysing the bacteria with Qiagen lysis buffer (Qiagen, Hilden, Germany) and a combination of lysozyme (Sigma). The DNA was purified using Qiagen genomic tips. DNA purity and concentration were determined using a spectrophotometer (Molecular Devices, Sunnyvale, CA) at 260 and 280 nm wavelengths. DNA purity, that is, the relation between L260 and L280, was 1.83, which is well within expected 1.7 and 2. Phosphorothioate-modified oligonucleotides 1668, 1668 m, and 1720 (all 6 μg) or i.p. administration of antisense (120 μg), L -NMMA or L -NAME was injected intracisternally into mice and rats. CpG ODN (5 μg) or i.p. administration of antisense (100 μg) was reduced from 5.5% to 0.6% (18). Control mice received the same volume of PBS in the same way (19).

**Neutrophil depletion.** BALB/c mice were injected i.p. with 1 mg mAb RB6-8C5, or the IgG rat anti-OVA mAb as a control, 2 h before injection of ODN 1668. Analysis of blood smears showed that the mAb RB6-8C5 depletes the monocyte/macrophage population by 90%. In addition, for direct depletion of macrophages in meninges and brain, the simultaneous intracisternal inoculation of etoposide (1.25 mg/kg) and CpG ODN (6 μg) into CSF was performed.

**Selectin blockade.** Fucoidin (10 mg/kg body weight) was administered i.v. or s.c. 10 min before intracisternal injection of CpG ODN, and 12 h later. Control mice received the same volume of PBS in the same way (19).

**Complement depletion.** CVF was injected i.p. at the dosage of 0.8 μg/g body weight every 48 h. Interaction with complement receptor 1 (CR1; CD35) was mediated by i.p. injection with either 400 μg IgG rat antinouse CR1 Abs (8C12), or with control, 400 μg IgG rat anti-OVA, 24 h before intracisternal injection of CpG ODN (20).

**Inhibition of NO synthase (NOS).** L -NMMA or L -NAME was injected i.p. at the dosage of 0.4 mg/kg, 0.1 mg/g body weight 2 h before intracisternal injection of CpG ODN or PBS (21).

**Administration of antisense of NF-κB.** Mice received an intracisternal injection of CpG ODN (6 μg) + antisense of NF-κB (6 μg) or p.i. administration of antisense (120 μg). Control mice received only an intracisternal injection with ODN. The sequences of phosphorothioate oligonucleotides were as previously described (22): murine p65 antisense 1, 5'-TACTGTCGTTTTGTCGTTTTGTCGTT-3' and murine p65 antisense 2, 5'-TTC ATG CT-3'.

**Assessment of endotoxin levels**

Endotoxin concentration in each stock of the *E. coli*, *S. aureus*, calf thymus DNA, and oligonucleotide was assayed using the endpoint chromogenic *Limulus* amebocyte lysate assay (BioWhitaker, Walkersville, MD). Preparation of *S. aureus* DNA, calf thymus DNA, and oligodeoxynucleotides (ODN) had levels of LPS < 0.025 EU/ml. *E. coli* DNA preparation had levels of LPS < 1 EU/ml.

**Injection protocol**

Mice and rat were anesthetized with mixture of hypnorm, dormicum, and distilled water (1:1:2), and placed on clean table. After disinfection of injecting area, 10 μl of either DNA, ODN, LPS, PGN, or PBS was injected intracisternally into mice and rats.

Macrophage depletion was induced by s.c. injection of 12.5 mg/kg body weight of etoposide in a volume of 100 μl, into the nuchal region on 3 consecutive days before and 2 consecutive days after injection of CpG ODN; control mice received the same volume of the vehicle diluted in PBS (16). Analysis by FACS showed that the etoposide depletes the monocyte/macrophage population by > 90%. In addition, for direct depletion of macrophages in meninges and brain, the simultaneous intracisternal inoculation of etoposide (1.25 mg/kg) and CpG ODN (6 μg) into CSF was performed.

**Neuraptin deficiency.** BALB/c mice were injected i.p. with 1 mg mAb RB6-8C5, or the IgG rat anti-OVA mAb as a control, 2 h before injection of ODN 1668. Analysis of blood smears showed that the mAb RB6-8C5 depletes the granulocyte population by > 90% (17).

NK cell depletions were performed in C57BL/6 mice 1 day before injection of CpG ODN and repeated 2 days after injection of CpG ODN. C57BL/6 mice were administered with i.p. injection of either 200 μg PK 136 mAb or the IgG O1C5.B2 as a control. Flow cytometry analysis revealed that the population of NK 1.1 spleen cells from mice injected with a single dose of PK 136 mAb was reduced from 5.5% to 0.6% (18).

**Selectin blockade.** Fucoidin (10 mg/kg body weight) was administered i.v. or s.c. 10 min before intracisternal injection of CpG ODN, and 12 h later. Control mice received the same volume of PBS in the same way (19).

For evaluation of the role of TNF-α in CpG ODN-triggered meningitis, α-MSH (10 or 20 μg) or LPS (20 μg) were intracisternally injected into CSF. CpG ODN (6 μg) or LPS (20 μg) alone was used as a control. In addition, α-MSH (50 μg) alone was also injected i.p. 3 days after the inoculation. α-MSH with CpG ODN provoked a similar chemotactic response as CpG ODN alone.

**Complement depletion.** CVF was injected i.p. at the dosage of 0.8 μg/g body weight every 48 h. Interaction with complement receptor 1 (CR1; CD35) was mediated by i.p. injection with either 400 μg IgG rat antinouse CR1 Abs (8C12), or with control, 400 μg IgG rat anti-OVA, 24 h before intracisternal injection of CpG ODN (20).

**Inhibition of NO synthase (NOS).** L -NMMA or L -NAME was injected i.p. at the dosage of 0.4 mg/kg, 0.1 mg/g body weight 2 h before intracisternal injection of CpG ODN or PBS (21).

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GAAACAGATCGTCCATGTG-3'; murine p65 antisense 2, 5'-GAGGGG AACAAGATGCTCCATGTG-3'; murine p65 sense, 5'-ACCATOGAGCAT CTGTTC-3'; murine p65 nonsense, 5'-GTACTACTCTGAGCAAGG A-3'. All of these phosphorothioate oligonucleotides were synthesized by Scandi-
navian Gene Synthesis.

Assessment of blood-brain barrier integrity and leukocyte numbers in CSF
To assess the integrity of the blood-brain barrier in animals exposed to CpG ODN, Evans blue was used. Meningitis was induced in SD rats as described above, but 1 h before termination of the experiment, 1 ml 1% Evans blue (Sigma) was injected i.v. (23). CSF was collected from SD rats injected with CpG ODN (12 µg) or PBS. Evans blue concentration in the CSF was determined by measuring the absorbance at 650 nm with a spec-
trophotometer (Molecular Devices); serially diluted Evans blue in PBS served as a standard. In other rats, the CSF was collected following intra-
cisternal injection with CpG ODN (12 µg) or PBS, and centrifuged (1500 × g, 10 min). Total and differential leukocyte counts of CSF were done immediately after the CSF collection.

Histopathological and immunohistochemical examination of brain
Histopathological examination of brain was performed after routine fixa-
tion and paraffin embedding. Tissue sections from brain were cut to 5 µm thick and stained with H&E. All the slides were coded and evaluated blindly.

For immunohistochemical examination, the brain was removed, mounted on cryostat casts, frozen in isopentane prechilled in liquid ni-
trogen, and kept at −70°C until cryosectioned. Serial cryosections, 6 µm thick, were stained with rat mAbs directed against mouse CD11b (Mac-1), CD4 (GK1.5), CD8 (53.6.7), and Mac-3, followed by incubation with biotinylated secondary Abs and avidin-biotin-peroxidase complexes and

In situ hybridization for cytokine mRNA detection
In situ hybridization was conducted to detect mRNA expression of TNF-α, IL-1β, IL-12, as well as monocyte chemoattractant protein-1 (MCP-1), as previously detailed (14). Briefly, synthetic oligonucleotide probes for TNF-α, IL-1β, IL-12 (gift from T. Olsson, Karolinska Institute, Stock-
holm, Sweden), and MCP-1 were labeled at the 3' end using terminal deoxynucleotidyl transferase (Advanced Biotechnologies, Leatherhead, U.K.) and [α-35S]ATP (DuPont Scandinavia, Stockholm, Sweden). Sections of 4-µm-thick freshly frozen brain were thaw mounted onto slides and were hybridized with 1 × 106 cpm of labeled probe/100 µl hybrid-
ization mixture. After emulsion autoradiography, development, and fix-
tation, the coded slides were examined by dark field microscopy for positive cells containing >15 silver grains/cell in a starlike distribution.

Assessment of TNF-α levels in culture supernatants, CSF
Splereens from C57BL/6 mice were obtained aseptically and passed through a nylon mesh. Erythrocytes were depleted by hypotonic lysis. The resulting single cell suspension was resuspended in Iscove’s complete medium (10% FCS, 5 × 10⁻³ M 2-ME, 2 mM l-glutamine, and 50 µg/ml gentamicin). Subsequently, 1 × 10⁶ cells/ml were incubated with 1 µM ODN 1668, 1668 (1 µM) + α-MSH (0.2 mg/ml), 1668 (1 µM) + antisense p65 (1 µM), 1668 (1 µM) + sense p65 (1 µM), 1668 (1 µM) + nonsense (1 µM), respectively. The cultures were kept in 24-well plates (Nunc A/S, Roskilde, Denmark) at 37°C in 5% CO₂ and 95% humidity. The supernatants were collected after 18 h for the detection of TNF-α.

TNF-α levels of supernatants and CSF were determined using a TNF-α ELISA kit from R&D Systems (Minneapolis, MN). The assay was performed as recommended by the manufacturer.

Results
Bacterial DNA and synthetic ODN containing unmethylated CpG dinucleotides (CpG ODN) induce meningitis
To study the impact of bacterial DNA in mediating brain inflamma-
tion, we injected intracisternally DNA and CpG ODN (ODN 1668) into the cisterna magna of mice and rats, and found that meningitis was induced by bacterial DNA and CpG ODN (Fig. 1, A–D), but not by vertebrate DNA or PBS (Fig. 1E). In addition, we used another ODN, ODN 2006, containing unmethylated CpG se-
quences. In this case, 9 of 10 mice injected intracisternally with ODN 2006 developed meningitis. To exclude possibility of LPS contamination in DNA preparations, we injected intracisternally both types of bacterial DNA and CpG ODN to LPS nonresponder C3H/HeJ mice. There was no difference in the incidence or score of inflammatory cell infiltrate induced after the injection of bac-
terial DNA and CpG ODN in LPS-resistant strain C3H/HeJ mice compared with congeneric LPS-respondent strain C3H/HeN mice. In contrast, intracisternal injection of LPS induced meningitis in C3H/HeN, but not in C3H/HeJ mice (Fig. 2A). To further rule out other bacterial contaminants in our DNA, the purified DNA was digested with DNase I (Sigma). Intact bacterial DNA induced meningitis, while DNase-exposed bacterial DNA did not (Fig. 2B).

These data indicate that induction of meningitis was due to bac-
terial DNA and CpG ODN rather than to LPS or other contami-
ants. We used CpG ODN (ODN 1668) as representative of bac-
terial DNA in all the following experiments unless noted.

To further evaluate meningitis triggered by bacterial DNA, we assed potential changes of blood-brain permeability by measur-
ing influx of systemically injected Evans blue to CSF and total and differential leukocyte counts in CSF in SD rats exposed to CpG ODN. Rats rather than mice were used in this experiment since CSF from the former species can be more easily collected. Table I shows that blood-brain permeability was markedly increased in CSF from rats injected with CpG ODN compared with control rats. In addition, CpG ODN-triggered meningitis was characterized by an increase of mononuclear cells in CSF (Table I).

A requirement for the induction of meningitis is that the bacte-
rial DNA should be deposited locally in the cisterna rather than

Table I. Mean CSF leukocyte counts and CSF Evans blue concentration and score of inflammatory cell infiltrate in SD rats injected intracisternally with ODN 1668 (12 µg) or PBS

<table>
<thead>
<tr>
<th>CSF index</th>
<th>ODN 1668</th>
<th>PBS</th>
<th>ODN 2006</th>
<th>ODN 2041</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytes (total) (10³/ml)</td>
<td>603 ± 292*</td>
<td>3 ± 5</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Polymorphonuclear</td>
<td>4 ± 3</td>
<td>0</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Mononuclear cells</td>
<td>599 ± 293*</td>
<td>3 ± 5</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Score of inflammatory cell infiltrate in brain tissue</td>
<td>2.25 ± 0.5*</td>
<td>0 ± 0</td>
<td>1.4 ± 0.7</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

| Evans blue (µg/ml) | 7.3 ± 2.5* | 0.08 ± 0.04 | NA | NA |

* CSF was collected from SD rats 3 days after injection with ODN 1668. n = 4 per group for CSF leukocyte counts. Evans blue applied i.v. was quantified in CSF by spectrophotometry at 650 nm and used as a measure of blood-brain integrity. Evans blue experimental group (n = 3) and control group (n = 2). C57BL/6 mice injected intracisternally with ODN 2006 (6 µg/mice, n = 10) and with ODN 2041 (6 µg/mice, n = 5). % p < 0.05 vs PBS controls. The score of infiltration in the subarachnoid space was scored by a predetermined scheme: 0 = no infiltration; 1 = occasional occurrence of inflammatory cells; 2 = inflammatory cells forming an infiltrate not involving the entire depth of the subarachnoid space; 3 = inflammatory infiltrate involving the entire subarachnoid space, NA, not analyzed.
administered systemically. Indeed, i.p. injection of mice with either 10 nmol (i.e., 60 μg) or 20 nmol (i.e., 120 μg) CpG ODN did not produce histological evidence of meningitis. In contrast, the CpG ODN given intracisternally at a dose of 1 or 10 nmol (6 or 60 μg) gave rise to meningitis in close to 100% of mice (data not shown).

We found that 1 nmol (6 μg) CpG ODN was the lowest dose for triggering meningitis (data not shown). The score of inflammatory cell infiltrate was dose dependent (Fig. 1, B–D). Bacterial DNA- and CpG ODN-triggered meningitis was inducible in six mouse strains, including NMRI, BALB/c, C57BL/6, C3H/HeN, C3H/HeJ, and CB17, as well as SD rats (data not shown). Phosphorothioate-modified CpG ODN induced meningitis 3 days after inoculation, and did so at a magnitude higher than that of phosphodiester CpG ODN (data not shown).

Meningitis as analyzed by histopathology appeared within 12 h and lasted for at least 14 days. The maximal incidence and score of inflammatory cell infiltrate were noted on day 3 after injection of CpG ODN (Fig. 2C). There was hypertrophy of the leptomeninges, and infiltrating leukocytes were found in the meningeal

FIGURE 3. Cellular requirement for induction of CpG ODN-triggered meningitis. A–D, Immunohistochemical analysis of brain, showing meningeal infiltration of cells expressing Mac-1 (brown)-positive cells (A), absence of CD4 (B), CD8 (C) staining, and high frequency of Mac-3⁺ cells (D) in meninges and around blood vessels of a mouse killed 3 days after intracisternal inoculation with ODN 1668 (6 μg). E and F, Incidence and score of inflammatory cell infiltrate (severity) in BALB/c mice depleted of neutrophils using mAb RB6-8C5 (n = 10 per group), C57BL/6 mice depleted of NK cells using mAb PK136 (n = 10 per group), SCID mice deficient in T and B cells and congenic CB17 control mice (n = 11 per group), and C57BL/6 mice depleted of macrophages using etoposide treatment by s.c. injection or intracisternal injection (i.c.) (n = 10 per group). All values are expressed as mean ± SD. All the mice were killed 3 days after intracisternal inoculation of 6 μg ODN 1668.
lining cell layer as well as around blood vessels. This meningeal infiltration was apparent around the basal cisterns, on the cortical surface, and in the longitudinal fissure of the cerebral hemispheres, with higher density around blood vessels (Fig. 1A–D).

Which property of bacterial DNA is responsible for induction of meningitis induced by bacterial DNA?

To answer this question, one needs to consider the structure of bacterial DNA and eukaryotic DNA. Unmethylated Cpg motifs are present at the expected frequency of one per sixteen dinucleotides in the bacterial DNA, but are underrepresented (Cpg suppression) and predominantly methylated in eukaryotic DNA (24). This structural difference seems to explain the finding that bacterial DNA and certain synthetic oligonucleotides containing unmethylated Cpg dinucleotides activate immune cells (9, 10, 12). To determine whether the methylation of Cpg dinucleotides protects against the induction of meningitis, we used oligonucleotides containing either an embedded unmethylated Cpg dinucleotide (1668), a methylated Cpg dinucleotide of the same sequence (1668 m), or a Cpg inverted to nonmethylated Cpg dinucleotide (1720) in mice and SD rat. Only ODN 1668 was able to cause meningitis (Figs. 1, B, F, and G, and 2B). In agreement, only in minority of cases (one of four mice) methylated ODN 2006 triggered meningitis. Thus, oligonucleotides containing unmethylated Cpg dinucleotides are responsible for the induction of meningitis.

The role of different immunocompetent cells in meningitis induced by CpG ODN

To study the role of different immune cells in meningitis induced by CpG ODN, we first checked which immune cells participated in meningitis using immunohistochemistry. Immunohistochemical analysis of mouse brain injected with CpG ODN demonstrated that there was abundance of Mac-1+ and Mac-3+ mononuclear cells at all stages of meningitis, but lack of T lymphocytes (Fig. 3, A–D). These data suggest that cells of monocyte/macrophage lineage may exert a role in development of this condition.

We treated mice i.p. with a cytotoxic drug (etoposide) known to selectively deplete monocyte/macrophage population to assess the role of this cell lineage in CpG ODN-triggered meningitis (25). Depletion of monocytes almost completely abrogated CpG ODN-triggered meningitis (Fig. 3, E and F). Also, simultaneous inoculation of CpG ODN and etoposide into cerebral ventricles inhibited development of meningitis (Fig. 3, E and F). These results strongly indicate that the monocyte/macrophage lineage is responsible for the induction of CpG ODN-triggered meningitis.

To decide the role of other immune cells such as neutrophils, NK cell, and T and B cells, we depleted neutrophil and NK cells in the mice and also used SCID mice. As shown in Fig. 3, E and F, the incidence and score of inflammatory cell infiltrate were not significantly different between neutrophil or NK cell-depleted mice or SCID mice and their control mice. They reflect that neutrophil, NK cells, and T and B cells do not affect development of meningitis induced by CpG ODN. Taken together, these studies provided strong evidence for the role of macrophages in initiation and development of meningitis triggered by CpG ODN.

The role of NF-κB in meningitis induced by CpG ODN

What factor controls and regulates the activity of macrophages in CpG ODN-triggered meningitis? To answer this question, we investigated the role of NF-κB, a major intracellular factor controlling and regulating gene expression of proinflammatory cytokines at the transcriptional level (26, 27). Importantly, previous studies have shown that bacterial DNA and CpG ODN induce NF-κB activation in macrophages (12). For this purpose, we locally administered antisense phosphorothioate oligonucleotides targeted against the translation initiation site of the p65 subunit of NF-κB, which has previously been shown to abrogate experimental colitis in mice (22). Phosphorothioate oligonucleotides were administered to mice either as a single i.p. injection or as a single intracisternal injection. We found that a single i.p. injection of p65 antisense oligonucleotide (60 or 120 μg) did not decrease the incidence and score of the inflammatory cell infiltrate (data not shown). In contrast, a single intracisternal inoculation of the p65 antisense oligonucleotide (6 μg) significantly decreased the incidence and score of the inflammatory cell infiltrate of CpG ODN-triggered meningitis (Table II). As expected, significant changes of incidence and score of inflammatory cell infiltrate were not observed in mice treated with control oligonucleotides (6 μg p65 sense and nonsense). However, there was an obvious toxicity resulting in an increased mortality when the dose of p65 antisense was higher than 6 μg (data not shown). Notably, mRNA expression of TNF-α and IL-1β was significantly reduced in brains of mice treated with p65 antisense (Fig. 4G). Also, in vitro antisense-mediated down-regulation of p65 expression in CpG ODN-stimulated mononuclear cells was accompanied by significantly reduced secretion of TNF-α. This effect was absent using various control oligonucleotides (data not shown). Taken together, the above evidence implies that NF-κB p65 is a major transcriptional regulator of expression of CpG ODN-triggered meningitis.

The role of cytokines, chemokines, and complement in meningitis induced by CpG ODN

To analyze how macrophages trigger meningitis, we studied effect of products released from macrophages that might be important in induction of meningitis. For this purpose, we first measured mRNA expression for cytokines in mouse brain using in situ hybridization. Fig. 4, A and B, shows that mRNA expression for the cytokines TNF-α, IL-1β, IL-12, and MCP-1 was increased in brains exposed to CpG ODN compared with brains exposed to calf thymus DNA or PBS.

As TNF-α, a cytokine mainly produced by activated monocytes/macrophages, is one of major mediators of bacterial meningitis (7, 28, 29), and bacterial DNA and CpG ODN is known to stimulate macrophages to release TNF-α (12), we assessed its impact on the course of meningitis. We collected CSF samples from SD rats injected with CpG ODN and analyzed TNF-α in CSF by ELISA. TNF-α levels in CSF were clearly elevated in rats injected with CpG ODN compared with un.injected controls (Fig. 4C). In contrast, only background levels of TNF-α were found in CSF from control rats inoculated with calf thymus DNA or with PBS (data not shown). In addition, intracisternal administration of TNF-α

### Table II. Impact of local p65 antisense treatment on meningitis induced by CpG ODN

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>No. of Mice</th>
<th>Incidence of Meningitis (%)</th>
<th>Score of Inflammatory Cell Infiltrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>ODN 1668</td>
<td>4</td>
<td>100</td>
<td>2.8 ± 0.5</td>
</tr>
<tr>
<td>1668 + antisense 1</td>
<td>9</td>
<td>22</td>
<td>0.2 ± 0.4</td>
</tr>
<tr>
<td>1668 + antisense 2</td>
<td>8</td>
<td>50</td>
<td>0.6 ± 0.7</td>
</tr>
<tr>
<td>1668 + sense</td>
<td>3</td>
<td>100</td>
<td>2.3 ± 0.6</td>
</tr>
<tr>
<td>1668 + nonsense</td>
<td>3</td>
<td>100</td>
<td>2.3 ± 0.6</td>
</tr>
<tr>
<td>Antisense 1 alone</td>
<td>4</td>
<td>0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Antisense 2 alone</td>
<td>4</td>
<td>0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>p65 sense alone</td>
<td>3</td>
<td>33</td>
<td>0.7 ± 1.2</td>
</tr>
<tr>
<td>Nonsense alone</td>
<td>3</td>
<td>0</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

*Incidence and score of inflammatory cell infiltrate in C57BL/6 mice treated with intracisternal injection of p65 antisense of NF-κB sense, nonsense (all 6 μg/mouse). After 3 days, the mice were killed, and brains were examined by histopathology.
FIGURE 4. The role of TNF-α in development of meningitis. A, Cytokine and chemokine mRNA expression in C57BL/6 mice 3 days after intracisternal inoculation with 6 μg CpG ODN per mouse. Values are the mean ± SEM of number of positive cells/cm² (n = 5 mice per group). CT-DNA, calf thymus DNA. B, In situ hybridization of inflamed meningeal tissue showing TNF-α mRNA expression 3 days after inoculation of ODN 1668. Magnification ×40. C, Since CSF from rats can be more reproducibly harvested than from mice, rats were used for collection of CSF and measured for TNF-α levels. CSF TNF-α levels in SD rat on days 0, 3, and 5 after intracisternal inoculation with ODN 1668 (12 μg) (n = 4 per group). D and E, Incidence and score of inflammatory cell infiltrate (severity) in C57BL/6 mice, treated with α-MSH given intracisternally (10 or 20 μg) or i.p. (50 μg), 3 days after inoculation with ODN 1668 (6 μg) (n = 8 per group). Values are the mean ± SD score of inflammatory cell infiltrate score. F, α-MSH inhibits TNF-α release from mononuclear cells stimulated by ODN 1668 (n = 4 per group). G, Effects of p65 antisense 1 treatment on brain cytokine mRNA expression of TNF-α and
and B, incidence of meningitis and the score of inflammatory cell infiltrate were only marginally affected in IL-12 knockout mice compared with control congenic mice.

Next, we analyzed the effect of complement because the complement anaphylatoxins C5a and C3a are powerful chemoattractants that recruit macrophages and polymorphonuclear cells into the inflammatory site. In addition, monocytes/macrophages can produce almost all of the complement components in a functionally active form, and bear CR1 and CR3 (31–33). To assess the role of complement cascade in CpG ODN-triggered meningitis, the complement system was depleted using CVF. In another set of experiments, CR1 was blocked using rat anti-mouse CR1 Ab (8C12). Results from these experiments revealed that the complement system does not participate in development of meningitis because the incidence and score of inflammatory cell infiltrate were not significantly different between depleted and control mice (Fig. 5, A and B).

The role of NO in meningitis induced by CpG ODN

Since NO plays a detrimental role in bacterial meningitis (34), we decided to assess its role in CpG ODN-triggered meningitis. To ascertain whether NO elicits neurotoxicity in this condition, we administered inhibitors of NOS. TNF-α and IL-1β are potent inducers of inducible NOS (iNOS) (35). In our study, iNOS was inhibited using l-NMMA or l-NAME, injected i.p. (Fig. 5, A and B), shows that the incidence and score of inflammatory cell infiltrate were markedly down-regulated in mice treated with l-NMMA or l-NAME compared with control mice. These observations suggest a critical role of NO in development of CpG ODN-triggered meningitis.

The role of selectins in meningitis induced by CpG ODN

How do monocytes migrate to the subarachnoid space from blood vessels? To understand this process, we analyzed the role of selectins since these molecules mediate leukocyte rolling, and thus control early steps of extravasation of leukocytes during inflammation (36). To this end, we used fucoidin, which has the ability to block P- and L-selectins (19, 37, 38). Mice were treated with fucoidin either i.v. or s.c. Histopathological results 24 h after inoculation of CpG ODN demonstrated that the score of inflammatory cell infiltrate and incidence of meningitis were markedly reduced in mice treated with fucoidin compared with control mice (Fig. 6, A and B), indicating that selectins are instrumental in development of meningitis.

Synergy of bacterial DNA with LPS and PGN

Since LPS from Gram-negative bacteria and PGN from Gram-positive bacteria and bacterial DNA stimulate macrophages by different signal pathways (9, 39, 40), and any of these compounds may be present during bacterial meningitis, we wished to analyze whether LPS or PGN together with CpG ODN act synergistically in inducing meningitis. There were an intermediate incidence and score of inflammatory cell infiltrate when suboptimal amounts of either LPS or PGN were injected intracisternally. In contrast, a combination of a suboptimal dose of CpG ODN together with above doses of either LPS or PGN led to a high incidence and score of inflammatory cell infiltrate (Table III).

FIGURE 5. The role of macrophage products in development of meningitis. A and B, Incidence and score of inflammatory cell infiltrate (severity) in IL-12 knockout mice (n = 10 per group), depleted of complement using CVF treatment, neutralized CR1 using CR1-specific mAbs (n = 9 per group), inhibited iNOS using l-NMMA or l-NAME (n = 10 per group). All mice were killed 3 days after inoculation with ODN 1668 (6 µg). Values are the mean ± SD score of inflammatory cell infiltrate.

(0.2 ng; Genzyme, Cambridge, MA) in mice triggered meningitis (data not shown), further confirming role of this cytokine for the induction of brain inflammation. Furthermore, we used α-MSH, which can abrogate the effects of TNF-α-mediated brain inflammation (30). The incidence and score of inflammatory cell infiltrate of CpG ODN-triggered meningitis were significantly lower in mice treated with α-MSH than in control mice (Fig. 4, D and E). In addition, in vitro experiments displayed that the level of TNF-α was significantly decreased in supernatants from CpG ODN-stimulated mononuclear cells containing α-MSH compared with control supernatants (Fig. 4F). Collectively, these results indicate that TNF-α is operational in CpG ODN-triggered meningitis.

To study the role of IL-12 in induction of meningitis, we used IL-12 knockout mice to determine whether this cytokine is required for induction of meningitis. However, as shown in Fig. 5, A
Discussion

Our results indicate that intracisternally deposited bacterial DNA in general and unmethylated CpG oligonucleotides in particular trigger meningitis. This condition was not only assessed as inflammatory infiltrates in the meningeal tissue, but also proved to trigger pleocytosis and increased permeability of blood-brain barrier. Meningitis was induced within 12 h after intracisternal injection and lasted for at least 14 days. The maximal incidence and score of the inflammatory cell infiltrate were noted on day 3 after the injection of bacterial DNA or CpG ODN. Unmethylated CpG dinucleotides were responsible for induction of meningitis triggered by bacterial DNA. The magnitude of meningeal inflammation triggered by CpG ODN may be synergistically enhanced by the inflammatory response to LPS and PGN. Meningitis following injection of bacterial DNA was not due to surgical trauma or non-DNA bacterial contamination, since intracisternal inoculation of PBS, calf thymus DNA, or ODN lacking unmethylated CpG motifs did not trigger meningitis. In addition, bacterial DNA and CpG ODN triggered meningitis equally well both in LPS nonresponder and in LPS-sensitive mice. Finally, destruction of bacterial DNA using DNase I treatment abolished the induction of meningitis. These studies suggest that unmethylated CpG containing bacterial DNA may be a potent trigger of inflammatory cell infiltration in meningitis.

Macrophages play an important role in the pathogenesis of experimental bacterial (41, 42) and human meningitis (43), such as tuberculous meningitis. In children, tuberculous meningitis is associated with <50% mortality; most of the survivors have permanent neurologic sequelae and experience considerable disability. There are several lines of evidence that support the importance of macrophages also in CpG ODN-mediated meningitis. First, histochemical analysis of brain tissue demonstrated that meningitis was characterized by influx of mononuclear, Mac-3+ cells. More importantly, our experiments demonstrated that absence of neutrophils, NK cells, and T/B cells did not affect development of meningitis. In contrast, depletion of monocytes totally abrogated development of inflammation. Third, leukocyte differential count demonstrated that mononuclear cells predominated in CSF. Fourth, the pattern of mRNA expression of cytokines in the inflamed brain was indicative of macrophage products, such as the expression of TNF-α. In addition, previous study has demonstrated that phosphodiester and phosphorothioate ODN predominantly bind to the surface of leukocytes expressing Mac-1+ and, this binding inhibits migration of polymorphonuclear cells (44).

What are the mechanisms of bacterial DNA-mediated meningitis? Previous studies showed that bacterial DNA and CpG ODN directly activate macrophages (12). The first step of activation encompasses uptake of bacterial DNA or synthetic oligonucleotides by macrophages in a saturable, sequence-independent, temperature- and energy-dependent manner (45, 46) into an acidified intracellular compartment, in which DNA becomes degraded to ODN (47). Once there, unmethylated CpG dinucleotides activate within minutes the stress-kinase/jun pathway, yielding NF-κB (48). This transcription factor controls mRNA expression of a variety of cytokines and secretion of proinflammatory cytokines, such as TNF-α, IL-1β, IL-6, as well as MCP-1 (26, 49). TNF-α and IL-1β initiate meningeal inflammation (6, 7), elicit selectin expression on the endothelium (50), and promote synthesis of NO (35), as well as activate NF-κB (51, 52).

TNF-α is mainly released from monocyte and macrophage, and acts as a mediator of inflammation (53). It causes endothelial cell activation, up-regulates expression of adhesion molecules; and stimulates macrophage production of IL-1 and IL-6 (53). IL-1 and TNF-α display synergistic actions and stimulate the release of each other, thereby amplifying the cascade of other inflammatory mediators (54). A high level of TNF-α and IL-1 is found in the CSF of patients suffering from bacterial meningitis and experimental bacterial meningitis (55). Also, in case of CpG ODN-triggered meningitis, the levels of intrathecal TNF-α were clearly elevated. It has been shown that murine tTNF-α is able to induce meningitis (7, 56). Indeed, neutralization of TNF-α can attenuate bacterial meningitis (55). Inhibition of TNF-α release by α-MSH decreased the incidence and score of inflammatory cell infiltrate triggered by

### Table III. CpG ODN acts synergistically with LPS and PGN in the induction of meningitis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of Mice</th>
<th>Incidence of Meningitis (%)</th>
<th>Score of Inflammatory Cell Infiltrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>5</td>
<td>0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>LPS</td>
<td>5</td>
<td>60</td>
<td>0.8 ± 0.8</td>
</tr>
<tr>
<td>PGN</td>
<td>4</td>
<td>25</td>
<td>0.5 ± 1.0</td>
</tr>
<tr>
<td>1668</td>
<td>5</td>
<td>80</td>
<td>0.8 ± 0.5</td>
</tr>
<tr>
<td>LPS + 1668</td>
<td>6</td>
<td>100</td>
<td>2.7 ± 0.5</td>
</tr>
<tr>
<td>PGN + 1668</td>
<td>6</td>
<td>100</td>
<td>1.5 ± 0.8</td>
</tr>
</tbody>
</table>

*C57BL/6 mice were injected intracisternally with LPS (0.1 μg), PGN (1 μg), ODN 1668 (0.6 μg), a combination of LPS (0.1 μg) and ODN 1668 (0.6 μg), or a combination of PGN (1 μg) and ODN 1668 (0.6 μg). After 3 days, the mice were killed, and brains were examined by histopathology.*
CpG ODN. It has been demonstrated that TNF-α can initiate meningeval inflammation (7), resulting in breach of the blood-brain barrier leading to brain edema and increased intracranial pressure (2); and it plays a critical role in neuronal apoptosis in the hippocampus (57).

Nitrite levels are usually significantly elevated in CSF of patients with bacterial meningitis and in experimental meningitis (34, 58). Inhibition of iNOS has been shown to attenuate alteration of the blood-brain barrier permeability and meningeal inflammation (58). As TNF-α is a crucial mediator in meningitis triggered by CpG ODN, it was an expected finding that iNOS inhibition blocked the development of CpG ODN-triggered meningitis.

Selectins are cell surface glycoproteins that mediate the initial adhesion and rolling, a transient contact leading to a deceleration of leukocytes along the vascular endothelium. This is the first and essential step in the process of leukocyte extravasation into inflamed sites (31, 36). In bacterial meningitis, treatment with L- and P-selectin-binding fucoidin (37, 38) has been shown to attenuate meningeal inflammation, abolish pleocytosis, and inhibit the release of TNF-α and IL-1 in a rabbit model of meningitis induced by pneumococcal cell wall fragments (19, 59). The fact that fucoidin did not completely abrogate meningitis induced by CpG ODN indicates that E-selectin might also be required for development of the meningitis. Indeed, in TNF-α-induced meningitis, E-selectin has been shown to participate in the development of meningitis since CSF leukocyte influx and permeability were partially inhibited in P-selectin-deficient mice, and near complete inhibition of these parameters was displayed in mice being double deficient with respect to P- and E-selectin (56).

One of the major control mechanisms of gene expression occurs at the transcriptional level, and all of these proinflammatory cytokines have been shown to be regulated by the NF-κB via binding of NF-κB family members to their individual promoters (27, 60). CpG ODN and bacterial DNA cause nuclear translocation of the transcription factor NF-κB, including p65 and p50 (12). Ordinarily, NF-κB is sequestered in the cytoplasm by the inhibitory protein I-κB, and controls the promoter activity of gene of proinflammatory cytokines in macrophages (26, 27). TNF-α and IL-1β have the ability to mediate NF-κB activity in cells of the blood-brain barrier in response to localized inflammation (51, 52). Our data that local administration of p65 antisense to NF-κB abolished meningitis triggered by CpG ODN show a predominant role of the p65 subunit of NF-κB in this condition. This antisense effect was previously shown to be highly specific. The most likely mechanism by which p65 antisense oligonucleotides influence CpG ODN-induced meningitis is by reduced local production of cytokines. This is supported by in vitro experiments demonstrating that mononuclear cells exposed to CpG ODN produced less TNF-α if the culture contained p65 antisense, and in vivo experiments demonstrating that mRNA expression of TNF-α and IL-1β was significantly reduced in brain of mice treated with p65 antisense. In further support of this suggestion, it has been shown that local administration of antisense to the p65 subunit of NF-κB abrogated established experimental colitis in mice (22). Overall, these data suggest that NF-κB plays a regulatory role in CpG ODN-triggered inflammatory disorders such as meningitis, possibly providing an attractive target for future therapeutic interventions in bacterial meningitis.

Our observations provide the first evidence that bacterial DNA containing unmethylated CpG motifs induces meningitis, and suggest an important pathogenic role for bacterial DNA in bacterial meningitis. Macrophages and their products such as TNF-α exert an important role in development of inflammatory disease triggered by bacterial DNA; this type of inflammation can be regulated by p65 subunit of NF-κB. These findings may have implications for the treatment of bacterial meningitis. Thus, eradication of bacteria using antibiotics might not be an efficient enough procedure to clear the disease since released bacterial DNA from resident bacteria is proinflammatory, and in concert with LPS or PGN might promote a continuous inflammatory response. Indeed, anti-inflammatory treatment in conjunction with antibiotics has been shown to reduce inflammation and its clinical sequelae in both experimental and clinical studies (4, 61, 62). Future treatment strategies should include attempts to minimize bacterial growth together with blockade of the proinflammatory effects of bacterial DNA.

References
22. Neurath, M. F., S. Pettersson, K. H. Meyer zum Buschenfelde, and W. Strober. 1996. Local administration of antisense phosphorothioate oligonucleotides to the


