**Stimulation of Toll-Like Receptor 9 by Chronic Intraventricular Unmethylated Cytosine-Guanine DNA Infusion Causes Neuroinflammation and Impaired Spatial Memory**

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**Abstract**

Bacterial DNA contains a high frequency of unmethylated cytosine-guanine (CpG) motifs that have strong immunostimulatory properties; they are recognized by mammalian Toll-like receptor 9 (TLR9). Because accumulating data suggest that chronic inflammatory processes are involved in the pathogenesis of neurodegenerative diseases, we hypothesized that inflammatory responses stimulated by CpG DNA might contribute to neurodegeneration and brain dysfunction. To assess the effects of continuous CpG DNA exposure in the brain, C57BL/6 (n = 21) and TLR9-deficient mice (n = 15) were given intracerebroventricular infusions of CpG DNA or saline for 28 days. Spatial memory assessed weekly by Morris water maze demonstrated impairment in CpG-treated wild-type mice but not in TLR9-deficient or control-treated mice. Motor function was not affected. Immunohistochemical analysis revealed marked microglial activation and acute axonal damage surrounding the ventricles, ependymal disruption, and reactive astrogliosis within the hippocampal formation in the CpG-treated wild-type but not TLR9-deficient mice or saline-infused controls. These results suggest that the unfavorable effects of CpG DNA are dependent on TLR9 signaling and that exposure to bacterial DNA may contribute to impaired neural function, neuroinflammation, and subsequent neurodegeneration.

**Key Words:** Bacterial infection, CpG DNA, Innate immunity, Memory, Neurodegeneration, Neuroinflammation, TLR9.

**INTRODUCTION**

Accumulating data suggest that chronic inflammatory processes contribute to the pathogenesis and progression of neurodegenerative diseases such as Alzheimer disease (AD) and Parkinson disease (1–3). Microglia, the resident innate immune cells of the brain, may be involved in pathogenic chronic inflammatory processes because activated microglia can aggravate inflammation, become phagocytic, and cause neuronal degeneration (4–8). Infections (e.g. by bacteria) might contribute to a chronic state of inflammation because once pathological inflammation is initiated, it can persist in the central nervous system (CNS) without further stimulation by peripheral inflammatory mediators (9).

Unlike mammalian DNA, bacterial DNA is characterized by a high frequency of unmethylated cytosine-guanine (CpG) motifs that have strongly immunostimulatory properties. Cytosine-guanine DNA enters TLR9-expressing immune cells (e.g. dendritic cells and B cells) by endocytosis and binds to its receptor localized at the endoplasmic reticulum. A myeloid differentiation protein 88 (MyD88)-dependent pathway is initiated that finally induces the activation of nuclear factor-κB or mitogen-activated protein kinase pathways. This process is followed by an increased innate immune response with release of proinflammatory cytokines and chemokines that induce crosstalk with TLR1-type cells and natural killer cells (12–14). Murine microglial cells express TLR9 and respond to CpG DNA by the synthesis of nitric oxide (NO) (4). Previous studies suggest the therapeutic use of CpG-containing oligonucleotides that act as TLR9 ligands as vaccines or antiallergic or cancer adjuvants, but little is known about the effects of repeated or continuous CpG DNA exposure on the brain.

This study was designed to investigate whether long-term intraventricular infusion of CpG DNA leads to neuropathologic alterations in the brain such as activation of microglia and whether certain brain regions are more vulnerable to CpG DNA stimulation than others. Motor and memory performances were assessed to evaluate whether such changes are of functional significance. Finally, to see if the hypothesized unfavorable effects are dependent on TLR9 signaling, mice deficient for the CpG DNA receptor were also exposed to long-term intraventricular CpG DNA infusion.

**MATERIALS AND METHODS**

**Experimental Design**

Male C57BL/6 mice (n = 22, weighing 19–23 g, aged 2–3 months) and male TLR9-deficient mice (n = 16) were used for chronic infusion of either 0.9% NaCl or CpG DNA.
The TLR9-deficient mice were kindly provided by H. Wagner, Institute for Medical Microbiology, Immunology and Hygiene, Munich, Germany, and backcrossed on the C57BL/6j background for at least 10 generations. The TLR9-deficient mice were originally created by S. Akira, Osaka University (10). The 4 treatment groups were composed as follows: 1) infusion of CpG DNA (CpG oligodeoxynucleotide 1668 TCC ATG ACG TTC CTG ATG CT; TIB Molbiol, Berlin, Germany) in C57BL/6j mice (n = 10); 2) control group of C57BL/6j mice with 0.9% NaCl infusion (n = 11); 3) infusion of CpG DNA in TLR9-deficient mice (n = 8); and 4) control group of TLR9-deficient mice with 0.9% NaCl infusion (n = 7). In addition, another set of C57BL/6j mice received either CpG (n = 6) or 0.9% NaCl (n = 5) infusion for 1 week.

All mice were anesthetized with 150 mL of 7% chloral hydrate (Sigma-Aldrich, St Louis, MO) intraperitoneally and positioned in a stereotaxic frame (Stoelting, Wood Dale, IL) with an adaptor for mice. The scalp was incised along the midline from ear to ear, and the skull was exposed and carefully disinfected. The cannula of the brain infusion kit II (Alzet, Cupertino, CA) was aseptically implanted into the right lateral ventricle according to the following coordinates: −0.096 mm mediolaterally and −0.022 mm dorsoventrally relative to the bregma. The infusion system was connected to a mini osmotic pump (Model 2004, Alzet), which was placed subcutaneously between the scapulae. Mice were infused with a rate of 1 μg/d CpG DNA for 4 weeks. One C57BL/6j mouse died because of complications during the operation, and 1 TLR9-deficient mouse died because of unknown reasons 2 weeks after implantation of the pump; both were excluded from further analysis. Assessment of motor and memory function was performed as described later. After 35 days, all animals were deeply anesthetized, blood samples were taken and perfused with 4% formalin, and the brains were removed for immunohistochemical analysis. Animal experiments were approved by the Animal Care Committee of the University Hospital of Göttingen and by the District Government of Braunschweig, Lower Saxony, Germany.

**Motor Performance**

Once a week, motor performance of mice was evaluated by the tight rope test. Briefly, mice were placed in the middle of a tight rope, the time to reach the platform at the end of the rope was measured, and a score was calculated according to the needed time and whether the animal reached the platform (15, 16). In addition, motor performance was investigated once weekly by rotarod testing. A rotarod treadmill (Neuroscience Inc, Tokyo, Japan) was used to measure motor disability such as loss of balance, coordination, and motor control. Mice were placed on the cylinder at a slow rotational speed that was slowly increased from 4 to 40 rpm within a period of 5 minutes. The time the animals remained on the rotarod was recorded automatically. If the mouse remained on the rod for 5 minutes, the test was completed and scored as 300 seconds.

**Morris Water Maze**

The water maze task was performed in a white painted pool (diameter, 104 cm; height, 35 cm) containing water maintained at a temperature of 22°C. A transparent platform 10 cm in diameter was located 1 cm beneath the water surface. The pool was surrounded by several visual cues that were external to the maze. A video camera mounted to the ceiling above the pool was linked to a computer (TSE Systems, Bad Homburg, Germany), and the swim tracks and time required to escape from the water were recorded. Before implantation of the intraventricular pump, mice were trained to find the hidden platform within less than 90 seconds (18 trials for 3 days), and the latency, swim speed, and distance to find the submerged platform were evaluated. The platform remained in the same location throughout all tasks. Beginning 1 week after start of the intrathecal infusions, the water maze task was repeated once a week until the end of experiment.

**Primary Mouse Microglial Cell Culture**

Primary cultures of microglial cells were prepared from brains of newborn C57BL/6j and TLR9-deficient mice, as previously described (17). After removal of the meninges, cells were mechanically dissociated and suspended in Dulbecco modified Eagle medium with Glutamax I (Gibco Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were plated at a density of 2 brains per 75 cm² culture flask (Corning Costar GmbH, Wiesbaden, Germany) and incubated at 37°C in a humid atmosphere with 5% carbon dioxide. Culture medium was changed twice a week. After 10 to 14 days, the confluent mixed glial cultures were shaken 200× per minute for 30 minutes. Microglial cells in the supernatant were replated in 96-well cell culture plates at a density of 75,000 cells per well. After 3 hours, the enriched microglial cultures were exposed to the TLR4 agonist endotoxin (lipopolysaccharide [LPS] from Escherichia coli serotype 026:B6; Sigma, Taufkirchen, Germany) and the TLR9 agonist CpG (cytosine-guanosine oligodeoxynucleotide 1668; TCC ATG ACG TTC CTG ATG CT; TIB Molbiol) for 24 hours in the presence of interferon-γ (100 U/mL; Sigma). Control cultures were treated with interferon-γ only. To assess microglial activation, NO release of microglial cells was quantified by measurement of nitrite (one of its stable reaction products) in the cell culture supernatant. For this purpose, 100 μL of supernatant were mixed with 100 μL Griess reagent (equal volumes of 1% sulfanilamide in 30% acetic acid and 0.1% N-[1-naphthyl] ethylenediamine in 60% acetic acid) in a 96-well plate. After 10 minutes, the optical density at 570 nm was measured with a Genios multiplate reader (Tecan, Crailsheim, Germany). Concentrations were calculated by comparison of absorptions with a standard curve.

**Measurement of Interleukin-1β in Plasma and Cerebellum**

Frozen cerebelli were homogenized in 1 mL lysis buffer containing a protease inhibitor cocktail (The Genetics Company, Schlieren, Switzerland). After incubation for 2 hours at 4°C, homogenates were centrifuged at 18,000 × g for 10 minutes, and supernatants were stored at −80°C. Interleukin-1 β (IL-1β) concentrations in supernatants of cerebellar homogenates and in plasma were measured using
the Quantikine Mouse IL-1β/IL-1F2 Immunoassay (R&D Systems, Wiesbaden, Germany) according to the manufacturer’s instructions.

**Immunohistochemistry**

Formalin-fixed and paraffin-embedded 1-µm brain sections were examined. Briefly, deparaffinized sections were pretreated with 5 × 3 minutes of microwaving in citric acid buffer, 10 mmol/L, pH 6.0. After blocking with 10% FCS/PBS, primary antibodies were applied at the concentrations indicated and permitted to bind overnight at 4°C (for calretinin) or for 90 minutes at room temperature (for doublecortin, amyloid precursor protein [APP], isolectin B4, glial fibrillary acidic protein [GFAP]). Neuronal differentiation was detected by polyclonal rabbit anticalretinin antibody (1:2000; Swant, Bellinzona, Switzerland) and polyclonal goat antidoublecortin (1:50; Santa Cruz Biotechnology, Santa Cruz, CA). Axonal injury was investigated with a monoclonal mouse antibody against APP (1:2000; Chemicon, Temecula, CA). Microglia/macrophages were identified with *Griffonia simplicifolia* isolectin B4 (1:40; Sigma-Aldrich) and astrocytes with polyclonal rabbit anti-GFAP antibody (1:1000; DAKO, Glostrup, Denmark). For light microscopy, sections were incubated with appropriate biotinylated secondary antibodies (Amersham, Buckinghamshire, UK) followed by avidin-peroxidase treatment (Sigma-Aldrich). Detection of APP was performed with the alkaline phosphatase-antialkaline phosphatase method. Diaminobenzidine was used as the chromogen (Roche, Mannheim, Germany) for immunohistochemistry. Control sections were incubated with appropriate biotinylated secondary antibodies (Amersham, Buckinghamshire, UK) followed by avidin-peroxidase treatment (Sigma-Aldrich). Detection of APP was performed with the alkaline phosphatase-antialkaline phosphatase method. Diaminobenzidine was used as the chromogen (Roche, Mannheim, Germany) for doublecortin, isolectin B4, and GFAP. The APP and calretinin were visualized with new fuchsin. Control sections were incubated with isotype control antibodies or in the absence of primary antibodies. Coronal brain sections were stained with hematoxylin and eosin for the detection of necrosis and for the evaluation of the ependyma.

**In Situ Tailing (Labeling of DNA Fragmentation)**

Deparaffinized and hydrated 1-µm sections were treated with 50 µg/mL proteinase K (Sigma-Aldrich) for 15 minutes at 37°C in a reaction mixture containing 10 µL of 5× tailing buffer, 1 µL digoxigenin DNA labeling mix, 2 µL cobalt chloride, 12.5 U terminal transferase, and the necessary amount of distilled water to give a volume of 50 µL. After washing, the sections were incubated with 10% FCS for 15 minutes at room temperature and then washed again. A solution of alkaline phosphatase–labeled antidigoxigenin antibody in 10% FCS (1:250) was placed on the sections for 60 minutes at 37°C. The color reaction (black) was developed with 4-nitroblue-tetrazolium-chloride/5-bromine-4-chloride-3-indolyl-phosphate. The sections were counterstained with nuclear fast-red-aluminum hydroxide (all reagents from Roche).

**Histopathology**

All assessments were performed by an independent blinded investigator using a 40× objective. Immunostaining was examined with an Olympus BX51 microscope (Olympus, Hamburg, Germany). For quantification of cell proliferation and differentiation within the dentate gyrus, only immunoreactive cells within the granule cell layer and subgranular zone of the dentate gyrus were counted. An Analysis Software Imaging System (microscope BX51; Olympus; software AnalySIS 3.2; Soft Imaging System GmbH, Münster, Germany) was used to measure the area of the dentate granule cell layer. The densities of immunolabeled cells were expressed as the number of marked cells per square millimeter of the area measured. The density of labeled cells was evaluated in 3 coronal sections from each mouse. In situ–tailed cells within the upper and lower blades of the dentate gyrus were only counted if additional criteria such as apoptotic morphology (cell shrinkage, fragmented and condensed nuclei) were observed at a 60× oil magnification.

**Statistical Analysis**

All values are expressed as median and interquartile range. For statistical comparison, the nonparametric Mann-Whitney U test was used. Data from APP staining was analyzed by the Fisher exact test. Nitric oxide release of microglia is expressed as mean ± SD, and data were compared by unpaired t-test. A value of p < 0.05 was considered statistically significant. All statistical analyses were conducted using Prism software (GraphPad Software 4.0, San Diego, CA).

**RESULTS**

**CpG DNA–Induced Neuropathologic Alterations After 4 Weeks**

**CpG DNA Induced Strong Microglial Activation Surrounding All Ventricles and the Hippocampal Formation in C57BL/6 Mice**

Activation of microglia was assessed by staining with isolectin B4, which recognizes α-β-galactose–containing glycoconjugates on the membranes of microglial cells (18). After intraventricular CpG DNA infusion, there was distinct accumulation of microglial cells along the borders of the ventricles, particularly on the CpG DNA-infused right side (Fig. 1A). Activated microglia were also observed in neighboring brain regions such as the hippocampal formation (Fig. 1B). These findings were observed in all CpG DNA-exposed C57BL/6 mice but to a much smaller extent in nearly all TLR9-deficient mice 4 weeks after CpG DNA infusion (Figs. 1C, D). Because these alterations did not develop in mice infused with saline (Figs. 1E, F), they were not attributable to mechanical injury or the presence of the infusion pumps. Immunohistochemistry with markers for B cells (B220) and T cells (CD3) showed that only few B and T cells were present along the ventricles, and these cells were not observed in the hippocampal formation (data not shown).

**Evidence for Axonal Damage After Prolonged Exposure to CpG DNA**

Amyloid precursor protein immunostaining was used as a marker for acute axonal damage. The APP undergoes anterograde axonal transport in neurons; it accumulates and persists in the proximal end of transected axons for less than 30 days (19–21). Cytosine-guanine DNA-exposed C57BL/6 mice had significantly more acute axonal damage...
FIGURE 1. Pronounced microglial activation along all ventricles after cytosine-guanine (CpG) exposure. (A, B) Staining is most prominent around the CpG DNA-infused right ventricle (A); accumulation of microglial cells invading into the neighboring hippocampal formation (B) detail of [A]). (C, D) Prolonged infusion of CpG DNA in a Toll-like receptor 9 (TLR9)-deficient mouse resulted in very mild microglial activation (C) that only partly damaged the ependyma of the right lateral ventricle (D) detail of [C]). (E, F) Overview (E) and detail of the right lateral ventricle (F) isoelectin B4 staining showing no substantial microglial infiltration in a C57BL/6 mouse 4 weeks after intraventricular 0.9% NaCl infusion. Scale bars = (A, C, E) 500 μm; (B, D, F) 50 μm.
than saline-treated animals ($p < 0.0001$). The damaged axons were predominantly in the vicinity of the right lateral ventricle (Fig. 2A), but they were occasionally seen in the vicinity of the contralateral and third ventricles and the contralateral hemisphere next to the hippocampal formation (Fig. 2B). The TLR9-deficient mice that had been exposed to CpG DNA also had significantly more axonal damage than the saline-treated controls ($p = 0.009$).

**CpG DNA Infusion Led to Damage of the Choroid Plexus and Ependyma**

Evaluation of the lateral and third ventricles by hematoxylin and eosin staining revealed pronounced damage of both the choroid plexus and the ependyma in CpG DNA-exposed C57BL/6 mice; the choroid plexus was completely lost in nearly all of these mice. There was an almost complete loss of cilia of the ependyma in the CpG-exposed mice in contrast to the fully ciliated ependyma of the control animals. Furthermore, the adjacent ependymal and the subependymal cells were severely affected; in some cases, swelling of ependymal cells was observed. These changes seemed to be necrotic rather than apoptotic because the ependymal and subependymal cells were not labeled by in situ tailing. The TLR9-deficient mice and all saline-treated controls did not display these alterations (Figs. 3A, B).

**No Detection of Neuropathologic Changes Typical for Alzheimer Disease**

Because chronic neuroinflammation is associated with neurodegenerative diseases, staining with markers typical for alterations observed in Alzheimer disease (i.e. thioflavin-S, Congo red, and Bielschowsky silver staining) was also performed (22, 23). No labeling by any of those methods was observed in any treatment group (data not shown).
CpG DNA-Induced Alterations on NO and IL-1 Release

CpG DNA Did Not Stimulate TLR9-Deficient Microglia

To quantify microglial activation upon treatment with the TLR4 agonist LPS and the TLR9 agonist CpG DNA, NO release was measured. Maximum NO release from both wild-type and TLR9-deficient microglial cells was achieved by treatment with 1 μg/mL LPS (10%). After CpG DNA treatment, microglial cells of wt mice (open bars) produced nitrite amounts similar to that after LPS stimulation, whereas the TLR9-deficient mice microglia (black bars) were not stimulated upon CpG DNA exposure.

CpG DNA-Induced Alterations in the Dentate Gyrus

Reactive Astrogliosis in the Dentate Gyrus of C57BL/6 Mice After Prolonged CpG DNA Infusion

Analysis of GFAP-immunoreactive cells in the dentate granule cell layer revealed significantly more astrocytes per square millimeter in C57BL/6 mice exposed to CpG DNA than in saline-treated controls (p = 0.0008). In contrast, TLR9-deficient mice did not display significant differences in the density of astrocytes between the groups (p = 0.46; Figs. 5A–C).

No Change in the Density of Young Neurons in the Dentate Gyrus After Exposure to CpG DNA

Neither C57BL/6 nor TLR9-deficient mice exposed to CpG DNA or 0.9% NaCl showed any significant differences in the density of cells expressing the early neuronal marker doublecortin within the subgranular and granule cell layer of the dentate gyrus (p = 0.92 and p = 0.61, respectively). The medians (25th/75th percentiles) for CpG DNA and controls in C57BL/6 mice were 173.0 (135.5/303.2)/mm² versus 135.5 (303.2/173.0) (p = 0.0008).

FIGURE 4. Nitrite concentrations as measures of nitric oxide release of primary microglial cells from Toll-like receptor 9 (TLR9)-deficient and wild-type (wt) mice after stimulation with lipopolysaccharide (LPS) and cytosine-guanine (CpG) DNA. Nitrite concentration in the supernatant is expressed as percentage after stimulation with 1 μg/mL LPS (100%). After CpG DNA treatment, microglial cells of wt mice (open bars) produced nitrite amounts similar to that after LPS stimulation, whereas the TLR9-deficient mice microglia (black bars) were not stimulated upon CpG DNA exposure.

FIGURE 5. (A–C) Astrocyte responses to cytosine-guanine (CpG) DNA. (A, B) There are more glial fibrillary acidic protein (GFAP)-expressing astrocytes in the dentate gyrus of a C57BL/6 mouse treated with CpG DNA (B) than in a saline-treated control (Co) mouse (A). Scale bar = 50 μm. (C) Comparison of densities of GFAP-labeled cells in the dentate gyrus of C57BL/6 mice and Toll-like receptor 9 (TLR9)-deficient mice after 4 weeks of intraventricular CpG DNA infusion. There was a significant increase of astrocytes in C57BL/6 animals after CpG DNA exposure (p = 0.0008). Cytosine-guanine DNA-stimulated TLR9-deficient mice did not display any significant difference compared with saline-treated wild-type mice (p = 0.46, median and interquartile range).
181.2 (143.3/213.7)/mm² and in TLR9-deficient mice were 170.3 (103.2/287.4)/mm² versus 149.9 (28.0/218.7)/mm². Similarly, the density of calretinin-positive neurons did not significantly differ among the 4 groups (medians [25th/75th percentiles]: 103.7 [52.8/176.8]/mm² vs 103.2 [71.5/170.5]/mm² and 46.6 [28.9/83.9]/mm² vs 37.4 [17.9/148.2]/mm² [p = 0.90 and p = 0.78], respectively).

No Change in the Density of Apoptotic Cells in the Granule Cell Layer of the Dentate Gyrus After Exposure to CpG DNA

Analysis of in situ–tailed cells with additional morphologic criteria of apoptosis within the dentate granule cell layer revealed no significant differences in the densities of apoptotic cells in both C57BL/6 and TLR9-deficient mice exposed to either CpG or 0.9% NaCl (medians [25th/75th percentiles]: 103.7 [52.8/176.8]/mm² vs 103.2 [71.5/170.5]/mm² and 46.6 [28.9/83.9]/mm² vs 37.4 [17.9/148.2]/mm² [p = 0.90 and p = 0.78], respectively).

Neuropathologic Alterations After 1 Week of CpG DNA Infusion

To gain insight into the dynamic of the neuropathologic process and to evaluate whether the observed changes after 4 weeks of CpG DNA exposure represent maximum or declining alterations, C57BL/6 mice were investigated after 1 week of CpG DNA or 0.9% NaCl infusion. At this time point, microglial activation surrounding all ventricles was observed in 5 of 6 CpG-infused mice, whereas no activation was observed in control animals. The degree of activation, however, was not as marked as after 4 weeks, and the hippocampal formation was free from microglial infiltration after short-term infusion. In addition, axonal damage was evident in 5 of 6 CpG DNA mice, mostly in the vicinity of the right lateral ventricle; the degree of axonal damage after short-term CpG DNA exposure was also not as strong as after long-term exposure. Similarly, the ependyma of the lateral and third ventricles was already severely damaged after 1 week of CpG DNA infusion, although not as marked as at 4 weeks; all animals displayed loss of cilia, and in nearly all mice, there was loss of integrity of the covering ependyma. No axonal or ependymal surface alterations were observed in the control mice. There were also more GFAP-immunoreactive cells in the dentate gyrus of CpG DNA-treated mice than in saline-exposed animals, but this difference did not reach statistical significance (p = 0.329). The medians (25th and 75th percentiles) were as follows: 77.1 (42.1/77.1) versus 105.8 (48.3/159.4).

Effects of CpG DNA on Motor Performance and Spatial Memory

No Change in Motor Performance During CpG DNA Infusion

Comparison of scores obtained from the tight rope test of C57BL/6 and TLR9-deficient mice with either CpG DNA or saline infusion revealed comparable motor scores of all groups, that is, there was no impairment in motor function caused by pump implantation or prolonged CpG DNA infusion detected (p = 0.69 and p = 0.49). All groups of mice demonstrated similar motor coordination, balance, and latency to remain on the cylinder in the accelerating rotarod test (medians [25th/75th percentile] of the time on rod of C57BL/6 and TLR9-deficient mice [CpG DNA and 0.9% NaCl]: 226.6 [224.3/237.6] vs 211.8 [189.3/234.2] and 252.4 [239.8/255.4] vs 259.2 [239.0/267.0] [p = 0.49 and p = 0.7], respectively).

CpG Exposure Results in Impaired Spatial Memory in Morris Water Maze in C57BL/6 Mice

Before implantation of the pump, all mice were trained to find the hidden platform within less than 90 seconds. Evaluation of latency, swim velocity, and distance traveled to find the submerged platform of the initial training revealed no significant differences between the mice that were later exposed to CpG DNA or 0.9% NaCl. After implantation of the pump, the water maze task was repeated weekly; C57BL/6 mice with CpG DNA infusion performed significantly worse than their saline-treated controls. These mice took significantly longer to reach the hidden platform (p = 0.007), and the distance traveled to locate the submerged platform was significantly greater (p = 0.005); the swim velocities were not significantly different (p = 0.477, global overall comparison) (Figs. 6A–C). The difference between CpG DNA- and 0.9% NaCl-infused mice expanded in parallel with the duration of exposure. In contrast, TLR9-deficient mice with intraventricular CpG DNA or 0.9% NaCl infusion did not perform significantly differently in the water maze with respect to latency to reach the platform (p = 0.53), distance of the swim track (p = 0.28), and swim speed (p = 0.23) (data not shown).

DISCUSSION

During bacterial infection, activation of TLR9 stimulates protective immunity by initiation of adaptive immune responses via dendritic cells in lymph nodes and by the promotion of T-cell activation via cytokine induction. Because of these immunostimulatory effects, treatment with the TLR9 ligand CpG DNA has been investigated as a possible adjuvant therapy for allergies or cancer. Despite promising observations in mice, repeated daily injections of CpG DNA evoked abnormalities of the morphology and function of lymphoid organs and led to liver necrosis and hemorrhagic ascites 20 days after start of treatment (24). Data on the effects of CpG DNA in the brain are still limited.

Because accumulating data suggest that activated microglia can cause detrimental reactions in autoimmune and neurodegenerative diseases, we investigated the effects of chronic CpG DNA stimulation in the CNS. Chronic intrathecal CpG DNA exposure induced neuroinflammation with microglial activation surrounding the borders of all ventricles and reactive astrogliosis in the hippocampal formation. This was associated with acute periventricular axonal damage and loss of the ependymal surfaces. Moreover, prolonged stimulation with CpG DNA caused a significant deficit in spatial memory.

The activation of microglia we observed is in accordance with other studies. Cytosine-guanine DNA was shown to activate microglia, stimulate the synthesis of NO and tumor necrosis factor, enhance phagocytic activity in vitro (4, 17, 25, 26), and stimulate cytokine synthesis after a single
intraventricular injection in vivo (26). Similarly, intraventricular administration of other immunomodulatory and proinflammatory substances such as LPS and IL-2 also resulted in microglial activation and reactive astrogliosis (27, 28). Absence of neutrophil, natural killer cells, or B and T cells by depletion did not affect the development of the inflammatory immune response after a single intracisternal injection of CpG DNA in mice (29). We also detected no major contribution of B or T cells to the inflammatory process.

The continuous intraventricular infusion of CpG DNA significantly impaired the performance in a memory task. Because TLR9-deficient mice did not display any alteration in memory performance upon CpG DNA infusion, the effects were dependent on TLR9 signaling. Others have also observed memory deficits in conditions of chronic neuroinflammation. For example, chronic intrathecal infusion of LPS led to neuropsychological deficits in the form of attenuated long-term potentiation within the dentate gyrus of rats (30), whereas intraventricular stimulation with IL-2 resulted only in transient and moderate spatial memory impairment (27). Because of the apparent selective vulnerability of the hippocampal formation to the harmful effects of CpG DNA exposure in this study, we postulated that neuronal necrosis, increased neuronal apoptosis, or changes in the number of young neurons within the dentate gyrus might be associated with the impaired spatial memory, but we did not detect loss of pyramidal cells within the areas CA1-3, necrotic neurons, or evidence of increased neuronal apoptosis in the dentate gyrus. Similarly, terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling-immunoreactive cells were absent in the temporal lobe after intraventricular LPS stimulation (28). In contrast, LPS infusion into the basal forebrain of young rats led to increased activity of caspases 3, 8, and 9 in the ventral caudate and putamen (31). The CpG DNA-induced memory impairment was also not associated with alterations in the density of young doublecortin- and calretinin-expressing neurons in the dentate granule cell layer in this study. Whether cognitive function is correlated with changes in adult hippocampal neurogenesis is currently controversial, and recent data in mice suggest that improvement of spatial memory in response to an enriched environment does not require increased hippocampal neurogenesis (32). We speculate that the reactive astrogliosis and axonal injury may have affected the spatial memory functioning in the mice, that is, the activated microglia may have released toxic compounds. Moreover, changes in gene expression and synthesis of neurotransmitters or their receptors may also contribute to memory dysfunction: chronic neuroinflammation induced by LPS administration was associated with loss of N-methyl-D-aspartate receptors in the temporal lobe region (33) and decreased choline acetyltransferase activity in the basal forebrain of rats (31).

Chronic neuroinflammation as a consequence of intrathecal administration of LPS mimics some aspects of AD including the neuropathologic and behavioral alterations that are seen in affected patients (34). In the present study, however, the neuroinflammation induced by CpG DNA did not lead to the typical neuropathologic changes associated with AD such as amyloid deposits and plaques. In this context, 4 weeks of induced chronic inflammation might not have been long enough to induce the changes that become evident in AD patients. Although AD-associated alterations were absent, acute

FIGURE 6. (A–C) Time course of water maze performance of 0.9% NaCl-treated (white bars) and cytosine-guanine (CpG) DNA-exposed (gray bars) C57BL/6 mice from 7 to 28 days after start of intraventricular infusion. (A) Cytosine-guanine DNA-treated mice took significantly more time to reach the hidden platform (p = 0.007). (B) The distance traveled to locate the platform was significantly longer in these mice (p = 0.005). (C) There was no significant difference in swim speed between CpG DNA-treated and control mice (p = 0.47, median and interquartile range).
axonal damage was evident after CpG DNA exposure, predominantly in the vicinity of the lateral ventricles.

In multiple sclerosis, the numbers of activated microglia and macrophages have been positively correlated with the extent of axonal damage (35). Impaired learning and memory performance in rats with closed head injury was associated with acute axonal damage (36). The APP-immunoreactive areas were also observed in TLR9-deficient mice, although to a much lesser extent than in wild-type mice. Because axonal damage was observed in both strains, we speculate that the axon-damaging effects of CpG DNA may at least in part be independent of TLR9 signaling. Mechanical irritation caused by the presence of the intraventricular pump is unlikely to have substantially contributed to these alterations because no saline-treated animal exerted APP immunoreactivity.

In summary, our results show that chronic stimulation of the CNS with CpG DNA caused a pronounced inflammatory response with microglial activation and reactive astroglia. These neuropathologic changes were accompanied by impairment in spatial memory performance. The results further support the accumulating data that suggest that subacute or chronic neuroinflammatory processes can induce pathologic and harmful microglial activation, thereby contributing substantially to the pathogenesis in CNS diseases. In this context, the impairment of cognitive function reported here is of particular importance. Moreover, repeated administration of CpG DNA as a potent vaccine adjuvant or as an adjunctive cancer treatment in spatial memory performance. The results further support the accumulating data that suggest that subacute or chronic neuroinflammatory processes can induce pathologic and harmful microglial activation, thereby contributing substantially to the pathogenesis in CNS diseases. In this context, the impairment of cognitive function reported here is of particular importance. Moreover, repeated administration of CpG DNA as a potent vaccine adjuvant or as an adjunctive cancer therapy may have serious adverse effects on brain function.

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REFERENCES

5. Hayes GM, Woodroofe MN, Cuzner ML. Microglia are the major cell type expressing MHC class II in human white matter. J Neurol Sci 1987;80:25–37

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