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CpG Oligodeoxynucleotides Enhance Neonatal Resistance to Listeria Infection

Shuichi Ito,* Ken J. Ishii,† Mayda Gursel,* Hidekazu Shirotra,* Atsushi Ihata,* and Dennis M. Klinman1*

Infection by Listeria monocytogenes causes serious morbidity and mortality during the neonatal period. Previous studies established that immunostimulatory CpG oligodeoxynucleotides (ODN) can increase the resistance of adult mice to many infectious pathogens, including Listeria. This work examines the capacity of CpG ODN to stimulate a protective immune response in newborns. Results indicate that dendritic cells, macrophages, and B cells from 3-day-old mice respond to CpG stimulation by secreting IFN-γ, IL-12, and/or TNF-α. Spleen cells from CpG-treated neonates produce large amounts of cytokine and NO when exposed to bacteria in vitro. Newborns treated with CpG ODN are protected from lethal Listeria challenge and generate Ag-specific CD4 and CD8 T cells that afford long-term protection against subsequent infection. These results demonstrate that cellular elements of the neonatal immune system respond to stimulation by CpG ODN, thereby reducing host susceptibility to infectious pathogens. The Journal of Immunology, 2005, 174: 777–782.

Listeria monocytogenes is a Gram-positive intracellular bacterium that commonly causes infection in neonates (1, 2). Neonatal L. monocytogenes infection can have grave consequences, with mortality from disseminated infection ranging from 42 to 72% despite appropriate antibiotic therapy (2–5). In adults, rapid activation of the innate immune system limits the early spread of infectious pathogens such as Listeria, allowing the host to subsequently mount a sterilizing adaptive immune response (6, 7). The innate immune system is stimulated when TLR expressed by immune cells recognizes pathogen-associated molecular patterns (PAMPs) expressed by infectious microorganisms (8, 9). CpG motifs present in bacterial DNA are one example of a PAMP (10–13), interacting with TLR9 to trigger an innate immune response in which lymphocytes, dendritic cells (DC), and macrophages are stimulated to produce immunoprotective cytokines and chemokines (11–18). Previous studies of adult mice showed that CpG oligodeoxynucleotide (ODN) treatment increased host resistance to infection for a period that typically peaks several days after administration and persists for several weeks (19–21).

Immaturity of the immune system may limit the ability of newborns to recognize PAMPs and mount a protective Th1 response (22–24). It is well established that neonates are at increased risk from a variety of pathogens, including Listeria (22). This study examines the effect of CpG ODN in newborn mice using Listeria infection as a relevant model for monitoring the induction of protective immunity. Results indicate that CpG ODN can stimulate the innate immune system of neonates, improving their ability to survive Listeria challenge.

Materials and Methods

**Reagents**

Stimulatory (GCTAGACGTAGCGT) and control (GCTAGACGTTAG XCT) phosphorothioate ODN were synthesized at the Center for Biologics Evaluation and Research (CBER) core facility. All ODN were free of endotoxin and protein contamination, as determined by chromogenic Limulus amoebocyte lysate and bicinchoninic acid protein assays (Pierce). Polypeptides from the listeriolysin O protein (LLO) encoding a CD8-specific epitope (LLO_{91-99}) and a CD4-specific epitope (LLO_{169-200}) were also synthesized at the CBER core facility.

**Animals**

BALB/c mice were purchased from the National Cancer Institute (Frederick, MD). Animals were housed in sterile microisolator cages in a barrier environment in the CBER-specific pathogen-free animal facility. All experiments were Animal Care and Use Committee approved. Newborns were injected i.p. with 20 μg of CpG or control ODN in 20 μl of PBS on day 3 and challenged i.p. with Listeria at various times thereafter (19–21).

**Preparation of bacteria**

Listeria monocytogenes strain EGD (ATCC 15313; American Type Culture Collection) was grown in brain-heart infusion broth (BD Biosciences). Titered aliquots previously demonstrated to be infective in adult mice were frozen at −70°C and thawed immediately before use. The severity of infection was monitored by culturing serial 10-fold dilutions of homogenized liver and spleen preparations in brain-heart infusion agar plates at 37°C (19–21).

**Cytokine ELISPOT assays**

Single-cell suspensions were prepared from mouse spleens. In brief, 5 × 10^5 splenocytes/well were stimulated for 8 h with 1 μM CpG or control ODN in flat-bottom 96-well Immunol 2 plates previously coated with mAbs against IFN-γ, IL-12, or TNF-α (25). Alternatively, 5 × 10^5 cells were cultured in round-bottom 96-well microtiter plates with 2.5 × 10^{-5} M CD4 or CD8-specific LLO peptides for 6–8 h and then transferred to anti-IFN-γ or anti-IL-4 Ab-coated plates. Cells were incubated overnight at 37°C with 2.5 × 10^{-5} M CD4 or CD8-specific LLO peptides. The plates were then washed and treated with biotinylated cytokine-specific mAb followed by streptavidin-alkaline phosphatase. Spots were visualized by the...
temperature. Macrophages were identified by surface expression of F4/80, B cells by B220+/CD11c- expression, non-plasmacytoid DC (pDC) as being B220-/CD11c+, and pDC as being B220+/CD11c+. Samples were washed and analyzed (20,000–40,000 events) on a FACSCalibur flow cytometer (BD Biosciences) after gating on live cells with proper electronic compensation. The data were analyzed using CellQuest software (BD Immunocytometry Systems).

RT-PCR analysis

Single-cell suspensions were prepared from mouse spleens. In brief, 1 × 10^7 splenocytes/well were stimulated for 6 h with HKLM (5 × 10^5 CFU/ml). Total RNA was extracted from spleen cells using TRIzol reagent (Invitrogen Life Technologies) according to the protocol from the manufacturer. Five micrograms of total RNA was reverse-transcribed in first-strand buffer (50 mM Tris-HCl (pH 7.5), 75 mM KCl, and 2.5 mM MgCl_2) containing 25 μg/ml oligo(dT)_{12–18}, 200 U Moloney leukemia virus reverse transcriptase, 2 mM dNTP, and 10 mM DTT. The reaction was conducted at 42°C for 1 h. One microliter of the cDNA was subjected to the standard PCR for 33 cycles by using primers as: mouse inducible NO synthase 2 (iNOS2); sense, 5'-CTT CCG AAG TTT CTG GCA GCA-3', antisense, 5'-GAG CCT GCT GGC TTT GGG CTC CTC-3'; mouse β-actin, sense, 5'-GACATGGAGAAGATCTGGCAACCACA-3', antisense, 5'-ATTCCTCTGCTCGAAAGTCTAGAACCACA-3'. Aliquots of PCR were separated on a 1.5% agarose gel and visualized with UV light after ethidium bromide staining.

Statistics

Differences in survival rates were determined using χ^2 and Wilcoxon rank sum tests. All experiments involving cytokines or NO production were repeated at least three times and involved at least three independently studied mice per group per experiment. Statistical significance was evaluated using a two-tailed Student’s t test or Mann-Whitney U test. Values of p < 0.05 were considered to be significant.

Results

Spleen cells from newborn mice respond to CpG ODN in vitro

Neonates have a reduced capacity to mount Ag-specific Th1 immune responses (22–24). To evaluate whether TLR9-mediated activation of the innate immune system is similarly compromised, spleen cells from newborn and adult BALB/c mice were stimulated in vitro with CpG or control ODN. Analysis of the induced immune response focused on the production of IFN-γ, IL-12, and TNF-α, since these cytokines 1) are elicited by CpG treatment of adults and 2) help protect the host against bacterial infection (26–31). As seen in Fig. 1, there was a significant increase in the production of all three cytokines when spleen cells from 3-day-old mice were stimulated with CpG (but not control) ODN (p < 0.01). In the case of IL-12 and TNF-α, the magnitude of this response rivaled that of cells from adult mice, whereas both spontaneous and CpG-induced IFN-γ production was significantly lower in neonatal vs adult animals (p < 0.001).

Table 1. Phenotype of CpG-activated cytokine-secreting cells

<table>
<thead>
<tr>
<th>% IL-12-Secreting Spleen Cells</th>
<th>% TNF-secreting spleen cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Macrophage</td>
</tr>
<tr>
<td>Phenotype of IL-12-Secreting Cells (%) total</td>
<td>B cell</td>
</tr>
<tr>
<td>Newborn</td>
<td>32 ± 2*</td>
</tr>
<tr>
<td>Adult</td>
<td>9 ± 3</td>
</tr>
<tr>
<td>Phenotype of TNF-secreting cells (%) total</td>
<td>B cell</td>
</tr>
<tr>
<td>Newborn</td>
<td>8 ± 2*</td>
</tr>
<tr>
<td>Adult</td>
<td>2 ± 2</td>
</tr>
</tbody>
</table>

* Splenocytes from 4-day-old or adult mice were cultured in medium supplemented with 3 μM control or CpG ODN plus brefeldin A. The cells were stained for surface expression of various phenotype-specific markers (see Materials and Methods), and for intracytoplasmic expression of IL-12 and TNF-α. Results represent the average ± SD of two independently studied donors per group.

\[ \text{Statistics:} \quad \text{Differences in survival rates were determined using } \chi^2 \text{ and Wilcoxon rank sum tests. All experiments involving cytokines or NO production were repeated at least three times and involved at least three independently studied mice per group per experiment. Statistical significance was evaluated using a two-tailed Student’s t test or Mann-Whitney U test. Values of } p < 0.05 \text{ were considered to be significant.} \]

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The phenotype of the cells activated to secrete these cytokines was evaluated. As seen in Table I, CpG ODN induced an 8- to 25-fold increase in the number of cells actively secreting TNF-α and IL-12 in both newborn and adult mice. DC were the primary source of TNF-α following CpG stimulation in animals of both ages. B cells, macrophages, and DC all contributed to the production of IL-12, although relatively more macrophages and fewer DC from neonates produced this cytokine when compared with adult mice (p < 0.05).

**Neonatal mice respond to CpG ODN in vivo**

Neonatal and adult mice were treated with control or CpG ODN, and the number of spleen cells activated to secrete cytokine in vivo was examined 3 days later. CpG ODN administration stimulated a significant increase in the number of cells producing IFN-γ, IL-12, and TNF-α in both young and old mice (Fig. 2). Although the magnitude of the IL-12 and TNF-α response in neonates rivaled that of adults, the number of IFN-γ-secreting cells was significantly lower in neonatal than adult animals (Fig. 2, p < 0.001).

The ability of immune cells from CpG-treated mice to respond to HKLM was then examined. As seen in Fig. 3, the production of NO (a potent antibacterial agent) was significantly greater in cells from mice treated with CpG vs control ODN (Fig. 3A, p < 0.01) (32, 33). This finding was confirmed in studies of iNOS2 mRNA expression by spleen cells from these animals (Fig. 3B).

**CpG ODN improve the survival of newborn mice challenged with *L. monocytogenes***

Previous studies in adult mice established that the protective immune response induced by CpG ODN peaks 3 days after ODN administration and persists for several weeks (19–21). To examine whether protection was elicited in neonatal mice, animals were treated with control or CpG ODN and then challenged with the same stock of *L. monocytogenes* used in previous studies of adult animals (20, 21). This challenge model was selected because *Listeria* is a relevant neonatal pathogen, the cytokines and NO induced by CpG ODN are known to protect against *Listeria* (26–33), and earlier studies showed that CpG ODN could protect normal adult mice from *Listeria* (19–21). Whereas most (21 of 26) newborns treated with control ODN succumbed to infection by 20 LD₅₀ of *L. monocytogenes*, a majority (18 of 29) of those treated with CpG ODN survived challenge (p < 0.001 vs control ODN, Fig. 4). When the infectious dose was increased to 100 LD₅₀, all control ODN-treated mice were dead by day 7. By comparison, 40% of CpG-treated mice were alive at day 7, with 20% surviving indefinitely (p < 0.05, data not shown).

**Effect of CpG treatment on bacterial proliferation**

To examine the effect of CpG ODN treatment on *Listeria* proliferation in vivo, the number of bacteria present in the liver and spleen (the primary loci of *Listeria* infection (19, 20)) of challenged mice was monitored. Three-day-old animals were treated with control or CpG ODN and then challenged with 10 LD₅₀ of *L. monocytogenes*. The number of microorganisms present in mice

![FIGURE 2.](image1) Neonates respond to CpG ODN stimulation in vivo. BALB/c mice were injected i.p. with 20 μg (newborn) or 100 μg (adult) of CpG or control ODN. The activation of cytokine-secreting cells in the spleen was monitored 3 days later by ELISPOT assay. Data represent the mean ± SEM of seven independently studied mice in two independent experiments. ***p < 0.001; **p < 0.01; *p < 0.05 (Student’s t test).

![FIGURE 3.](image2) CpG ODN prime host cells to produce NO when subsequently exposed to bacteria. Three-day-old BALB/c mice were treated with 20 μg of control or CpG ODN. Peritoneal cells were isolated 3 days later and cultured with 5 × 10⁵ CFU/ml of HKLM for 48 h. A, Nitrite levels in culture supernatants, mean ± SEM of six independently studied mice in two independent experiments. B, Relative levels of iNOS2 mRNA expression was examined by PCR. ***, p < 0.01 (Student’s t test).

![FIGURE 4.](image3) Effect of CpG ODN treatment on the survival of newborn mice challenged with *L. monocytogenes*. Newborn BALB/c mice were injected i.p. with 20 μg of control (n = 26) or CpG (n = 29) ODN at 3 days of age. Mice were challenged with 20 LD₅₀ of *L. monocytogenes* 3 days after treatment, and their survival was monitored for 3 wk. ***p < 0.001 (Wilcoxon rank sum test).
injected with control ODN increased rapidly over time, with most animals succumbing to overwhelming infection by 4 days after challenge (Fig. 5). In contrast, the rate of proliferation of microorganisms in mice treated with CpG ODN was much slower, and these animals eventually resolved their infection. These effects are consistent with the improved survival of CpG-treated neonates observed in the higher challenge dose study presented above.

Kinetics of CpG-induced resistance to Listeria

The onset and persistence of CpG-dependent protection was examined. Significant protection was not achieved until 3 days after in vivo administration of CpG ODN, consistent with the time course of protection previously observed in adult mice (Fig. 6) (19–21). Protection persisted through day 7, but returned to near baseline levels within 2 wk.

Effect of CpG ODN treatment on the development of pathogen-specific immunity

Adult mice that survive Listeria challenge following CpG ODN treatment develop long-lasting pathogen-specific immunity (20, 21). To determine whether adaptive immunity also develops in neonatal mice, the presence of Ag-specific T cells in CpG-treated survivors of Listeria infection was examined. Spleen cells from 4-wk-old survivors were stimulated in vitro with CD4- or CD8-specific LLO peptides, and the number of cells stimulated to secrete IFN-γ and IL-4 was determined by ELISPOT assay. The data show the mean ± SD increase in the number of cytokine-secreting cells over unstimulated controls from five to six individually tested mice per group from two independent experiments.

To establish the efficacy of this long-term immunity, newborns treated with CpG ODN that survived Listeria challenge were rechallenged with 100 LD50 of Listeria at 8 wk of age (after the protective effect of CpG ODN had waned). Forty-four (88%) of 16 of these animals survived rechallenge, whereas all naive age-matched controls succumbed (p < 0.01, Table II). These findings indicate that CpG ODN elicit a rapid innate immune response in neonates and that subsequent pathogen exposure leads to the development of long-lasting Ag-specific immunity (20, 21).

Table II. Induction of long-term immunity following Listeria challenge of CpG-treated newborns

<table>
<thead>
<tr>
<th>Listeria Challenge</th>
<th>Naive Adults</th>
<th>CpG-Treated Survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 LD50</td>
<td>0/5</td>
<td>5/5**</td>
</tr>
<tr>
<td>100 LD50</td>
<td>0/5</td>
<td>9/11**</td>
</tr>
</tbody>
</table>

*Normal adults and mice treated at 3 days of age with CpG ODN that survived Listeria infection on day 6 were challenged at 8 wk of age with 10–100 LD50 of L. monocytogenes. Data represent the number of survivors per total number challenged. **p < 0.01, (χ² test).
Discussion
This work is the first to establish that CpG ODN can induce an innate immune response in newborn mice that improves survival following bacterial infection. Neonates are at increased risk from many pathogens due to the immaturity of their immune system (22). In particular, previous studies showed that Ag-specific Th1 and cell-mediated immune responses are reduced in newborns (22–24), while the likelihood of developing tolerance is increased (34, 35). Current results indicate that 1) immune cells from newborn mice respond to CpG ODN by secreting IFN-γ, IL-12, and TNF-α; 2) CpG ODN “prime” immune cells to produce NO when subsequently exposed to bacterial products; and 3) newborn mice treated with CpG ODN resist infection by L. monocytogenes.

CpG ODN were previously shown to decrease the susceptibility of adult animals to various bacterial, viral, and parasitic pathogens (19–21). However, their ability to confer protection to newborn mice was unknown. Induction of protective Th1 responses is generally compromised in neonates, reflecting the reduced expression of IL-12R, STAT4, and T-bet by immature immune cells (42, 43). Yet current results indicate that CpG ODN treatment can protect newborns from infection using Listeria as a model pathogen (19–21). Initial findings showed that spleen cells from newborn mice responded to in vitro stimulation with CpG (but not control) ODN by producing TNF-α and IL-12 at levels similar to those observed in adults (Fig. 1). The phenotype of the cells activated to secrete these cytokines was similar in newborn and adult mice, although fewer DC and more macrophages were triggered to produce TNF-α in the neonates (Table I).

IFN-γ production was also elicited, but at levels significantly lower than that of adults, perhaps reflecting the defects in IL-12R and T-bet expression noted above (42, 43). Indeed, IFN-γ levels in neonates were so low that we were unable to detect that cytokine by intracytoplasmic staining. The pattern of cytokine production observed in this work confirms and extends the observations of Kovarik et al. (44) and Sun et al. (45) that spleen cells from newborn mice produce IL-12 in response to CpG ODN in vitro. Current studies also establish that CpG ODN trigger cytokine production in vivo (Fig. 2) and prime spleen cells to produce large amounts of NO when exposed to bacteria in vitro (Fig. 3).

Of greatest importance, CpG treatment was found to significantly improve the survival of neonates challenged with Listeria (Fig. 4). As in adults, the immunoprotective cascade elicited by CpG administration required 3 days to reach optimal activity (Fig. 6 and Refs. 19–21). Yet both the magnitude and duration of protection induced in neonates was lower than that observed in adults challenged with the same stock of Listeria (19–21). CpG treatment protected approximately two-thirds of neonates from challenge with 10–20 LD50 of Listeria and the same treatment protected nearly 100% of adult mice from 100 LD50 of Listeria (20, 21). Indeed, although CpG treatment significantly prolonged the survival of neonates challenged with 100 LD50 of Listeria, only 20% of these mice survived long-term (compared with no survivors among mice treated with control ODN). The lower level of protection observed in CpG-treated neonates vs adults may reflect the poorer IFN-γ response of newborn mice (Fig. 1), since production of this cytokine correlates with resistance to neonatal listeriosis (22, 26–28, 31). Alternatively, the ability to generate an adaptive immune response capable of providing sterilizing immunity may be compromised in newborn mice (34, 35).

In this context, newborns as well as adults treated with CpG ODN and then challenged with Listeria developed LLO-reactive CD4 and CD8 T cells and resisted subsequent Listeria challenge (Table II and Fig. 7). These T cells preferentially produced IFN-γ, consistent with the known ability of CpG ODN to promote Th1-biased immune responses (14). This constellation of findings suggests that CpG ODN trigger an innate immune response that improves host resistance to initial pathogen challenge and promotes the subsequent development of pathogen-specific immunity (20, 21).

Although current studies indicate that CpG stimulation activates the same cell types in neonates as adults (Table I), the specific cell type(s) responsible for CpG-mediated protection in newborns was not established. Ongoing research in our laboratory suggests that pDC play a key role in CpG-mediated protection in adults (K. J. Ishii, S. Ito, J. Conover, T. Tamura, H. Hemmi, K. Ozato, S. Akira, and D. M. Klinman, manuscript in preparation). pDC isolated from CpG-treated mice transferred protection to naïve mice, and mice genetically deficient in pDC were not protected by CpG treatment. Yet additional cell types may also contribute to CpG-mediated immunity, including NK cells, macrophages, and B cells (11, 15).

Current findings support the hypothesis that activation of the innate immune system through TLR9 can significantly improve host resistance to infection. Combined with evidence that CpG ODN appear to be safe when administered at therapeutically useful doses (no adverse events were observed among the CpG-treated mice in the current study (6, 21)), these findings support the further development of CpG-based therapeutics for the prevention of infectious diseases.

References


