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Maternal treatment with a high dose of CpG ODN during gestation alters fetal craniofacial and distal limb development in C57BL/6 mice☆

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Abstract

Synthetic oligodeoxynucleotides (ODN) containing CpG motifs, characteristic of bacterial DNA, are currently being evaluated as vaccine adjuvants for inducing protective immunity. Recently, there is increasing pressure to vaccinate pregnant women against maternally transmitted diseases including AIDS and tetanus, as well as against potential bio-weapons such as anthrax. CpG vaccines are effective because they trigger transient increases in T_H1 cytokine production. Recent literature suggests, however, that a shift toward a T_H1 cytokine profile during pregnancy may increase the risk of fetal morphologic defects. On this basis, we hypothesized that exposure to CpG motifs during pregnancy could result in T_H1 inflammation leading to adverse effects on fetal development. To address this hypothesis, pregnant C57BL/6 mice were injected with CpG ODN (0–300 μg/dam) and maternal and fetal outcomes were determined. Injection of dams with the highest dose of CpG ODN resulted in markedly increased fetal resorptions and craniofacial/limb defects, while lower doses had little, if any effects. Histological examination of placentas revealed cellular necrosis with mixed inflammation and calcification in the spongiotrophoblast layer and dysregulation of labyrinthine vascular development. Concomitant elevations in maternal serum cytokine levels were observed including interleukin (IL)-2, IL-10 and IL-12. Treatment with 300 μg of non-CpG ODN did not cause any adverse effects. The 300 μg dose of CpG ODN used in the present study is 30-fold higher than the highest dose that has been administered to humans during clinical trials. These results suggest that the induction of T_H1 cytokines during pregnancy by CpG motifs may potentially increase the risk of fetal loss and morphologic defects in mice, at least at high doses, and support the need for further investigation of teratogenesis that may result from exposure to vaccine adjuvants designed to produce T_H1 cytokine profile shifts.

Keywords: CpG ODN; Immunostimulatory DNA; Fetal malformation; Craniofacial and distal limb development; Abortion; Th1 cytokine shift

1. Introduction

The Toll-like receptor (TLR) family recognizes specific pathogen-associated molecular patterns such as bacterial lipopolysaccharides (LPS) and bacterial DNA. Activation of immune cells though TLR binding produces a myriad of cytokines and chemokines important in orchestrating

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immune responses [1,2]. TLR-9 recognizes bacterial DNA through unmethylated CpG motifs (unmethylated deoxycytidyl-deoxyguanosin dinucleotides) that are present in much higher frequency in prokaryotic DNA than eukaryotic DNA [2–5]. Synthetic oligonucleotides (ODNs) containing unmethylated CpG motifs are capable of recapitulating the immune response to bacterial DNA, and as such their potential immunotherapeutic uses have been the focus of intensive research (reviewed in [6]). Potential therapeutic uses include activation of protective immunity [7,8], asthma immunotherapy [9,10], cancer therapy [11,12] and improvement of vaccine efficacy [13].

Maternal vaccination has been proposed as a means to provide neonates and young infants with sufficient immunity to resist potentially fatal infections [14]. As such, new vaccine technologies could result in fetal exposure to CpG motifs present in plasmid DNA vaccines and also directly through the use of CpG ODNs as vaccine adjuvants. In addition, significant workplace exposure to bacterial products occurs in numerous occupations where pregnant women are present including farming [15], laboratory animal care [16] and metal fabrication where exposure to microbial contaminated metalworking fluids occurs [17]. Occupational exposure could also result from vaccination of military personnel with plasmid vaccines or CpG adjuvant vaccines directed against bioterrorism agents including anthrax. In addition, animal studies have demonstrated transplacental transfer of DNA following maternal exposure via oral [18] and parenteral routes [19,20], suggesting the possibility for fetal exposure to foreign DNA subsequent to therapeutic use and environmental/occupational exposure. As such, safety concerns exist regarding therapeutic uses of CpG ODNs and/or CpG DNA exposure during pregnancy. Since interaction of CpG motif-rich bacterial DNA and synthetic ODNs with TLR-9 initiates a signaling cascade culminating in immune cell activation and increased production of predominantly inflammatory cytokines including IL-12, IFNγ, IL-2, and TNFα, therapeutic or occupational exposure to CpG motifs during pregnancy could potentially produce deleterious effects on maternal-fetal health. The present studies were conducted to determine the effects of maternal treatment with CpG ODN during gestation on fetal survival and development.

2. Materials and methods

2.1. CpG oligonucleotides

ODNs were synthesized by TriLink Biotechnologies (San Diego, CA) using an endonuclease-resistant phosphorothioate backbone, which extends the half-life and activity of CpG ODNs in vivo [5,21]. The immunostimulatory ODN (ODN 1826), hereafter referred to as CpG ODN, has the sequence 5′-TCCATGA*CG*TTCCTGA*CG*TT-3′ with the CpG motifs underlined. The control ODN, hereafter referred to as non-CpG ODN, has the sequence 5′-TCCATGA*GC*TTCCTGA*GTC*T-3′ where the CpG motifs have been rearranged resulting in an alteration of immunostimulatory response towards T_H2 immunity [22]. ODNs were reconstituted in pyrogen-free phosphate buffered saline (PBS) at 10 mg/ml and subsequently analyzed for LPS content, which was <0.05 EU/mg as tested using Endosafe® (Charles River Laboratories, Charleston, SC).

2.2. Animals and treatment

Male and female specific-pathogen-free C57BL/6 mice were obtained at 6 weeks of age from Jackson Laboratories (Bar Harbor, ME). Males were housed individually and females in groups of three per cage for an acclimatization period of 2 weeks. A microisolator caging system was used to maintain the specific-pathogen-free status of the animals and prevent exposure to microbes. Food and water were supplied ad libitum and the animals were maintained in the AAALAC accredited NIOSH animal facility under controlled conditions (21 ± 2 °C and 50 ± 10% relative humidity) using a 12 h light/dark cycle. All studies were conducted using protocols (02-ML-M-013, 03-VJ-M-013) approved by the Institutional Animal Care and Use Committee under the guidelines of the Public Health Services Policy on Humane Care and Use of Laboratory Animals. Two study designs that were conducted in parallel were used to examine the effects of gestational treatment with CpG ODN on fetal malformation (design #1) and pregnancy outcome (design #2). Upon commencement of breeding, one male was added to each cage of three females. Females were examined for vaginal plugs (evidence of copulation) the following morning at the beginning of the light cycle for a maximum of 4 days. The morning that the plug was found was considered day 0 of gestation and the females were then housed individually with nesting material. Pregnant C57BL/6 mice were administered 200 μl of PBS (vehicle), non-CpG ODN (300 μg/dam) or CpG ODN (3, 30, 300 μg/dam) by intraperitoneal injection (i.p.) on gestation day 6 at 9:00 a.m.

A total of 15 female mice per treatment group were mated for study design #1 and on the morning of gestation day 18, pregnant dams were sacrificed via CO_2 inhalation (number of gravid uteri per group was PBS, $n = 8$; non-CpG ODN, $n = 10$; 3 μg CpG ODN, $n = 8$; 30 μg CpG OND, $n = 9$; 300 μg CpG ODN, $n = 5$). Blood was collected from the abdominal aorta and serum stored at −80 °C for cytokine analysis. The gravid uteri were removed and weighed. Placentas and fetuses were removed from the uterine horns and preserved in 4% PBS-buffered paraformaldehyde, pH 7.4. Placental tissues were transected perpendicular to the long axis of the disc, paraffin-embedded, sectioned at 5 μm, and stained with hematoxylin and eosin for evaluation.

Pregnancy success rate, offspring survival and body weight gain were determined using a parallel experiment in which the dams were allowed to deliver and the offspring were examined for changes in immune function. A total of 35 females were mated per treatment group for study design