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A High Dose of IMT504, the PyNTTTTGT Prototype Immunostimulatory Oligonucleotide, Does Not Alter Embryonic Development in Rats

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Synthetic oligodeoxynucleotides (ODNs) are currently being evaluated as vaccine adjuvants for inducing protective immunity. As maternal vaccination is becoming increasingly common, the potential risk of vaccine formulation using ODN adjuvants should be warranted. A recent study performed in mice suggests that exposure to CpG motifs during pregnancy could result (although at very high doses as compared to the ones proposed for human vaccination) in fetal loss and morphological defects. PyNTTTTGT ODNs are immunostimulatory ODNs not bearing CpG motifs, which are very efficient vaccine adjuvants. In this report, we analyzed the potential teratogenic effect of its prototype IMT504 in rats. This animal model was chosen because PyNTTTTGT ODNs are barely active in mice. Intraperitoneal injection of IMT504 at a dose of 20 mg/kg (more than 1000 times higher than the one proposed for a vaccine dose in humans) at day 6 of pregnancy did not produce a significant decrease in the mean number of implanted fetuses or in the number of live pups delivered. Neither the fetuses nor the offspring presented malformations.

Introduction

Immunostimulatory oligodeoxynucleotides (ODNs) are synthetic molecules that stimulate cells of the immune system and are being assayed as adjuvants in vaccines and as medicines in the therapy of cancer and allergy (Barchet et al., 2008). ODNs that are active on human cells are grouped into 2 major classes: (1) the CpG ODNs, characterized by the presence of at least one active site bearing an unmethylated CpG in a given context (Krieg et al., 1998), and (2) the PyNTTTTGT ODNs that have at least one active site with the sequence PyNTTTTGT, in which Py is C or T and N is A, T, C, or G (Elias et al., 2003; Rodriguez et al., 2006). *In vitro*, both kinds of ODNs act on B cells and plasmacytoid dendritic cells at similar doses, causing activation, proliferation, immunoglobulin secretion, and expression of costimulatory molecules, respectively. However, some major differences between them have been described: (1) CpG ODNs induce the secretion of IFN α (Krug et al., 2001), while phosphorothiote (PS) PyNTTTTGT ODNs do not (Elias et al., 2003), and (2) PyNTTTTGT are potent stimulatory signals for mesenchymal stem cell expansion, whereas CpG ODNs are not effective (Hernando-Insúa et al., 2007).

Several toxicity studies have been performed using both antisense and immunostimulatory oligonucleotides, mainly in their PS nuclease resistance clinically useful form (Cornish et al., 1993; Galbraith et al., 1994; Monteith et al., 1997). However, only few of them have focused on the reproductive toxicology of these ODNs. In particular, one study described a significant increased in fetal resorptions and craniofacial/limb defects in the offspring of pregnant female mice intraperitoneally injected with 300 μg/dam of a CpG ODN on day 6 of gestation (Prater et al., 2006). As immunostimulators, ODNs could be used either as adjuvants or as therapy for cancer treatment. Generally, the doses used for vaccine applications are low and their safety concern is associated with local reactions. Antisense doses may be considerably higher. The present study was conducted to determine the effect of maternal treatment with IMT504, the prototype of the PyNTTTTGT class of immunostimulatory ODNs, on fetal survival and development during gestation in a species in which this ODN is pharmacologically active. The teratogenic potential of IMT504 was assayed in rats, because we have previously observed that PyNTTTTGT are barely active in mice (Elias et al., 2003, 2005).

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Materials and Methods

Oligonucleotides

Oligonucleotide IMT504 (5′-TCATCATTTTGTCATTTT GTCATT-3′) having phosphorothioate internucleotide linkages was purchased from Oligos ETC (Bethel, Maine), produced under GMP rules and purified by high performance liquid chromatography (HPLC). The ODN was suspended in depyrogenated water, assayed for lipopolysaccharide (LPS) contamination using the Limulus test and kept at −20°C until used. Purity was assessed by HPLC and PAGE assays. IMT504 preparations were used if LPS levels were undetectable.

Animals and treatments

Female Sprague Dawley (SD) 7-week-old rats were obtained from FUCAL Laboratories. After 2–3 days, pregnant animals were relocated in a facility at Immunotech S.A., Buenos Aires, Argentina. Animals were lodged in individual cages in an acclimatized room (21°C–24°C) with 55%–65% relative humidity and a light rate of 14 hours light to 10 hours darkness. All the animals had free access to water and were fed with a balanced diet. Rats were observed daily and the general state, attitude, response to food and water and any abnormal sign of each animal were recorded. Animals were weekly weighed. At day 6 of pregnancy, rats were administered 500 µL of either PBS or IMT504 (20 mg/kg) in PBS by intraperitoneal (i.p.) injection. Control and treated groups were divided into 2 subgroups (1 and 2) containing 10 animals each. Animals in subgroup 1 were euthanized at day 18 of pregnancy and blood was collected and stored at –80°C. Placentas and fetuses were removed from the uterine horns, inspected for anomalies, and preserved in 4% PBS-buffered paraformaldehyde, pH 7.4. The gravid uteri were also removed, weighed, and preserved in 4% PBS-buffered paraformaldehyde, pH 7.4. Animals in subgroup 2 were not euthanized and served to evaluate pregnancy success rate, offspring survival and body weight gain. Animal care and use were according to international guidelines (*Guide to the Care and Use of Experimental Animals*. Canadian Council on Animal Care, 1993. Vol 1. 2nd Ed. pp. 221. Ottawa, ON, Canada).

Flow cytometry

In order to assess drug exposure, a group of four animals was tested for CD40 activation in CD19-positive cells (B lymphocytes) taken from peripheral blood mononuclear cells (PBMC). At day 6 of pregnancy, rats were injected i.p. with either 20 mg/kg of IMT504 or saline. After 72 hours, animals were anesthetized and a blood sample was taken by cardiac puncture. Peripheral mononuclear blood cells (PMBC) were isolated by density gradient and staining was performed as previously described (Elias et al., 2003). Anti-CD19 and anti-CD40 antibodies (Serotec, Raleigh, NC) were used. Flow cytometric data for 20,000 cells/sample were acquired on a FACScan (BD Biosciences, San Jose, CA). Data were analyzed using Win MDI, 2.8, Interface Flow Cytometry Application software (Joseph Trotter, copyright 1993–1998).

Histopathological analysis

Placentas and fetuses were paraffin-embedded, and sections were stained and examined by light microscope in order to identify abnormalities in embryonic development.

Statistics

Differences between means were analyzed by one-way ANOVA, followed by Tukey's HSD test. $P < 0.05$ was considered statistically significant.

Results and Discussion

Pregnancy outcome at day 18 of gestation

Data from dams euthanized on day 18 of gestation are shown in Table 1. Maternal treatment with 20 mg/kg of IMT504 intraperitoneally injected at day 6 of pregnancy did not produce a significant decrease in the number of pups. On the other hand, no malformations were detected in fetuses either by macroscopic observation or by histopathological analysis. Drug exposure was confirmed by CD40 activation in CD19-positive cells from PBMC. The Median Fluorescence Intensity (MFI) was 117.0 ± 1.86 (M + SEM) in the control group versus 162.8 ± 9.01 in the ODN-treated animals.

Furthermore, maternal and fetal body weight were not significantly modified by the treatment (data not shown). Fetal resorptions were 1.1% in the control group and 5.2% in the animals injected with IMT504. These values are within the spontaneous fetal resorption rate reported for SD rats (Lang, 1993).

Pregnancy success rate, offspring survival, and body weight gain

Data at day 21 after gestation are shown in Table 1. Neither the number of pups nor the offspring weight showed a significant decrease after maternal treatment with 20 mg/kg of IMT504 intraperitoneally injected at day 6 of pregnancy (data not shown). In addition, malformations were not detected by macroscopic observation.

Immunostimulatory oligonucleotides are entering clinical trials in vaccines, cancer and, in the short-term, may

Table 1. Pregnancy Outcome and Litter in ODN IMT504-Treated Rats

	Gestation day 18		
		Implants	
Group	*No. females*	*M*	*SD*
IMT504 (20 mg/kg)	n:29	15.1	2.8
Saline	n:7	15.5	2.0
		t:0.77	
	Deliver on time		
		Live pups	
Group	*No. females*	*M*	*SD*
IMT504 (20 mg/kg)	n:9	13.1	2.4
Saline	n:8	14.1	2.7
		t:0.31	

The average number of implanted fetuses at day 18 of gestation and live pups at delivery time in rats inoculated with ODN IMT504 (20 mg/kg) at day 6 of gestation compared to control rats injected with saline. Differences were not statistically significant.

enter clinical trials in tissue repair procedures (Hernando-Insúa et al., 2007; Coronel et al., 2008). Useful doses in each of these applications may be very different, ranging from 0.007 mg/kg in vaccines to 10 mg/kg and higher in cancer applications (Murad et al., 2007; Jurk and Vollmer, 2007; Dorn and Kippenberger, 2008; Gupta and Cooper, 2008). One report has warned about the toxic reproductive potential of a CpG immunostimulatory ODN in mice (Prater et al., 2006). In this study, a significant increase in fetal resorptions and craniofacial/limb defects was observed with a single i.p. injection of the CpG ODN of 15 mg/kg at day 6 of pregnancy. Anomalies were not observed with a 10 times lower dose or when a non-CpG ODN was used. In the present study, we studied the toxic reproductive potential of IMT504, the prototype of the PyNTTTTGT class of immunostimulatory ODNs, which has been proven to be effective as adjuvant in rats when combined with the HbsAg (Elias et al., 2005). The study was conducted in rats because these ODNs are barely active in mice (Elias et al., 2003; Elias et al., 2005) and the dose was 20 mg/kg (a dose more than 1000 times higher than the one proposed for a vaccine in humans) administered in one i.p. injection at day 6 of pregnancy. In these conditions, no changes were detected in the size or the morphology of the offspring. Since the animal model and doses were different from the study performed in mice with CpG ODNs (Prater et al., 2006), it is difficult to speculate about the theratogenic potential of both classes of immunostimulatory ODNs. However, the results here presented strongly suggest that IMT504 is a safe drug in case it were administered to pregnant women as adjuvant in vaccines. On the other hand, since susceptibility to any drug can be very different between humans and rodents, a careful follow-up both during and after clinical trials is mandatory.

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