International Immunopharmacology 11 (2011) 1032–1037

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Aggregation and secondary loop structure of oligonucleotides do not determine their ability to inhibit TLR9

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ARTICLE INFO

Article history:
Received 18 November 2010
Received in revised form 10 January 2011
Accepted 16 February 2011
Available online 2 March 2011

Keywords:
Toll like receptor 9
B lymphocytes
Inhibitory oligonucleotides
CpG-DNA

ABSTRACT

Toll-like receptor 9 (TLR9) is an endosomal DNA sensor that warns us of the presence of infectious danger and triggers a rapid pro-inflammatory response in dendritic cells, macrophages, and B cells. The consequences of uncontrolled TLR9 activation can be detrimental for the host, contributing to the pathogenesis of bacterial septic shock or autoimmune diseases, such as systemic lupus erythematosus. Therefore, we need to develop TLR9 antagonists. We and others have created inhibitory oligonucleotides (INH-ODN) that are capable of sequence-dependent inhibition of TLR9-induced activation in both human and mouse cells. However, it is not clear whether marked differences in INH-ODN activity related to base sequence derived from polymerization of INH-ODNs or their ability to complex with stimulatory CpG-oligonucleotides (ST-ODN). Furthermore, the 5′ end of INH-ODNs may assume a particular loop configuration that may be needed for binding to a critical site on TLR9. Here, we show that 1) G-tetrads required for ODN stacking were compatible with INH-ODN activity but were not necessary; 2) there was no relationship between activity and self-association at endosomal pH; 3) there was no evidence for direct binding between ST-ODNs and INH-ODNs; 4) when a 3G sequence was disrupted, despite a preserved stem-loop formation, INH-ODN activity was abolished. These results support the conclusion that certain features of the primary linear sequence are critical for TLR9 inhibition, but changes in secondary structure or in ODN aggregation are irrelevant.

1. Introduction

An important early warning of the presence of bacteria and viruses is provided by the recognition of their DNA and RNA by toll-like receptors (TLRs) 3, 7, 8, and 9 [1]. These TLRs differ from the other TLRs in that they are active in endosomes rather than on the cell surface [2,3]. Thus, the response of TLRs to nucleic acids in the environment depends on the delivery of nucleic acids to endosomes either by passive uptake [4], via B cell receptor for antigen [5,6], or (as immune complexes) by the Fc receptors of dendritic cells [7,8]. A main reason why self DNA and RNA fail to trigger TLRs in B cells is their normal inability to access endosomes. When this barrier is overcome, as through the co-engagement of B cell receptor for antigen, they become stimulatory [6,9,10]. Once stimulatory DNA is in the endosome, TLR9 is recruited there from storage in the endoplasmic reticulum [11], an event facilitated by the cytoplasmic protein UNC93b1 [12,13].

The structural requirements for triggering TLR9 have been elucidated, largely by the study of synthetic single-stranded DNA [14]. Base sequence and demethylation of certain cytosines proved critical as an optimal stimulatory motif was described as "purine, purine, unmethylated cytosine, guanine, pyrimidine, pyrimidine" (the "CpG motif") [15]. Six base sequences containing this motif appear in mammalian DNA about 1/25 as frequently as in bacterial DNA, whereas other six-base sequences had roughly equal frequency [15]. Other sequence features were described that further increased the rarity of the CpG motif [16] but never as much as 2 logs, raising the fear that high concentrations of mammalian DNA might still stimulate TLR9 (as in crush injuries). It would be comforting to have a protective inhibitor in the system to prevent activation by self DNA.

Indeed, it was shown that poly G-containing deoxyribonucleotides (ODN) could block DNA-induced activation, including that driven by stimulatory (ST-) ODN bearing the CpG motif [17,18]. This effect was otherwise ODN sequence non-specific, required high micromolar concentrations of inhibitory (INH-) ODN, and also blocked signaling pathways other than TLR9 [19]. INH-ODNs with greater sequence and TLR9 specificity were then described by Krieg et al. [20,21], culminating in our extensive structure–function studies of single-stranded ODN to define the differences between INH-ODN and ST-ODN [16,22–25]. 1) ST-ODNs require the unmethylated CpG, whereas INH-ODNs do not. 2) For inhibition or stimulation, a CC is required at the 5′ end. An extra 5′ T is required for stimulation but not for inhibition. 3) A sequence of 3Gs is

Abbreviations: TLR, toll-like receptor; INH-ODN, inhibitory oligonucleotide; ST-ODN, stimulatory CpG oligonucleotide; pO, phosphodiester; pS, phosphorothioate; nM, nanomolar.

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doi:10.1016/j.intimp.2011.02.023

required for INH-ODN, with 3–5 covalently linked bases intervening between the 3Gs and the 5′ CC [16]. At two of these intervening positions, Cs greatly reduce activity [23]. 4) Base choice at the other positions is much less important [23]. 5) The shortest ODN with substantial inhibitory activity is thus a 10-mer: CCx (not C) (not C) xGGGG, where x is any base [23]. 6) Duplication of the 5′ end can enhance activity 3–6 fold [25]. 7) Orientation is critical, for when the order of bases was reversed, INH-ODN activity was 99.9% lost [24].

What do these strict sequence requirements tell us about the mechanism of action of INH-ODN? There is evidence that avidity of TLR9 binding is not strictly dependent on base sequence and is a poor predictor of biologic activity [26]. So, could the sequence requirements be necessary for INH-ODN to polymerize, complex with ST-ODN, or assume a particular secondary structure needed to bind to a critical site?

2. Materials and methods

2.1. Oligonucleotides

Sigma Genesis (St. Louis, MO) and Integrated DNA Technologies (Coralville, IA) synthesized and purified our phosphorothioate (pS) and phosphodiester (pO) ODN.

The prototype ST-ODN sequence for human cells is 5′-TCGTCGTTTTGTCGTTTTGTCGTT-3′, numbered 2006 with a phosphorothioate (pS-) backbone and 2059 with a native phosphodiester (pO-) backbone [27,28]. The standard concentrations used were 100 nM for 2006 and 10 μM for 2059, chosen for their ability to provide equal suboptimal stimulation.

The prototype inhibitory sequence is 2114: 5′-TCCTGGAGGG-GAAGT-3′. The pS-form of this sequence is a strong inhibitor of ST-ODN in both mouse and human cells used in previous studies [23,24]. Titrations were routinely performed with 8 concentrations 0.5 log apart from 0.3 to 1000 nM. The pO-form of this same sequence, 4228, was also used at 8 concentrations 0.5 log apart, but the range used was higher: 185–15000 nM. Sequences of the other INH-ODN used appear in Figs. 1 and 3.

2.2. Definitions

"Potency" is the term for the concentration of an INH-ODN which inhibits the optimal stimulation by 50%. "% activity" refers to potency of the prototype INH-ODN (2114 or 4228)/potency of the test INH-ODN. Thus, the % activity of 2114 is set at 100%.

"Critical areas" refer to 3 pairs of bases in the INH-ODN sequence where single base changes caused the most profound decrease in

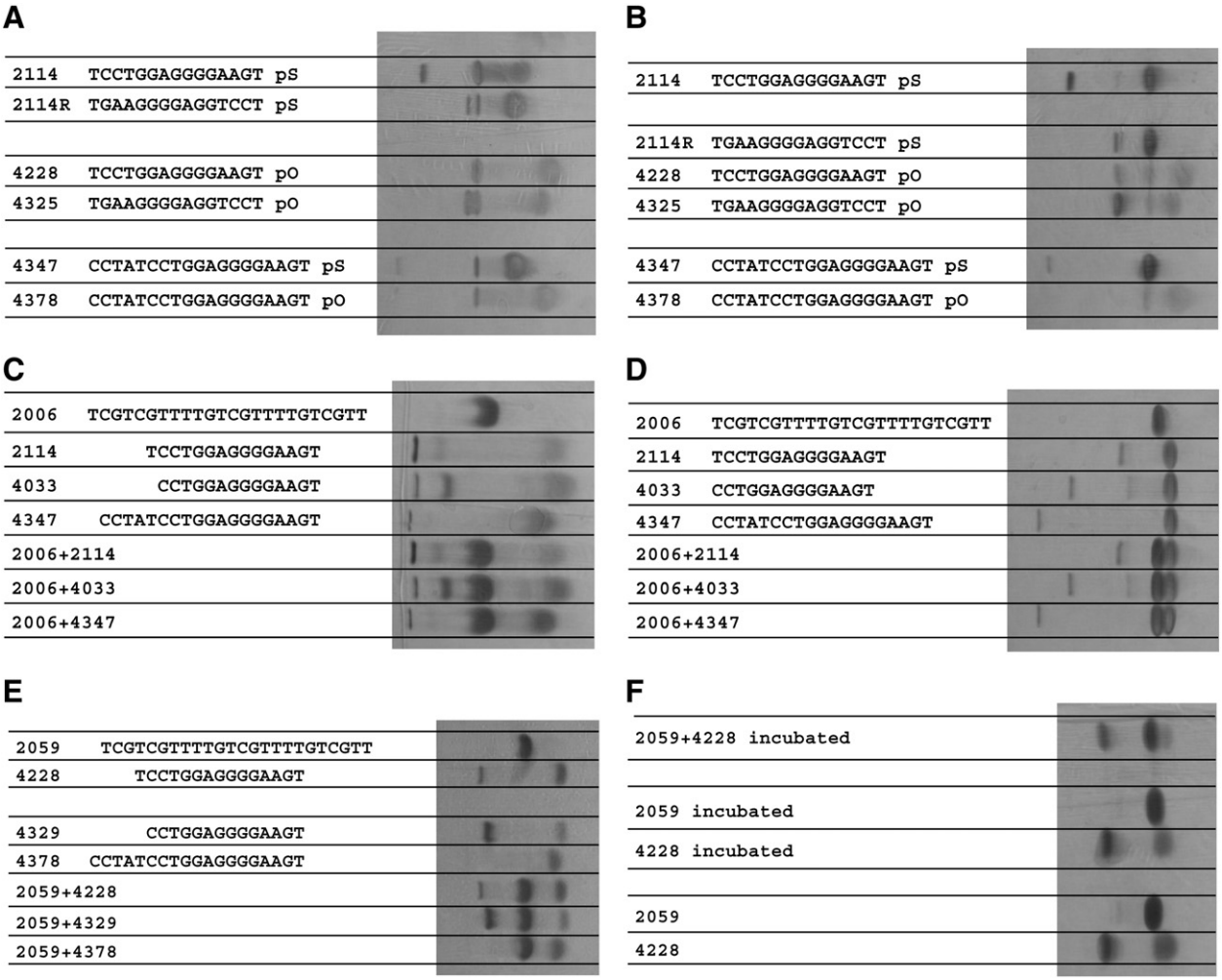

Fig. 1. Search for molecular associations between ODN. To investigate the self-association of INH-ODN or their inactive variants (panel A, pH 6.0; panel B, pH 6.5) and their ability to bind ST-ODN (Panel C, pS backbone, pH 6.0; Panel D, pS backbone, pH 7.0; Panel E, pO backbone, pH 6.0), ODN preparations were assayed by native 20% PAGE. Origin is at the left. In Panel F, pH 6.0, ST-ODN 2059 and INH-ODN 4228, both with pO backbones, were mixed and co-incubated or incubated separately for 48 h at 37 °C before assay on PAGE. For comparison, fresh 2059 and 4228 are also shown.