BINDING AND SIGNALING STUDIES OF SYNTHETIC OLIGODEOXYNUCLEOTIDES IN CYTOTOXIC CELLS

by

HARJEET KAUR

(Under the Direction of Donald L. Evans)

ABSTRACT

CpG motifs are the immunostimulatory component of bacterial DNA. Synthetic oligodeoxynucleotides (ODNs) containing CpG motifs activated natural killer (NK) cells. The present study was designed to investigate the binding and signaling effects of single base oligodeoxyguanosine on fish cytotoxic cells (Nonspecific cytotoxic cells; NCC) and human cytotoxic cells (NK cells). 20-mers of phosphodiester single base oligodeoxynucleotides (dG20, dC20, dA20 and dT20) and conventional dinucleotide CpG ODN were used in this study.

Tissue distribution studies in fish demonstrated that ODN dG20 bound to fish cytotoxic cells (NCC) in the anterior kidney (i.e. fish bone marrow equivalent), spleen and liver. dG20 binding to fish cytotoxic cells (NCC), human NK cells (YT-INDY), mouse macrophages (RAW 264.7) and human monocyte-macrophage cells (THP-1) was saturable and specific demonstrated by inhibition with unlabeled dG20 and CpG ODNs but not by dC20, dA20 or dT20. Southwestern blots of cell membrane proteins obtained from fish and human cytotoxic cells, as well as mouse and human macrophages

demonstrated two different mw species (14-18 kDa and 29-34 kDa) of dG20 and CpG binding proteins.

The other protein that binds polyguanosines is Scavenger receptor-A. Approximately 15-25% of purified NCC expressed Scavenger receptor-A. This receptor contributed to only 50% of dG20 binding by NCC.

Activation studies revealed that dG20 activated a 2-fold upregulation of membrane binding of homologous dG20-biotin in fish cytotoxic cells. dG20 also stimulated increased membrane expression of NCCRP-1(the activation marker of fish cytotoxic cells) and expression of cytosolic FasL that was released into the culture supernatants. dG20 and CpG treatment of human NK cells induced cellular DNA synthesis and dG20 increased cell numbers by approximately 10% at 10 hours post-treatment. Both dG20 and CpG ODN binding induced a calcium flux in human NK cells within seconds of treatment. Phosphodiester single base dG20 thus binds and mediates activation of fish and human cytotoxic cells and as such may comprise a new type of bacterial ligand important in initiation of innate immune responses.

INDEX WORDS: Oligodeoxynucleotides, Nonspecific cytotoxic cells, NK cells, FasL, Pattern recognition receptors, Pathogen associated molecular patterns, Scavenger receptor

BINDING AND SIGNALING STUDIES OF SYNTHETIC OLIGODEOXYNUCLEOTIDES IN CYTOTOXIC CELLS

by

HARJEET KAUR

B.V.Sc. & A.H., Gobind Ballabh Pant University of Agriculture and Technology, India, 1997

M.V.Sc., Gobind Ballabh Pant University of Agriculture and Technology, India, 1999

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2003

BINDING AND SIGNALING STUDIES OF SYNTHETIC

OLIGODEOXYNUCLEOTIDES IN CYTOTOXIC CELLS

by

HARJEET KAUR

Approved:

Major Professor: Donald L. Evans

Committee: Frederick Quinn
Harry W. Dickerson
Liliana Jaso-Friedmann
Thomas Krunkosky

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
August 2003

DEDICATION

I dedicate this dissertation to my family and my husband. Their unconditional love, support and understanding were integral to the completion of my studies.

ACKNOWLEDGEMENTS

I would like to thank number of people, for without them this degree would never have been possible.

First I would like to extend my sincere gratitude to my major professor, Dr. Donald L. Evans. His endless patience, guidance and encouragement enabled me to achieve this degree. I sincerely believe that his guidance has started my career on the path to success. I would also like to thank Dr. Liliana Jaso-Friedmann, a committee member, whose patience, support and invaluable suggestions not only helped me in completing my degree but also helped me through some rough times of life. I also extend my thanks to my committee members Drs. Frederick Quinn, Harry W. Dickerson and Thomas Krunkosky. Their suggestions have been invaluable. Drs. Frederick Quinn and Harry W Dickerson were gracious enough to join my committee at the eleventh hour.

A special thanks goes to the research co-ordinator of our laboratory, John Leary III, for helping me through out this period in my experiments. I also thank my colleague Kesavnair Praveen for helping me in some of my major experiments. Without his help it would not have been possible for me to achieve this goal. I wish to thank Dr. Zacharie Brahmi for providing me the cell line that I used for most of my studies.

I thank the entire department of Microbiology and Parasitology for their role in my academic endeavors at UGA.

Last but certainly not the least, I wish to thank my husband, Niraj, whose encouragement, support and love were extremely helpful during these four years. I also

thank my parents, brothers and sisters for all their love and support. I needed this from them and could not have achieved this goal otherwise. I also express my gratitude for my friend Devendra K. Sharma who helped me in bagging this opportunity to come to UGA for PhD in Immunology.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Cells of the innate immune system [i.e. Natural killer cells (NK cells), macrophages, etc.] must be activated in order to trigger the generation of optimal adaptive immune responses. These cells lack the highly specific antigen receptors of T and B cells, instead they rely on expression of a set of germline encoded receptors to recognize ligands. Pattern recognition receptors (PRRs) are one group of germline encoded receptors of innate immunity (Gordon, 2002) that are expressed constitutively and/or their expression is rapidly increased upon infection. CD14, mannose receptors, Scavenger receptors and a novel protein family of Toll-like receptors (TLRs) are some of the pattern recognition receptors. There are ten known family members of the TLR family (TLR1-10). NK cells are known to express some of these TLR family receptors (Hornung *et al*., 2002). Pattern recognition receptors have a general ability to detect certain molecular structures present in pathogens but not in self-tissues (Dempsey *et al*., 19961; Kumar *et al*., 1997). These are usually referred to as pathogen associated molecular pattern (PAMP) ligands (Medzhitov and Janeway, 1998) and include bacterial components like lipopolysaccharide (LPS), peptidoglycan (PGN), lipoteichoic acid (LTA), lipoproteins and bacterial DNA.

The immune system appears to use the presence of PAMPs as a danger signal that indicates the presence of infection and activates appropriate defense pathways. There is ample evidence that certain bacteria activate innate immunity in mammals utilizing the PAMP LPS (Zhang and Ghosh, 2000; Werling and Jungi, 2003). Binding of LPS by cells results in activation and induction of proinflammatory cytokine secretion. Another PAMP that recently evoked much interest in the field of DNA immunization is prokaryotic DNA (Krieg, 2002). Although prokaryotic DNA is known to have immune

stimulatory effects, it is only a few years since the discovery that immune stimulatory effects depended on the presence in bacterial DNA of unmethylated CpG (cytosine-phosphate-guanosine) dinucleotides in particular base motifs called CpG islands (Wagner, 1999). These effects could be reproduced by synthetic oligodeoxynucleotides (ODNs) containing these "CpG motifs" (CpG ODNs) (Yamamoto *et al*., 1994b). CpG motif containing DNA is referred to as CpG DNA. It is now generally accepted that immune activation by CpG motifs evolved as an immune defense against infection. In eukaryotic DNA, CpG motifs are relatively rare and are heavily methylated (Bird, 1993). Eukaryotic DNA thus has a subtly different molecular pattern compared to prokaryotic DNAs, and because it lacks these unmethylated CpG "danger signals," eukaryotic DNA does not activate innate immunity.

CpG DNA stimulates dendritic cells (DCs), macrophages, B cells and NCC directly, i.e. by binding to their membranes. CpG co-stimulates T cells and possibly B cells in the apparent absence of antigen presenting cells (APCs) and also stimulates NK cells indirectly by activating second party cells (DCs) to secrete cytokines. CpG ODNs containing hexamer palindromic sequences [5'-purine-purine-CpG-pyrimidine-pyrimidine-3'] mimic bacterial DNA and induce IFN-γ and other Th-1 type cytokines (Iho *et al*., 1999; Yamamoto *et al*., 1994a). Some ODNs that do not contain CpG motifs are also immunostimulatory. These include polyguanosine oligonucleotides (poly G) (Pisetsky and Reich, 1998; Lipford *et a*l., 2000) and some species-specific GpC oligonucleotides (Oumouna *et al*., 2002). These are referred to as non-CpG ODNs. Together immunostimulatory CpG and non-CpG ODNs are referred to as immunostimulatory sequences. Although the molecular mechanisms of CpG and non-

CpG DNA/ODNs induced immune activation are still incompletely understood, it appears from studies of mammalian cells that the CpG DNA undergoes receptor-mediated endocytosis, which then leads to the activation of mitogen-activated protein kinases, NFκB and other cell-signaling pathways (Hu *et al*., 2003). One CpG DNA receptor recently identified is a member of Toll like receptor family (TLR) and is known as TLR9 (Wagner, 2002). Evidence for the presence of other DNA receptors comes from the fact that different ODNs (CpG and non-CpG ODNs) produce different binding patterns. The identity of these non-TLR receptors is unknown and remains to be established.

Under physiological conditions, CpG DNA/ODNs promote professional APC function by causing autonomous maturation/activation of these cells. Under pathophysiological conditions, CpG DNA is involved in the production of septic shock. By promoting professional APC function and by co-stimulating lymphocytes, CpG-ODN also functions as a natural adjuvant (Kaisho and Akira, 2002; Zimmermann *et al*., 2003). One characteristic of their adjuvanticity is the induction of Th1-type humoral and cell-mediated responses to proteinaceous antigens and to plasmid DNA vaccines. The dominance of the Th1 response generated by immunostimulatory DNA is demonstrated in their ability to protect against Th2-type allergic diseases (e.g. asthma) by converting Th2 responses into protective Th1 responses (Kitagaki *et al*., 2002). Another therapeutic potential of CpG DNA includes anti-tumor immunity. Thus, CpG-DNA/ODNs appear to represent a previously underestimated pathogen-associated molecular pattern ligand recognized by pattern recognition receptors that influence both innate and adaptive immune responses.

Most of the information available describing the immune potential of immunostimulatory sequences is based on studies in mammalian models. The role of immunostimulatory sequences in activation of immune cells in lower vertebrates is still not fully understood. Nonspecific cytotoxic cells (NCC) are the teleost equivalent of mammalian NK cells. NCC play an important role in anti-protozoan and anti-bacterial immunity in fish. CpG DNA is one of the pathogen associated molecular patterns recognized by NCC (Oumouna *et al*., 2002). Only a few studies have been conducted in teleosts that demonstrate that CpG DNA/ODNs directly activate cells (Oumouna *et al*., 2002; Jorgensen *et al*., 2001). These studies did not provide any information regarding the identity of the receptors that bind CpG or non-CpG DNA/ODNs. Therefore, studying the role of CpG/non-CpG ODNs in lower vertebrate cells like fish NCC would enhance our understanding of innate immunity not only from an evolutionary point of view but may also provide information regarding the identity of the receptors involved in the signaling pathways activated by these pathogen associated molecular patterns. Similarly, studies of these same ODNs in their mammalian counterparts (NK cells) may provide additional information regarding the evolution of DNA binding membrane protein structures/sequences that are necessary for anti-bacterial immunity. In this dissertation, studies were focused on bacterial immunity and comparisons were made between aquatic and terrestial vertebrates.

The objective of the research comprising this dissertation was to test the hypothesis that synthetic olideoxynucleotides bind to and activate cytotoxic cells of fish and humans. To accomplish this objective the following specific aims were tested:

1) Determine the binding and activation potential of synthetic ODNs on nonspecific cytotoxic cells (NCC).
2) Determine the binding and activation potential of synthetic ODNs on mammalian NK cells.
3) Identify and characterize the ODN binding proteins.
4) Study the signaling induced by cross-linking of ODN receptor in cytotoxic cells.

REFERENCES

Bird A. P. 1993. Functions for DNA methylation in vertebrates. Cold Spring harbor Symp. Quant. Biol. **58:**281-285.

Dempsey P. W., M. E. Allison, S. Akkaraju, C. C. Goodnow, and D. T. Fearon. 1996. C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. Science **271:**348–350

Gordon S. 2002. Pattern recognition receptors: doubling up for the innate immune response. Cell. **111:**927-930.

Hornung V., S. Rothenfusser, S. Britsch, A. Krug, B. Jahrsdorfer, T. Giese, S. Endres, and G. Hartmann. 2002. Quantitative expression of Toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J Immunol. **168:**4531-4537.

Hu Z., S. Sun, and F. Zhou. 2003. The binding of CpG-oligodeoxynucleotides to cell-surface and its immunostimulatory activity are modulated by extracellular acidic pH. Vaccine. **21:**485-490.

Iho S., T. Yamamoto, T. Takahashi, and S. Yamamoto. 1999. Oligodeoxynucleotides containing palindrome sequences with internal 5'-CpG-3' act directly on human NK and activated T cells to induce IFN-gamma production in vitro. J Immunol. **163:**3642-3652

Jorgensen J. B., J. Zou, A. Johansen, and C. J. Secombes. 2001. Immunostimulatory CpG oligodeoxynucleotides stimulate expression of IL-1beta and interferon-like cytokines in rainbow trout macrophages via a chloroquine-sensitive mechanism. Fish Shellfish Immunol. **11:**673-682.

Kaisho T., and S. Akira. 2002. Toll-like receptors as adjuvant receptors. Biochim Biophys Acta. **1589:**1-13.

Kitagaki K., V. V. Jain, T. R. Businga, I. Hussain, and J. N. Kline. 2002. Immunomodulatory effects of CpG oligodeoxynucleotides on established Th2 responses. Clin Diagn Lab Immunol. **9:**1260-1269.

Krieg A. M. 2002. CpG motifs in bacterial DNA and their immune effects. Annu Rev Immunol. **20:**709-760.

Kumar A., Y. L. Yang, V. Flati, S. Der, S. Kadereit, A. Deb, J. Haque, L. Reis, C. Weissmann, and B. R. Williams. 1997. Deficient cytokine signaling in mouse embryo fibroblasts with a targeted deletion in the PKR gene: role of IRF-1 and NF-kappaB. EMBO J. **16:**406–416

Lipford G. B., S. Bendigs, K. Heeg, and H. Wagner. 2000. Poly-guanosine motifs costimulate antigen-reactive CD8 T cells while bacterial CpG-DNA affect T-cell activation via antigen-presenting cell-derived cytokines. Immunology. **101:**46-52.

Medzhitov R., and C. A. Janeway Jr. 1998. Innate immune recognition and control of adaptive immune responses. Semin Immunol. **10:**351-353.

Oumouna M., L. Jaso-Friedmann, and D. L. Evans. 2002. Activation of nonspecific cytotoxic cells (NCC) with synthetic oligodeoxynucleotides and bacterial genomic DNA: binding, specificity and identification of unique immunostimulatory motifs. Dev Comp Immunol. **26:**257-269.

Pisetsky D. S., and C. F. Reich. 1998. The influence of base sequence on the immunostimulatory properties of defined oligonucleotides. Immunopharmacology. **40:**199-208.

Wagner H. 1999. Bacterial CpG DNA activates immune cells to signal infectious danger. Adv Immunol. **73:**329-368

Wagner H. 2002. Interactions between bacterial CpG-DNA and TLR9 bridge innate and adaptive immunity. Curr Opin Microbiol. **5:**62-69.

Werling D., and T. W. Jungi. 2003. Toll-like receptors linking innate and adaptive immune response. Vet Immunol Immunopathol. **91:**1-12.

Yamamoto T., S. Yamamoto, T. Kataoka, K. Komuro, M. Kohase, and T. Tokunaga. 1994a. Synthetic oligonucleotides with certain palindromes stimulate interferon production of human peripheral blood lymphocytes in vitro. Jpn J Cancer Res. **85:**775-779.

Yamamoto T., S. Yamamoto, T. Kataoka, and T. Tokunaga. 1994b. Ability of oligonucleotides with certain palindromes to induce interferon production and augment natural killer cell activity is associated with their base length. Antisense Res Dev. **4:**119-122.

Zhang G., and S. Ghosh. 2000. Molecular mechanisms of NF-kappaB activation induced by bacterial lipopolysaccharide through Toll-like receptors. J Endotoxin Res. **6:**453-457.

Zimmermann S., K. Heeg, and A. Dalpke. 2003. Immunostimulatory DNA as adjuvant: efficacy of phosphodiester CpG oligonucleotides is enhanced by 3' sequence modification. Vaccine. **21:**990-995.

CHAPTER 2

LITERATURE REVIEW

NONSPECIFIC CYTOTOXIC CELLS AND NATURAL KILLER CELLS

Nonspecific cytotoxic cells (NCC) are the teleost equivalent of natural killer (NK) cells. These cells share many functional characteristics with their mammalian counterparts (Graves *et al*., 1984). Both NK cells and NCC are necessary participants in innate immunity during acute stress responses. In contrast to freshly obtained mammalian NK cells, fresh (non-cytokine induced) NCC kill a wide array of targets, including virus-infected cells (YAC-1) (Evans *et al*., 1984), tumor cell lines (K562, Daudi, HL-60) (Evans *et al*., 1984) and parasites (*Tetrahymena pyriformis*) (Graves *et al*., 1985). Several different activatory and inhibitory receptors are known to be associated with the functional activity of NK cells (Middleton *et al*., 2002). NCC do not utilize these receptors in target cell killing but instead express a Type III antigen receptor for its functional activation. This receptor is referred to as nonspecific cytotoxic cells receptor protein-1 (NCCRP-1) (Friedmann *et al*., 2001). It recognizes a discrete ligand referred to as natural killer target antigen (NKTag) which is found on many different types of target cells and protozoan parasites. NCCRP-1 expression is up-regulated when cells are activated by various stimuli (Evans *et al*., 1990). Activated NK cells utilize various molecules to kill the target cells that include perforin, granzyme and FasL (Trapani and Smyth, 1993; Vujanovic, 2001). On the other hand, the only molecule that has been identified so far in NCC is FasL (Evans *et al*., 2000; Friedmann *et al*., 2000; Bishop *et al*., 2002; Evans *et al*., 2001).

Cells of innate immune system like NK and NCC can be differentially activated by variety of stimuli. These include tumor cells, protozoan parasites and bacterial components like lipopolysaccharide, peptidoglycan, flagella and bacterial DNA and viral

RNA. These antigens activate cells of immune system and are called pathogen associated molecular pattern (PAMP) ligands (Medzhitov and Janeway, 1998). PAMP are recognized by a set of receptors called pattern recognition receptors (PRR). The most conserved family of PRR are known as Toll like receptors (TLR) (Takeda *et al*., 2003). There are ten known members (TLR-1-10) of this family (Takeda *et al*., 2003). Recognition of bacterial DNA is mediated by a member of the Toll like receptor family known as TLR9 (Wagner, 2002). Engagement of this receptor by bacterial DNA induces cell signaling and subsequently triggers a pro-inflammatory cytokine response and a predominantly Th1-type immune response. The immunostimulatory potential of bacterial DNA resides in the dinucleotide motifs called cytosine-phosphate-guanosine (CpG) (Wagner, 1999). The bacterial DNA analogue is referred to as CpG DNA. The synthetic analogues of bacterial DNA are single-stranded oligodeoxynucleotides (ODNs) containing specific phosphodiester or phosphorothioate derivatized CpG dinucleotide motifs (Yamamoto *et al*., 1994a).

BACTERIAL DNA AND THE IMMUNE RESPONSE

The first evidence that bacterial DNA had immunostimulatory potential was provided by the work of Tokunaga and colleagues (Tokunaga T *et al*., 1984; Yamamoto *et al*., 1988). They reported that DNA prepared from BCG activated IFN secretion and anti-tumor activity by NK cells. Others (Yamamoto *et al*., 1992a) later showed that bacterial DNA, but not eukaryotic DNA induced activation of NK cells. Extensive studies to determine the structural requirements of this immune stimulatory activity revealed that bacterial DNA contained specific sequences arranged in a general context of Purine-Purine-CpG-Pyrimidine-Pyrmidine ("CpG motifs") (Krieg *et al*., 1995; Ballas *et al*., 1996).

Vertebrate DNA and microbial DNA differ in the content and methylation status of CpG motifs. Bacterial DNA contains four fold more CpG motifs compared to eukaryotic DNA (Bird, 1987). The CpG motifs are non-methylated in bacteria, virus and retrovirus whereas these are methylated in vertebrates at 5'-cytosine (Bird, 1993). The exception occurs in the CpG islands found in the promoters of housekeeping genes where they are assumed to act as origins of DNA replication (Antequera and Bird, 1999). Spontaneous deamination of methylated 5'-cytosine can escape detection of the DNA repair system. This makes methylated CpG islands as mutational hotspots. It was predicted that as a consequence and over evolutionary time, methylated CpGs tend to be lost from the genome of vertebrates. This phenomenon has been referred to as CpG suppression (Schorderet *et al*., 1992).

Mammalian immune cells of both innate and adaptive immune systems are responsive to bacterial DNA (CpG-DNA) (Lipford *et al*., 1998; Heeg *et al*., 1998; Sparwasser *et al*., 1998; Sparwasser *et al*., 1997; Stacey *et al*., 1996; Hacker *et al*., 2000a; Pisetsky *et al*., 1995). The immunostimulatory activity of bacterial DNA is restricted to 12-20 base sequences containing the CpG dinucleotides with selective flanking bases (Yamamoto *et al*., 1994b). Therefore, synthetic ODNs consisting of CpG motifs/ CpG nests and appropriate flanking bases are used to study the effects of bacterial DNA on the innate and adaptive immune systems.

FACTORS INFLUENCING IMMUNOSTIMULATION BY CpG ODNs

Base sequence. CpG ODN mediated activation of immune cells is dependent on base sequences. It is suggested that the TLR9 reads CpG DNA sequence from the 5'- to the

3'-end, and the modifications that block the 5'-end abrogates immunostimulation (Kandimalla *et al*., 2002; Bhagat *et al*., 2003). ODNs with different numbers of CpG motifs and variable flanking sequences produce different effects on immune cells (Bauer *et al*., 1999; Pisetsky, 1999; Ballas *et al*., 2001; Gursel *et al*., 2002; Krieg *et al*., 1998a; Blazar *et al*., 2001; Messina *et al*., 1993). Inversion of CpG to GpC (guanosine-phosphate-cytosine) or methylation of the CpG motifs in the ODN abrogated the immunostimulatory potential of these CpG ODNs in murine and human cells (Schluesener *et al*., 2001). A recent study indicated that inversion of CpG to GpC did not abrogate the interferon inducing capacity of CpG (Magnusson *et al*., 2001). Similarly, the inversion of CpG to GpC caused only a partial reduction of interleukin-12 (IL-12) and interferon-gamma (IFN-γ) inducing activity of these ODNs in murine cells (Parronchi *et al*., 1999). In teleosts, both CpG and GpC ODNs are shown to be equally effective in activation of cytotoxic activity of NCC (Oumouna *et al*., 2002). *In vivo* studies in pigs have shown that CpG as well as GpC ODNs significantly enhanced the antigen specific and the mitogen induced proliferation of cells in different lymphoid tissues (Van der Stede *et al*., 2002). CpG dinucleotides in certain base contexts are shown to have a neutralizing effect on the activation potential of other immunostimulatory CpG ODNs. An example of this would be CpG dinucleotides preceded by a G/C or followed by a G/C (Krieg *et al*., 1998b; Zhao *et al*., 2000).

Flanking sequences. The CpG dinucleotide motif as well as the flanking sequences influence immune cell activation (Bauer *et al*., 1999) by ODNs. The minimal essential structure required for interferon induction in murine spleen cells was shown to be the

hexamer sequence AACGTT or GACGTT and for human spleen cells GTCGTT (Sonehara *et al*., 1996; Yamamoto *et al*., 1994b). Optimal activation of B-cell, dendritic cell, macrophage and NK cells was produced by CpG motifs flanked by two 5' purines (preferably a GpA or ApA dinucleotide) and two 3' pyrimidines (preferably a TpC or TpT dinucleotide) (Krieg *et al*., 1995; Iho *et al*., 1999; Sonehara *et al*., 1996; Yamamoto *et al*., 1994a; Boggs *et al*., 1997; Yamamoto *et al*., 1994b; Pisetsky and Reich, 1993). Certain palindromic hexamer sequences like GACGTT, AGCGCT and AACGTT were active for NK cells whereas non-palidromic sequences were inactive (Yamammoto *et al*., 1992b). Similar to mammals, the hexamer palindrome sequence of 5'-purine-purine-CpG-pyrimidine-pyrimidine-3' were stimulatory for teleosts. For this species, the 5' purines were preferably GpT and 3' pyrimidines were TpT (Oumouna *et al*., 2002).

Sequences at 5' and 3' end of active hexamer. Among compounds with an active hexamer, the extent of cell activation varies with flanking sequence. Of the four types of nucleotides, guanosine (in the form of poly G runs) was the most immunostimulatory (Ballas *et al*., 1996; Pisetsky and Reich, 1998; Ballas *et al*., 2001; Lang et al., 1999). The effects of polyguanosine (poly G) runs, however, depended on the location (Lee *et al*., 2000), the chemical property of the ODN backbone (Lee *et al*., 2000; Ballas *et al*., 2001) and the target cell type (Ballas *et al*., 2001). Uptake and binding of CpG ODNs are strongly enhanced by the addition of flanking poly G runs at the 3' end of the ODN (Yi and Krieg, 1998; Lee *et al*., 2000; Pisetsky and Reich, 1998).

Length. The length of the ODNs also affects the immunostimulatory potential of ODNs. The optimum length for effective immunostimulation was shown to be between 18-30 bases (Yamamoto *et al*., 1994b). ODNs as short as 15 bases in length (Ballas *et al*., 1996) and as long as 45 bases (Tokunaga *et al*., 1992) were also effective in inducing NK cell lytic activity *in vitro* and IFN-γ production.

Backbone specific. Besides the "CpG motif" and the flanking sequences, there are some species-specific and cell-specific effects of backbone modifications of CpG DNA/ODNs. The same ODN might not be stimulatory for all the cells (Ballas *et al*., 2001; Gursel *et al*., 2002). The unmodified backbone of ODNs is composed of phosphodiester (Po) linkages whereas the modified backbone is referred to as phosphorothioate (Ps) derivitized. It contains sulfur in place of a non-bridging oxygen of the phosphodiester. The Ps backbone conferred both nuclease resistance and improved cellular uptake of the ODN. Nuclease resistance, conferred by the Ps backbone alone, was not sufficient for enhanced ODN potency (Zimmermann *et al*., 2003). An alternative approach for conferring nuclease resistance in CpG ODNs has recently been determined. This was accomplished by the tethering of Po poly G runs on 3' end of CpG ODNs (Zimmermann *et al*., 2003). The Ps backbone had both enhancing and inhibitory effects on macrophage responses to CpG DNA (Sester *et al*., 2000). Ps derivitized ODNs were active at 10-to100-fold lower concentrations than corresponding phosphodiester ODNs in maintenance of macrophage viability in the absence of colony stimulating factor-1 (CSF-1); in induction of nitric oxide production; and in the activation of the IL-12 promoter (Liang *et al*., 1996). Marked B cell activation was also seen with Ps ODNs compared to

Po ODNs (Liang *et al*., 1996). Unlike macrophages and B cells, NK cell activity was reduced following treatment with Ps derivitized ODNs (Ballas *et al*., 2001). An ODN with the chimeric backbone Po-Ps-Po linkage, in combination with poly G tails, was a potent inducer of NK lytic activity but had little effect on cytokine secretion or B cell proliferation (Ballas *et al*., 2001).

Species and cell specific. In various cell types, differential requirement regarding the CpG ODN sequences between human and mice are seen. In general, GACGTT was stimulatory for murine spleen cells (Jones *et al*., 1999) whereas GTCGTT (Jones *et al*., 1999) was stimulatory for human and bovine spleen cells (Zhang *et al*., 2001). Certain hexamer palindromes with CpG dinucleotides, for example GACGTC, that did not activate mouse NK cells (Ballas *et al*., 1996) were found to be active for human NK cells (Iho *et al*., 1999). The Purine-purine-CpG-pyrimidine-pyrimidine sequences that were immunostimulatory for mouse spleen cells, such as AACGCT or AACGTC (Krieg *et al*., 1995), did not directly activate mouse (Ballas *et al*., 1996) and human NK cells (Iho *et al*., 1999). Hexameric CpG ODN known to be actively stimulatory for mouse B cells (AACGTT) (Lee *et al*., 2000; Pisetsky and Reich, 1998) were not active for human B cells (Liang *et al*., 2000). For monocytes and macrophages, hexamer palindromes behaved actively regardless of the species (Yamamoto *et al*., 1994b; Sato *et al*., 1996).

OTHER IMMUNOSTIMULATORY DNA SEQUENCES/ SINGLE BASE ODNs

Although CpG motifs are the immunologically active component of immunostimulatory sequences (ISS), other DNA sequences also induce immune cell activation or potentiate responses induced by ISS.

Single base guanosine ODNs. ODNs containing stretches of deoxyguanosine (dG)/Poly G runs (Guanosine-rich or G-rich ODNs) displayed enhanced immunological activities (Sasaki *et al*., 1996; Lipford *et al*., 2000; Pisetsky and Reich, 1998; Messina *et al*., 1993; Pisetsky and Reich, 1993; Verthelyi *et al*., 2001; Kimura *et al*., 1994; Filion *et al*., 2003). Unlike CpG-ODNs that mimic the structural characteristics of bacterial DNA, G-rich ODNs have no accepted naturally occurring counterpart associated with the bacterial genome. However, the telomeric ends of vertebrate chromosomes contain G-rich ODNs (McElligott *et al*., 1997; Wright *et al*., 1997) and G-rich regions are known to occur in the promoter regions of certain housekeeping genes (Siddiqui-Jain *et al*., 2002). The immunostimulatory effects of G-rich ODN may result from the variant structures that dG can form because of their ability to form Hoogsteen base pairs. This type of base pairing forms structures known as quadruplex DNA, G-quadruplexes or G-tetrads and are composed of four stranded arrays in either parallel or antiparallel orientations (Venczel *et al*., 1993; Sen and Gilbert, 1990). These conformation dependent ligands however bind to pattern recognition proteins that have been identified in the present research. The immunological effects of poly G ODNs extend from antigen presenting cells (APC) that include B cells, macrophages and dendritic cells (DC) (Pisetsky and Reich, 1998) to cytotoxic cells (NK cells) (Kimura *et al*., 1994).

ODNs containing guanosine analogs. Another class of small DNA molecules with immunostimulatory activity is composed of ODNs containing modified guanine ribonucleosides (Lee *et al*., 2003; Kandimalla et al., 2003). An ODN containing C8-substituted and N7, C8-disubstituted guanine ribonucleosides (Lee *et al*., 2003) stimulated both humoral and cellular immune responses in a variety of animal models. The antiviral actions of these guanosine analogs were attributed to their ability to induce Type I IFNs. The molecular mechanisms by which the guanosine analogs potentiate immune responses are not known but the role of toll like receptor 7 (TLR7) is implicated. Similarly, another ODN containing a synthetic CpR motif (R = 2'-deoxy-7-deazaguanosine) was a more potent immunostimulatory agent than the naturally occurring CpG homolog (Kandimalla *et al*., 2003) in induction of cytokine secretion from murine spleen cells, anti-tumor activity and reversal of T helper 2 (Th2) type immune response to T helper 1 (Th1) type.

Non-CpG ODNs. Recent studies have shown that ODNs lacking CpG (non-CpG) motifs are also immunostimulatory (Sano *et al*., 2003; Uhlmann and Vollmer, 2003; Wang and Krieg, 2003). Non-CpG ODNs when conjugated to ovalbumin (OVA) antigen enhanced the immune responses by improving the capture of ODN conjugated antigen and antigen presentation by APCs, and induction of Th2 differentiation (Sano *et al*., 2003). These effects were not dependent on the sequence of ODN. Non-CpG ODNs rich in thymidine had immunostimulatory effects on human leucocytes (Vollmer *et al*., 2002). In the same study immunostimulatory effects were observed with methylated CpG ODNs also,

specifically when the ODN length was increased from 18 to 24 or more nucleotides (Vollmer *et al*., 2002).

Naturally occurring immunostimulatory DNA. Besides synthetic ODNs, naturally occurring forms of immunostimulatory non-CpG DNA also exist. Genomic DNA from dying and necrotic cells (Ishii *et al*., 2001) induced maturation of antigen presenting cells. DNA from certain plants was immunostimulatory for B cells, macrophages and DC (Wang et al., 2002). Similarly, non-CpG ODNs and methylated vertebrate genomic DNA synergized with specific antigen (hen egg lysozyme/HEL) in stimulating HEL-specific B cells to proliferate, to express the early activation marker CD69 and to activate the NF-kappaB pathway (Wang and Krieg, 2003).

EFFECTS OF CpG DNA/ODNs AND SINGLE BASE ODNs ON CELLS OF THE IMMUNE SYSTEM

Monocytes, macrophages and dendritic cells. Antigen-presenting cells (APC) including dentritic cells (DC) (Sparwasser *et al*., 1998); macrophages (Stacey *et al*., 1996; Sparwasser *et al*., 1997) and B cells (Hacker *et al*., 2000a) are directly stimulated by CpG DNA. CpG DNA induced APC maturation (Schattenberg *et* a*l*., 2000; Bauer *et al*., 2001a), cytokine production (Halpern *et al*., 1996; Gilkeson *et al*., 1998) and up-regulation of major histocompatibility complex (MHC) molecules. Co-stimulatory molecules on APC such as B7.1, B7.2 and CD40 (Lipford *et al*., 1997) were also upregulated by CpG DNA. Synthetic CpG ODNs directly stimulated DC (Hartmann *et al*., 1999; Vogel *et al*., 2000; Sparwasser *et al*., 2000; Krug *et al*., 2001a; Wang *et al*.,

2002) and macrophages (Sester *et al*., 1999; Pisetsky and Reich, 2000; Stacey *et al*., 2000; Zhang *et al*., 2001; Schluesener *et al*., 2001) to release various cytokines such as tumor necrosis factor (TNF-α), IL-1, IL-6, IL-10, IL-12 and granulocyte-macrophage colony-stimulating factor (GM-CSF). In addition, CpG-ODNs induced production of interferons (IFN-α/β) from dendritic cells (Krug *et al*., 2001a) and nitric oxide from macrophages (Shoda *et al*., 2001; Utaisincharoen *et al*., 2002). In lower vertebrates such as fish, CpG ODNs induced activation of macrophages determined by induction of IL-1β and IFN-like cytokine secretion (Jorgensen *et al*., 2001a; Jorgensen *et al*., 2001b). The inclusion of poly G runs in ODNs bearing CpG or non-CpG motifs increased the binding and uptake of these ODNs by macrophages and DCs (Dalpke *et al*., 2002; Lee *et al*., 2002). CpG ODN rich in guanosine induced sustained proliferation of macrophage-like cells in contrast to non-G-rich CpG ODNs (Lang *et al*., 1999) and induced production of IL-6, IL-12 and TNF-α from macrophages and DC (Zimmermann *et al*., 2003; Lee *et al*., 2000). Single base poly guanosine ODN induced DNA synthesis in macrophages (Sasaki *et al*., 1996). The G-rich ODN prevented ongoing disease and induced protective immune response in infected mice (Zimmermann *et al*., 2003). Contrary to the growth promoting effects of G-rich ODNs, reports have been published showing the inhibitory effects on cellular proliferation produced by ODNs containing G quartets (Saijjo *et al*., 1997; Castier *et al*., 1998; White *et al*., 1996).

B cells. B cells are directly stimulated by CpG DNA (Pisetsky and Reich, 1993; Hacker *et al*., 2000a) to differentiate into plasma cells secreting antibodies (Brown *et al*., 1998; Shoda *et al*., 2001), to secrete IL-6 and IL-10 and induce proliferation and polyclonal

immunoglobulin production (Krieg *et al*., 1995; Goeckeritz *et al*., 1999; Hartmann and Krieg, 2000; Hartmann *et al*., 2000; Chen *et al*., 2001). The immunological effects of G-rich ODNs extend to B cells. CpG motifs with poly G runs were mitogenic for B cells under conditions in which other synthetic homopolymers as well as mammalian DNA were inactive (Pisetsky and Reich, 1993; Pisetsky and Reich, 1998; Pisetsky and Reich, 1999). Binding and uptake of CpG ODNs in B cells was strongly enhanced by the addition of flanking poly G runs at the 3' end of the ODN (Pisetsky and Reich, 1998). ODNs flanked by poly G runs at 3' end induced IL-12 production from B cells whereas those flanked by other three nucleotide runs did not (Lee *et al*., 2000). Conflicting results have been reported for the effects of single base ODNs on B cells (Liang *et al*, 1996; Pisetsky and Reich, 1998). Liang et al. (1996) reported that neither Po nor Ps forms of any of the four single base ODNs induced B cell activation. In contrast, Pisetsky and Reich (1998) showed that a 30-mer of dG induced B cell proliferation and up-regulated CD69 expression. Nonetheless, both these studies showed that single base guanosine ODN bound B cells and T cells (Pisetsky and Reich, 1998).

NK cells. NK cells are stimulated both directly (Iho *et al*., 1999) and indirectly (Kimura *et al*., 1994) by CpG DNA. Direct activation of NK cells resulted in increased production and secretion of IFN-γ by these cells which in turn enhanced NK cell lytic activity (Iho *et al*., 1999). Indirect activation of NK cells by CpG DNA/ODNs also resulted in enhanced NK cell-mediated cytotoxicity and release of IFN-γ (Kimura *et al*., 1994; Chace *et al*., 1996; Ballas *et al*., 1996; Iho *et al*., 1999; Yamamoto *et al*., 2000; Hafner *et al*., 2001). This stimulatory effect, however, required the presence of adherent cells which produced

IL-12, TNF-α and type I interferons. These cytokines together with CpG DNA stimulated NK cells (Chace *et al*., 1996). Stimulatory effect on NK cells was demonstrated following G-rich ODN binding (Kimura *et al*., 1994; Ballas *et al*., 1996; Verthelyi *et al*., 2001). Inclusion of dG sequences in ODNs bearing CpG or non-CpG motifs increased the cytolytic activity and secretion of IFN-γ by murine NK cells (Kimura *et al*., 1994). The potentiation of cytokine production was the result of increased ODN binding to macrophages mediated by Scavenger receptors (SR). CpG ODNs flanked by poly G runs on both 3' and 5' ends induced NK cell activation at a dose much smaller than the CpG ODN that were flanked by poly A runs (Ballas *et al*., 1996; Ballas *et al*., 2001). It was recently shown that CpG ODN activated NCC had increased cytolytic activity against tumor target cells (Oumouna *et al*., 2002). Similar to CpG ODNs, single base poly G ODN also increased the cytolytic activity of NCC (Oumouna *et al*., 2002).

T cells. CpG DNA/ODNs have multiple effects on T cell function *in vivo* and *in vitro* (Mannon *et al*., 2000; Sun *et al*., 2000; Warren *et al*., 2000; Brunner *et al*., 2000; Lipford *et al*., 2000; Storni *et al*., 2002). T cells are activated either directly (Mannon *et al*., 2000) or indirectly by cytokines from APC (Stacey *et al*., 1996; Sun *et al*., 2000; Warren *et al* 2000; Brunner *et al* 2000; Storni *et al* 2002; Krug et al., 2003) or by co-stimulation with CpG DNA/ODNs and cytokines (Lipford *et al*., 2000). Co-stimulation is active against CD8 but not CD4 T cells. ODN mediated co-stimulation resulted in IL-2-driven T-cell proliferation and activation of cytolytic T cells (CTLs). Similarly, a costimulatory effect was seen in CD8 T cells following poly G ODN binding (Lipford *et al*., 2000).

APCs activated by CpG ODN induced CTL responses that were fully protective against infection with lymphocytic choriomeningitis virus or recombinant vaccinia virus (Storni *et al*., 2002).

APOPTOSIS AND CpG ODNs

The pro- and anti-apototic effects of CpG ODNs may vary according to the cell type. For example, CpG provided a survival signal to antigen presenting cells (DCs and B cells) (Yi AK *et al*., 1999; Yi and Krieg, 1998a; Wang *et al*., 1997; Park *et al*., 2002). This might be a mechanism by which bacterial DNA stimulated and maintained innate immune responses. On the other hand, CpG ODNs produced pro-apoptotic effects in transformed cells. *In vivo* antitumor studies by Shen and colleagues (2002) showed that ODNs containing the pro-apoptotic polyG motifs significantly inhibited prostate tumor growth by inducing apoptosis in these cells. CpG motifs were not essential for anti-tumor activity of these ODNs but the effects were indirectly enhanced by CpG immune activating sequences. Tidd *et al* (2003) established that treatment of the human leukemia line MOLT-4 with CpG oligodeoxynucleotide 5mers composed of the sequence CGNNN (N = A, G, C or T) rapidly induced apoptosis/cell cycle arrest. The presence of the 5'-CpG motif was obligatory for these effects. Induction of apoptosis in MOLT-4 cells did not require new protein synthesis and was insensitive to caspase 3 inhibitors. CpG ODNs participated in limiting the spread of pathogens by activating the effectors of the innate immune response and promoting an adaptive immune response (Verthelyi *et al*., 2003). Verthelyi *et al* (2003) demonstrated that CpG ODN treatment of rhesus macaques significantly reduced the severity of the lesions caused by a challenge with Leishmania.

Leishmania superinfection is common in immunocompromised hosts, particularly those infected with HIV.

CLASSIFICATION OF SYNTHETIC ODNs AND CROSS-INHIBITORY POTENTIAL OF ODNs

CpG oligonucleotides (ODN) are classified in two structurally different classes: CpG-D/A type and CpG-K/B type. This classification is based on the type of immune response initiated by these ODNs. CpG-K/B type phosphorothioate (Ps) ODNs expressing multiple CpG motifs stimulate immune cells to proliferate and secrete IL-6 and IgM (Bohle *et al*., 2001; Liang *et al*., 2000; Hartmann and Krieg, 2000; Gursel *et al*; 2002). CpG-D/A type ODNs, which contain a phosphodiester (Po) pur-pyr-CpG-pur-pyr motif, capped at each end by a Ps polyG tail, stimulate NK cells to produce IFN-γ (Verthelyi *et al*., 2001; Ballas *et al*., 2001; Gursel *et al*; 2002) and stimulate dendritic cells to produce large amounts of IFN-α compared to the CpG-K/B type (Krug *et al*., 2001a; Hemmi *et al*., 2003). Kerkmann *et al*. (2003) showed that CpG-A ODN had higher and prolonged kinetics of Type I IFN production whereas CpG-B ODN was more active in stimulating IL-8 production and increasing expression of costimulatory and Ag-presenting molecules in dendritic cells. This suggested that CpG-A and CpG-B trigger distinct regulatory pathways in peripheral dendritic cells. Gursel *et al*. (2002) showed that both types of ODNs bind to and are internalized by B cells, NK cells and monocytes. They also showed that in monocytes these two types of ODNs cross-inhibited each other activity and the intracellular localization and the functional activity of these ODNs differed from each other.

Apart from these two classes, single base ODNs represent the third class of ODN that has varied immunological effects on cells (Liang *et al*, 1996; Pisetsky and Reich, 1998; Zhu *et al*., 2002a; Zhu *et al*., 2002b). Single base ODNs inhibited the binding (Zhu *et al*., 2002a) and immunostimulatory activity of bacterial DNA or CpG-ODNs (Halpern and Pisetsky, 1995; Zhu *et al*., 2002a; Zhu *et al*., 2002b). This effect was seen with 30-mer Po dG and Ps ODNs (dA, dT, dG, and dC). These ODNs blocked the production of nitric oxide in macrophages (Zhu *et al*., 2002b) and blocked IL-12 and nitric oxide production by DC (Zhu *et al*., 2002a) induced by bacterial DNA, an immunostimulatory CpG ODN and LPS. Furthermore, these compounds inhibited up-regulation of costimulatory molecules CD40 and CD86 as well as major histocompatibility complex-II molecules (Zhu *et al*., 2002a) indicating that ODNs had an effect on DC maturation. 20-mers and 5-mers of Ps dG inhibited cytokine production by mouse splenocytes by bacterial DNA (Halpern and Pisetsky, 1995).

PATTERN RECOGNITION RECEPTORS (PRR) FOR BACTERIAL DNA/ODNs

A number of receptors are described that bind bacterial DNA and synthetic ODNs (Hemmi *et al*., 2000; Kimura *et al*., 1994; Benimetskaya *et al*., 1997). These membrane proteins are broadly referred to as pattern recognition receptors (PRR). The most well characterized of these are: a member of the Toll like-receptor (TLR) family known as TLR-9 (Hemmi *et al*., 2000; Bauer *et al*., 2001b; Kirschning and Bauer, 2001; Takeshita *et al*., 2001; Ashkar and Rosenthal, 2002; Chuang *et al*., 2002; Wagner, 2002); Scavenger receptor (SR) (Kimura *et al*., 1994; Biessen *et al*., 1998); and Mac-1 (CD11b/CD18) (Benimetskaya *et al*., 1997). Similar to the requirements of cellular activation, binding of

CpG ODNs also depended on the base sequence. Some ODNs had a higher propensity to bind cells than others. Human TLR9 bound to CpG DNA via species-specific CpG motif recognition (Jones *et al*., 1999). TLR9 is a 120 kDa protein (Hemmi *et al*., 2000) and is expressed by most immune cells including NK cells, B cells, macrophages and dendritic cells (Hornung *et al*., 2002). The optimal CpG motif for human TLR9 recognition is GTCGTT, whereas the optimal murine sequence is GACGTT.

The presence of dG sequences also affected the binding of ODNs to the cell surface when they flank six base hexamer CpG motifs (Kimura *et al*., 1994; Pisetsky and Reich, 1998; Yi *et al*., 2001). Single base oligodeoxynucleotides also bound cells. Those consisting of homopolymer of guanosine had the highest binding activity compared to homopolymers composed of the other bases (Liang *et al*, 1996; Pisetsky and Reich, 1998). It is known that G-rich ODNs bind a receptor on mammalian monocytes, macrophages, B lymphocytes, capillary endothelial cells, platelets and adipocytes and fish macrophages (Dannevig *et al*., 1994; Frøystad *et al*., 1998; Frøystad *et al*., 2002); sinusoidal and endocardial endothelial cells (Gjøen *et al*., 1992; Sørensen *et al*., 1998); liver parenchymal cells (Malerod *et al*., 2002); and NCC (Kaur *et al*., in press). At least one receptor responsible for this binding is referred to as a Scavenger receptor-A (SR-A) (Pearson *et al*., 1996; Zhu *et al*., 2001). Scavenger receptor-A belongs to a family of receptors known as Scavenger receptor (SR) family. This family is classified, based on the structure, into six different classes (class A-F) (Nakamura *et al*., 2001). Members of some of these classes (A, C and E) are implicated in bacterial binding and uptake (Peiser *et al*., 2002). Scavenger receptors do not bind native DNA, but instead recognize ODNs based on conformation of the ligand. The ligand pattern is formed by intrachain and

interchain Hoogsteen base pairing between multiple guanosine bases (e.g., quadruplex DNA) (Wloch *et al*., 1998; Prasad *et al*., 1999). The specificity of this binding to SR depended on the base-quartet-stabilized G-quadruplex structure (Pearson *et al*., 1993). Besides poly G, SR also bind to a variety of other macromolecules that include bacterial lipopolysaccharide, acetylated lipoproteins, dextran sulfate and polyvinyl sulfate (Fitzgerald *et al*., 2000; Ramet *et al*., 2001; Brown *et al*., 1980; Pearson *et al*., 1996). This receptor contributed to the binding of G-rich ODNs on various cells but is not essential for the uptake and immunostimulatory activity of these ODNs (Zhu *et al*., 2001).

Multiple other DNA-binding proteins have been described. They ranged in size from 20 to 143 kDa and were precipitated from the cell surfaces using chemical cross-linking with biotinylated ODNs followed by detection with Avidin-peroxidase (Bennett *et al*., 1985; Yakubov *et al*., 1991; Beltinger *et al*., 1995; Laktionov *et al*., 2001). These proteins have not been identified at the sequence level and their immunostimulatory activities are still unknown. Some have proposed that these proteins might be involved in the transport of ODNs into cells (Laktionov *et al*., 1999). Perhaps the best characterized of these proteins is Mac-1 (CD11b/CD18). This protein is a cell-surface receptor that is expressed by polymorphonuclear leukocytes, macrophages and natural killer cells (Benimetskaya *et al*., 1997). Mac-1 bound ODNs and mediated their internalization in these cell types (Benimetskaya *et al*., 1997).

Finally, competitive binding inhibition studies between CpG DNA and bacterial DNA and between CpG ODNs and ODNs composed of poly G runs (oligodeoxyguanosine) have shown that more than one receptor exists (on cells) for

bacterial DNA/ODNs (Zhu *et al*., 2002a; Zhu *et al*., 2002b; Oumouna *et al*., 2002). These studies suggested that an overlapping array of receptor(s) exist for CpG, bacterial DNA and single base oligodeoxyguanosine.

SIGNAL TRANSDUCTION BY CPG DNA/ODNS AND SINGLE BASE ODNs

There are conflicting reports in the literature concerning the mechanism of CpG ODN binding to cells and the initiation of subsequent signaling responses. Unlike lipopolysaccharide (LPS) which does not require endocytosis to functionally associate with the membrane expressed TLR4/MD2 complex (i.e. the PRR for LPS), internalization and endosomal maturation is conditional for CpG-DNA to activate TLR9 (Ahmad-Nejad *et al*., 2002). The two PAMPs (LPS and CpG-DNA) thus trigger signaling from two different cellular locations: the former at the cell membrane and the latter at the lysosomal compartment. Except for two reports on B cells (Liang *et al*., 1996; Pisetsky and Reich, 1998), most of the literature supported the hypothesis that binding of CpG DNA/ODNs is carried out by surface receptors. However, the activation of cell signaling pathways occurred only after internalization of the CpG DNA, along with co-internalization of its receptor and their acidification in the endosomal compartment (Macfarlane and Manzel, 1998; Hacker *et al*., 1998; Yi *et al*., 1998; Manzel and Macfarlane, 1999; Hacker, 2000; Ahmad-Nejad *et al*., 2002; Hu *et al*., 2003). The two reports on B cells (Liang *et al*., 1996; Pisetsky and Reich, 1998), however, showed that internalization is not necessary for ODN induced B cell activation. Binding of CpG ODNs to murine splenocytes and human peripheral blood mononuclear cells and subsequent triggering of CpG-related signaling was pH-dependent (Hu *et al*., 2003).

That is, more binding occurred and with greater specificity at acidic pH (6.4) compared to near neutral pH of 7.4. Drugs such as chloroquine and quinacrine that interfered with the endosomal processing/maturation (a pH-dependent step) specifically blocked all the immune stimulatory effects of CpG DNA (Macfarlane and Manzel, 1998; Hacker *et al*., 1998; Yi *et al*., 1998). Once ODNs are internalized, within 15-30 minutes the cell-signaling pathways that are induced by CpG DNA lead to enhanced nuclear transcription of molecules like IL-12, TNF-α, IFNα/β, IFN-γ, IL-6, IL-18 and IL-1β. The best understood signaling pathway initiated by CpG DNA is mediated by TLR9 binding (Bauer *et al*., 2001; Chuang *et al*., 2002; Wagner, 2002; Kirschning and Bauer, 2001; Takeshita *et al*., 2001; Hacker, 2000; Schnare *et al*., 2000; Hacker *et al*., 2000b; Hartmann and Krieg, 2000, Lenert *et al*., 2001; Yi *et al*., 2002). TLR9 was internalized along with the bound CpG DNA/ODN. This initiated a signaling cascade that culminated in transcription of multiple cytokines and proto-oncogenes that are thought to mediate other immune effects of CpG DNA.

The specific cellular signaling pathways for TLR9 have been extensively studied. Toll like receptors have a cytoplasmic domain similar to IL-1R. This domain is referred to as the Toll/IL-1 cytoplasmic domain (TIR). A cytoplasmic adaptor protein, myeloid differentiation factor 88 (MyD88), is recruited through TIR to TLR9 (Chuang *et al*., 2002; Hacker *et al*., 2000b; Hartmann and Krieg, 2000). Interleukin-1 receptor associated kinase (IRAK) is then recruited to TIR by MyD88 (Chuang *et al*., 2002). IRAK is autophosphorylated and subsequently activates adaptor protein tumor necrosis factor receptor associated factor-6 (TRAF6) (Chuang *et al*., 2002; Takeshita *et al*., 2001; Hartmann and Krieg, 2000) that results in oligomerization of TRAF6. Downstream

kinases such as the mitogen activated protein (MAP) kinases that include extracellular signal regulated kinase (ERK) (Yi and Krieg, 1998b; Yi *et al*., 2002; Akhtar *et al*., 2003), stress kinase c-Jun N-terminal kinase (JNK) (Wloch *et al*., 1998), p38 (Yi *et al*., 2002; Choudhury *et al*., 2002) and IκB kinase (IKK) complex (Lenert *et al*., 2001) are activated by TRAF6. Transcription factors activator protein-1 (AP-1) (Hornung *et al*., 2002; Hacker *et al*., 1998; Spiegelberg and Raz, 2002) and NFkappaB (Yi *et al*., 1999*;* Yi *et al*., 2001; Yi *et al*., 1998; Lenert *et al*., 2001; Hornung *et al*., 2002) are also activated by these kinases. Acidification of endosomal CpG DNA was coupled to the rapid generation of intracellular reactive oxygen species (Yi *et al*., 1998). The CpG DNA-induced reactive oxygen species burst was linked to the degradation of IκB and the activation of NFkappaB. This in turn induced leukocyte gene transcription and cytokine secretion (Yi *et al*., 1998).

Heat shock proteins have recently been shown to be associated with CpG signaling and recognition (Bandholtz *et al*., 2003; Kumaraguru *et al*., 2003; Zhu and Pisetsky, 2001). Hsp90 acted by binding the CpG ODNs and acting as a ligand transfer molecule (Bandholtz *et al*., 2003).

IMPORTANCE OF CpG DNA AND SINGLE BASE ODNs

CpG ODN binding to APC causes the secretion of cytokines that favor the amplification of Th1 responses (induction of IL-12 and IFN-γ). This type of cytokine profile may also initiate switching of an established Th2 response to a Th1 response (Van der Stede *et al*., 2002; Yi *et al*., 2002; Chiaramonte *et al*., 2000; Kline *et al*., 2002). *In vivo* studies following ODN injection have demonstrated a tendency of treated animals to produce a

higher IFN-γ (Th1-like) mRNA expression compared to controls (Van der Stede *et al*., 2002). The combination treatments of CpG ODN and allergen effectively reversed an established atopic asthma and eosinophilic airway disease response. This occurred partially through redirecting a Th2 to a Th1 response by production of IFN-γ that inhibits production of IL-5 (Kline *et al*., 2002; Kitagaki *et al*., 2002) and by inhibiting IgE synthesis (Bohle *et al*., 1999; Bohle, 2002). The type of response initiated by CpG was recently shown to depend on the signaling pathway that was activated by CpG (Yi *et al*., 2002). CpG DNA-induced p38 activity was required for the Th1 type response in APCs. In contrast, ERK played a central negative regulatory role in the CpG DNA-mediated Th1 type response by promoting production of the Th2 type cytokine, IL-10 (Yi *et al*., 2002).

The ability to induce Th1 type responses demonstrated the appropriateness of CpG-ODN as an immunostimulatory adjuvant in vaccines (Kojima *et al*., 2002; Van der Stede *et al*., 2002, Kline *et al*., 2002; Dalpke *et al*., 2002; McCluskie *et al*., 2002; Spiegelberg and Raz, 2002; Oxenius *et al*., 1999; Krieg, 1999; Davis, 2000; Brunner *et al*., 2000; Zimmermann *et al*., 2003). Pigs immunized with OVA (Van der Stede *et al*., 2002) or outer membrane lipoprotein-A (OmlA) (Alcon *et al*., 2003) supplemented with CpG ODNs had a significantly enhanced primary antibody response compared to controls that received OVA or OmlA without ODN. Similarly, CpG ODNs were potent adjuvants when used in association with hepatitis B surface protein administered by parenteral or mucosal routes to BALB/c mice (McCluskie *et al*., 2002). CpG exerted additional adjuvant effects by activating the cells that are normally inferior APC to participate in immune induction by inducing release of heat shock proteins (Hsp) from macrophages.

These Hsp bound the antigens and cross-primed the inferior APC which inturn activated CTLs (Kumaraguru *et al*., 2003). The presence of guanosine in ODNs was shown to increase the adjuvanticity of CpG DNA/ODN (Dalpke *et al*., 2002; Zimmermann *et al*., 2003).

Besides, its role as an adjuvant for vaccines, CpG DNA also has anti-tumor activity that appears to be unrelated to the production of any secondary nonspecific toxicity (Davila and Celis, 2000; Auf *et al*., 2001; Weiner, 2000; Vicari *et al*., 2002; Sandler *et al*., 2003) and protective anti-infection activity (Krieg *et al*., 1998a). Thus, CpG DNA produces an array of complex immunologic effects ranging from direct innate immune responses to direct and indirect augmentation of adaptive immune responses. It is a single agent that can be used as a vaccine adjuvant (Uhlmann and Vollmer, 2003; Zimmermann *et al*., 2003); as an anti-cancer therapeutic agent (Baral *et al*., 2003; Carpentier *et al*., 2003); it may participate in protection against allergic responses (Choudhury *et al*., 2002); it has been used as chemotherapeutic and immunoprotective agents (Krieg *et al*., 1998; Verthelyi *et al*., 2003).

REFERENCES

Ahmad-Nejad, P., H. Hacker, M. Rutz, S. Bauer, R. M. Vabulas, and H. Wagner. 2002. Bacterial CpG-DNA and lipopolysaccharides activate Toll-like receptors at distinct cellular compartments. Eur J Immunol. **32:**1958-1968.

Akhtar, M., J. L. Watson, A. Nazli, and D. M. McKay. 2003. Bacterial DNA evokes epithelial IL-8 production by a MAPK-dependent, NFkappaB-independent pathway. FASEB J. May 8 (in press)

Alcon V. L., M. Foldvari, M. Snider, P. Willson, S. Gomis, R. Hecker, L. A. Babiuk, and M. E. Baca-Estrada. 2003. Induction of protective immunity in pigs after immunisation with CpG oligodeoxynucleotides formulated in a lipid-based delivery system (Biphasix). Vaccine. **21:**1811-1814.

Antequera F., and A. Bird. 1999. CpG islands as genomic footprints of promoters that are associated with replication origins. Curr Biol. **9:**661-667.

Ashkar A. A., and K. L. Rosenthal. 2002. Toll-like receptor 9, CpG DNA and innate immunity. Curr Mol Med. **2:**545-556.

Auf G., A.F. Carpentier, L. Chen, C. Le Clanche, and J. Y. Delattre. 2001. Implication of macrophages in tumor rejection induced by CpG-oligodeoxynucleotides without antigen. Clin Cancer Res. **7:**3540-3543.

Ballas Z. K., A. M. Krieg, T. Warren, W. Rasmussen, H. L. Davis, M. Waldschmidt, and G. J. Weiner. 2001. Divergent therapeutic and immunologic effects of oligodeoxynucleotides with distinct CpG motifs. J Immunol. **167:**4878-4886.

Ballas Z. K., W. L. Rasmussen, and A. M. Krieg. 1996. Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. J Immunol **157:**1840–1845.

Bandholtz L., Y. Guo, C. Palmberg, K. Mattsson, B. Ohlsson, A. High, J. Shabanowitz, D. F. Hunt, H. Jornvall, H. Wigzell, B. Agerberth, and G. H. Gudmundsson. 2003. Hsp90 binds CpG oligonucleotides directly: implications for hsp90 as a missing link in CpG signaling and recognition.Cell Mol Life Sci. **60:**422-429.

Baral R. N., A. Saha, S. A. Chatterjee, K. A. Foon, A. A. Krieg, G. A. Weiner, and M. Bhattacharya-Chatterjee. 2003. Immunostimulatory CpG oligonucleotides enhance the immune response of anti-idiotype vaccine that mimics carcinoembryonic antigen. Cancer Immunol Immunother. **52:**317-327.

Bauer M., K. Heeg, H. Wagner, and G. B. Lipford. 1999. DNA activates human immune cells through a CpG sequence-dependent manner. Immunology. **97:**699-705.

Bauer M., V. Redecke, J. W. Ellwart, B. Scherer, J. P. Kremer, H. Wagner, and G. B. Lipford. 2001a. Bacterial CpG-DNA triggers activation and maturation of human CD11c-, CD123+ dendritic cells. J Immunol. **166:**5000-5007.

Bauer S., C. J. Kirschning, H. Hacker, V. Redecke, S. Hausmann, S. Akira, H. Wagner, and G. B. Lipford. 2001b. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. Proc Natl Acad Sci U S A. **98:**9237-9242.

Beltinger C., H. U. Saragovi, R. M. Smith, L. LeSauteur, N. Shah, L. DeDionisio, L. Christensen, A. Raible, L. Jarett, and A..M. Gewirtz. 1995. Binding, uptake, and

intracellular trafficking of phosphorothioate-modified oligodeoxynucleotides. J Clin Invest. **95:**1814-1823.

Benimetskaya L., J. D. Loike, Z. Khaled, G. Loike, S. C. Silverstein, L. Cao, J. el Khoury, T. Q. Cai, and C. A. Stein. 1997. Mac-1 (CD11b/CD18) is an oligodeoxynucleotide-binding protein. Nat Med. **3:**414-420.

Bennett R. M., G. T. Gabor, and M. M. Merritt. 1985. DNA binding to human leukocytes. Evidence for a receptor-mediated association, internalization, and degradation of DNA. J Clin Invest. **76:**2182-2190.

Bhagat L., F. G. Zhu, D. Yu, J. Tang, H. Wang, E. R. Kandimalla, R. Zhang, and S. Agrawal. 2003. CpG penta- and hexadeoxyribonucleotides as potent immunomodulatory agents. Biochem Biophys Res Commun. **300:**853-861.

Biessen E. A., H. Vietsch, J. Kuiper, M. K. Bijsterbosch, and T. J. Berkel. 1998. Liver uptake of phosphodiester oligodeoxynucleotides is mediated by scavenger receptors. Mol Pharmacol. **53:**262-269.

Bird A. P. 1987. CpG islands are markers in the vertebrate nucleus. Trends Genet. **3:**342-347.

Bird A.P. 1993. Functions for DNA methylation in vertebrates. Cold Spring harbor Symp. Quant. Biol. **58:**281-285.

Bishop G. R., S. Taylor, L. Jaso-Friedmann, and D. L. Evans. 2002. Mechanisms of nonspecific cytotoxic cell regulation of apoptosis: cytokine-like activity of Fas ligand. Fish Shellfish Immunol. **13:**47-67.

Blazar B. R., A. M. Krieg, and P. A. Taylor. 2001. Synthetic unmethylated cytosine-phosphate-guanosine oligodeoxynucleotides are potentstimulators of antileukemia responses in naive and bone marrow transplant recipients. Blood. **98:**1217-1225.

Boggs R. T., K. McGraw, T. Condon, S. Flournoy, P. Villiet, C. F. Bennett, and B. P. Monia. 1997. Characterization and modulation of immune stimulation by modified oligonucleotides. Antisense Nucleic Acid Drug Dev. **7:**461-471.

Bohle B., B. Jahn-Schmid, D. Maurer, D. Kraft, and C. Ebner. 1999. Oligodeoxynucleotides containing CpG motifs induce IL-12, IL-18 and IFN-gamma production in cells from allergic individuals and inhibit IgE synthesis in vitro. Eur J Immunol. **29:**2344-2353.

Bohle B. 2002. CpG motifs as possible adjuvants for the treatment of allergic diseases. Int Arch Allergy Immunol. **129:**198-203.

Brown M. S., S. K. Basu, J. R. Falck, Y. K. Ho, and J. L. Goldstein. 1980. The scavenger cell pathway for lipoprotein degradation: specificity of the binding site that mediates the uptake of negatively-charged LDL by macrophages. J Supramol Struct. **13:**67-81.

Brunner C., J. Seiderer, A. Schlamp, M. Bidlingmaier, A. Eigler, W. Haimerl, H. A. Lehr, A.M. Krieg, G. Hartmann, and S. Endres. 2000. Enhanced dendritic cell maturation by TNF-alpha or cytidine-phosphate-guanosine DNA drives T cell activation in vitro and therapeutic anti-tumor immune responses in vivo. J Immunol. **165:**6278-6286.

Carpentier A. F., G. Auf, and J. Y. Delattre. 2003. CpG-oligonucleotides for cancer immunotherapy : review of the literature and potential applications in malignant glioma. Front Biosci. **8:**E115-27.

Castier Y., E. Chemla, J. Nierat, D. Heudes, M. A. Vasseur, C. Rajnoch, P. Bruneval, A. Carpentier, and J. N. Fabiani. 1998. The activity of c-myb antisense oligonucleotide to prevent intimal hyperplasia is nonspecific. J Cardiovasc Surg (Torino). **39:**1-7.

Chace J. H., N. A. Hooker, K. L. Mildenstein, A. M. Krieg, and J. S. Cowdery. 1997. Bacterial DNA-induced NK cell IFN-gamma production is dependent on macrophage secretion of IL-12. Clin Immunol Immunopathol. **84:**185-193.

Chen W., Y. Yu, C. Shao, M. Zhang, W. Wang, L. Zhang, and X. Cao. 2001. Enhancement of antigen-presenting ability of B lymphoma cells by immunostimulatory CpG-oligonucleotides and anti-CD40 antibody. Immunol Lett. **77:**17-23.

Chiaramonte M. G., M. Hesse, A. W. Cheever, and T. A. Wynn.2000. CpG oligonucleotides can prophylactically immunize against Th2-mediated schistosome egg-induced pathology by an IL-12-independent mechanism. J Immunol. **164:**973-985.

Choudhury B. K., J. S. Wild, R. Alam, D. M. Klinman, I. Boldogh, N. Dharajiya, W. J. Mileski, and S. Sur. 2002. In vivo role of p38 mitogen-activated protein kinase in mediating the anti-inflammatory effects of CpG oligodeoxynucleotide in murine asthma. J Immunol. **169:**5955-5961.

Chuang T. H., J. Lee, L. Kline, J. C. Mathison, and R. J. Ulevitch. 2002. Toll-like receptor 9 mediates CpG-DNA signaling. J Leukoc Biol. **71:**538-544.

Dalpke A. H, S. Zimmermann, I. Albrecht, and K. Heeg. 2002. Phosphodiester CpG oligonucleotides as adjuvants: polyguanosine runs enhance cellular uptake and improve immunostimulative activity of phosphodiester CpG oligonucleotides in vitro and in vivo. Immunology. **106:**102-112.

Dannevig B. H., A. Lauve, C. M. Press, and T. Landsverki. 1994. Receptor-mediated endocytosis and phagocytosis by rainbow trout head kidney sinusoidal cells. Fish and shellfish. **4:**3-18.

Davila E., and E. Celis. 2000. Repeated administration of cytosine-phosphorothiolated guanine-containing oligonucleotides together with peptide/protein immunization results in enhanced CTL responses with anti-tumor activity. J Immunol. **165:**539-547.

Davis H. L. 2000. CpG motifs for optimization of DNA vaccines. Dev Biol (Basel). **104:**165-9.

Evans D. L., S. S. Graves, D. Cobb, and D. L. Dawe. 1984. Nonspecific cytotoxic cells in fish (Ictalurus punctatus). II. Parameters of target cell lysis and specificity. Dev Comp Immunol. **8:**303-312.

Evans D. L., D. T. Harris, D. L. Staton, and L. Jaso-Friedmann. 1990. Pathways of signal transduction in teleost nonspecific cytotoxic cells. Dev Comp Immunol. **14:**295-304.

Evans D. L., S.L. Taylor, J. H. Leary III, G. R. Bishop, A. Eldar, and L. Jaso-Friedmann. 2000. In vivo activation of tilapia nonspecific cytotoxic cells by Streptococcus

iniae and amplification with apoptosis regulatory factor(s). Fish Shellfish Immunol. **10:**419-434.

Evans D. L., J. H. Leary III, and L. Jaso-Friedmann. 2001. Nonspecific cytotoxic cells and innate immunity: regulation by programmed cell death. Dev Comp Immunol. **25:**791-805.

Filion M. C., N. Saha, M. Gueddi, and N. C. PhilliPs. 2003. Development of short non-CpG phosphodiester oligonucleotides as immune stimulatory agents. Vaccine. **2:**983-989.

Froystad M. K., M. Rode, T. Berg, and T. Gjoen. 1998. A role for scavenger receptors in phagocytosis of protein-coated particles in rainbow trout head kidney macrophages.Dev Comp Immunol. **22:**533-549.

Froystad M. K., V. Volden, T. Berg, and T. Gjoen. 2002. Metabolism of oxidized and chemically modified low density lipoproteins in rainbow trout--clearance via scavenger receptors. Dev Comp Immunol. **26:**723-733.

Gilkeson G. S., J. Conover, M. Halpern, D. S. Pisetsky, A. Feagin, and D. M. Klinman. 1998. Effects of bacterial DNA on cytokine production by (NZB/NZW)F1 mice. J Immunol. **161:**3890-3895.

Gjoen T., and T. Berg. 1992. Metabolism of low density lipoproteins in rainbow trout. Fish physiology and biochemistry. **9:**453-461.

Goeckeritz B. E., M. Flora, K. Witherspoon, Q. Vos, A. Lees, G. J. Dennis, D. S. Pisetsky, D. M. Klinman, C.M. Snapper, and J. J. Mond. 1999. Multivalent cross-linking of membrane Ig sensitizes murine B cells to a broader spectrum of CpG-

containing oligodeoxynucleotide motifs, including their methylated counterparts, for stimulation of proliferation and Ig secretion. Int Immunol. **11:**1693-1700.

Graves S. S., D. L. Evans, D. Cobb, and D. L. Dawe. 1984. Nonspecific cytotoxic cells in fish (Ictalurus punctatus). I. Optimum requirements for target cell lysis. Dev Comp Immunol. **8:**293-302.

Graves S. S., D. L. Evans, and D. L Dawe. 1985. Antiprotozoan activity of nonspecific cytotoxic cells (NCC) from the channel catfish (Ictalurus punctatus). J Immunol. **134:**78-85.

Gursel M., D. Verthelyi, I. Gursel, K. J. Ishii, and D. M. Klinman. 2002. Differential and competitive activation of human immune cells by distinct classes of CpG oligodeoxynucleotide. J Leukoc Biol. **71:**813-20.

Hacker H., H. Mischak, T. Miethke, S. Liptay, R. Schmid, T. Sparwasser, G. B. Lipford, K. Heeg, and H. Wagner. 1998. CpG-DNA-specific activation of antigen-presenting cells requires stress kinase activity and is preceded by non-specific endocytosis and endosomal maturation. EMBO J. **17:**6230-6240.

Hacker H. 2000. Signal transduction pathways activated by CpG-DNA. Curr Top Microbiol Immunol. **247:**77-92.

Hacker G., V. Redecke, and H. Hacker. 2000a. Activation of the immune system by bacterial CpG-DNA. Immunology. **105:**245-251.

Hacker H., R. M. Vabulas, O. Takeuchi, K. Hoshino, S. Akira, and H. Wagner. 2000b. Immune cell activation by bacterial CpG-DNA through myeloid differentiation marker 88 and tumor necrosis factor receptor associated factor (TRAF)6. J Exp Med. **192:**595-600.

Hafner M., R. Zawatzky, C. Hirtreiter, W. A. Buurman, B. Echtenacher, T. Hehlgans, and D. N. Mannel. 2001. Antimetastatic effect of CpG DNA mediated by type I IFN. Cancer Res. **61:**5523-5528.

Halpern M. D., R. J. Kurlander, and D. S. Pisetsky. 1996. Bacterial DNA induces murine interferon-gamma production by stimulation of interleukin-12 and tumor necrosis factor-alpha. Cell Immunol. **167:**72-78.

Halpern M. D., and D. S. Pisetsky. 1995. In vitro inhibtion of murine IFNγ production by phosphorothioate deoxyguanosine oligomers. Immunopharmacology. **29:**47-52.

Hartmann G., and A. M. Krieg. 2000. Mechanism and function of a newly identified CpG DNA motif in human primary B cells. J Immunol. **164:**944-953.

Hartmann G., R. D. Weeratna, Z. K. Ballas, P. Payette, S. Blackwell, I. Suparto, W. L. Rasmussen, M. Waldschmidt, D. Sajuthi, R. H. Purcell, H. L. Davis, and A. M. Krieg. 2000. Delineation of a CpG phosphorothioate oligodeoxynucleotide for activating primate immune responses in vitro and in vivo. J Immunol. **164:**1617-1624.

Hartmann G., G. J. Weiner, and A. M. Krieg. 1999. CpG DNA: a potent signal for growth, activation, and maturation of human dendritic cells. Proc Natl Acad Sci U S A. **96:**9305-9310.

Heeg K., T. Sparwasser, G. B. Lipford, H. Hacker, S. Zimmermann, and H. Wagner. 1998. Bacterial DNA as an evolutionary conserved ligand signalling danger of infection to immune cells. Eur J Clin Microbiol Infect Dis. **17:**464-469.

Hemmi H., T. Kaisho, K. Takeda, and S. Akira. 2003. The roles of Toll-like receptor 9, MyD88, and DNA-dependent protein kinase catalytic subunit in the effects of two distinct CpG DNAs on dendritic cell subsets.J Immunol. **170:**3059-3064.

Hemmi H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira. 2000. A Toll-like receptor recognizes bacterial DNA. Nature. **408:**740-745.

Hornung V., S. Rothenfusser, S. Britsch, A. Krug, B. Jahrsdorfer, T. Giese, S. Endres, and G. Hartmann. 2002.Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J Immunol. **168:**4531-4537.

Hu Z., S. Sun, and F. Zhou. 2003. The binding of CpG-oligodeoxynucleotides to cell-surface and its immunostimulatory activity are modulated by extracellular acidic pH. Vaccine. **21:**485-490.

Iho S., T. Yamamoto, T. Takahashi, and S. Yamamoto. 1999. Oligodeoxynucleotides containing palindrome sequences with internal 5'-CpG-3' act directly on human NK and activated T cells to induce IFN-gamma production in vitro. J Immunol. **163:**3642-3652

Ishii K. J., K. Suzuki, C. Coban, F. Takeshita, Y. Itoh, H. Matoba, L. D. Kohn, and D. M. Klinman. 2001. Genomic DNA released by dying cells induces the maturation of APCs. J Immunol. **167:**2602-2607.

Jaso-Friedmann L., J. H. Leary III, and D. L. Evans. 2000. Role of nonspecific cytotoxic cells in the induction of programmed cell death of pathogenic protozoans: participation of the Fas ligand-Fas receptor system. Exp Parasitol. **96:**75-88.

Jaso-Friedmann L., J. H. Leary III, and D. L. Evans. 2001. The non-specific cytotoxic cell receptor (NCCRP-1): molecular organization and signaling properties. Dev Comp Immunol. **25:**701-711.

Jones T. R., N. Obaldia III, R. A. Gramzinski, Y. Charoenvit, N. Kolodny, S. Kitov, H. L. Davis, A. M. Krieg, and S. L. Hoffman. 1999. Synthetic oligodeoxynucleotides containing CpG motifs enhance immunogenicity of a peptide malaria vaccine in Aotus monkeys. Vaccine. **17:**3065-3071.

Jorgensen J. B., A. Johansen, B. Stenersen, and A. I. Sommer. 2001a. CpG oligodeoxynucleotides and plasmid DNA stimulate Atlantic salmon (Salmo salar L.) leucocytes to produce supernatants with antiviral activity. Dev Comp Immunol. **25:**313-321.

Jorgensen J. B., J. Zou, A. Johansen, and C. J. Secombes. 2001b. Immunostimulatory CpG oligodeoxynucleotides stimulate expression of IL-1beta and interferon-like cytokines in rainbow trout macrophages via a chloroquine-sensitive mechanism. Fish Shellfish Immunol. **11:**673-682.

Kandimalla E. R., L. Bhagat, D. Wang, D. Yu, F. G. Zhu, J. Tang, H. Wang, P. Huang, R. Zhang, and S. Agrawal. 2003. Divergent synthetic nucleotide motif recognition pattern: design and development of potent immunomodulatory oligodeoxyribonucleotide agents with distinct cytokine induction profiles. Nucleic Acids Res. **31:**2393-2400.

Kandimalla E. R., L Bhagat, D. Yu, Y. Cong, J. Tang, and S. Agrawal. 2002. Conjugation of ligands at the 5'-end of CpG DNA affects immunostimulatory activity. Bioconjug Chem. **13:**966-974.

Kaur H., L. Jaso-Friedmann, and D. L. Evans. 2003. Identification of a scavenger receptor homologue on nonspecific cytotoxic cells and evidence for binding to oligodeoxyguanosine. Fish and Shellfish. In press.

Kerkmann M., S. Rothenfusser, V. Hornung, A. Towarowski, M. Wagner, A. Sarris, T. Giese, S. Endres, and G. Hartmann. 2003. Activation with CpG-A and CpG-B Oligonucleotides reveals two distinct regulatory pathways of Type I IFN synthesis in human plasmacytoid dendritic cells. J Immunol. **170:**4465-4474.

Kimura Y., K. Sonehara, E. Kuramoto, T. Makino, S. Yamamoto, T. Yamamoto, T. Kataoka, and T. Tokunaga. 1994. Binding of oligoguanylate to scavenger receptors is required for oligonucleotides to augment NK cell activity and induce IFN. J Biochem (Tokyo) **116:**991-994.

Kirschning C. J., and S. Bauer. 2001. Toll-like receptors: cellular signal transducers for exogenous molecular patterns causing immune responses. Int J Med Microbiol. **291:**251-260

Kitagaki K., V. V. Jain, T. R. Businga, I. Hussain, and J. N. Kline. 2002. Immunomodulatory effects of CpG oligodeoxynucleotides on established Th2 responses. lin Diagn Lab Immunol. **9:**1260-1269.

Kline J. N., K. Kitagaki, T. R. Businga, and V. V. Jain. 2002. Treatment of established asthma in a murine model using CpG oligodeoxynucleotides. Am J Physiol Lung Cell Mol Physiol. **283:**L170-179.

Kojima Y., K. Q. Xin, T. Ooki, K. Hamajima, T. Oikawa, K. Shinoda, T. Ozaki, Y. Hoshino, N. Jounai, M. Nakazawa, D. Klinman, and K. Okuda. 2002. Adjuvant effect of multi-CpG motifs on an HIV-1 DNA vaccine.Vaccine. **20:**2857-2865.

Krieg A. M., L. Love-Homan, A. K. Yi, and J. T. Harty. 1998a. CpG DNA induces sustained IL-12 expression in vivo and resistance to Listeria monocytogenes challenge. J Immunol. **161:**2428-2434.

Krieg A. M., T. Wu, R. Weeratna, S. M. Efler, L. Love-Homan, L. Yang, A. K. Yi, D. Short, and H. L. Davis. 1998b. Sequence motifs in adenoviral DNA block immune activation by stimulatory CpG motifs. Proc Natl Acad Sci U S A. **95:**12631-12636.

Krieg A. M., A. K. Yi, S. Matson, T. J. Waldschmidt, G. A. Bishop, R. Teasdale, G. A. Koretzky, and D. M. Klinman. 1995. CpG motifs in bacterial DNA trigger direct B-cell activation. Nature. **374:**546-549.

Krieg A. M.1999. Direct immunologic activities of CpG DNA and implications for gene therapy. J Gene Med. **1:**56-63.

Krug A., S. Rothenfusser, V. Hornung, B. Jahrsdorfer, S. Blackwell, Z. K. Ballas, S. Endres, A. M. Krieg, and G. Hartmann. 2001a. Identification of CpG oligonucleotide sequences with high induction of IFN-alpha/beta in plasmacytoid dendritic cells. Eur J Immunol. **31:**2154-2163.

Krug A., A. Towarowski, S. Britsch, S. Rothenfusser, V. Hornung, R. Bals, T. Giese, H. Engelmann, S. Endres, A. M. Krieg, and G. Hartmann. 2001b. Toll-like receptor expression reveals CpG DNA as a unique microbial stimulus for plasmacytoid dendritic cells which synergizes with CD40 ligand to induce high amounts of IL-12. Eur J Immunol. **31:**3026-3037.

Krug A., S. Rothenfusser, S. Selinger, C. Bock, M. Kerkmann, J. Battiany, A. Sarris, T. Giese, D. Speiser, S. Endres, and G. Hartmann. 2003. CpG-A oligonucleotides

induce a monocyte-derived dendritic cell-like phenotype that preferentially activates CD8 T cells. J Immunol. **170:**3468-3477.

Kumaraguru U., C. D. Pack, and B. T. Rouse. 2003. Toll-like receptor ligand links innate and adaptive immune responses by the production of heat-shock proteins. J Leukoc Biol. **73:**574-83.

Laktionov P., B. Chelobanov, E. Rykova, and V. V. Vlassov. 2001. Interaction of oligonucleotides with cellular proteins.Nucleosides Nucleotides Nucleic Acids. **20:**859-862.

Laktionov P. P., J. E. Dazard, E. Vives, E. Y. Rykova, J. Piette, V. V. Vlassov, and B. Lebleu. 1999. Characterisation of membrane oligonucleotide-binding proteins and oligonucleotide uptake in keratinocytes. Nucleic Acids Res. **27:**2315-2324.

Lang R., L. Hultner, G. B. Lipford, H. Wagner, and K. Heeg. 1999. Guanosine-rich oligodeoxynucleotides induce proliferation of macrophage progenitors in cultures of murine bone marrow cells. Eur J Immunol. **29:**3496-3506.

Lee J., T. H. Chuang, V. Redecke, L. She, P. M. Pitha, D. A. Carson, E. Raz, and H. B. Cottam. 2003. Molecular basis for the immunostimulatory activity of guanine nucleoside analogs: Activation of Toll-like receptor 7. Proc Natl Acad Sci U S A. May 8

Lee S. W., M. K. Song, K. H. Baek, Y. Park, J. K. Kim, C. H. Lee, H. K. Cheong, C. Cheong, and Y. C. Sung. 2000. Effects of a hexameric deoxyriboguanosine run conjugation into CpG oligodeoxynucleotides on their immunostimulatory potentials. J Immunol. **165:**3631-3639.

Lenert P., L. Stunz, A. K. Yi, A. M. Krieg, and R. F. Ashman. 2001. CpG stimulation of primary mouse B cells is blocked by inhibitory oligodeoxyribonucleotides at a site proximal to NF-kappaB activation. Antisense Nucleic Acid Drug Dev. **11:**247-256.

Liang H., Y. Nishioka, C. F. Reich, D. S. Pisetsky, and P. E. LiPsky. 1996. Activation of human B cells by phosphorothioate oligodeoxynucleotides. J Clin Invest. **98:**1119-1129.

Liang H., C. F. Reich, D. S. Pisetsky, and P. E. LiPsky. 2000. The role of cell surface receptors in the activation of human B cells by phosphorothioate oligonucleotides. J Immunol. **165:**1438-1445.

Lipford G. B., S. Bendigs, K. Heeg, and H. Wagner. 2000. Poly-guanosine motifs costimulate antigen-reactive CD8 T cells while bacterial CpG-DNA affect T-cell activation via antigen-presenting cell-derived cytokines. Immunology. **101:**46-52.

Lipford G. B., K. Heeg, and H. Wagner. 1998. Bacterial DNA as immune cell activator. Trends Microbiol. **6:**496-500

Macfarlane D. E., and L. Manzel. 1998. Antagonism of immunostimulatory CpG oligodeoxynucleotides by quinacrine, chloroquine, and structurally related compounds. J Immunol. **160:**1122-1131.

Magnusson M., S. Magnusson, H. Vallin, L. Ronnblom, and G. V. Alm. 2001. Importance of CpG dinucleotides in activation of natural IFN-alpha-producing cells by a lupus-related oligodeoxynucleotide. Scand J Immunol. **54:**543-550.

Malerod L., K. Juvet, T. Gjoen, and T. Berg. 2002. The expression of scavenger receptor class B, type I (SR-BI) and caveolin-1 in parenchymal and nonparenchymal liver cells. Cell Tissue Res. **307:**173-80.

Mannon R. B., and C. Nataraj, D. S. Pisetsky. 2000. Stimulation of thymocyte proliferation by phosphorothioate DNA oligonucleotides. Cell Immunol. **201:**14-21.

Manzel L., and D. E. Macfarlane. 1999. Lack of immune stimulation by immobilized CpG-oligodeoxynucleotide. Antisense Nucleic Acid Drug Dev. **9:**459-64.

McCluskie M. J., and R. D. Weeratna, P J Payette, H L Davis. 2002. Parenteral and mucosal prime-boost immunization strategies in mice with hepatitis B surface antigen and CpG DNA. FEMS Immunol Med Microbiol. **32:**179-185.

McElligott R., and R. J. Wellinger. 1997. The terminal DNA structure of mammalian chromosomes. EMBO J. **16:**3705-3714.

Medzhitov R., and C. A. Janeway Jr. 1998. Innate immune recognition and control of adaptive immune responses. Semin Immunol. **10:**351-353.

Messina J. P., G. S. Gilkeson, and D. S. Pisetsky. 1993. The influence of DNA structure on the in vitro stimulation of murine lymphocytes by natural and synthetic polynucleotide antigens. Cell Immunol. **147:**148-157.

Middleton D., M. Curran, and L. Maxwell. 2002. Natural killer cells and their receptors. Transpl Immunol. **10:**147-164.

Nakamura K., H. Funakoshi, F. Tokunaga, and T. Nakamura. 2001. Molecular cloning of a mouse scavenger receptor with C-type lectin (SRCL)(1), a novel member of the scavenger receptor family. Biochim Biophys Acta. **1522:**53-58.

Oumouna M., L. Jaso-Friedmann, and D. L. Evans. 2002. Activation of nonspecific cytotoxic cells (NCC) with synthetic oligodeoxynucleotides and bacterial genomic DNA: binding, specificity and identification of unique immunostimulatory motifs. Dev Comp Immunol. **26:**257-269.

Oxenius A., M. M. Martinic, H. Hengartner, and P. Klenerman. 1999. CpG-containing oligonucleotides are efficient adjuvants for induction of protective antiviral immune responses with T-cell peptide vaccines. J Virol. **73:**4120-4126.

Park Y., S. W. Lee, and Y. C. Sung. 2002. CpG DNA inhibits dendritic cell apoptosis by up-regulating cellular inhibitor of apoptosis proteins through the phosphatidylinositide-3'-OH kinase pathway. J Immunol. **168:**5-8.

Parronchi P., F. Brugnolo, F. Annunziato, C. Manuelli, S. Sampognaro, C. Mavilia, S. Romagnani, and E. Maggi. 1999. Phosphorothioate oligodeoxynucleotides promote the in vitro development of human allergen-specific CD4+ T cells into Th1 effectors. J Immunol. **163:**5946-5953.

Pearson A. M., A. Rich, and M. Krieger. 1993. Polynucleotide binding to macrophage scavenger receptors depends on the formation of base-quartet-stabilized four-stranded helices. J Biol Chem. **268:**3546-3554.

Pearson A. M. 1996. Scavenger receptors in innate immunity. Curr Opin Immunol. **8:**20-28.

Peiser L., S. Mukhopadhyay, and S. Gordon. 2002. Scavenger receptors in innate immunity. Curr Opin Immunol. **14:**123-128.

Pisetsky D. S., and C. Reich. 1993. Stimulation of in vitro proliferation of murine lymphocytes by synthetic oligodeoxynucleotides. Mol Biol Rep. **18:**217-221.

Pisetsky D. S., C. Reich, S. D. Crowley, and M. D. Halpern. 1995. Immunological properties of bacterial DNA. Ann N Y Acad Sci. **772:**152-163.

Pisetsky D. S., and C. F. Reich. 1998. The influence of base sequence on the immunostimulatory properties of defined oligonucleotides. Immunopharmacology. **40:**199-208.

Pisetsky D. S., and C. F.. Reich. 1999. Influence of backbone chemistry on immune activation by synthetic oligonucleotides. Biochem Pharmacol. **58:**1981-1988.

Pisetsky D S. 1999. The influence of base sequence on the immunostimulatory properties of DNA. Immunol Res. **19:**35-46.

Pisetsky D. S, and C. F. Reich. 2000. Inhibition of murine macrophage IL-12 production by natural and synthetic DNA. Clin Immunol. **96:**198-204.

Prasad V., S. Hashim, A. Mukhopadhyay, S. K. Basu, and R. P. Roy. 1999. Oligonucleotides tethered to a short polyguanylic acid stretch are targeted to macrophages: enhanced antiviral activity of a vesicular stomatitis virus-specific antisense oligonucleotide. Antimicrob Agents Chemother. **43:**2689-2696.

Ramet M., A. Pearson, P. Manfruelli, X. Li, H. Koziel, V. Gobel, E. Chung, M. Krieger, and R. A. Ezekowitz. 2001. Drosophila scavenger receptor CI is a pattern recognition receptor for bacteria.Immunity. **15:**1027-1038.

Saijo Y., B. Uchiyama, T. Abe, K. Satoh, and T. Nukiwa. 1997. Contiguous four-guanosine sequence in c-myc antisense phosphorothioate oligonucleotides inhibits cell growth on human lung cancer cells: possible involvement of cell adhesion inhibition. Jpn J Cancer Res. **88:**26-33.

Sandler A. D., H. Chihara, G. Kobayashi, X. Zhu, M. A. Miller, D. L. Scott, and A. M. Krieg. 2003. CpG oligonucleotides enhance the tumor antigen-specific immune response of a granulocyte macrophage colony-stimulating factor-based vaccine strategy in neuroblastoma. Cancer Res. **63:**394-399.

Sano K., H. Shirota, T. Terui, T. Hattori, and G. Tamura. 2003. Oligodeoxynucleotides without CpG motifs work as adjuvant for the induction of th2 differentiation in a sequence-independent manner. J Immunol. **170:**2367-2373.

Sasaki T., S. Horiuchi, M. Yamazaki, and S. Yui. 1996. Stimulation of macrophage DNA synthesis by polyanionic substances through binding to the macrophage scavenger receptor. Biol Pharm Bull. **19:**449-455.

Sato Y., M. Roman, H. Tighe, D. Lee, M. Corr, M. D. Nguyen, G. J. Silverman, M. Lotz, D. A. Carson, and E. Raz 1996. Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. Science. **273:**352-354.

Schattenberg D., M. Schott, G. Reindl, T. Krueger, D. Tschoepe, J. Feldkamp, W. A. Scherbaum, and J. Seissler. 2000. Response of human monocyte-derived dendritic cells to immunostimulatory DNA. Eur J Immunol. **30:**2824-2831.

Schluesener H. J., K. Seid, M. Deininger, and J. Schwab. 2001. Transient in vivo activation of rat brain macrophages/microglial cells and astrocytes by immunostimulatory multiple CpG oligonucletides. J Neuroimmunol. **113:**89-94.

Schnare M., A. C. Holt, K. Takeda, S. Akira, and R. Medzhitov. 2000. Recognition of CpG DNA is mediated by signaling pathways dependent on the adaptor protein MyD88. Curr Biol. **10:**1139-1142.

Schorderet D. F., and S. M. Gartler. 1992. Analysis of CpG suppression in methylated and nonmethylated species. Proc Natl Acad Sci U S A. **89:**957-961.

Sen D., W. Gilbert. 1990. A sodium-potassium switch in the formation of four-stranded G4-DNA. Nature **344:**410-414.

Sester D. P., S. Naik, S. J. Beasley, D. A. Hume, and K. J. Stacey. 2000.. Phosphorothioate backbone modification modulates macrophage activation by CpG DNA. J Immunol. **165:**4165-4173.

Sester D. P., K. J. Stacey, M. J. Sweet, S. J. Beasley, S. L. Cronau, and D. A. Hume. 1999. The actions of bacterial DNA on murine macrophages. J Leukoc Biol. **66:**542-548.

Shen W., M. Waldschmidt, X. Zhao, T. Ratliff, and A. M. Krieg. 2002. Antitumor mechanisms of oligodeoxynucleotides with CpG and polyG motifs in murine prostate cancer cells: decrease of NF-kappaB and AP-1 binding activities and induction of apoptosis. Antisense Nucleic Acid Drug Dev. **12:**155-164.

Shoda L. K., K. A. Kegerreis, C. E. Suarez, W. Mwangi, D. P. Knowles, and W. C. Brown. 2001. Immunostimulatory CpG-modified plasmid DNA enhances IL-12, TNF-alpha, and NO production by bovine macrophages. J Leukoc Biol. **70:**103-112.

Siddiqui-Jain A., C. L. Grand, D. J. Bearss, and L. H. Hurley. 2002. Direct evidence for a G-quadruplex in a promoter region and its targeting with a small molecule to repress c-MYC transcription. Proc Natl Acad Sci U S A. **99:**11593-11598.

Sonehara K., H. Saito, E. Kuramoto, S. Yamamoto, T. Yamamoto, and T. Tokunaga. 1996. Hexamer palindromic oligonucleotides with 5'-CG-3' motif(s) induce production of interferon. J Interferon Cytokine Res. **16:**799-803.

Sorensen K. K., J. Melkko, and B. Smedsrod. 1998. Scavenger-receptor-mediated endocytosis in endocardial endothelial cells of Atlantic cod Gadus morhua.J Exp Biol. **201:**1707-1718.

Sparwasser T., E. S. Koch, R. M. Vabulas, K. Heeg, G. B. Lipford, J. W. Ellwart, and H. Wagner. 1998. Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. Eur J Immunol. **28:**2045-2054.

Sparwasser T., T. Miethke, G. Lipford, A. Erdmann, H. Hacker, K. Heeg, and H. Wagner. 1997. Macrophages sense pathogens via DNA motifs: induction of tumor necrosis factor-alpha-mediated shock. Eur J Immunol. **27:**1671-1679.

Sparwasser T., R. M. Vabulas, B. Villmow, G. B. Lipford, and H. Wagner. 2000. Bacterial CpG-DNA activates dendritic cells in vivo: T helper cell-independent cytotoxic T cell responses to soluble proteins. Eur J Immunol. **30:**3591-3597.

Spiegelberg H. L., and E. Raz.2002. DNA-based approaches to the treatment of allergies. Curr Opin Mol Ther. **4:**64-71.

Stacey K. J., D. P. Sester, M. J. Sweet, and D. A. Hume. 2000. Macrophage activation by immunostimulatory DNA. Curr Top Microbiol Immunol. **247:**41-58.

Stacey K. J., M. J. Sweet, and D. A. Hume. 1996. Macrophages ingest and are activated by bacterial DNA. J Immunol. **157:**2116-2122.

Storni T., F. Lechner, I. Erdmann, T. Bachi, A. Jegerlehner, T. Dumrese, T. M. Kundig, C. Ruedl, and M. F. Bachmann. 2002. Critical role for activation of antigen-presenting cells in priming of cytotoxic T cell responses after vaccination with virus-like particles. J Immunol. **168:**2880-2886.

Sun S., X. Zhang, D. Tough, and J. Sprent. 2000. Multiple effects of immunostimulatory DNA on T cells and the role of type I interferons. Springer Semin Immunopathol. **22:**77-84.

Takeda K., T. Kaisho, and S. Akira. 2003. Toll-like receptors. Annu Rev Immunol. **21:**335-376.

Takeshita F., C. A. Leifer, I. Gursel, K. J. Ishii, S. Takeshita, M. Gursel, and D. M. Klinman. 2001. Role of Toll-like receptor 9 in CpG DNA-induced activation of human cells. J Immunol. **167:**3555-3558.

Tidd D. M., C. M. Broughton, and R. E. Clark. 2003. CpG oligodeoxynucleotide 5mer-induced apoptosis in MOLT-4 leukaemia cells does not require caspase 3 or new protein synthesis. Nucleic Acids Res. **31:**2297-2304.

Tokunaga T., H. Yamamoto, S. Shimada, H. Abe, T. Fukuda, Y. Fujisawa, Y. Furutani, O. Yano, T. Kataoka, and T. Sudo. 1984. Antitumor activity of deoxyribonucleic acid fraction from Mycobacterium bovis BCG. I. Isolation, physicochemical characterization, and antitumor activity. J Natl Cancer Inst. **72:**955-962.

Tokunaga T., O. Yano, E. Kuramoto, Y. Kimura, T. Yamamoto, T. Kataoka, and S. Yamamoto. 1992. Synthetic oligonucleotides with particular base sequences from the cDNA encoding proteins of Mycobacterium bovis BCG induce interferons and activate natural killer cells. Microbiol Immunol. **36:**55-66.

Trapani J. A., and M. J. Smyth. 1993. Killing by cytotoxic T cells and natural killer cells: multiple granule serine proteases as initiators of DNA fragmentation. Immunol Cell Biol. **71:**201-208.

Uhlmann E., and J. Vollmer. 2003. Recent advances in the development of immunostimulatory oligonucleotides. Curr Opin Drug Discov Devel. **6:**204-217.

Utaisincharoen P., N. Anuntagool, P. Chaisuriya, S. Pichyangkul, and S. Sirisinha. 2002. CpG ODN activates NO and iNOS production in mouse macrophage cell line (RAW 264.7). Clin Exp Immunol. **128:**467-473.

Van der Stede Y., F. Verdonck, S. Vancaeneghem, E. Cox, and B.. M Goddeeris. 2002. CpG-oligodinucleotides as an effective adjuvant in pigs for intramuscular immunizations. Vet Immunol Immunopathol. **86:**31-41.

Venczel E. A., and D. Sen. 1993. Parallel and antiparallel G-DNA structures from a complex telomeric sequence. Biochemistry **32:**6220-6228.

Verthelyi D., M. Gursel, R. T. Kenney, J. D. Lifson, S. Liu, J. Mican, and D. M. Klinman. 2003. CpG oligodeoxynucleotides protect normal and SIV-infected macaques from Leishmania infection. J Immunol. **170:**4717-4723.

Verthelyi D., K. J. Ishii, M. Gursel, F. Takeshita, and D. M. Klinman. 2001. Human peripheral blood cells differentially recognize and respond to two distinct CPG motifs. J Immunol. **166:**2372-2377.

Vicari A. P., C. Chiodoni, C. Vaure, S. Ait-Yahia, C. Dercamp, F. Matsos, O. Reynard, C. Taverne, P. Merle, M. P. Colombo, A. O'Garra, G. Trinchieri, and C. Caux. 2002. Reversal of tumor-induced dendritic cell paralysis by CpG

immunostimulatory oligonucleotide and anti-interleukin 10 receptor antibody.J Exp Med. **196:**541-549.

Vogel J. C., and M. C. Udey. 2000.Activation of skin dendritic cells by immunostimulatory DNA. Springer Semin Immunopathol. **22:**45-54.

Vollmer J., A. Janosch, M. Laucht, Z. K. Ballas, C. Schetter, and A. M. Krieg. 2002. Highly immunostimulatory CpG-free oligodeoxynucleotides for activation of human leukocytes. Antisense Nucleic Acid Drug Dev. **12:**165-175.

Vujanovic N. L. 2001. Role of TNF family ligands in antitumor activity of natural killer cells. Int Rev Immunol. **20:**415-437.

Wagner H. 1999. Bacterial CpG DNA activates immune cells to signal infectious danger. Adv Immunol. **73:**329-368

Wagner H. 2002. Interactions between bacterial CpG-DNA and TLR9 bridge innate and adaptive immunity. Curr Opin Microbiol. **5:**62-69.

Wang Z., J. G. Karras, T. P. Colarusso, L. C. Foote, and T. L. Rothstein. 1997. Unmethylated CpG motifs protect murine B lymphocytes against Fas-mediated apoptosis. Cell Immunol. **180:**162-167.

Wang Y., W. Wang, N. Li, Y. Yu, and X. Cao. 2002. Activation of antigen-presenting cells by immunostimulatory plant DNA: a natural resource for potential adjuvant. Vaccine. **20:**2764-2771.

Wang Y., and A. M. Krieg. 2003. Synergy between CpG- or non-CpG DNA and specific antigen for B cell activation. Int Immunol. **15:**223-231.

Warren T. L., S. K. Bhatia, A. M. Acosta, C. E. Dahle, T. L. Ratliff, A. M. Krieg, and G. J. Weiner. 2000. APC stimulated by CpG oligodeoxynucleotide enhance activation of MHC class I-restricted T cells. J Immunol. **165:**6244-6251.

Weiner G. J. 2000. The immunobiology and clinical potential of immunostimulatory CpG oligodeoxynucleotides. J Leukoc Biol. **68:**455-463.

White J. R., E. C. Gordon-Smith, and T. R. Rutherford. 1996. Phosphorothioate-capped antisense oligonucleotides to Ras GAP inhibit cell proliferation and trigger apoptosis but fail to downregulate GAP gene expression. Biochem Biophys Res Commun. **227:**118-124.

Wloch M. K., S. Pasquini, H. C. Ertl, and D. S. Pisetsky. 1998. The influence of DNA sequence on the immunostimulatory properties of plasmid DNA vectors. Hum Gene **9:**1439-1447.

Wright W. E., V. M. Tesmer, K. E. Huffman, S. D. Levene, and J. W. Shay. 1997. Normal human chromosomes have long G-rich telomeric overhangs at one end. Genes Dev. **11:**2801-2809.

Yakubov L., L. Yurchenko, M. Nechaeva, E. Rykova, V. Karaymshev, J. Tonkinson, V. Vlassov, and C. A. Stein. 1991. Interaction of oligonucleotides with cellular receptors. Nucleic Acids Symp Ser. **24:**311.

Yamamoto S., E. Kuramoto, S. Shimada, and T. Tokunaga. 1988. In vitro augmentation of natural killer cell activity and production of interferon-alpha/beta and -gamma with deoxyribonucleic acid fraction from Mycobacterium bovis BCG. Jpn J Cancer Res. **79:**866-873.

Yamamoto S., T. Yamamoto, S. Shimada, E. Kuramoto, O. Yano, T. Kataoka, and T. Tokunaga. 1992a. DNA from bacteria, but not from vertebrates, induces interferons, activates natural killer cells and inhibits tumor growth. Microbiol Immunol. **36:**983-997.

Yamamoto S., T. Yamamoto, T. Kataoka, E. Kuramoto, O. Yano, and T. Tokunaga. 1992b. Unique palindromic sequences in synthetic oligonucleotides are required to induce IFN [correction of INF] and augment IFN-mediated [correction of INF] natural killer activity.J Immunol. **148:**4072-4076.

Yamamoto T., S. Yamamoto, T. Kataoka, and T. Tokunaga. 1994a. Ability of oligonucleotides with certain palindromes to induce interferon production and augment natural killer cell activity is associated with their base length. Antisense Res Dev. **4:**119-122.

Yamamoto T., S. Yamamoto, T. Kataoka, K. Komuro, M. Kohase, and T. Tokunaga. 1994b Synthetic oligonucleotides with certain palindromes stimulate interferon production of human peripheral blood lymphocytes in vitro. Jpn J Cancer Res. **85:**775-779.

Yamamoto S., T. Yamamoto, S. Iho, and T. Tokunaga. 2000. Activation of NK cell (human and mouse) by immunostimulatory DNA sequence. Springer Semin Immunopathol. **22:**35-43.

Yi A. K., and A. M. Krieg. 1998a. CpG DNA rescue from anti-IgM-induced WEHI-231 B lymphoma apoptosis via modulation ofI kappa B alpha and I kappa B beta and sustained activation of nuclear factor-kappa B/c-Rel. J Immunol. **160:**1240-1245.

Yi A. K., and A. M. Krieg. 1998b. Rapid induction of mitogen-activated protein kinases by immune stimulatory CpG DNA. J Immunol. **161:**4493-7449.

Yi A. K., R. Tuetken, T. Redford, M. Waldschmidt, J. Kirsch, and A. M. Krieg. 1998. CpG motifs in bacterial DNA activate leukocytes through the pH-dependent generation of reactive oxygen species. J Immunol. **160:**4755-4761.

Yi A. K., D. W. Peckham, R. F. Ashman, and A. M. Krieg. 1999. CpG DNA rescues B cells from apoptosis by activating NFkappaB and preventing mitochondrial membrane potential disruption via a chloroquine-sensitive pathway. Int Immunol. **11:**2015-2024.

Yi A. K., J. G. Yoon, S. C. Hong, T. W. Redford, and A. M. Krieg. 2001. Lipopolysaccharide and CpG DNA synergize for tumor necrosis factor-alpha production through activation of NF-kappaB. Int Immunol. **13:**1391-1404.

Yi A. K., J. G. Yoon, S. J. Yeo, S. C. Hong, B. K. English, and A. M. Krieg. 2002. Role of mitogen-activated protein kinases in CpG DNA-mediated IL-10 and IL-12 production: central role of extracellular signal-regulated kinase in the negative feedback loop of the CpG DNA-mediated Th1 response. J Immunol. **168:**4711-4720.

Zhang Y., L. K. Shoda, K. A. Brayton, D. M. Estes, G. H. Palmer, and W. C. Brown. 2001. Induction of interleukin-6 and interleukin-12 in bovine B lymphocytes, monocytes, and macrophages by a CpG oligodeoxynucleotide (ODN 2059) containing the GTCGTT motif. J Interferon Cytokine Res. **21:**871-881.

Zhao H., S. H. Cheng, and N. S. Yew. 2000. Requirements for effective inhibition of immunostimulatory CpG motifs by neutralizing motifs. Antisense Nucleic Acid Drug Dev. **10:**381-389.

Zhu F. G., and D. S. Pisetsky. 2001. Role of the heat shock protein 90 in immune response stimulation by bacterial DNA and synthetic oligonucleotides.Infect Immun. **69:**5546-5552.

Zhu F. G., C. F. Reich, and D. S. Pisetsky. 2001. The role of the macrophage scavenger receptor in immune stimulation by bacterial DNA and synthetic oligonucleotides. Immunology. **103:**226-234.

Zhu F. G., C. F. Reich, and D. S. Pisetsky. 2002a. Inhibition of murine dendritic cell activation by synthetic phosphorothioate oligodeoxynucleotides.J Leukoc Biol. **72:**1154-1163.

Zhu F. G., C. F. Reich, and D. S. Pisetsky. 2002b. Inhibition of murine macrophage nitric oxide production by synthetic oligonucleotides. J Leukoc Biol. **71:**686-694.

Zimmermann S., K. Heeg, and A. Dalpke. 2003. Immunostimulatory DNA as adjuvant: efficacy of phosphodiester CpG oligonucleotides is enhanced by 3' sequence modification. Vaccine. **21:**990-995.

CHAPTER 3

IDENTIFICATION OF A SCAVENGER RECEPTOR HOMOLOGUE ON NONSPECIFIC CYTOTOXIC CELLS AND EVIDENCE FOR BINDING TO OLIGODEOXYGUANOSINE[1]

[1] Kaur, H., L. Jaso-Friedmann and D. L. Evans. Accepted by *Fish and Shellfish Immunology*. Reprinted here with permission of publisher, 5/5/2003.

Abstract

In mammals scavenger receptors (SR) are expressed by monocytic-macrophage lineage cells and B-cells. Studies of various teleost species have indirectly demonstrated the presence of SR receptors on phagocytic or endothelial cells by showing the uptake of SR ligands [i.e. derivatized (acetylated) lipoproteins] by these cells. In the present study nonspecific cytotoxic cells (NCC) were examined for membrane expression of an SR-like protein. Approximately 15-25% of purified NCC expressed SR-A demonstrated by binding by a monoclonal (2F8) specific for mouse SR-A (types-I, -II). Flow cytometric analysis determined that SR binding cells had the same size and "side scatter" characteristics as NCC. Two color flow analysis of NCC demonstrated that only a subset of NCC expressed the SR-A-like protein and non-NCC were SR-A negative. Membrane expression of SR on NCC was confirmed by fluorescence microscopy. Analysis of the tissue distribution of SR bearing cells demonstrated that in both catfish and tilapia, SR-A was expressed by NCC in the peripheral blood, spleen and anterior kidney. Experiments were also done to determine if the ligands known to bind mammalian SR-A had a similar specificity for the teleost receptor. Cold competition binding experiments determined that anti-SR-A antibody competed with and reduced biotinylated poly-guanosine 20-mer binding to NCC by approximately 40%. Two other types of ligands known to bind [mammalian] SR-A [i.e. polyvinyl sulphate and dextran sulphate] likewise decreased anti-SR-A antibody binding to NCC by 40%. These studies for the first time demonstrated that NCC express the teleost orthologue of mammalian SR-A, suggesting that NCC may participate in physiologic regulation of lipid metabolism in addition to functions of innate immunity.

Keywords: Scavenger receptor; Oligodeoxyguanosine; Nonspecific cytotoxic cells; polyvinyl sulphate; dextran sulphate; G-tetrad

1. Introduction

Scavenger receptor-A (type-I) (SR-A) is a trimeric membrane glycoprotein of approximately 220 kDa [1] expressed predominantly on (mammalian) cells of the monocytic lineage. This receptor is only one of a class of SR proteins composed of SR-B-1, scavenger receptor C, Marco and CD36 [2]. In mammals SR is expressed on macrophages, endothelial cells [3] such as liver sinusoidal cells and B-cells [4]. Indirect evidence suggests that these receptors are also found on NK cells [5]. Although the physiological functions of SR are not known, their ligand specificities suggest a very broad range of innate immune potential. These include recognition of highly (polyanion) negatively charged molecules such as synthetic polymers (polyvinyl sulphate) and dextran sulphate. Guanosine-rich oligodeoxynucleotides bind to SR to increase DNA synthesis [6] and stimulate macrophage progenitors to undergo proliferation [7]. Others have shown that G-rich oligodeoxynucleotides (ODNs) inhibit cell growth [8], prevent hyperplasia [9] and trigger apoptosis [10]. G-rich ODNs in the form of poly- (dG12) "Runs" (e.g. 5' and/or 3' extensions of poly-G) next to CpG motifs [5] and hexameric dG6 [11] have immunostimulatory potential for IL-2 induced T-cell proliferation and IL-12 and TNF-alpha secretion (respectively). These studies indicated that ODNs containing various numbers of flanking poly-guanosine nucleic acids participated as potent stimulators of many different characteristics of innate, as well as adaptive, immune responses.

Although less well characterised, the expression of SR by teleost and lamprey cells has been previously reported [12-19]. Among the cell types that bound or phagocytosed a wide variety of acetylated or otherwise highly charged SR ligands were sinusoidal lining cells in the anterior kidney [14], macrophages [14,18] and endothelial [14] and epithelial cells [13].

Scavenger receptor-A binds to base quartet stabilised four-stranded helices (e.g. G-tetrads) of oligodeoxyguanosine [5]. The biological activities of G-tetrads or G-rich ODNs have been postulated to depend on the formation of intramolecular quartets that confer nuclease resistance [20]. These G-tetrads are generated by intramolecular self-associations between multiple strands containing (minimally) four contiguous guanosine nucleotides (G4-DNA) [21-25]. G-tetrads may occur "naturally" and the formation of their unique three-dimensional shape has also been previously associated with conformational dependent binding activity against HIV-1 [20] and inhibition of telomerase activity [23, 24].

In the present study a subpopulation of NCC was identified that express a membrane protein that is crossreactive with a monoclonal against mouse SR-A (types-I, -II). This protein binds two different highly charged ligands composed of polyvinyl sulphate and dextran sulphate. Competitive binding was performed using a commercially available anti-human SR-A and a synthetic oligodeoxynucleotide composed of a [(poly)guanosine 20-mer (dG20)]. These studies indicated that similar to the function on mammalian cells, the teleost SR-A is expressed on NCC and as such recognizes G-tetrads containing oligodeoxynucleotides.

2. Materials and methods

2.1. Animals

Ictalurus punctatus (catfish) of both sexes, were obtained from local commercial farms. *Oreochromis niloticus* (tilapia) of both sexes were obtained from Americulture, Animas, NM. Catfish were maintained in flow-through 300 gallon aquaria at ambient temperature and tilapia were kept in temperature controlled (23-26 °C) flow-through aquaria. Fish were fed commercial fish pellets (Purina Catfish Startena, Ralston Purina Co., St. Louis, MO). Water quality was monitored for temperature, nitrite, ammonia nitrogen and chlorine contents.

2.2. Media, cells, reagents and antibodies

The mouse macrophage/monocyte line J774A.1 (ATCC TIB-67) was a gift from Dr. R. Sharma (Univ. Ga.). Cells were cultured in RPMI-1640 (Cellgro, Media Tech, Washington, DC) supplemented with L-glutamine, sodium pyruvate, MEM vitamin solution, MEM essential [amino acid] solution, non-essential [amino acid] solution (Cellgro), 50 mg ml^{-} gentamicin (Schering-Plough Animal health Corp., Kenilworth, NJ) and 10% foetal bovine serum (FBS) (Atlanta biologicals, Norcross, GA). Washing buffer consisted of 1X phosphate buffered saline (PBS), 0.1% sodium azide (Sigma Chemical Co.) and 1% bovine serum albumin (Sigma Chemical Co.) and was called PAB. Reagents used were: calf thymus DNA, Phorbol 12-Myristate 13-Acetate (PMA) (# P8139, Sigma Chemical Co., St. Louis, MO), calcium ionophore-A23187 (# C7522, Sigma), polyvinyl sulphate (# P-6000, Sigma), CHAPS (# C-3023, Sigma), EDTA (# E-4884, Sigma) and Extravidin-phycoerythhrin (PE) conjugate (# E4011, Sigma).

Monoclonal antibody 5C6 (IgM isotype) specific for a 32-kDa receptor protein (i.e. NCCRP-1) [26] was prepared in-house. Other antibodies were: FITC conjugated rat anti-mouse scavenger receptor antibody (SR-AI/II) 2F8 (#1322F, Serotec Inc., Raleigh, NC); rat anti-mouse scavenger receptor antibody (SR-AI/II) 2F8 (#1322, Serotec); rat anti-mouse IgG2b-FITC isotype control (Southern Biotechnology Associates Inc., Birmingham, AL.); anti-mouse IgM-FITC and biotin-anti-IgM conjugates (Sigma Immunochemicals, St. Louis, MO).

2.3. Purification of NCC

Fish weighing 20-60g were net captured and sacrificed by submersion in anaesthetic (3-aminobenzoic acid ethyl ester; Sigma Chemical Co.). Anterior kidney (AK) tissue was removed aseptically and passed through screen mesh to obtain single cell suspensions in complete RPMI-1640 containing 10% FBS. Red cells were first removed by one cycle of centrifugation through Ficoll-Hypaque, and NCC were purified by density gradient centrifugation over a 45.5 % Percoll cushion. Cells at the interface were collected, washed once with RPMI and resuspended in complete RPMI. Cells in all assays were 70-90% mab 5C6 positive [26].

2.4. Oligonucleotides

The 20-mer oligodeoxyguanosine (dG20) was purchased from MWG-Biotech (High Point, NC). The dG20 was synthesised as a phosphodiester using standard methods. Prior to use, it was resuspended in PBS prepared in endotoxin free water. 3'end biotinylated dG20 was purchased from MWG-Biotech.

2.5. Determination of endotoxin content

The endotoxin content of all reagents used in the cellular assays was determined by a *Limulus amebocyte lysate* assay using the conditions established by the manufacturer (kit # 210-A; Sigma Chemical Co.). The sensitivity of the *Limulus* amebocyte lysate assay was 0.015 endotoxin units (EU) ml^{-}.

2.6. Flow cytometry

Analysis was performed using an EPICS XL-MCL four color analyser (Coulter Electronics Corp, Hileah, FL), equipped with 15 mW air cooled argon-ion laser operating at 488 nm wavelength. Two-parameters consisting of forward scatter (FS; size) and side scatter (SS; granularity) analysis were used to establish "scatter gates" and cells were positively identified by mab 5C6 staining and backgating into the scatter plot. 10,000 to 15,000 events were collected per sample. Conjugate and isotype controls were included to set the baseline fluorescence and to determine nonspecific binding of antibodies. FITC was detected using 525nm bandpass filter by photomultiplier tube 1 (PMT1) and the PE signal was detected with 575nm bandpass filter by photomultiplier tube 2 (PMT2). Data was analysed using Coulter's System II software, version 3.0.

2.7. Determination of scavenger receptor (SR-A) expression

To determine SR-A expression, cells (J774.A1 and NCC) were incubated with saturating concentrations of 2F8-FITC, isotype –FITC conjugated control mab or anti-NCCRP-1 specific mab 5C6 [26] for 1h on ice (4 °C). Cells stained with primary mab-5C6 (IgM isotype) were next incubated with anti-IgM-FITC for 30 min (4 °C), washed twice with

PAB and analysed by single color flow cytometry. To determine SR-A expression by mixtures of cells from the anterior kidney, spleen or liver (e.g. unfractionated cells), red blood cells were first removed by centrifugation at 1200 rpm for 10 min over Ficoll-Hypaque and cells were next incubated with the appropriate mabs for 1 h, washed twice with PAB and analyzed by single-color flow cytometry.

Staining for two color analysis was carried out by the simultaneous addition of each monoclonal or isotype control (i.e. mab 5C6, 2F8-FITC) and the appropriate conjugate (e.g. Biotin-anti-IgM and Extravidin-PE) for 1 h on ice. Cells were washed twice and analysed by single- and two-color flow cytometry. In experiments to evaluate stimulus induced up-regulation/modulation of SR-A, purified NCC were incubated with dG20, PMA/A23187 (50 ng ml^- /250 ng ml^-) or media alone for 12, 24, 36, 48 or 60 h. At each time point 25 x 10^3 cells were stained with 2F8-FITC conjugate or isotype mab-FITC conjugate and analysed by single-color flow cytometry.

2.8. Competition assays

Freshly prepared NCC were incubated in PAB at 4 °C for 2 h. 25 x 10^3 cells were stained with a 50% saturating concentration of biotinylated-dG20 and increasing concentrations of 2F8 simultaneously for 1 h on ice. This was followed by washing and incubation with Extravidin-PE for 30 min on ice. Stained cells were analysed by flow cytometer.

To assess competitive inhibition of 2F8-FITC binding of NCC by polyvinyl sulphate (PVS) or dextran sulphate, NCC were pre-incubated with varying concentrations of each for 30 min on ice followed by washing and incubation with 2F8-FITC conjugate

for 1 h on ice. After washing to remove unbound 2F8-FITC mab, cells were analysed by single-color flow cytometry.

2.9. Immunofluorescence

Purified NCC and J774.A1 cells were incubated with PAB at 4 °C for 2 h. 200 X 10^3 cells were stained with saturating concentration of 2F8-FITC for 1h on ice. Cells were washed to remove unbound 2F8-FITC mab and analysed by Zeiss microscope (Axiovert 35, Carl Zeiss Inc., Thornwood, NY). Images were captured with RT Color camera (Diagnostic Instruments, Sterling Heights, MI) and processed with Adobe Photoshop software (Adobe systems, San Jose, CA).

2.10. Western blotting

For Western blot analysis of J774.A1 cells with 2F8 mab, cell lysates were prepared in CHAPS lysis buffer (10mM CHAPS, 0.15M NaCl, 10mM Tris-Cl, pH 7.6, 1mM EDTA). Lysate was boiled in SDS sample buffer (0.25M Tris-Cl, pH 6.8, 8% SDS, 40% glycerol) with (reducing) or without (non-reducing) dithiothreitol (Fisher Scientific, Fair Lawn, NJ) and electrophoresed on 12.5% gel and transferred to nitrocellulose filter. Filter was blocked for 30 min at room temperature (RT) with blocking buffer (5% non-fat dry milk in TBS (25mM Tris-HCl, pH 7.5, 0.15M NaCl) containing 0.05% Tween-20), incubated with primary (mab 2F8 diluted 1:200 in blocking buffer) and secondary (anti-IgG-Horseradish Peroxidase conjugate diluted 1:10000 in blocking buffer) antibodies for 1 h and detected with chemiluminiscent substrate (SuperSignal® West Pico Chemiluminescent, Pierce, Rockford, IL).

3. Results

3.1 size (FS) and "granularity" (SS) characteristics of anterior kidney cells expressing NCCRP-1 and SR-A (types I,II)

In order to determine the distribution of SR-A binding cells in unfractionated anterior kidney (AK) tissue, red blood cells (RBCs) were first removed by Ficoll Hypaque density gradient centrifugation and size and side scatter profiles were obtained. Three major populations were identified (Fig. 3.1A). Unfractionated cells were stained with mab 5C6 (specific for NCCRP-1; Fig. 3.1B) and mab 2F8-FITC (specific for SR-A; Fig. 3.1C) to determine the percentage positive cells in each of the three scatter populations. Monoclonal antibody binding cells in each histogram were then backgated to "scatter plots" in order to identify the size and scatter characteristics of each positive population. Fig. 3.1D-E shows the histograms derived from this "reverse" (backgating) protocol and demonstrates that the cells expressing NCCRP-1 and SR-A overlap within the same general area of the scatter histogram.

To determine the specific cell type in the catfish AK tissue that bound to the SR-A mab, NCC were purified and two-color flow analysis was conducted by staining with both mab 5C6 and 2F8-FITC. In Fig. 3.2, all SR-A positive cells are also double positive for binding to mab 5C6 (mab 5C6 binds NCCRP-1) [26]. The majority of NCC were single positive for mab 5C6 (70.4%; quadrant 4; Fig. 3.2A). 18.4% of the NCC were double positive (quadrant 2; Fig. 3.2A). A total of 88.8% (quadrants 2 and 4; Fig. 3.2A) of all cells analyzed were positive for mab 5C6. Fig. 3.2B and 3.2C are single color flow analysis of mab 5C6 and 2F8-FITC binding (respectively) to confirm the purity of NCC in the two-color analysis.

3.2. Fluorescence microscopy and specificity of monoclonal antibody 2F8

To confirm the membrane location of SR-A on NCC and to compare the pattern of SR-A expression with J774.A1 cells, fluorescence microscopy experiments were conducted. Binding of 2F8 mab was observed on the cell surface of both NCC (Fig. 3.3A) and J774.A1 cells (Fig. 3.3B). J774.A1 cells displayed a scattered distribution of SR-A antibody binding on cell surface. NCC on the other hand, had more of a localized binding. NCC are much smaller cells (3 micron diameter) compared to the J774.A1 cells (8 micron diameter).

To identify the specificity of mab 2F8 for the SR-A proteins (types-I, -II), and to confirm published data [28], Western blot analysis was done. Analysis of lysates of J774.A1 cells (Fig. 3.4) revealed that the reduced SR was approximately 80 kDa (lane 1, Fig. 3.4) and the non-reduced form approximately 220 kDa (lane 3, Fig. 3.4). Binding specificity of mab 2F8 was also determined by flow cytometric analysis of 2F8 binding to J774.A1 cells (data not shown).

3.3. SR -A (TYPES-I,II) on NCC binds "conventional" ligands and oligodeoxyguanosine (dG20)

Initial characterisation of SR-A on NCC utilized competition binding experiments between mab 2F8-FITC and polyvinyl sulphate (PVS), a known ligand of SR. To accomplish this, purified NCC were first incubated with different concentrations of PVS followed by the addition of mab 2F8-FITC. Fig. 3.5A demonstrates saturable competition of PVS with mab 2F8-FITC binding. Similar experiments were conducted to determine the ability of dextran sulphate to compete with mab 2F8-FITC binding to

NCC. Fig. 3.5B shows that addition of increased concentrations of dextran sulphate produced a competition binding curve similar to that of PVS.

The same experimental approach was taken to determine if mab 2F8-FITC competed with dG20 binding. NCC were first incubated for 2 h with PBS containing sodium azide. Monoclonal 2F8-FITC and biotinylated dG20 were then added and the mixture incubated for one h (4 °C). Flow cytometric analysis showed (Fig. 3.6) that mab 2F8-FITC reduced dG20 binding by approximately 40-50% compared to isotype control. The increase in dG20 binding at high concentrations of antibody (i.e. 300 ng) was (probably) produced by nonspecific protein aggregate binding to cells.

3.4. Tissue distribution of cells expressing SR-A

Experiments were next conducted to determine the tissue distribution of NCC expressing the SR-A protein. Table 3.1 demonstrates that NCC from AK, spleen as well as liver express the SR-A like protein. Tissue from both catfish and tilapia were examined to determine whether SR-A expression was unique to one species of teleost. Equivalent levels of expression were observed on NCC from both catfish and tilapia.

3.5. SR-A expression by NCC is not up-regulated by oligonucleotide or phorbol ester/calcium ionophore treatments

To determine a possible signaling capacity of the expressed SR-A-like protein, purified NCC were treated for varying lengths of time with dG20 or PMA/A23187. NCC were examined by flow cytometry for increased expression of SR-A determined by mab 2F8-

FITC binding at 12-60h post-treatment (Fig. 3.7). Expression of SR-A by treated NCC did not differ from media controls.

Table 3.1. Tissue distribution of cells that bind to Scavenger Receptor-A monoclonal 2F8.

	Percentage Positive Cells	
Tissue[1]	*Ictalurus punctatus*	*Oreochromis niloticus*
Anterior Kidney	16	6
Spleen	21	9
Liver	14	16

[1] Red blood cells were removed by Ficoll-Hypaque prior to analysis.

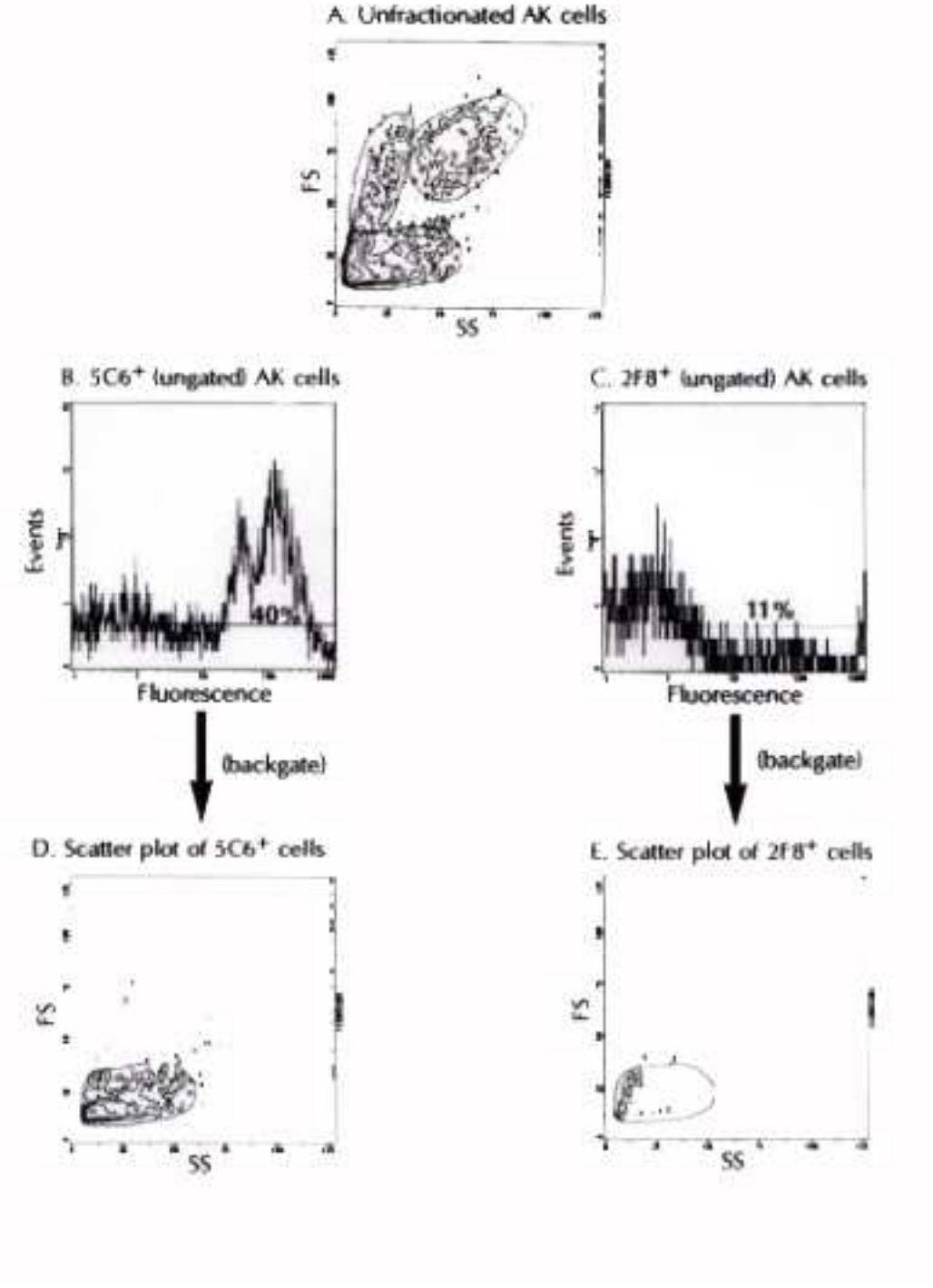

A. Unfractionated AK cells
FS
SS
B. 5C6+ (ungated) AK cells
Events
40%
Fluorescence
C. 2F8+ (ungated) AK cells
Events
11%
Fluorescence
(backgate)
(backgate)
D. Scatter plot of 5C6+ cells
FS
SS
E. Scatter plot of 2F8+ cells
FS
SS

Fig. 3.1. Identification of the scatter characteristics of scavenger receptor and 5C6 positive cells in unfractionated anterior kidney cells. 25 x 10^3 unfractionated anterior kidney cells were incubated with mab 5C6 (plus anti-mouse IgM-FITC) or 2F8-FITC for one h on ice. Stained cells were analyzed by flow cytometry for size and scatter characteristics (A). 5C6 (B) and 2F8 (C) positive cells were identified in ungated AK cells and fluorescing cells in each population were backgated for comparisons of size and scatter properties (D and E). Representative of two different experiments.

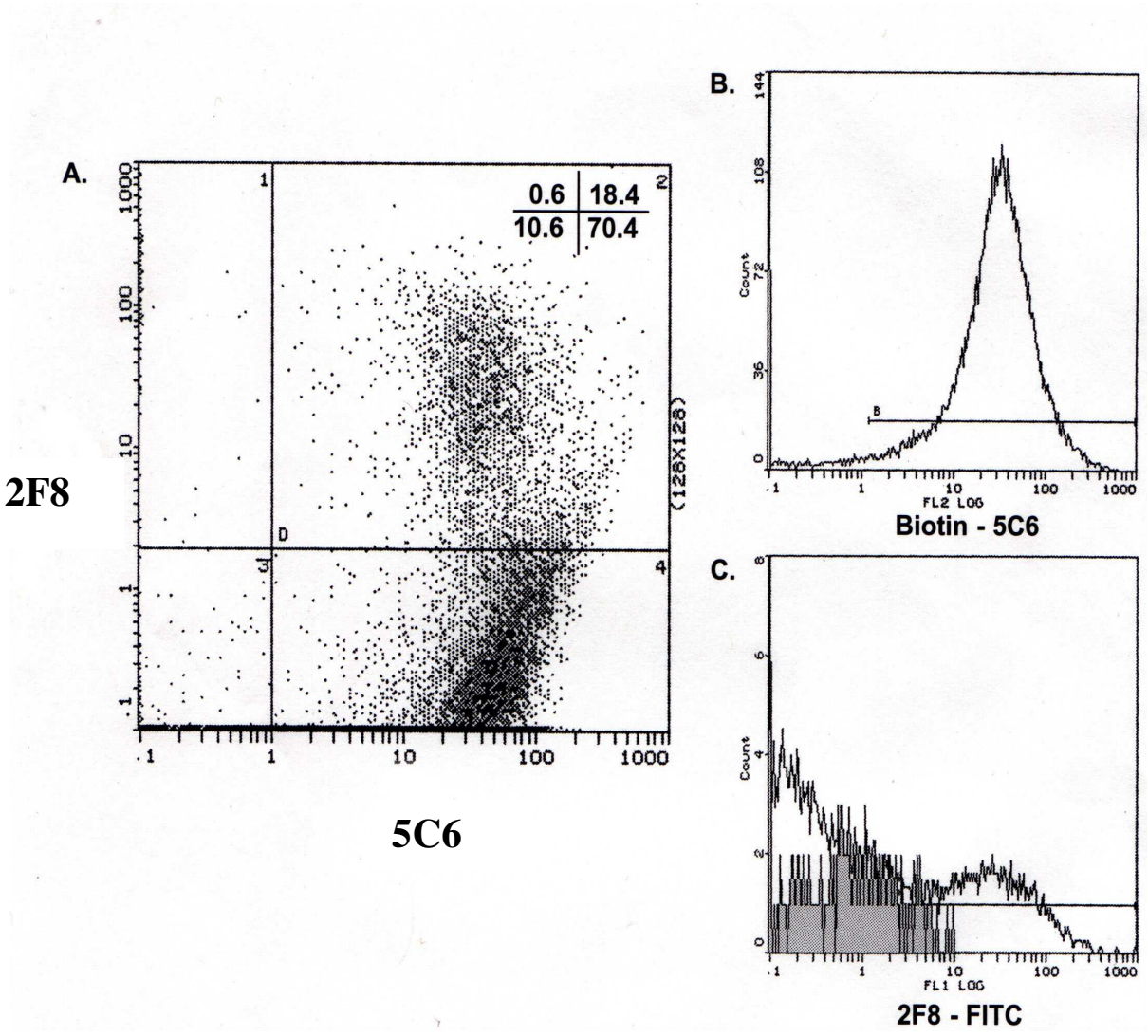


Fig. 3.2. Expression of scavenger receptor by NCC. Purified NCC were stained with mab 5C6 (plus anti-IgM-biotin and extravidin-PE) and 2F8-FITC and analyzed by two-color flow cytometry. Percent positives in each quadrant are shown. Double positives are shown in (A) and single positive Biotin-5C6 and 2F8-FITC are shown in (B) and (C) respectively.

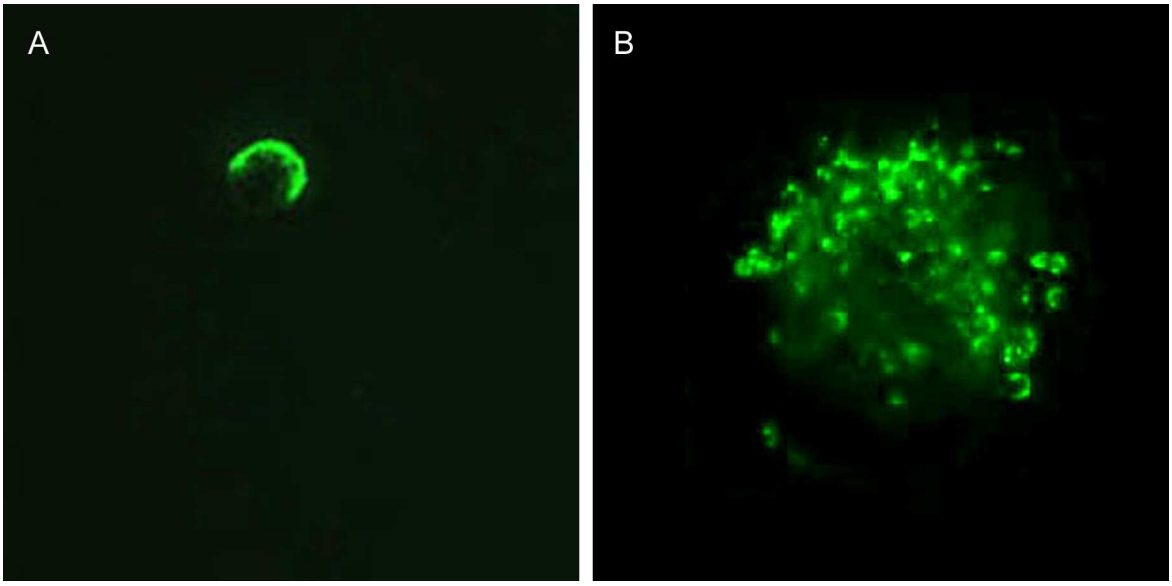


Fig. 3.3. Detection of 2F8 binding on NCC and J774.A1 cells by immunofluorescence microscopy. Purified NCC (A) and J774.A1 (B) cells were incubated with PAB at 4 °C for 2 h. 200 X 10^3 cells were stained with saturating concentration of 2F8-FITC for 1h on ice. Cells were washed to remove unbound 2F8-FITC mab and analysed by fluorescent microscope (x 1000).

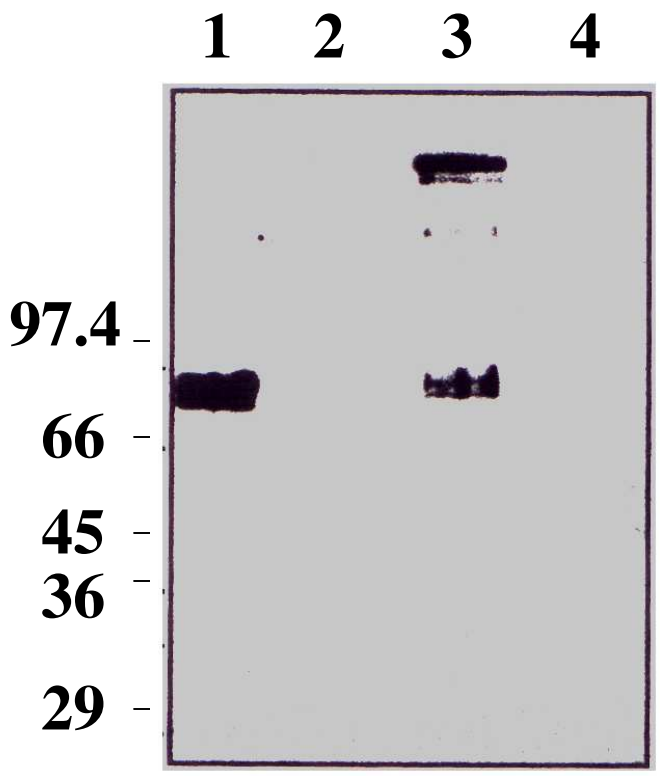


Fig. 3.4. Western blot analysis of J774.A1 cells with 2F8. J774.A1 cell lysate was prepared as described in Material and Methods and proteins in the reduced (lanes 1 and 2) and non-reduced (lanes 3 and 4) lysates were resolved by SDS-PAGE and transferred onto nitrocellulose. Western blot analysis was done by adding monoclonal antibody 2F8 (1: 10000) to lanes 1 and 3 and anti-IgG-peroxidase conjugate to lanes 2 and 4 for 1 h . The bound antibody in lanes 1 and 3 was detected by anti-IgG-peroxidase conjugate (1 h) and chemiluminescent substrate.

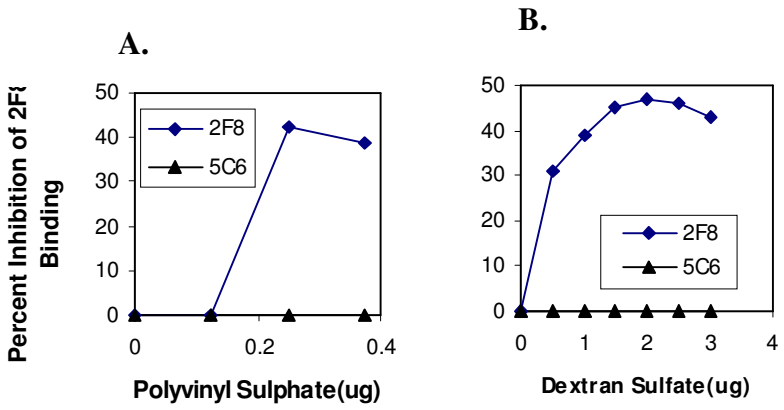


Fig. 3.5. Polyvinyl sulphate and dextran sulphate competitively inhibited binding of scavenger receptor antibody 2F8 to NCC. Purified NCC (25 x 10^3 cells) were incubated with different concentrations of polyvinyl sulphate (A) or dextran sulphate (B) for 30 min (4 °C), washed with PAB and incubated with 2F8-FITC (300ng/) for 1 h. Cells were washed with PAB (2x) and analyzed by flow cytometry. Percent inhibition was calculated by subtracting binding in the presence of inhibitor from total 2F8 binding. Representative of three independent experiments.

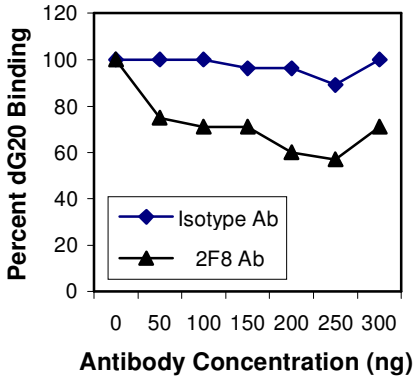


Fig. 3.6. Scavenger receptor antibody 2F8 competes with dG20 binding to NCC. Purified NCC were incubated with PAB for 2 h. 25 x 10^3 cells were then incubated with different amounts of anti-SR antibody 2F8 (as indicated) and 0.25µg of biotinylated dG20 for 1 h (4 °C). Cells were washed and incubated with PE-Avidin conjugate for 30 min and analyzed by flow cytometry. Representative of three independent experiments.

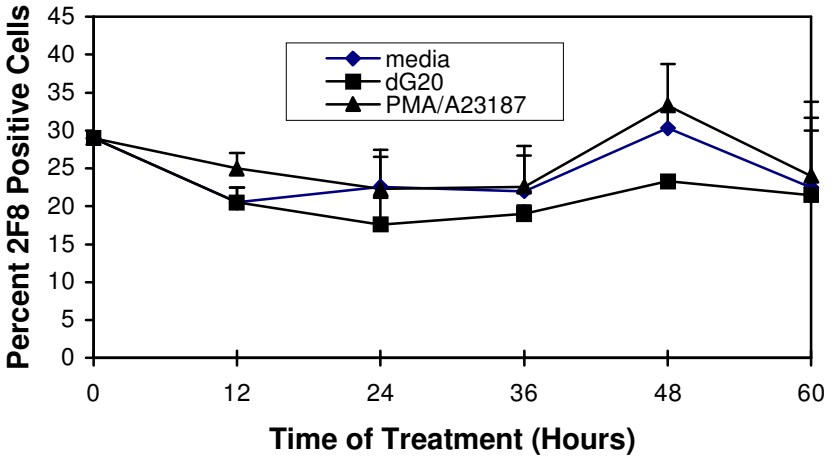


Fig. 3.7. Scavenger-Receptor-A expression is not up-regulated by ODN or phorbol ester/calcium ionophore. Purified NCC were incubated with dG20, PMA/A23187 or media for 0 to 60 h at room temperature. At each time point 25 x 10^3 cells were harvested, washed with PAB and stained with 2F8-FITC conjugate for 1 h on ice. Cells were washed twice with PAB and analyzed by flow cytometry. Mean of three different experiments is shown.

4. Discussion

Scavenger Receptors (SR) are expressed on a diverse spectrum of teleost cells including, macrophages [12], liver sinusoidal [14] and endocardial endothelial cells [17]. The ligands of the SR expressed by these cells are equally diverse and may be composed of modified plasma proteins, derivatised lipoproteins and G-tetrads formed by intrachain folded guanosine-rich oligodeoxynucleotides. Previous reports [5, 27, 28] have shown that tetramers of stabilised polyguanosine (i.e. G-tetrads) bind to scavenger receptors (SR-A type-I) on various types of mammalian cells. It has been demonstrated previously [29] that dG20 binding to NCC was receptor mediated and that NCC expressed several different specificities for ODNs including receptors for guanosine-phosphate-cytosine (GpC)/cytosine-phosphate-guanosine (CpG) and dG20. These studies also showed that bacterial DNA and the dinucleotide ligands composed of GpC or CpG motifs bound to the same receptor. However, sODNs expressing these motifs could not compete with dG20 for binding to NCC [29]. Because previous reports [5, 27, 28] have shown that one ligand recognised by SR-A (type-I) is an sODN composed of G-tetrads, it was predicted that all or a portion of the dG20 binding capacity of NCC depended on expression of SR-A. Such a finding would suggest that NCC expressed SR-A (types-I, -II) or some teleost equivalent. Others have previously shown that sODNs containing poly-guanosine flanking "Runs" or "extensions" bound to scavenger receptors on APCs [11] and CD8 positive T-cells [30]. In these studies, binding initiated cellular secretions of TNF-alpha and IL-12 [30] and activated T-cell proliferation and cytotoxicity [11].

Synthetic ODNs may bind to multiple different receptors on APC [31, 32]. One species of these receptors that binds bacterial nonmethylated DNA is the Toll-like

receptor-9 (TLR-9) [31]. However, there has not been any evidence that TLR-9 binds G-tetrads and the only cross-reactive ligand that binds both SR-A and any TLR family member is LPS [27].

Although not all types of teleost tissue were examined, the present study identified a member of the SR family that was found on NCC and not on other lymphocytes or tissue macrophages. Both direct and indirect evidence was presented that NCC expressed a membrane receptor similar to SR-A (types-I, -II). These experiments were accomplished by phenotype analysis using mab 2F8 (Fig. 3.1 and 3.2), i.e. flow cytometry, by immunofluorescence microscopy (Fig. 3.3) and by demonstrating that known ligands of SR (i.e. polyvinyl sulphate, dextran sulphate and dG20) competitively inhibited 2F8 binding to NCC (Fig. 3.5 and 3.6 respectively). The majority of cell types expressing SR are APC. Finding of SR on the teleost equivalent of mammalian NK cells (i.e. NCC) may provide an important phylogenetic clue regarding the evolutionary differentiation of cell types from a potential common precursor. Interestingly, these findings further support our hypothesis that NCC contribute an important role in anti-bacterial innate immunity.

Expression of SR-A (types I and II), SR-B as well as CD36 is restricted to APC, certain T-cells and macrophages in mammals. The function of SR in these species facilitates phagocytosis by APC and signaling of T-cells in the presence of negatively charged polyanions/ligands such as acetylated low-density lipoproteins, dextran sulphate and polyvinyl sulphate. However, these receptors (apparently) lack a clearly defined antigen specificity. It is this negative charge density of certain ligands and the corresponding receptor promiscuity that has contributed to the conclusions in many

studies of teleosts [12-18] as well as more primitive vertebrates [e.g. sea lamprey (*Petromyzon marinus*), [19] that SR are involved in innate immunity. In all these lower vertebrates yet studied, direct evidence for SR expression (i.e. protein sequence, anti-SR mab Western blot reactivity, mRNA or cDNA detection) has not been presented. Although transcripts (mRNA) for Drosophila [33] and sea urchin scavenger receptor cysteine-rich (SRCR) domains [34] have been described, the expression of SR on cells from these phylogenetic ancient species has not been reported. The gene exists in invertebrates but the protein has not been described.

In mammals, studies of SR-like function have also indicated an important role for this family of receptors in innate immune function. In addition to recognition of polyanions, SR found principally on mammalian macrophages also bind to oligonucleotides composed of (poly)oligodeoxyguanosine (dG) or to ODNs consisting of flanking dG runs [27]. An ODN composed of a –AACpGTT- nest flanked by 12 guanosine nucleotides activated mouse macrophages to mediate gamma interferon activated NK cell lysis [5] of YAC-1 targets. Similar to the present study, indirect evidence was presented suggesting that G-tetrads bound to Type-I SR on macrophages. Results from ligand competition assays also suggested a unique participation of poly-G [30] as a direct co-stimulant of CD8 cytotoxicity and proliferation. Conjugation of dG "Runs" to a CpG (i.e. dG6) produced an ODN [11] that bound to mouse APC and stimulated the release of various cytokines. In that study, an ODN composed of dG20 competed with the binding of the dG6 to splenic dendritic cells. Finally, evidence for a more direct influence of guanosine-rich sODNs (-TTGGAGGGGGTGGTGGGG-) [7] on

proliferation responses was shown in a study demonstrating the expansion of macrophage-like cells from mouse bone marrow cells.

Multiple mechanisms and pathways of cellular activation have to be considered in order to explain these results. For SR activation, previous studies [3, 7, 27, 28] have shown that for dG20 to bind and initiate signaling, a base quartet stabilised four-stranded helix is formed. Compared to previous studies that indirectly demonstrated quadruplex binding of poly-G to SR using ligand competition, the present work for the first time showed specific competition for binding using the SR-A (types-I, -II) mab 2F8-FITC. These data established that not all NCC are SR positive (Fig. 3.2). This indicated that in addition to SR-A, other receptors are present on NCC that also bind dG20. To support this hypothesis, it was previously reported [29] (and data not shown) that dG20 crosslinks homologous membrane proteins (i.e. non-SR-A receptors) and activates NCC to increase membrane binding by biotinylated-dG20 and mab 5C6. These are characteristics of cellular activation processes dependent on membrane receptor protein crosslinkage.

Finally, because it was not possible to detect SR-A in NCC lysates by Western blot analysis using mab 2F8 (data not shown) it was surprising to find good binding by flow cytometry. This suggested that mab 2F8 may recognise a conformation dependent epitope on SR-A which is lost upon SDS-PAGE manipulation. The Western blot experiments were resolutely negative under all conditions attempted. Until these issues are resolved it is suggested that SR-A (type-I) as well as other members of the SR family have evolved in molecular structure from teleosts to mammals. This would suggest that only a certain degree of amino acid homology would be conserved in evolution and that

in order to discern the complete identity of the "teleost" SR, future sequencing studies will be required.

References

[1] Kodama, T., Reddy, P., Kishimoto, C. & Krieger, M. Purification and characterization of a bovine acetyl low density lipoprotein receptor. Proceedings of the National Academy of Sciences USA 1988; 5:9238-42.

[2] van Velzen, A. G., Suzuki, H., Kodama, T. & van Berkel, T. J. C. The role of scavenger receptor Class A in the adhesion of cells is dependent on cell type and cellular activation state. Experimental Cell Research 1999;250:264-71.

[3] Gugliucci, C. A. & Stahy, A. J. C. Assay for scavenger receptors on human monocyte-derived macrophages. Journal of Immunological Methods 1994;174:103-7.

[4] Singh, N., Bhatia, S., Abraham, R., Basu, S. K., Georg,e A., Bal, V. & Rath, S. Modulation of T cell cytokine profiles and peptide-MHC complex availability in vivo by delivery to scavenger receptors via antigen maleylation. Journal of Immunology 1998;160:4869-80.

[5] Kimura, Y., Sonehara, K., Kuramoto, E., Makino, T., Yamamoto, S., Yamamoto, T., Kataoka, T. & Tokunaga, T. Binding of oligoguanylate to scavenger receptors is required for oligonucleotides to augment NK cell activity and induce IFN. Journal of Biochemistry 1994;116:991-4.

[6] Sasaki, T., Horiuchi, S., Yamazaki, M. & Yui, S. Stimulation of macrophage DNA synthesis by polyanionic substances through binding to the macrophage scavenger receptor. Biological Pharmacology Bulletin 1996;19:449-55.

[7] Lang, R., Hultner, L., Lipford, G. B., Wagner, H. & Heeg, K. Guanosine-rich oligodeoxynucleotides induce proliferation of macrophage progenitors in cultures of murine bone marrow cells. European Journal of Immunology 1999;29:3496-506.

[8] Saijo, Y., Ychiyama, B., Abe, T., Satoh, K. & Nukiwa, T. Contiguous four-guanosine sequence in c-myc antisense phosphorothioate oligonucleotides inhibits cell growth on human lung cancer cells: possible involvement of cell adhesion inhibition. Japanese Journal of Cancer Research 1997;88:26-33.

[9] Castier, Y., Chemla, E., Nierat, J., Heudes, D., Vasseur, M. A., Rajnoch, C., Bruneval, P., Carpentier, A. & Fabiani, J. N. The activity of c-myb antisense oligonucleotide to prevent intimal hyperplasia is nonspecific. Journal of Cardiovascular Surgery (Torino) 1998;39:1-7.

[10] White, J. R., Gordon-Smith, E. C. & Rutherford, T. R. Phosphorothioate-capped antisense oligonucleotides to Ras GAP inhibit cell proliferation and trigger apoptosis but fail to downregulate GAP gene expression. Biochemical Biophysics Research Communication 1996;227:118-24.

[11] Lee, S. W., Song, M. K., Baek, K. H., Park, Y., Kim, J. K., Lee, C. H., Cheong, H. K., Cheong, C. & Sung, Y. Effects of a hexameric deoxyriboguanosine run conjugation into CpG oligodeoxynucleotides on their immunostimulatory potentials. Journal of Immunology 2000;165:3631-9.

[12] Froystad, M. K., Rode, M., Berg, T. & Gjoen, T. A role for scavenger receptors in phagocytosis of protein-coated particles in rainbow trout head kidney macrophages. Developmental & Comparative Immunology 1998;22:533-49.

[13] O'Donnell, G. B., Smith, P. R., Kilmartin, J. J. & Moran, A. P. Uptake and fate of orally administered bacterial lipopolysaccharide in brown trout (Salmo trutta). Fish and Shellfish Immunology 1994;4:285-99.

[14] Dannevig, B. H., Lauve, A., Press, C. M. & Landsverk, T. Receptor-mediated endocytosis and phagocytosis by rainbow trout head kidney sinusoidal cells. Fish and Shellfish Immunology 1994:4:3-18.

[15] Gjoen, T. & Berg, T. Metabolism of low density lipoproteins in rainbow trout. Fish Physiology & Biochemistry 1992;9:453-61.

[16] Dannevig, B. H. & Berg, T. Isolation of pronephros cells which endocytose chemically modified proteins in the rainbow trout. Developmental & Comparative Immunology 1986;10:25-34.

[17] Sorensen, K. K., Melkko, J. & Smedsrod, B. Scavenger-receptor-mediated endocytosis in endocardial endothelial cells of atlantic cod Gadus morhua. Journal of Experimental Biology 1998;20:1707-18.

[18] Dannevig, B. H., Struksnaes, G., Skogh, T., Kindberg, G. M. & Berg, T. Endocytosis via the scavenger- and the mannose-receptor in rainbow trout (Salmo gairdneri) pronephros is carried out by nonphagocytic cells. Fish Physiology & Biochemistry 1990;8:229-38.

[19] Mayer, W. E. & Tichy, H. A cDNA clone from the sea lamprey Petromyzon marinus coding for a scavenger receptor Cys-rich (SRCR) domain protein. Gene 1995;164:267-71.

[20] Bishop, J. S., Guy-Caffey, J. K., Ojwang, J. O., Smith, S. R., Hogan, M. E., Cossum, P. A., Random, R. F. & Chaudharym N. Intramolecular G-quartet motifs confer nuclease resistance to a potent anti-HIV oligonucleotide. Journal of Biological Chemistry 1996;271:5698-703.

[21] Sen, D. & Gilbert, L. Formation of parallel four-stranded complexes by guanine-rich motifs in DNA and its implications for meiosis. Nature 1988;334:364-6.

[22] Sundquist, W. I. & Heaphy, S. Evidence for interstrand quadruplex formation in the dimerization of human immunodeficiency virus 1 genomic RNA. Proceedings of the National Academy of Sciences USA 1993;90:3393-97.

[23] Liu, Z., Lee, A. & Gilbert, W. Gene disruption of a G4-DNA-dependent nuclease in yeast leads to cellular senescence and telomere shortening. Proceedings of the National Academy of Sciences USA 1995;92:6002-6.

[24] Zahler, A. M., Williamson, J. R., Cech, T. R. & Prescott, D. M. Inhibition of telomerase by G-quartet DNA structures. Nature 1991;350:718-20.

[25] Klobutcher, L. A. Developmentally excised DNA sequences in Euplotes crassus capable of forming G quartets. Proceedings of the National Academy of Sciences USA 1995;92:1979-83.

[26] Evans, D. L, Smith, E. E, Jaso-Friedmann, L., St. John, A., Koren, H. S. & Harris, D. T. Identification of a putative antigen receptor on fish nonspecific cytotoxic cells with monoclonal antibodies. Journal of Immunology 1988;141:324-32.

[27] Pearson, A. M., Rich, A. & Krieger, M. Polynucleotide binding to macrophage scavenger receptors depends on the formation of base-quartet-stabilized four-stranded helices. Journal of Biological Chemistry 1993;268:3546-54.

[28] Pearson, A. M. Scavenger receptors in innate immunity. Current Opinion in Immunology 1996;8:20-28.

[29] Oumouna, M., Jaso-Friedmann, L. & Evans, D. L. Activation of nonspecific cytotoxic cells (NCC) with synthetic oligodeoxynucleotides and bacterial genomic DNA: binding, specificity and identification of unique immunostimulatory motifs. Developmental & Comparative Immunology 2002;26:257-69.

[30] Lipford, G. B, Bendigs, S., Heeg, K. & Wagner, H. Poly-guanosine motifs costimulate antigen-reactive CD8 T cells while bacterial CpG-DNA affect T-cell activation via antigen-presenting cell-derived cytokines. Immunology 2000;101:46-52.

[31] Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K. & Akira, S. A toll-like receptor recognizes bacterial DNA. Nature 2000;408:740-5.

[32] Modlin, R. L. A toll for DNA vaccines. Nature 2000;408:659-60.

[33] Abrams, J. M., Lux, A., Steller, H. & Drieger, M. Macrophages in Drosophila embryos and L2 cells exhibit scavenger receptor-mediated endocytosis. Proceedings of the National Academy of Sciences U.S.A 1992;89:10375-79.

[34] Dangott, L. J., Jordan, J.E., Bellet, R.A. & Garbers, D.L. Cloning of the mRNA for the protein that crosslinks to the egg peptide speract. Proceedings of the National Academy of Sciences U.S.A 1989;86:2128-32.

CHAPTER 4

SINGLE BASE OLIGODEOXYGUANOSINE BINDING PROTEINS ON NONSPECIFIC CYTOTOXIC CELLS: IDENTIFICATION OF A NEW CLASS OF PATTERN RECOGNITION RECEPTORS[1]

[1] Kaur, H., L. Jaso-Friedmann and D. L. Evans. Submitted to *Journal of Immunology,* 5/20/2003.

Abstract

The present study was designed to identify membrane proteins on (teleost) nonspecific cytotoxic cells (NCC) and mammalian cells that bind single base oligodeoxynucleotide (ODN) ligands. These ODNs were composed of 20-mers of guanosine (dG20), adenosine (dA20), thymidine (dT20) or cytosine (dC20). ODNs were first examined for binding to NCC and to (mouse) RAW 264.7 and (human) THP-1 cells. Binding to NCC by dG20 was specific and saturable at 1.25 μg/ml. Saturable binding to RAW and THP-1 cells by dG20 occurred at 0.2 μg/ml and 0.8 μg/ml (respectively). dG20 bound to NCC in the anterior kidney (i.e. fish bone marrow equivalent), spleen and liver. Southwestern blots of whole cell lysates and immunoprecipitation of biotin labeled cell membranes from NCC, RAW 264.7 and THP-1 cells demonstrated two different mw species (14-18 kDa and 29-34 kDa) of binding proteins. These were crossreactive by Western blot examination with a polyclonal anti-histone-1 antibody. Function studies revealed that dG20 activated a 2-fold upregulation of membrane binding of homologous dG20-biotin. dG20 also stimulated NCC increased membrane expression of NCCRP-1. Additional experiments were conducted to determine the DNAse sensitivity of the different ODNs. dG20 appeared to be more resistant to DNase treatment compared to dC20, dA20 and dT20. The single base ODN binding proteins may represent a new class of pattern recognition receptors that are involved in innate anti-bacterial resistance mediated by NCC.

Introduction

The oligodeoxynucleotide (ODN) ligand specificities of mammalian NK, antigen presenting cells (APC)(B-cells, macrophages, dendritic cells) and T-cells have been extensively reviewed (1-8). Contact of these cells with certain nonmethylated (bacterial) DNA in the form of CpG immunostimulatory motifs resulted in the activation of cellular functions including cytokine release, DNA proliferation and in certain cases enhancement of cytolytic activity. ODNs composed of one to several CpG dinucleotides may now be considered as pathogen associated molecular pattern (PAMP) ligands that bind to pattern recognition receptors (PRR) composed of Toll-like receptor-9 (TLR-9) (9).

A phylogenetic linkage between mammals and cold blooded vertebrates (i.e. teleost fish) has recently been established (10) for defining the potential role of ISS ODNs in innate immunity. In these studies of cellular activation and binding of synthetic ODNs (sODN) to nonspecific cytotoxic cells (NCC), many differences were described compared to mammalian "canonical" dogma. The preferred binding motifs for mammalian cells are –GACGTT- (mice) and –GTCGTT- (humans) (1). These species generally do not bind the GpC dinucleotide. We determined that the optimum immunostimulatory motif for teleosts was either 5'-C/AT/AGCTT-3' or 5'-GTCGTT-3' (10). Methylation of cytosine inhibited both teleost and mammalian cell responses to sODNs. NCC were also activated by sODNs composed of dinucleotides flanked by consecutive deoxyguanosine residues (dG runs) in addition to the single base oligodeoxyguanosine 20-mer nucleotide (i.e. dG20) (10).

Although recognition of this particular ODN was unique for teleost cells, CpG palindromes containing flanking sequences or "runs" of 12 guanosine nucleotides (i.e.

dG12) have been shown by others to bind and activate mouse NK cells (11). In both mice (11) and catfish (10), binding was accompanied by finding a relatively large increased killing of YAC-1 and IM-9 targets respectively. The NK cell studies were further supported by previous data from others suggesting that sODNs containing poly-guanosine flanking runs or "guanosine extensions" bound to scavenger receptors on APCs (12) and CD8 positive T-cells (13). In these studies, binding initiated cellular secretions of TNF-alpha and IL-12 (13) as well as initiation of T-cell proliferation and cytotoxicity (12).

Because these previous reports (14) demonstrated that tetramers of stabilized polyguanosine quartets bound to scavenger receptors (SR-A type-I), we have also examined (15) the possibility that SR-A type-I was responsible for dG20 binding activity to NCC. Those studies demonstrated that total binding by dG20 to NCC could not be explained solely by expression of Scavenger Receptor-A (SR-A) because neither antibody to SR-A or SR-A ligands (i.e. dextran sulfate, polyvinyl sulfate) competed more than 40-50% of total dG20 binding. Based on these data, we predicted that NCC bound to single base ODNs utilizing a heterogeneous population of DNA receptors. In order to examine this possibility, the present study demonstrates that NCC express single base oligodeoxyguanosine binding proteins that may comprise a new class of pattern recognition receptors independent of Scavenger and Toll-like receptors.

Material and methods

Animals

Ictalurus punctatus (catfish) of both sex, were obtained from local commercial farms. Fish were maintained in flow-through 300 gallon aquaria at ambient temperature (17-25^{o}C) and fed commercial fish pellets (Purina Catfish Startena, Ralston Purina Co., St. Louis, MO). Water quality was monitored for temperature, nitrite, ammonia nitrogen and chlorine contents.

Media, reagents, cells and antibodies

The murine macrophage-like cell line, RAW264.7, and human monocytic cell line, THP-1, were obtained from American Type Culture Collection (ATCC, Bethesda, MD). NCC were prepared from anterior kidney (AK) of catfish. Cells were cultured in RPMI-1640 (Cellgro, Media Tech, Washington, DC) supplemented with L-glutamine, sodium pyruvate, MEM vitamin solution, MEM amino acid solution, MEM non-essential solution (Cellgro), 50 mg/ml gentamicin (Schering-Plough Animal health Corp., Kenilworth, NJ) and 10% fetal bovine serum (FBS, Atlanta biologicals, Norcross, GA). Washing media (PAB) consisted of 1X phosphate buffer saline (PBS), 0.1% sodium azide (Sigma Chemical Co., St. Louis, MO) and 1% bovine serum albumin (Sigma Chemical Co.). Calf thymus DNA, Phorbol 12-Myristate 13-Acetate (PMA, # P8139), calcium ionophore-A23187 (# C7522), DNase I (#D4263), Extravidin-phycoerythhrin (PE) conjugate (# E4011) and ExtrAvidin-Peroxidase Conjugate (#E2886) were obtained from Sigma Chemical Co. Monoclonal (mab) antibody 5C6 (IgM isotype) specific for a 32-kDa NCC receptor protein (NCCRP-1) was prepared in house (16). Unconjugated

(#1322) and FITC conjugated (#1322F) forms of rat anti-mouse scavenger receptor antibody (SR-AI/II) 2F8 were purchased from Serotec Inc. (NC, USA). The isotype rat anti-mouse IgG2b-FITC was purchased from Southern Biotechnology Associates Inc. (Birmingham, AL.). Anti-mouse IgM-FITC and biotin-IgM conjugates were obtained from Sigma Immunochemicals.

Purification of NCC

Fish weighing 20-60g were net captured and sacrificed by submersion in anesthetic (3-aminoenzoic acid ethyl ester; Sigma Chemical Co.). Anterior kidney (AK) tissue (mammalian bone marrow equivalent) was removed aseptically and passed through screen mesh to obtain single cell suspensions in complete RPMI-1640 containing 10% FBS. Cells were purified by density gradient centrifugation over a 45.5 % Percoll cushion. Cells at the interface were collected, washed once with RPMI and resuspended in complete RPMI.

Oligodeoxynucleotides

Oligodeoxynucleotides were purchased from MWG-Biotech (High Point, NC). They were synthesized as phosphodiesters using standard methods and resuspended in endotoxin-free water. 5'end biotinylated (dG20-biotin, dA20-biotin and dC20-biotin) and digoxigenin (DIG-dG20) labeled ODNs were purchased from MWG-Biotech. The sequence, characteristics and size of each ODN are shown in Table 4.1. ODNs and calf thymus DNA were resuspended in endotoxin free water. The endotoxin level was less

than 0.015 endotoxin units (EU)/ml for all reagents used in the study as determined by *Limulus Amebocyte lysate* assay (kit # 210-A; Sigma Chemical Co.).

Binding assays

Purified NCC (1 x 10^6/ml) were treated with dG20-biotin (50 µg/ml) for 0 h and 36 h. Cells were washed and incubated with PAB for 1 h. 1 x 10^5 cells were stained with saturating amounts of mab 5C6 and dG20-biotin for 1 h followed by washing twice with PAB and addition of anti-mouse-IgM-FITC (1:20 dilution) and Extravidin- PE conjugate (1:20 dilution) for 30 min. Cells were washed and analyzed by flow cytometry (two color analysis). Final resuspension of cells in all flow cytometric experiments was in 300 µl of PAB. For single color analysis of dG20-biotin binding, 1 x 10^5 purified NCC or 5 x 10^4 RAW264.7 and THP –1 cells were incubated at 4°C in PAB for 1 h followed by incubation with saturating amounts of dG20-biotin for 1 h and Extravidin-PE for 30 min. Cells were washed and analyzed by flow cytometer. For SR expression on RAW264.7 cells, 2.5 x 10^4 cells were incubated with 100 µl of 5 µg/ml of FITC conjugated anti-SR antibody 2F8 for 1 h, washed and analyzed by flow cytometry.

To determine the saturating amounts of dG20, 1 x 10^5 purified NCC or 5 x 10^4 RAW264.7 and THP-1 cells were incubated (PAB/4°C) with 100 µl of different concentrations of dG20-biotin for 1 h. Cells were washed twice with PAB and further incubated with Extravidin-PE conjugate for 30 min. Cells were washed and analyzed by flow cytometer.

Preparation of cell membranes

For membrane preparation, cells were washed three times with ice cold TBS (25mM Tris-Cl, pH 7.5, 150mM NaCl). Cells were resuspended in Dounce homogenization buffer (10mM Tris-Cl, pH 7.6, 0.5mM $MgCl_2$, 10 µg/ml leupeptin, 10 µg/ml pepstatin and 1mM PMSF) @ 2 x 10^7 cells/ml and incubated on ice for 15 min. 333µl of tonicity restoration buffer (10mM Tris-cl, pH 7.6, 0.5mM $MgCl_2$ and 0.6M NaCl) was added per ml of homogenization buffer and cells were spun at 500g for 5 min. Supernatant was collected and EDTA was added to 5mM. Supernatant was then spun at 13000 rpm for 10 min. Supernatant was discarded and pellet equivalent to1 x 10^6 cells was washed twice with cold TBS containing 10 µg/ml leupeptin, 10 µg/ml pepstatin and 1mM PMSF and finally resuspended in 100 µl of hot 1X SDS-sample buffer.

Competition inhibition of dG20 binding to NCC and RAW264.7

Cells (2.5 x 10^4) were incubated with dA20, dC20, dG20, dT20, CpG ODN and anti-SR antibody 2F8 (in case of RAW264.7) for 1 h on ice. Concentration of unlabeled dG20, dA20, dC20 and dT20 was 1- to 1000-fold excess (NCC and RAW264.7) and that of CpG was 1- to 1000-fold excess (NCC) or 4- to 4000-fold excess (RAW264.7) of 50% saturating amounts of dG20-biotin. For 2F8, 100 µl of following concentrations were used: 1 µg/ml, 2 µg/ml, 3 µg/ml and 4 µg/ml per 2.5 x 10^4 cells. Cells were washed twice to remove excess unbound ODNs/antibody and incubated with 50% saturating amounts of dG20-biotin for 1 h on ice. Cells were washed and further incubated for 30 min with Extravidin-PE conjugate and analyzed by flow cytometry.

Ligand (Southwestern) blot

NCC, THP-1 and RAW264.7 cell membrane proteins were resolved on 12.5 % SDS-PAGE gel and transferred onto nitrocellulose membrane at 100 v for 1h. Nitrocellulose filters were blocked with SuperBlock Dry Blend (TBS) blocking buffer (#37545, Pierce Chemical Co., Rockford, IL) containing 0.1% Tween for 30 min. Primary ligand incubation (dG20-biotin and dA20-biotin diluted 100ng/ml in blocking buffer) was done for 1 h, filters were washed with TBS containing 0.1% Tween -T (TBS-T) for 20 min (four times, 5 min each) and finally incubated with secondary conjugate (ExtrAvidin-peroxidase conjugate diluted 1:10000 in blocking buffer) for 1 h. After washing with TBS-T for 20 min, detection was done with chemiluminiscent substrate (SuperSignal® West Pico Chemiluminescent, Pierce Chemical Co.).

Surface labeling, ligand-precipitation and blotting

NCC were surface biotinylated using EZ-Link™ Sulfo-NHS-Biotin (#21217, Pierce Chemical Co.) according to manufacturer's instruction. Briefly, cells were washed three times with cold PBS, incubated with Sulfo-NHS-Biotin (50µg/ml) @ 1 x 10^7 cells/ml for 20 min at RT on rotator, washed four times with ice-cold PBS and lyzed with CHAPS lysis buffer (10mM CHAPS, 0.15M NaCl, 10mM Tris-Cl, pH 7.6, 1mM EDTA) containing protease inhibitors (1mM PMSF, 1µM Leupeptin and 1µM Pepstatin). NCC lysates prepared from surface biotinylated cells were subjected to ligand precipitation. For this, 200 µl of lysate was incubated for 2 h with 10µg of DIG-dG20 at 4°C followed by incubation with anti-digoxigenin-agarose beads (100µl of slurry, # A-3827, Sigma Chemical Co.) for 2 h at 4°C. Beads were pelleted, washed and bound proteins were

eluted with boiling in 1X SDS sample buffer. These proteins were resolved on 12.5% gel, transferred to nitrocellulose and probed with ExtrAvidin-peroxidase conjugate (1:15000) for 1 h. After washing with TBS-T for 20 min, detection was done with chemiluminescent substrate.

Western blot

Anterior kidney NCC membrane preparations were resolved on 12.5% gel, transferred onto nitrocellulose membrane and probed with biotinylated anti-histone H1 antibody (#M20151S, Biodesign, Saco, ME) (1 µg/ml) for 1 h. After washing with TBS-T for 20 min, filters were incubated with ExtrAvidin-peroxidase (1:50000) for 1 h, washed with TBS-T and proteins were detected with chemiluminescent substrate.

Receptor expression

To determine NCCRP-1 receptor expression, purified NCC (1x 10^6/ml) were treated with different ODNs (50 µg/ml), calf thymus DNA (5 µg/ml) and PMA/A23187 (0.5 µg/ml /2.5 µg/ml) for various time periods. 1 x 10^5 cells were harvested, washed with PAB and stained with saturating concentrations of 5C6 mab for 1 h on ice. Cells were washed twice with PAB, incubated with anti-mouse FITC-IgM for 30 min on ice, washed and analyzed by flow cytometer.

To determine ODN receptor expression, purified NCC (1x 10^6/ml) were treated with dG20 (50 µg/ml), PMA/A23187 (0.5 µg/ml /2.5 µg/ml) and media for different time periods as indicated. At each time point 1 x 10^5 cells were analyzed for ODN receptor expression. Cells were incubated in PAB for 1 h, pelleted, stained with saturating

concentration of dG20-biotin for 1 h on ice, washed twice followed by addition of Extravidin-PE for 30 min. Cells were washed and analyzed by flow cytometer.

DNAse assay

For DNAse treatment, 15µg of ODNs and 25 units of DNase I were dissolved together in 10 µl of 100mM Tris-Cl (pH 8) containing 5mM $MgCl_2$ and incubated at 37°C for 5h. The mixture was heated at 95°C for 5 min to inactivate the enzyme and chilled immediately. DNase I treated and non-treated ODNs were resolved on a 15% denaturing polyacrylamide gel at 180 v for 90 min. The gel was stained with ethidium bromide (0.5 µg/ml) for 30 min at room temperature. Gel pictures were obtained using a UV trans-illuminator.

Flow cytometry

Flow cytometry analysis was performed using an EPICS XL-MCL four color analyzer (Coulter Electronics Corp, Hileah, FL), equipped with 15 mW air cooled argon-ion laser operating at 488 nm wavelength. Two-parameters, forward scatter (FS; size) and side scatter (SSc; granularity), and backgating were used to positively identify each cell population. 10,000 to 15,000 events were collected per sample. Conjugate controls were included to set the baseline fluorescence. FITC was detected using 525nm bandpass filter by photomultiplier tube 1 (PMT1) and PE with 575nm bandpass filter by photomultiplier tube 2 (PMT2). Isotype control antibodies were included to determine non-specific binding. Data was analyzed using Coulter's System II software, version 3.0.

Results

Single base oligodeoxyguanosine 20-mer binds to NCC and mammalian cells

Experiments were first conducted to determine the cell type specificity and percentage positive cells of the dG20 constitutive binding levels. NCC were purified from the teleost bone marrow equivalent [i.e. the anterior kidney (AK)] and analyzed by flow cytometry for dG20 and mab 5C6 binding (specificity for NCCRP-1). 21% of NCC were positive for constitutive dG20 binding (Fig. 4.1A and 4.1B).

Table 4.2 provides a partial tissue distribution analysis of dG20, dC20 and dA20 binding. Both dG20 and dC20 bound to NCC in liver and AK tissue. The spleen contained the lowest percentage of positive cells. Mammalian cell lines THP-1 and RAW264.7 were 86% and 97% positive (respectively) for dG20 binding (Fig. 4.1A). Table 4.3 compares the binding of three single base ODNs to NCC, RAW 264.7 and THP-1 cells. As compared to dG20 the other two single base ODNs showed lower (dC20) or negligible (dA20) levels of binding.

Two color flow experiments were done with dG20-activated NCC to determine the up-regulated dG20 receptor expression (Fig. 4.1B). This was compared with constitutive dG20 binding to NCC (Fig. 4.1B). These data demonstrated that 26% and 67% (quadrant 2 as a percent of quadrant 1 plus 2) of purified NCC were positive for the dG20 binding at 0 h and 36 h respectively (Fig. 4.1B). This indicated an up-regulation of dG20 receptor expression on NCC after treatment with dG20.

Saturation and specificity of dG20 binding

Binding experiments were next done to determine whether binding by dG20 to NCC, RAW 264.7 and THP-1 satisfied the saturation requirements for receptor binding by a ligand. Purified NCC or tissue cultured cells were incubated with different concentrations of dG20 (in PAB/4°C) and percent positive binding was determined by flow cytometry (Fig. 4.2). Fifty percent saturation occurred at approximately 1.25 µg/ml NCC; 0.2 µg/ml RAW 264.7; and 0.8 µg/ml THP-1 cells.

Specificity of binding was determined by "cold" competition assays. NCC and RAW264.7 cells were first incubated with different fold excess of soluble unlabeled ODNs (dG20, dA20, dT20 and dC20), followed by fifty percent saturating amounts of dG20-biotin. Fig. 4.3A and 4.3C show that only unlabeled dG20 competed for binding with homologous ODN (dG20-biotin) in NCC and RAW264.7. dG20 binding in both these cell types was reduced to <30% in the presence of unlabeled dG20. Fig. 4.3B and 4.3D demonstrates that phosphodiester CpG competed partially for dG20 binding at high concentrations in both these cells. These results indicated that dG20 binding to NCC is saturable and specific.

Identification of dG20 binding proteins by ligand (Southwestern) blot

Purified NCC, THP-1 and RAW 264.7 were lysed and membrane preparations were analyzed by SDS-PAGE and dG20-biotin, dA20-biotin or ExtrAvidin-peroxidase conjugate binding using a Southwestern blotting technique. In Fig. 4.4A, lanes 1, 4 and 7 are NCC lysates; lanes 2, 5 and 8 THP-1 lysates; and lanes 3, 6 and 9 RAW 264.7 lysates. dG20-biotin was added to lanes 1-3; dA20-biotin added to lanes 4-6; and conjugate only

to lanes 7-9. NCC membranes had binding proteins of 14-16 and 29 kDa and THP-1 and RAW264.7 had almost identical binding patterns of 14-18 and approximately 33 kDa binding proteins. The high MW bands (~66kDa) seen in ODN-probed lanes as well as in conjugate control lanes indicated they might be avidin-binding proteins and, therefore, were nonspecific.

To confirm the membrane expression of these proteins, NCC were surface labeled with biotin and immunoprecipitation was done. Cell lysates were prepared from biotinylated cells. DIG-dG20 was added to biotinylated NCC lysates and immunoprecipitation was carried out using anti-digoxigenin agarose beads. Immunoprecipitates were resolved in 12.5% gels (Fig. 4.4B). An intense 29 kDa with faint 14 and 20 kDa signals were observed. The high MW band (~66kDa) is nonspecific as the same molecular weight band was e seen with Extravidin conjugate alone. Because previous (manuscript in preparation) data indicated a possible relationship between the low molecular weight DNA binding proteins and the histone family of DNA regulatory molecules (histone-like proteins), NCC membrane preparations were probed with polyclonal anti-histone-1 by Western blot analysis. Fig. 4.4C demonstrates that NCC membrane lysates probed with this antibody produced 14-18 and 29 kDa protein signals. The same anti-histone antibody, however, did not block dG20 binding to NCC (data not shown).

dG20 binding proteins on RAW264.7 cells are not Scavenger receptors

Previous experiments by others demonstrated the binding of single base oligodeoxyguanosine to Scavenger receptors (SR), thus experiments were conducted to

determine whether blocking of SR with anti-SR antibody on RAW 264.7 cells prevented dG20 binding. To accomplish this, competitive binding experiments were conducted. In Fig. 4.5A, SR antibody binding to RAW 264.7 cells is shown by flow cytometry. In Fig. 4.5B, pre-treatment of cells with 1-4 μg/ml of anti-SR antibody did not inhibit binding by dG20-biotin.

Binding of dG20 to NCC up-regulates expression of membrane proteins

Experiments were conducted to determine whether dG20 binding to NCC up-regulated (homologous) receptor protein expression. Purified NCC were incubated with dG20 for different time periods and cells were examined for increased expression of the homologous dG20 binding proteins. Up-regulation of dG20 binding proteins on NCC was measured by flow cytometry. Binding of dG20 to NCC increased homologous receptor expression on each NCC as demonstrated in Fig. 4.6A (insert) and 4.6B. Comparisons between media control and ODN-biotin (dG20) demonstrated that there was an increase in the percentage positive NCC at 36 h (26% increase), 48 h (28% increase) and 72 h (40% increase) post-treatment. Measures of mean fluorescence intensity (MFI) of these same cells (Fig. 4.6A) indicated that the percentage increase of MFI of dG20 treated cells (compared to media controls) was more than 100% [(79-21)/21 x 100)] after 36 h and approximately 35% [(76-56)/56 x 100)] after 48 h treatment.

All single base ODNs were next examined for their ability to up-regulate expression of NCCRP-1. This protein has been previously characterized as an activation/antigen receptor (16) and signaling protein on teleost NCC. Fig. 4.7 shows that dG20 produced the greatest increase in membrane expression of NCCRP-1 following

48 h and 72 h treatments. Positive and negative controls were PMA/A23197 and calf thymus DNA respectively.

Effects of DNase-I treatment on the oligodeoxynucleotides

One potential mechanism for dG20 binding and cellular activation may be dependent on the unique conformations (eg. G-quartet) attained by single stranded oligodeoxyguanosine that regulates binding to pattern recognition proteins. To examine the potential resistance of the single base oligodeoxynucleotides to DNase fragmentation and to (indirectly) confirm the complex structural conformations of dG20, equal amounts of each ODN were treated with DNase I and resolved by SDS-PAGE for effects on electrophoretic mobility (e.g. residual complex formation). In Fig. 4.8, lanes 1, 3, 5 and 7 contain ODNs that were not treated with DNase I compared to each treated ODN in: lane 2 (dG20), lane 4 (dC20), lane 6 (dA20) and lane 7 (dT20). A difference in the intensity of ODNs stained with ethidium bromide was noticed. This may be due to differential staining of different ODNs with ethidium bromide. SDS-PAGE and ethidium bromide staining demonstrated a difference in the staining profile of dG20 compared to both non-treated and DNase I treated ODNs. The DNA "smear" in lanes 1 and 2 represent relatively large molecular weight complexes of dG20 because of apparent increased resistance to DNase I treatment. These data suggested that soluble dG20 may form unique structural configurations and as such may bind to pattern recognition proteins on NCC.

Table 4.1. *Sequence of the oligodeoxynucleotides*

5'-Oligodeoxynucleotides-3'[a]	Name
CCCCCCCCCCCCCCCCCCCC	dC20
AAAAAAAAAAAAAAAAAAAA	dA20
TTTTTTTTTTTTTTTTTTTT	dT20
GGGGGGGGGGGGGGGGGGGG	dG20
TCGTCGTTGTCGTTGTCGTT	CpG

[a]Non-biotinylated and 5' end biotin and digoxigenin conjugated forms of ODNs were also used in the study.

Table 4.2. *ODN binding in various tissues of catfish containing NCC*

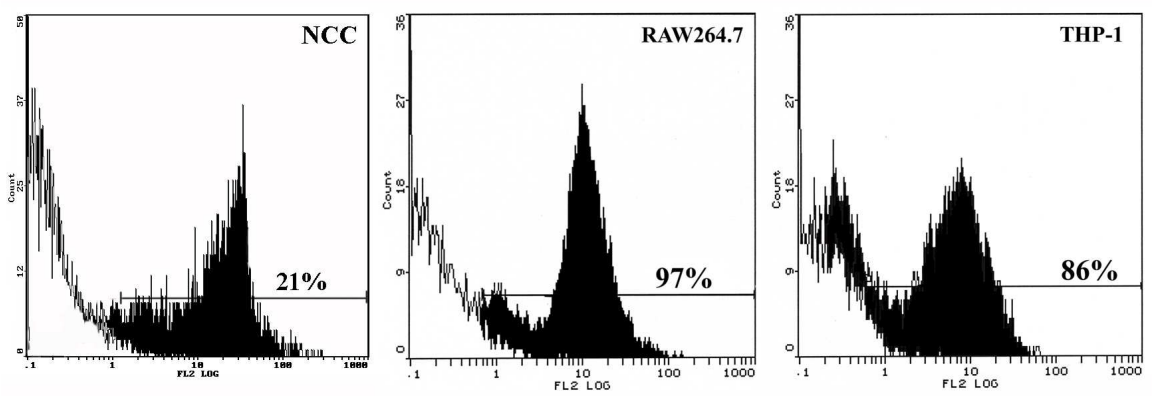
ODN[a]	**Anterior Kidney**[b]	**Spleen**[c]	**Liver**[c]
dG20	21[d]	18	67
dC20	14	7	45
dA20	2.5	2.5	2

[a]1 x 10^5 cells were incubated with PAB for 1 h on ice followed by incubation with saturating concentrations of biotinylated ODNs for 1 h and Extravidin-PE conjugates for 30 min and analyzed by flow cytometry. Percent binding is shown.
[b]Data shown is representative of three independent experiments.
[c]Data shown is representative of two independent experiments.
[d]The percentages shown were calculated by subtracting the control (background percent fluorescence) from the test binding. Background was usually less than 5%.

Table 4.3. *ODN binding on NCC, RAW264.7 and THP-1*

ODN[a]	**NCC**	**RAW264.7**	**THP-1**
dG20	21[b]	96	87
dC20	14	17	2
dA20	2	1	1

[a]1 x 10^5 cells were incubated with PAB for 1 h on ice followed by incubation with saturating concentrations of biotinylated ODNs for 1 h and Extravidin-PE conjugates for 30 min and analyzed by flow cytometry. Percent binding is shown. Data shown is representative of three independent experiments.
[b]The percentages shown were calculated by subtracting the control (background percent fluorescence) from the test binding. Background was usually less than 5%.


NCC
21%
RAW264.7
97%
THP-1
86%
Count
FL2 LOG

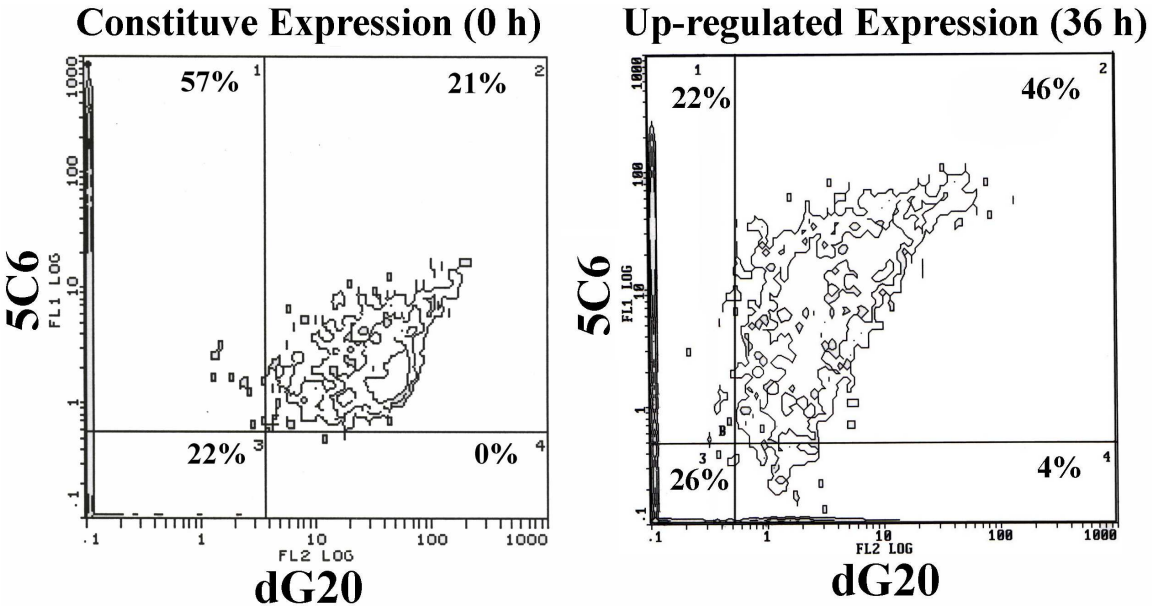

Constituve Expression (0 h)
57%
21%
22%
0%
5C6
FL1 LOG
FL2 LOG
dG20
Up-regulated Expression (36 h)
22%
46%
26%
4%
5C6
FL1 LOG
FL2 LOG
dG20

FIGURE 4.1. dG20 binds to NCC, RAW264.7 and THP-1. (A) Constitutive levels of dG20 binding. Purified NCC, RAW264.7 and THP-1 cells were incubated with PAB for 1 h/ 4°C followed by incubation with saturating amounts of dG20-biotin for 1 h on ice and Extravidin-PE conjugate for 30 min. Cells were washed twice and analyzed by flow cytometry. Histograms for conjugate alone (open) and cell specific (closed) dG20 binding are shown. (B) Purified NCC were treated with dG20 for 0 h and 36 h and up-regulation of dG20 binding determined by two-color analysis. Cells were washed twice with PAB and stained with mab 5C6 and dG20-biotin for 1 h followed by addition of FITC-anti-mouse-IgM and Extravidin-PE conjugates (30 min). Cells were analyzed by flow cytometry (two color analysis). Percent positive cells in each quadrant are shown.

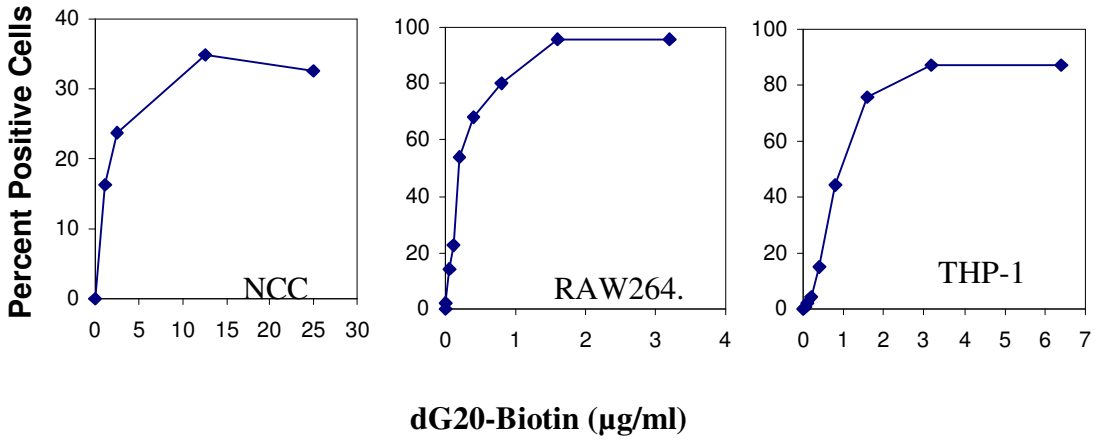


FIGURE 4.2. Saturation binding of dG20 to NCC, RAW264.7 and THP-1 cells. 1 x 105 purified NCC, and 5 x 104 RAW264.7 and THP-1 were incubated with PAB for 1 h/ 4°C followed by incubation with 100 ml of different concentrations of dG20-biotin (as indicated) for 1 h on ice. Cells were washed twice with PAB and further incubated with Extravidin-PE conjugate (30 min) and analyzed by flow cytometry. Percent binding is shown. Representative of three (NCC) or two (RAW264.7 and THP-1) independent experiments.

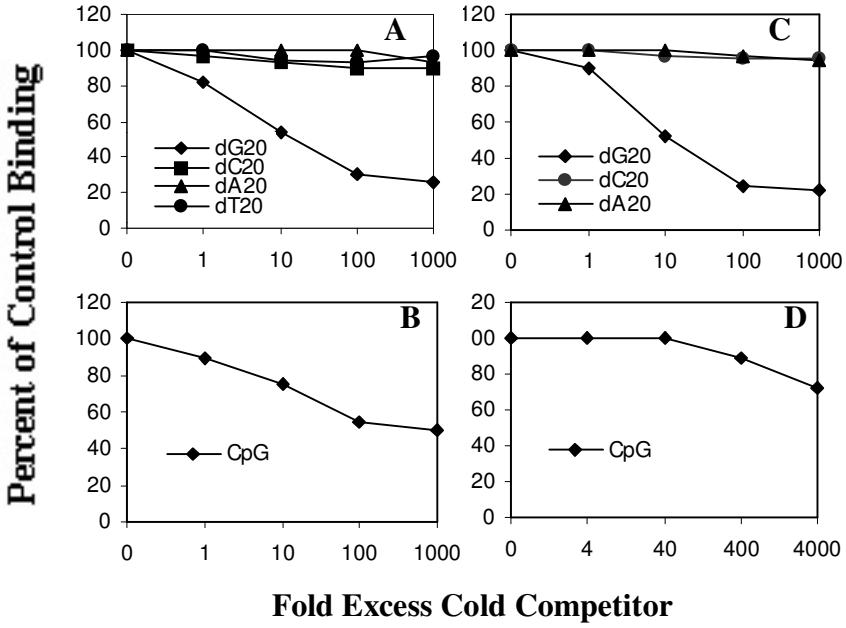
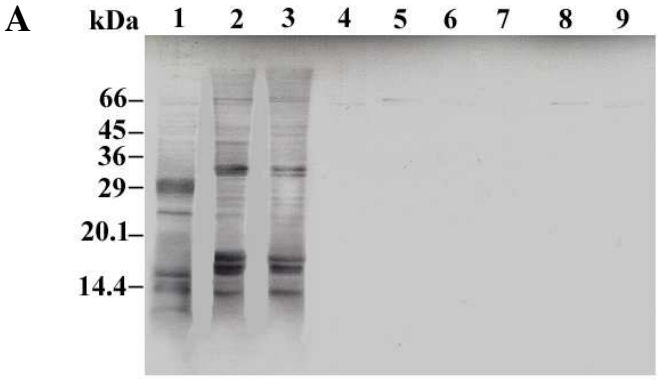
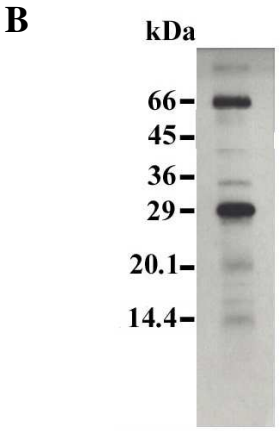


FIGURE 4.3. dG20 binding to NCC and RAW264.7 cells is competitively inhibited by cold homologous dG20 and CpG but not by dC20 or dA20. 2.5 x 10^4 NCC (A and B) or RAW264.7 (C and D) were incubated with unlabeled dA20, dC20, dG20, dT20 and CpG ODN (fold-excess of cold ODN as indicated) for 1 h on ice. Cells were washed to remove excess unbound ODNs and incubated with 50% saturating amounts of dG20-biotin for 1 h on ice. Cells were washed and further incubated for 30 min with Extravidin-PE conjugate and analyzed by flow cytometer. Data shown is representative of two independent experiments.


A
kDa
1
2
3
4
5
6
7
8
9
66
45
36
29
20.1
14.4


B
kDa
66
45
36
29
20.1
14.4

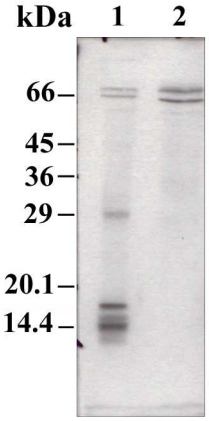

C
kDa
1
2
66
45
36
29
20.1
14.4

FIGURE 4.4. dG20 binds to low molecular weight proteins. (A) Membrane preparations of anterior kidney NCC (lanes 1, 4 and 7), THP-1 (lanes 2, 5 and 8) and RAW264.7 (lanes 3, 6 and 9) were resolved by reducing SDS-PAGE in 12.5% gel and transferred onto nitrocellulose. Ligand blotting was accomplished by adding dG20-biotin (100ng/ml) plus streptavidin-peroxidase conjugate to lanes 1, 2 and 3; dA20-biotin (100ng/ml) plus streptavidin-peroxidase conjugate to lanes 4, 5 and 6; ExtrAvidin-peroxidase conjugate alone to lanes 7, 8 and 9. (B) NCC lysates prepared from surface membrane biotinylated cells were subjected to ligand precipitation using DIG-dG20 and anti-digoxigenin agarose beads. Immunoprecipitates were resolved on 12.5% gel, transferred to nitrocellulose and probed with ExtrAvidin-peroxidase. (C) Anterior kidney NCC membrane preparations were resolved in 12.5% gel and transferred onto nitrocellulose and subjected to Western blotting with biotinylated (polyclonal) anti-histone H1 antibody (lane 1) followed by ExtrAvidin-peroxidase each for 1 h or ExtrAvidin-peroxidase alone (lane 2) for 1 h.

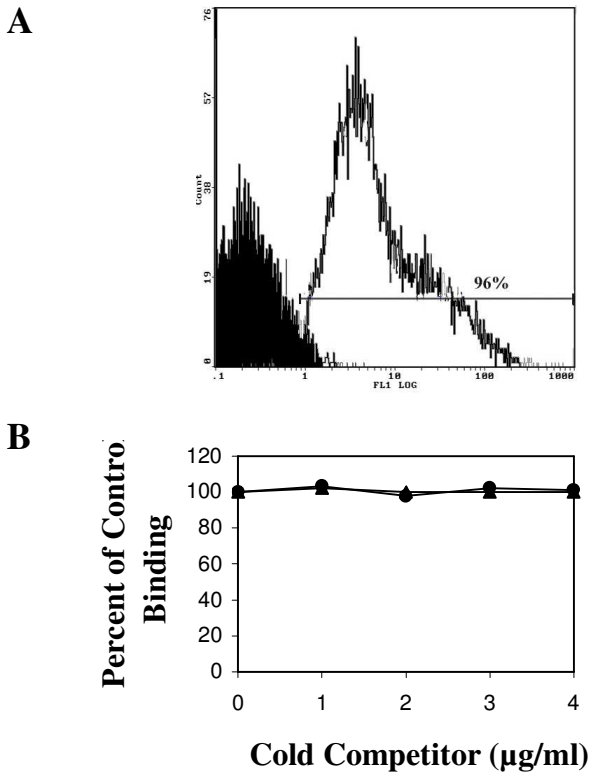


FIGURE 4.5. dG20 binding to RAW264.7 is not competitively inhibited by anti-Scavenger receptor (SR) antibody. (A) 2.5 x 10^4 cells were incubated with PAB for 1 h/ 4°C followed by incubation with 100 ml of 5 mg/ml of FITC conjugated anti-SR antibody or isotype control antibody for 1 h on ice. Cells were washed twice and analyzed by flow cytometer. Overlay histogram of anti-SR antibody (open) and isotype control antibody (close) is shown. (B) 2.5 x 10^4 cells were incubated with 100 ml of different concentrations (1 mg/ml, 2 mg/ml, 3 mg/ml and 4 mg/ml) of anti-SR antibody (•) or isotype control antibody (•) for 1h on ice. Cells were washed to remove excess antibody and further incubated with 50% saturating amounts of dG20-biotin for 1 h followed by Extravidin-PE for 30 min. Cells were washed and analyzed by flow cytometry.

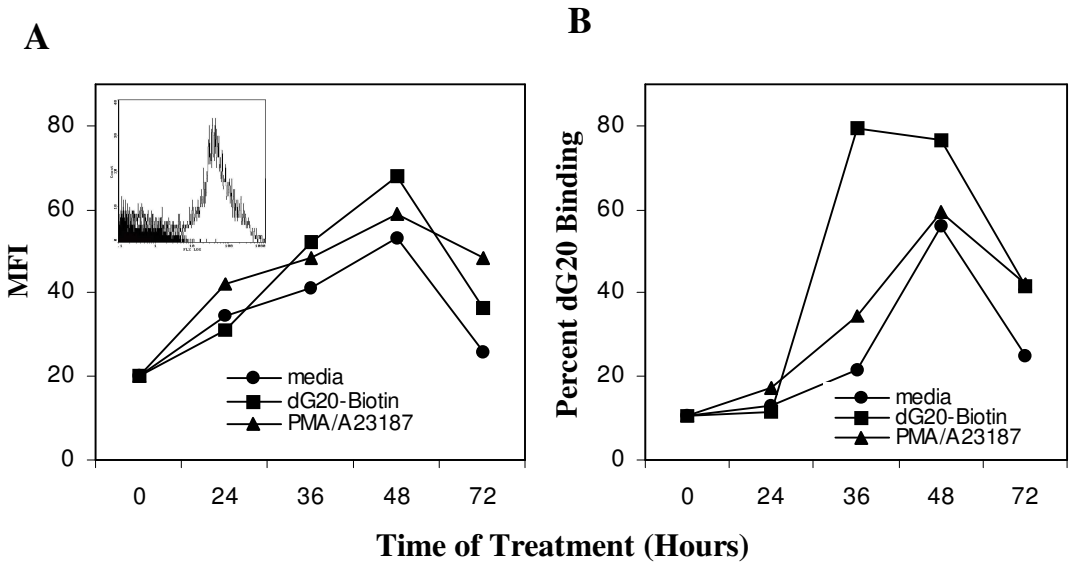


FIGURE 4.6. Binding of dG20 to NCC up-regulates the expression of the homologous receptor. Purified NCC (1x 106 cells/ml) were incubated with dG20 (50 mg/ml) (•), PMA/A23187 (0.5 mg/ml and 2.5 mg/ml respectively) (•) and media (•) for the indicated time periods. At each time point 1x 105 cells were harvested, washed with cold PAB and incubated with dG20-biotin for 1 h followed by the addition of Extravidin-PE conjugate for 30 min on ice. Cells were analyzed by flow cytometry. Mean fluorescence intensities (MFI) (A) and percent positive cells (B) at each time point are shown. Data are representative of three independent experiments. Insert: Histogram showing MFI of dG20 binding on NCC at 0 h (close) and after 36 h of dG20 treatment (open).

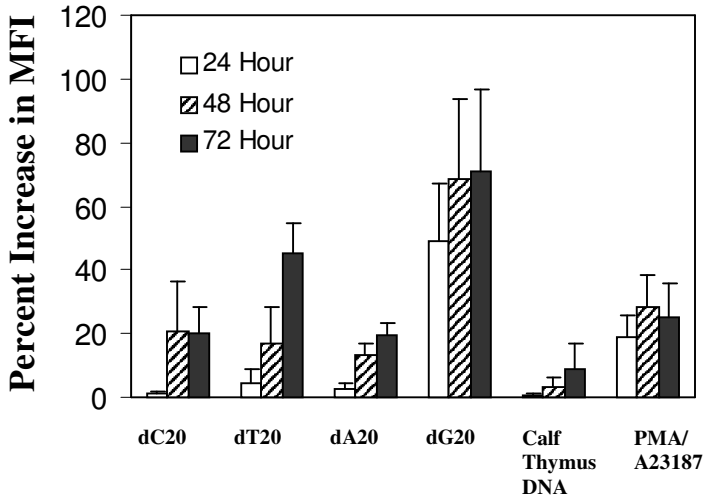


FIGURE 4.7. Synthetic oligodeoxynucleotides upregulate NCCRP-1 expression. Purified NCC (1x 10^6 cells/ml) were incubated with different sODNs (50 mg/ml), calf thymus DNA (5 mg/ml) and PMA/A23187 (0.5 mg/ml and 2.5 mg/ml respectively) for different time periods. At each time point, 1 x 10^5 cells were harvested, washed twice with PAB and stained with saturating concentrations of mab 5C6 (anti-NCCRP-1). Analysis was done by flow cytometry. Percent increase in MFI was compared with non-treated controls. The mean ± standard deviation of three independent experiments is shown.

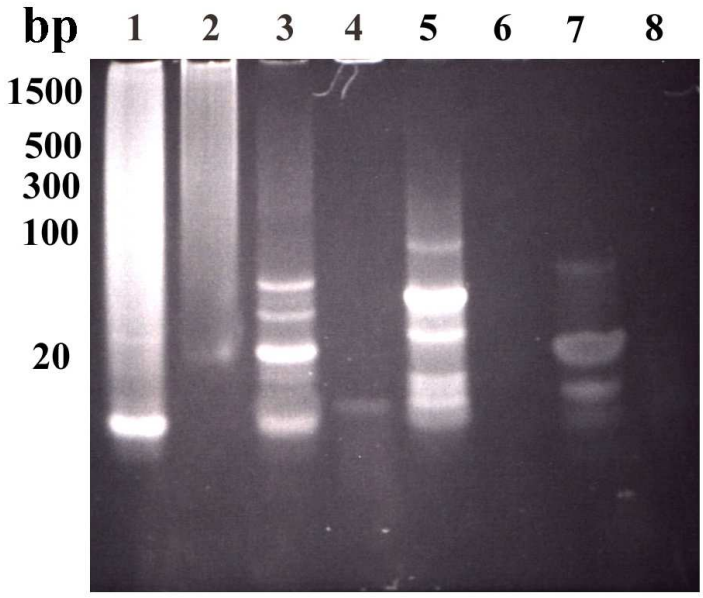


FIGURE 4.8. ODN dG20 forms complexes. Equal amounts of ODNs dG20 (lane 1 and 2), dC20 (lanes 3 and 4), dA20 (lanes 5 and 6) and dT20 (lanes 7 and 8) either non-treated (lanes 1,3,5 and 7) or treated (lanes 2,4,6 and 8) with DNase I were resolved in a 15% denaturing polyacrylamide gel at 180 v for 90 min. Gel was stained with ethidium bromide (0.5 mg/ml) for 30 min at room temperature. Staining patterns were resolved using UV trans-illuminator.

Discussion

We previously demonstrated that ODNs composed of dG20 bound to the teleost NK cell equivalent referred to as nonspecific cytotoxic cells (NCC) (10). Although recognition of this particular ODN was unique for teleost cells, CpG palindromes containing flanking sequences or "runs" of 12 guanosine nucleotides (i.e. dG12) have been shown by others to bind and activate mouse NK cells (11) and tetramers of stabilized polyguanosine quartets bind to scavenger receptors (SR-A type-I). We have previously shown (15) that NCC express SR-A (types I-II) and that one ligand for this receptor was dG20. Competition studies using anti-SR-A antibody and labeled dG20 however demonstrated that SR-A bound less than 50% of the total binding capacity of NCC for dG20. These data suggested that a heterogeneous group of DNA binding proteins (DBP) (i.e. pathogen recognition receptors) are expressed on NCC. In the present study we report that dG20 binds to at least three low molecular weight proteins on (teleost) NCC. These DBP are also expressed by mammalian THP-1, RAW264.7 and YT-Indy cells (data not shown).

Immunoregulation of cellular functions by single base ODNs as well as CpG dinucleotide containing ODNs have been reported (17-18). The chemistry/conformation of single base phosphodiester (Po) oligodeoxyguanosine exhibited unique qualities not characteristic of any other single base composition or structure. Among the Po single base ODNs, only dG30 was mitogenic for B-cells. All phosphorothioate (Ps) single base ODNs tested also had this activity (18). Interestingly, negative immunoregulatory activities have also been reported for single base ODNs as well as CpG ODNs. Pretreatment of J774 cells with Po dG30 inhibited E. coli DNA, LPS and CpG activation

of IL-12 production (18). All other Po single base ODNs (i.e. dA, dT, dC) tested did not produce this effect. Similar experiments (19) with the same target cell but measuring the effects of pre-treatment of J774 cells with Po 30-mers of dA, dC, dG or dT demonstrated that these ODNs had little to no effects on CpG, *E. coli* or LPS stimulation of nitric oxide production. In this same study however, the Ps derivitized single base ODNs completely inhibited nitrite production in a dose and time dependent fashion.

These findings, in addition to other research by this group (20) have clearly demonstrated that single base ODNs may have different effects based on whether they are derivitized (e.g. Ps or Po) and on the target cell type involved in the immunoregulatory response. The heterogeneity of effects elicited by ODN treatment was also suggested in a study (21) demonstrating that pretreatment of RAW 264.7 cells with Ps CpG produced hyporesponsiveness to subsequent CpG treatment effects. This type of preexposure significantly reduced cytokine secretion and cytokine mRNA by these cells. Thus both single base dG as well as Ps derivitized CpG elicite significant immunoregulatory effects on a diverse spectrum of mammalian cells.

Data in the present study suggested that the binding of single base dG20 to NCC, RAW264.7 and THP-1 cells is receptor mediated and (at least for RAW264.7) not dependent on SR binding (Fig. 4.5). The data for the first time suggested that single base ODN dG20 binds to a unique new family of low molecular weight (mw) proteins. The mw of the DBP also indicated that consideration of Toll or mannose receptor involvement was not feasible. Previous research by others (9, 22) has demonstrated that sODNs may bind to multiple different receptors on APC and that one species of these receptors is the TLR-9. However, there has not been any evidence for crossreactivity of

single base oligodeoxyguanosines with TLR-9 or SR (except for polyguanosine binding to SR). The only other ligand with binding specificity for both TLR and SR is LPS (23). Preliminary unpublished sequence data of the 18 kDa protein from NCC demonstrated that it is not a SR or TLR. Additionally, the mw of these receptors (220 kDa and 120 kDa respectively) are clearly different from the dG20 binding proteins reported in the present study. We are currently studying the physiological relevance of these low mw dG20 binding proteins and this work will be submitted as a separate manuscript.

Other techniques to study the effects of multiple nucleic acid containing sequences on immune function have been published. Conjugation of dG Runs to CpG (i.e. dG6) produced an ODN (12) that bound to mouse APC and stimulated the release of various cytokines. In that study, an ODN composed of dG20 competed with the binding of the dG6 to splenic dendritic cells. In another study (24) conjugation of dG Runs to 3' and 5' ends of CpG ODN enhanced NK cell lytic activity. Costimulatory effects of guanosine-rich (G-rich) ODNs were shown in T-cells where costimulation by G-rich ODNs resulted in IL-2-driven proliferation and induced cytolytic T cell function (13). Evidence for a more direct influence of G-rich sODNs [-TTGGAGGGGGTGGTGGGG-] (25) on proliferation responses was shown in a study demonstrating the expansion of macrophage-like cells from mouse bone marrow cells.

The ability of dG20 to bind and induce activation of cells can be attributed to the unique conformations (eg. G-tetrad) attained by single stranded oligodeoxyguanosine. The presence of 3 or more contiguous guanines in a single base oligodeoxynucleotide enables the possibility of intrachain binding stabilized by Hoogsteen base pairing (i.e. parallel-strand alignment). This type of base pairing indeed changes the conformation of

dG20. In fact, interchain base pairing can also occur from the stacking of G-tetrads to form G-quadruplexes. The intrachain and/or interchain base-paring may result in the formation of patterns that may be recognized by membrane proteins (or PRRs). The occurrence of G-tetrad or G-quadruplexes binding proteins adds further weight to their role as important signaling mediators. The combination of phosphodiester bonding with complex intra- and interchain configurations (may) impose important immunologic/adjuvant properties on the single base dG20. These may include: DNase resistance, *in vivo* retention without deleterious immunological consequences (i.e. induction of autoimmunity) and specific binding to cells from many different vertebrate species.

Finally, in the present study we hypothesized the presence of multiple dG20 binding proteins that may be distantly related to histone-like proteins (manuscript in preparation) and as such represent a new class of pattern recognition receptors. Although the traditional cellular location of histone proteins is associated with conformational stabilization of chromatin, studies performed in higher vertebrates have shown that these proteins may also be expressed on the membranes of a diversity of cell types. Human monocytes express membrane histones H2a and H2b (26-28); a human transformed B-cell line (Raji) expresses 14-18 kDa and 33-34 kDa histone-like membrane proteins (29); and T-cells express a 17 kDa membrane H2b (30-31) and a 29 kDa H3 (31-32). The mechanism of transport of these histones to the cell membrane is not understood, as these proteins do not contain traditional transmembrane domains. Additional examples of the relatively widespread expression of membrane histones and evidence that these proteins may be involved in innate antibacterial resistance are studies showing that neurons (33)

and macrophages (34) express 30-33 kDa histone (H1) proteins that bind LPS and thyroglobulin (respectively). These studies (26-32) have clearly demonstrated that histone-like proteins (e.g. histone-1 and core histones) are expressed by cells of immune system. The present study confirms these data (Fig. 4.4B) and suggests that these proteins may comprise a new type of pattern recognition receptor.

An additional question relates to the immunological/biological relevance of finding single base dG20 binding proteins on cells because such G-rich DNA structures occur in very low percentages in the genome of bacteria. Single-stranded and double-stranded G-rich strands, however, are found in much higher frequency in the telomeric regions of chromosomes (35) of all vertebrates, slime moulds, fungi and protozoa. Interestingly, DNA released from dying eukaryotic cells is shown to induce phenotypic and functional maturation of APC (36). A possibility exists that these G-rich strands expressed at the ends (telomeres) of linear chromosomes, released from the dying cells, can induce inhibitory or activatory signals in other cells of immune system. Previously, it was shown that activation of dendritic cells by necrotic/dying cells used the same signaling (TLR) pathway that was used by microbial agents like LPS and bacterial DNA (37). This suggested that G-rich eukaryotic DNA may cross-react with CpG binding receptors. The relevance of prokaryotic DNA binding to the same membrane proteins as dG20 can also be supported by our research. We (10) and others (19,20) have shown that single base ODNs competitively inhibit the CpG binding to cells. CpG/GpC dinucleotides and dG20 bind to the same proteins (10) on NCC and biotinylated CpG/GpC also bind to the same molecular weight proteins as dG20 determined by Southwestern blot analysis (unpublished data). These same proteins also bind bacterial DNA. The crossreactive

dG20 and CpG DNA binding proteins now comprise a third class (in addition to Scavenger receptors Types I and II and TLR-9) of pattern recognition receptors that recognize ODNs (3, 23, 25, 38-40). These studies place the single base dG20 binding as crossreactive and promiscuous. Binding appears to be conformation/configuration dependent, but also one that is characteristic of ligands that bind to pattern recognition receptors.

References

1. Krieg, A. M. 2000. The role of CpG motifs in innate immunity. *Curr. Opin. Immunol. 12:35.*

2. Krieg, A. M. 1999. Mechanisms and applications of immune stimulatory CpG oligodeoxynucleotides. *Biochim. Biophys. Acta. 1489:107.*

3. Pisetsky, D. S. 1996. Immune activation by bacterial DNA: a new genetic code. *Immunity 5:303.*

4. Krieg, A. M. 2000. Immune effects and mechanisms of action of CpG motifs. *Vaccine 19:618.*

5. Scheule, R. K. 2000. The role of CpG motifs in immunostimulation and gene therapy. *Adv. Drug Deliv. Rev. 44:119.*

6. Weiner, G. J. 2000. The immunobiology and clinical potential of immunostimulatory CpG oligodeoxynucleotides. *J. Leukoc. Biol. 68:455.*

7. Bendelac, A., and D. T. Fearon. 2000. Receptors and effectors of innate immunity. *Curr. Opin. Immunol. 12:11.*

8. Lipford, G. B., K. Heeg, and H. Wagner. 1998. Bacterial DNA as immune cell activator. *Trends Microbiol. 6:496.*

9. Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira. 2000A. Toll-like receptor recognizes bacterial DNA. *Nature 408:740*.

10. Oumouna, M., L. Jaso-Friedmann, and D. L. Evans. 2002. Activation of nonspecific cytotoxic cells (NCC) with synthetic oligodeoxynucleotides and bacterial genomic DNA: binding, specificity and identification of unique immunostimulatory motifs. *Dev. Comp. Immunol. 26:257*.

11. Kimura, Y., K. Sonehara, E. Kuramoto, T. Makino, S. Yamamoto, T. Yamamoto, T. Kataoka, and T. Tokunaga. 1994. Binding of oligoguanylate to scavenger receptors is required for oligonucleotides to augment NK cell activity and induce IFN. *J. Biochem. 116:991*.

12. Lee, S. W., M. K. Song, K. H. Baek, Y. Park, J. K. Kim, C. H. Lee, H-K. Cheong, C. Cheong, and Y. C. Sung. 2000. Effects of a hexameric deoxyriboguanosine run conjugation into CpG oligodeoxynucleotides on their immunostimulatory potentials. *J. Immunol. 165:3631*.

13. Lipford, G. B., S. Bendigs, K. Heeg, and H. Wagner. 2000. Poly-guanosine motifs costimulate antigen-reactive CD8 T cells while bacterial CpG-DNA affect T-cell activation via antigen-presenting cell-derived cytokines. *Immunology 101:46*.

14. Biessen, E. A., H. Vietsch, J. Kuiper, M. K. Bijsterbosch, and T. J. Berkel. 1998. Liver uptake of phosphodiester oligodeoxynucleotides is mediated by scavenger receptors. *Mol. Pharmacol. 53:262*.

15. Kaur, H., L. Jaso-Friedmann, and D. L. Evans, 2003. Identification of a Scavenger receptor homologue on Nonspecific cytotoxic cells and evidence for binding to oligodeoxyguanosine, Fish Shellfish Immunol. (in press).

16. Evans, D. L., E. E. Smith, L. Jaso-Friedmann, A. St. John, H. S. Koren, and D. T. Harris. 1988. Identification of a putative antigen receptor on fish nonspecific cytotoxic cells with monoclonal antibodies. *J. Immunol. 141:324*.

17. Pisetsky, D. S., and C. F. Reich III. 1999. Influence of backbone chemistry on immune activation by synthetic oligonucleotides. *Biochem. Pharmacol. 58:1981*.

18. Pisetsky, D. S., and C. Reich. 1993. Stimulation of in vitro proliferation of murine lymphocytes by synthetic oligodeoxynucleotides. *Mol. Biol. Rep. 18:217*.

19. Zhu, F. G., C. F. Reich, and D. S. Pisetsky. 2002. Inhibition of murine macrophage nitric oxide production by synthetic oligonucleotides. *J. Leukoc. Biol. 71:686*.

20. Zhu, F. G., C. F. Reich, and D. S. Pisetsky. 2002. Inhibition of murine dendritic cell activation by synthetic phosphorothioate oligodeoxynucleotides. *J. Leukoc. Biol. 72:1154*.

21. Yeo, S. J., G. Yoon, S. C. Hong, and A. K. Yi. 2003. CpG DNA induces self and cross-hyporesponsiveness of RAW264.7 cells in response to CpG DNA and lipopolysaccharide: alterations in IL-1 receptor-associated kinase expression. *J. Immunol. 170:1052*.

22. Modlin, R. L. 2000. A Toll for DNA vaccines. *Nature 408:659*.

23. Pearson, A. M., A. Rich, and M. Krieger. 1993. Polynucleotide binding to macrophage scavenger receptors depends on the formation of base-quartet-stabilized four-stranded helices. *J. Biol. Chem. 268:3546*.

24. Ballas, Z. K., W. L. Rasmussen, and A. M. Krieg. 1996. Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. *J. Immunol. 157:1840.*

25. Lang, R., L. Hultner, G. B. Lipford, H. Wagner, and K. Heeg. 1999. Guanosine-rich oligodeoxynucleotides induce proliferation of macrophage progenitors in cultures of murine bone marrow cells. *Eur. J. Immunol. 29:3496*.

26. Bennett, R. M., G. T. Gabor, and M. M. Merritt. 1985. DNA binding to human leukocytes. Evidence for a receptor-mediated association, internalization and degradation of DNA. *J. Clin. Invest. 76:2182*.

27. Holers, V. M., and B. L. Kotzin. 1985. Human peripheral blood monocytes display surface antigens recognized by monoclonal antinuclear antibodies. *J. Clin. Invest. 76:991*.

28. Emlen, W., V. M. Holers, W. P. Arend, and B. Kotzin. 1992. Regulation of nuclear antigen expression on the cell surface of human monocytes. *J. Immunol. 148:3042*.

29. Kubota, T., Y. Kanai, and N. Miyasaka. 1990. Interpretation of the cross-reactivity of anti-DNA antibodies with cell surface proteins: the role of cell surface histones. *Immunol. Lett. 23:187*.

30. Ojcius, D. M., S. Muller, C. S. Hasselkus-Light, J. D. Young, and S. Jiang. 1991. Plasma membrane-associated proteins with the ability to partially inhibit perforin-mediated lysis. *Immunol. Lett. 28:101*.

31. Watson, K., R. J. Edwards, D. C. Parmelee, S. Shaunak, N. J. Gooderham, and D. S. Davies. 1994. Histones located on the plasma membrane of T-cells. *Biochem. Soc. Trans. 22:199S*.

32. Watson, K., R. J. Edwards, S. Shaunad, D. C. Parmelee, C. Sarraf, N. J. Gooderham, and D. S. Davies. 1995. Extra-nuclear location of histones in activated human peripheral blood lymphocytes and cultured T-cells. *Biochem. Pharmacol. 50:299*.

33. Bolton, S. J., and V. H. Perry. 1997. Histone H1; a neuronal protein that binds bacterial lipopolysaccharide. *J. Neurocytol. 26:823*.

34. Brix, K., W. Summa, F. Lottspeich, and V. Herzog. 1998. Extracellularly occurring histone H1 mediates the binding of thyroglobulin to the cell surface of mouse macrophages. *J. Clin. Invest. 102:283*.

35. Zakian, V. A. 1995. Telomeres: beginning to understand the end. *Science 270:1601*.

36. Ishii, K. J., K. Suzuki, C. Coban, F. Takeshita, Y. Itoh, H. Matoba, L. D. Kohn, and D. M. Klinman. 2001. Genomic DNA released by dying cells induces the maturation of APCs.

J. Immunol. 167:2602.

37. Li, M., D. F. Carpio, Y. Zheng, P. Bruzzo, V. Singh, F. Ouaaz, R. M. Medzhitov, and A. A. Beg. 2001. An essential role of the NF-kappa B/Toll-like receptor pathway in induction of inflammatory and tissue-repair gene expression by necrotic cells. *J. Immunol. 166:7128*.

38. Bishop, J. S., J. K. Guy-Caffey, J. W. Ojawang, S. R. Smith, M. E. Hogan, P. A. Cossum, R. F. Rando, and N. Chaudhary. 1996. Intramolecular G-quartet motifs confer nuclease resistance to a potent anti-HIV oligonucleotide. *J. Biol. Chem. 271:5698*.

39. Pearson, A .M. 1996. Scavenger receptors in innate immunity. *Curr. Opin. Immunol. 8:20*.

40. Benimetskaya, L., M. Berton, A. Kolbanovsky, S. Benimetsky, and C.A. Stein. 1997. Formation of a G-tetrad and higher order structures correlates with biological activity of the RelA (NF-kappaB p65) 'antisense' oligodeoxynucleotide. *Nucleic Acids Res. 25:2648*.

CHAPTER 5

SINGLE BASE OLIGODEOXYGUANOSINE UPREGULATES FAS LIGAND RELEASE BY NONSPECIFIC CYTOTOXIC CELLS[1]

[1]Kaur, H., L. Jaso-Friedmann and D. L. Evans. Submitted to*Developmental and Comparative Immunology*, 6/20/2003.

ABSTRACT

Nonspecific cytotoxic cells (NCC) are the teleost equivalent of mammalian NK cells. In the present study a novel stimulus secretion model is described for catfish NCC utilizing single base oligodeoxyguanosine. Binding of guanosine 20-mers (dG20) to NCC activated increased expression of cytosolic FasL detected by an anti-human FasL monoclonal. *In vitro* treatment of purified NCC with dG20 produced a 7-fold increase in expression of soluble FasL (sFasL) after 4h. Antibody binding to NCC was saturable and approximately 30-35% of total NCC were positive for sFasL expression. The teleost FasL equivalent produced programmed cell death of appropriate FasR positive targets. Supernatants from dG20 activated NCC produced hypoploidy and annexin-V binding by FasR bearing HL-60 cells. Treatment of activated supernatants with immobilized anti-FasL monoclonal neutralized these activities. These studies demonstrated that an NK like cell (NCC) produces and secretes sFasL following binding by single base oligodeoxyguanosine.

Key Words. Nonspecific cytotoxic cells, single base oligos, oligodeoxyguanosine, soluble Fas ligand, Fas receptor, programmed cell death, HL-60, Annexin-V, DNA hypoploidy.

INTRODUCTION

Binding of membrane FasL to its receptor (Fas) plays an important role in homeostasis and self-tolerance in humans and mice [1]. Membrane FasL (mFasL) is an upregulated cytotoxic effector molecule of CTL and NK cells [2-3] however mFasL is constitutively expressed in select organs (testis and eyes) where it has been implicated in the property of immune privilege [4-5]. The Fas/FasL system also participates in the resolution of inflammation by phagocytosis where monocytes/macrophages can trigger apoptosis in other cells by regulated surface expression of FasL and by release of soluble FasL [6-7]. Although most studies describe FasL as a type II membrane protein [8-11], cleavage of this form by different metalloproteases releases an active soluble form. However, enzymatic generation of soluble FasL (sFasL) produces a somewhat different protein from the constitutive intracellular form which is stored in granules or microvesicles [10-13].

Constitutive expression of sFasL has been reported in the human T-cell line Jurkat [14-15], in neutrophils [16] and in the serum of patients with NK cell lymphomas [13,17]. The release of sFasL into the serum of patients with malaria [18] and atopic dermatitis [19] indicated the potential important role of this apoptotic factor in immunoregulation of many different types of responses. We have previously reported [20-25] that NCC from certain teleosts are Fas receptor (FasR) negative but constitutively express sFasL. In these studies, FasL produced activation induced programmed cell death of FasR bearing mammalian tumor cells. In the present study we demonstrate a stimulus secretion model where binding of single base oligodeoxyguanosine to NCC signals the release of sFasL.

MATERIAL AND METHODS

Animals. *Ictalurus punctatus* (catfish) of both sexes, obtained from local commercial farms, were maintained in flow-through 300 gallon aquaria at ambient temperature (17-25^{o}C) and fed commercial fish pellets (Purina Catfish Startena, Ralston Purina Co., St. Louis, MO). Water quality was monitored for temperature, nitrite, ammonia nitrogen and chlorine contents.

Cells, media and reagents. NCC were purified from anterior kidney (AK) of catfish. Human HL60 promyelocytic leukemia cells (ATCC CCL 240) (FasR positive) and K562 erythroleukemic cells (ATCC CCL 243) (FasR negative) were used as target cells for apoptosis studies. RPMI-1640 (Cellgro, Media Tech, Washington, DC) supplemented with L-glutamine, sodium pyruvate, MEM vitamin solution, MEM amino acid solution, MEM non-essential solution (Cellgro), 50 mg/ml gentamicin (Schering-Plough Animal health Corp., Kenilworth, NJ) and 10% fetal bovine serum (FBS, Atlanta biologicals, Norcross, GA) was used for cell culture. Washing media (PAB) consisted of 1X phosphate buffer saline (PBS), 0.1% sodium azide (Sigma Chemical Co., St. Louis, MO) and 1% bovine serum albumin (Sigma Chemical Co.). Calf thymus (CT) DNA, Phorbol 12-Myristate 13-Acetate (PMA, # P8139), calcium ionophore-A23187 (# C7522), Extravidin-phycoerythhrin (PE) conjugate (# E4011), Propidium Iodide (PI) (# P4170) were obtained from Sigma Chemical Co. Annexin-V-FITC (# 556419) was purchased from Pharmingen, Torreyana, CA. Antibodies used were: mouse anti-human FasL antibody (116-277 aa) (# F37720, Transduction Lab, Lexington, KY), anti-mouse IgG-FITC (# F-8264, Sigma Chemical Co.), mouse anti-NCCRP-1 antibody (5C6) (IgM isotype) specific for a 32-kDa receptor protein NCCRP-1 (prepared in house [26]),

biotinylated anti-mouse IgM (# B9265, Sigma Chemical Co.). Oligodeoxynucleotides (ODNs) used were: phosphodiester forms of 20-mers of single base guanosine (dG20) and cytosine (dC20) (MWG-Biotech, High Point, NC). Bacterial DNA was prepared from *Streptococcus iniae* (DAN14). ODNs and bacterial DNA were resuspended in endotoxin-free water (# 210-7, Sigma).

Purification and permeabilization of NCC. Anterior kidney (AK) tissue (mammalian bone marrow equivalent) was removed aseptically from anesthetized (3-aminoenzoic acid ethyl ester; Sigma Chemical Co.) catfish and passed through screen mesh to obtain single cell suspensions in complete RPMI-1640 containing 10% FBS. Cells were purified by density gradient centrifugation over a 45.5 % Percoll cushion. Cells at the interface were collected, washed twice with RPMI and resuspended in complete RPMI. Cell preparation higher than 80% 5C6 positive were used for the experiments. For permeabilization, cells were resuspended in ice-cold acetone for 20 min on ice. Cells were washed twice with PBS prior to staining with antibodies.

FasL kinetics and binding assays. Permeabilized cells were used for all binding assays. For saturation binding assay, 5 x 10^4 cells were incubated with different concentrations of mouse anti-human FasL antibody or isotype control antibody for 1 h, cells were washed and incubated with anti-mouse IgG-FITC for 30 min. Cells were washed and analyzed by flow cytometry. For FasL kinetics experiment, 1x 10^6 cells (NCC)/ml were treated with media alone, dG20 (50 µg/ml) and PMA+A23187 (0.05 µg/ml /0.25 µg/ml) for different time periods. 5 x 10^4 cells were analyzed for anti-FasL antibody binding at each of these time points. 5x 10^4 cells were incubated with saturating concentrations of anti- FasL antibody or isotype control antibody (where indicated) for 1

h, followed by washing and incubation with anti-mouse IgG-FITC for 30 min. For two-color flow cytometry, 1 x 10^5 cells were incubated with anti-FasL antibody and 5C6 for 1 h, followed by washing and incubation with biotinylated anti-mouse IgM for 30min. Cells were washed and incubated with anti-mouse IgG-FITC and Extravidin- PE for 30 min, washed and analyzed. All the incubations were done on ice. Nonspecific uptake of conjugate by permeabilized cells was determined by conjugate controls and statistics adjusted accordingly.

Supernatant generation from NCC and treatment of target cells with supernatants. 1x 10^6 cells/ml were incubated with media alone, ODNs (50 µg/ml), bacterial DNA (5 µg/ml), CT DNA (5 µg/ml) and PMA+A23187 (0.05 µg/ml /0.25 µg/ml) for 24 h at room temperature. For FasL neutralization experiments, supernatants were preincubated with anti-FasL antibody or isotype control antibody for 2 h/4°C followed by incubation with protein G-beads (# 20398, Pierce, Rockford, IL) for 2 h/4°C. Supernatants were collected after 24 h and stored at –20°C till further use.

Apoptosis detection in target cells. 5 x 10^5 target cells (HL60 or K562) were incubated with 500 µl of the supernatants obtained by treatment of NCC. Apoptosis was detected in target cells by Annexin-V binding (early 6 h) and DNA hypoploidy (late 24 h). For Annexin-V binding 5x10^4 cells were washed, resuspended in 100 µl of Annexin-V binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$, all reagents from Sigma) and incubated with 2.5 µl of Annexin-V-FITC and 5 µg/ml of PI for 15 min in dark and analyzed by flow cytometry. For DNA hypoploidy 5 x 10^4 cells were washed and treated with 400 µl of nuclear isolation media (NIM: 0.005% PI, 0.1% Triton X-100, and 0.1% RNase in PBS, all reagents from Sigma) for 5 min on ice in dark.

DNA hypoploidy was given as DNA fragmentation index (DFI). DFI is the ratio of sub G0/G1 to G0/G1 peak.

RESULTS

NCC express soluble Fas ligand. We previously reported that NCC were surface membrane negative for FasR and FasL [25]. In order to investigate the potential role of these apoptotic regulating proteins in NCC effector function, experiments were done to determine whether these cells constitutively expressed soluble intracellular FasL. NCC were purified, permeabilized with ice-cold acetone and stained with mouse anti-human FasL antibody or isotype control. Figure 5.1A demonstrates by two color flow analysis that soluble FasL is coexpressed with NCCRP-1 by NCC. NCCRP-1 has previously been identified as a membrane signaling protein on NCC. Figure 5.1B demonstrates saturable binding to approximately 30-35% of total NCC.

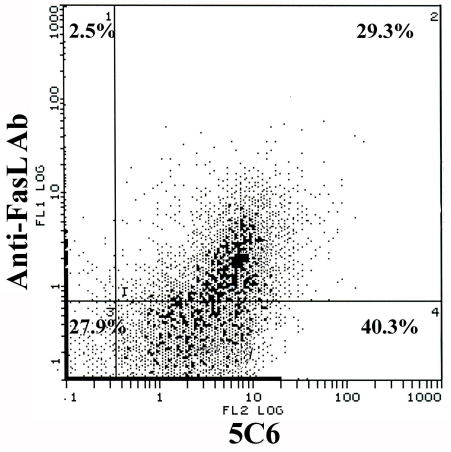
Although constitutive expression of FasL on 30-35% NCC was shown, it was of interest to study the possible up-regulation of expression following NCC activation with dG20. The kinetics of intracellular expression and the relative concentrations of sFasL in NCC following activation with dG20 was therefore examined. NCC were treated *in vitro* for different time periods and intracellular expression of FasL detected by flow cytometry. Figure 5.2 demonstrates a rapid and large increase in expression (i.e. mean fluorescence intensity/cell) of cytosolic FasL with maximum levels following 3 h treatment. However, the number of cells expressing FasL did not increase (data not shown).

FasL is secreted by dG20 activated NCC. To determine whether binding to NCC by single base oligodeoxynucleotides induced the secretion of sFasL, cells were treated *in vitro* for 24 h with dG20, (control) dC20, bacterial DNA, CT DNA and PMA+A23187 and tissue culture supernatants were examined for the presence of apoptotic inducing factors. FasR positive HL-60 cells and FasR negative K562 cells were utilized to detect the induction of apoptosis in the presence of these activated supernatants. Figure 5.3A shows that supernatants from dG20 treated NCC initiated the exterior membrane leaflet inversion of phosphotidylserine on HL-60 targets determined by annexin-V binding. Maximum annexin-V binding occurred on HL-60 cells following 6 h treatment with dG20 activated supernatants. Annexin-V binding was not detected on K562 cells following treatment with the same ODN activated supernatants. Activated supernatants were next examined for ability to induce DNA hypoploidy in FasR positive HL-60 and FasR negative K562 cells. Cells were treated for 24 h with activated supernatants and extent of DNA fragmentation was determined by cell cycle analysis (e.g. DNA hypoploidy). DNA hypoploidy was determined by measuring the concentration of 2N DNA in the presence of a concomitant increase in sub-G_0G_1 peak of treated HL-60 cells (Figure 5.3B). DNA reduction was expressed by a DNA fragmentation index.

Neutralization of FasL activity. To determine a direct relationship between the release of soluble FasL from NCC and the production of apoptotic DNA fragmentation (e.g. DNA hypoploidy) an anti-FasL antibody was used to neutralize/remove FasL from "activated" supernatants. Tissue culture supernatants were obtained from NCC previously treated *in vitro* with dG20, dC20, PMA-A23187, bacterial DNA or CT DNA. These supernatants were then mixed with protein-G immobilized anti-FasL or isotype

control monoclonal antibodies (mab). These "neutralized" supernatants were next added to FasR positive HL-60 cells for fragmentation of DNA characteristic of apoptosis. Figure 5.4 demonstrates that PMA, bacterial DNA and dG20 treatment of NCC produced supernatants that initiated large increases in DNA hypoploidy of HL-60 cells. Pretreatment of these supernatants with immobilized anti-FasL antibody neutralized this activity. Isotype control mab had no significant effect on the removal of apoptotic activity.

A. Two-color analysis for FasL binding to NCC



B.

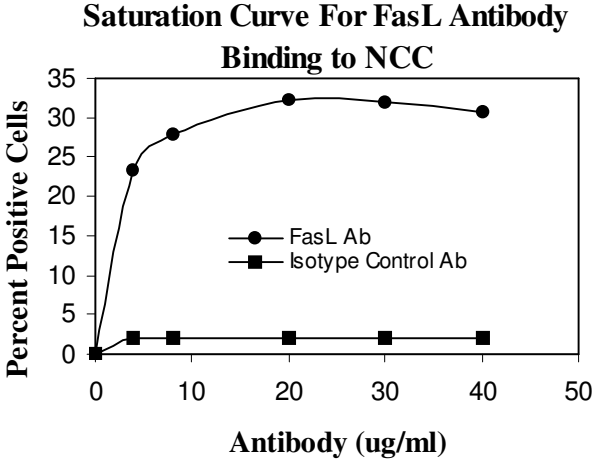


Figure 5.1. Soluble FasL is expressed by NCC. A) Permeabilized NCC (1 x 10^5) were incubated with anti-FasL antibody and mab 5C6 (1 h/ice), washed twice and incubated with biotinylated mouse anti-IgM (30 min/ice). Cells were washed and incubated with anti-mouse IgG-FITC (conjugate for anti-FasL) and Extravidin- PE (conjugate for mab 5C6) for 30 min/ice. Cells were analyzed by two-color flow cytometry. Isotype control values (<4% for FasL and <10% for 5C6 antibodies) have been subtracted. B) Purified NCC were treated with ice-cold acetone (20 min on ice), and washed twice with PBS. 5 x 10^4 cells were incubated with different concentrations of mouse anti-human FasL antibody or isotype control antibody (1 h/ice), cells were washed and incubated with anti-mouse IgG-FITC (30 min/ice). Cells were washed and analyzed by flow cytometry.

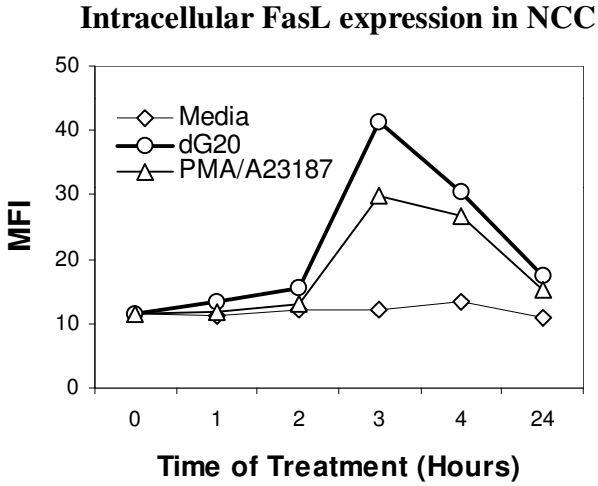
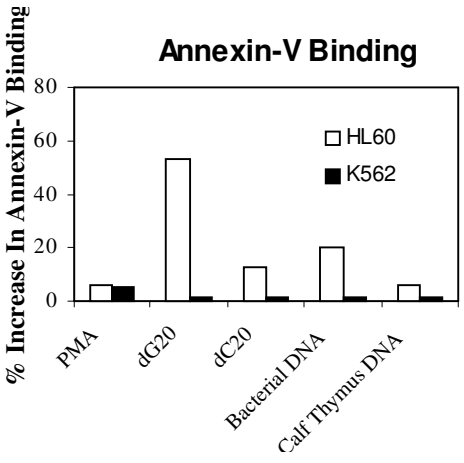


Figure 5.2. dG20 induces an increase in expression of intracellular FasL in NCC. 1x 10^6 cells /ml were treated with media alone, dG20 (50 mg/ml) and PMA+A23187 (0.05mg/ml /0.25mg/ml) for indicated time periods. 5 x 10^4 cells were harvested at each time point and permeabilized with ice-cold acetone, washed twice and incubated with anti- FasL antibody for 1h, followed by washing and incubation with anti-mouse IgG-FITC for 30 min. Cells were analyzed by flow cytometry. Representative of two independent experiments is shown.

A.


Annexin-V Binding
% Increase In Annexin-V Binding
80
60
40
20
0
HL60
K562
PMA
dG20
dC20
Bacterial DNA
Calf Thymus DNA

B.

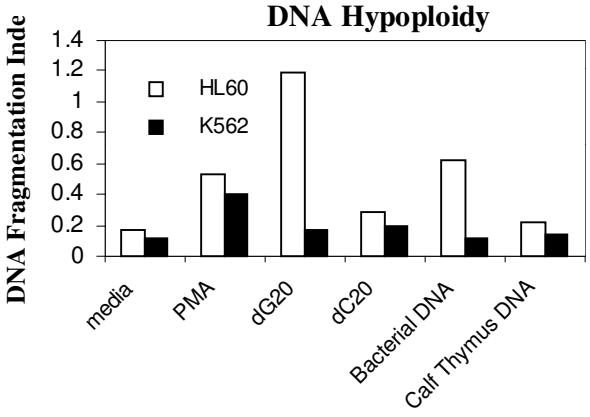
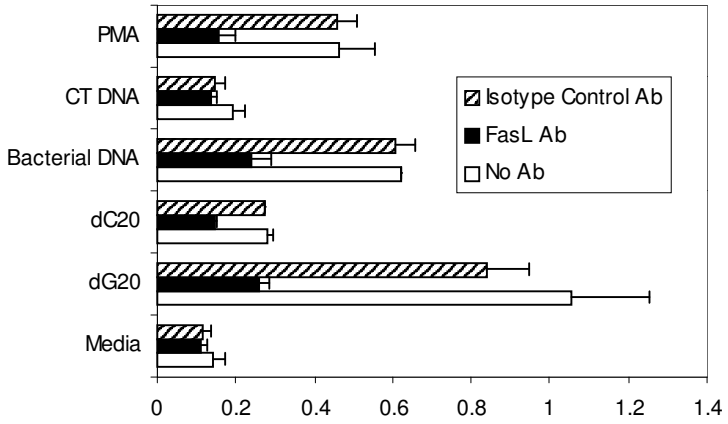

DNA Hypoploidy
DNA Fragmentation Inde
1.4
1.2
1
0.8
0.6
0.4
0.2
0
HL60
K562
media
PMA
dG20
dC20
Bacterial DNA
Calf Thymus DNA

Figure 5.3. Treatment of NCC with dG20 activated secretion of FasL. To determine whether supernatants from ODN treated NCC contained apoptosis inducing FasL, 1 x 10^6 NCC/ml were incubated with media alone, ODNs (50mg/ml), bacterial DNA (5mg/ml), CT DNA (5mg/ml) and PMA+A23187 (0.05mg/ml /0.25mg/ml) for 24 h at room temperature. Supernatants were collected after 24 h treatment and 500 ml was added to 5 x 10^4 HL60 or K562 cells. (A) After incubation with the "activated" supernatants, target cells were analyzed for annexin-V binding and PI uptake. For this, cells were washed, resuspended in 100ml of annexin-V binding buffer and incubated with 2.5 ml of annexin-V-FITC and 5 mg/ml of PI for 15 min in dark and analyzed by flow cytometry. Percent increase over media control in annexin-V single positive cells are shown. PI single positive and Annexin-V plus PI double positive cells (necrotic cells) were excluded from the analysis. (B) After 24 h incubation with the "activated" supernatants, HL60 were analyzed for late apoptosis by determination of extent of DNA hypoploidy. Cells were washed and treated with 400 ml of nuclear isolation media for 5 min on ice in dark and analyzed by flow cytometry. DNA fragmentation index (DFI) is shown. Representative of three independent experiments is shown.

Neutralization of FasL activity from "activated" supernatants.



DNA Fragmentation Index

Figure 5.4. Neutralization of FasL activity in supernatants. Supernatants generated from NCC treated (24 h at room temperature) with media alone, ODNs (50 mg/ml), bacterial DNA (5 mg/ml), CT DNA (5 mg/ml) and PMA+A23187 (0.05 mg/ml /0.25 mg/ml) were first incubated with anti-FasL antibody or isotype control antibody for 2 h/4°C. Antigen-antibody mixtures were then incubated with Protein G beads for 2 h/4°C. Beads were centrifuged and 500 ml of the supernatants were added to 5x 10^4 HL60 cells. After 24h incubation the HL-60 cells were washed and treated with 400 ml of nuclear isolation media (5 min on ice in dark) and analyzed by flow cytometry. DNA fragmentation index (DFI) is shown. Mean ± SE computed from two independent experiments.

DISCUSSION

Mammalian NK and lymphokine activated NK (LAK) cells express and use FasL as a second major effector molecule [27-28] in addition to granzyme-perforin pathways. In previous reports [29-31], stimulation of NK cells with interferon-γ, appropriate tumor target cells or Fc receptor ligands activated membrane expression of FasL. Activated NK cells also secreted a biologically active soluble form of FasL [13,32]. This form of the ligand was apparently not produced by metalloprotease cleavage of membrane FasL but rather represented a cytosolic vesicle-contained and secreted protein. Stimulus induced secretion of FasL was also characteristic of T-cells. Activation of Jurkats by PMA, ionomycin, anti-CD3 and bacterial superantigen caused the rapid release (15 min) of sFasL [33]. Secretion was not based on *de novo* synthesis because treatment with cycloheximide or actinomycin D did not affect early release [14].

Phagocytic cells contain relatively large (constitutive) concentrations of membrane and cytosolic soluble FasL. Both neutrophils and monocytes expressed membrane FasL and also contained large intracellular stores of FasL. Soluble FasL was released from neutrophils and monocytes less than 10 min following stimulation with immune complexes (i.e. FcγR ligation) [34-35]. This did not require *de novo* gene transcription or protein translation but represented the release of preformed soluble cytosolic FasL. These findings [36] suggested that phagocyte-derived sFasL might be released as an important "cytokine" mediator of cytotoxicity during inflammation.

Besides its role as a death inducing molecule, soluble FasL is known to have proinflammatory activities such as chemotactic stimulation for polymorphonuclear neutrophils [37]. Soluble FasL also induced neutrophil infiltration and rapid rejection of

FasR positive tumor cells [38]. Binding to FasL initiated both B and T cell proliferation [39, 40].

We previously described [20-25] the presence of a FasL-like protein in NCC that was released following membrane contact with certain target cells (e.g. HL-60). The apoptotic effects of the released/secreted substance were neutralized by anti-FasL antibody [25]. A second pathway in this stimulus-release model for FasL activity was identified in the present study. NCC are the teleost equivalent of NK cells from warm blooded vertebrates and as such represent a phylogenetic model for regulation of innate immunity. Studies of NK cells from evolutionary more recent species have not indicated a stimulus-response relationship between any type of oligodeoxynucleotide and synthesis/secretion/release of biologically active FasL. We demonstrated that FasL was upregulated in NCC by single base ODN and bacterial DNA (Fiure 5.4). Regulation occurred within a few hours of the response and the programmed cell death pathology was identified by finding both early phosphotidylserine leaflet inversion and late DNA hypoploidy in FasR positive HL-60 target cells. In the present study, FasL was identified as the stimulus for induction of apoptosis because anti-FasL antibody neutralized this activity. These data, combined with the knowledge of the role of FasL in inflammation, suggested that this ligand may be an important mediator of innate immune responses by two different mechanisms: by the direct killing of target cells after release from NCC and by the recruitment of phagocytic cells to the site of infection.

We demonstrated for the first time in any species that single base ODN binding activated increased concentrations of soluble biologically active FasL. This molecule was released/secreted by NCC and as such may be utilized as a second major cytotoxic

effector mechanism in inflammation and regulation of FasR bearing mammalian target cells. This indicated that teleost FasL has biological activities consistent with the mammalian Fas-FasL system. These data also provided a significant phylogenetic linkage between processes of innate immune regulation of cold-blooded vertebrates and analogous responses of mammals.

REFERENCES

[1] J.H. Russell, Activation-induced death of mature T-cells in the regulation of immune responses, Curr. Opin. Immunol. 7 (1995) 382-394.

[2] A.H. Montel, M.R. Bochan, W.S. Goebel, Z. Bramhi, Fas-mediated cytotoxicity remains intact in perforin and granzyme B antisense transfectants of a human NK-like cell line, Cell. Immunol. 165 (1995) 312-317.

[3] Y. Oshimi, S. Oda, Y. Honda, S. Nagata, S. Miyazaki, Involvement of Fas ligand and Fas-mediated pathway in the cytotoxicity of human natural killer cells, J. Immunol. 157 (1996) 2909-2915.

[4] T. Griffith, T. Brunner, S. Fletcher, D. Green, T. Ferguson, Fas-ligand induced apoptosis as a mechanism of immune privilege, Science 270 (1995) 1189-1192.

[5] D. Bellgrau, D. Gold, H. Selawry, J. Moore, A. Franzusoff, R.C. Duke, A role for CD95 ligand in preventing graft rejection, Nature 377 (1995) 630-632.

[6] J.H. Allison, M. Georgiou, A. Strasser, D.L. Vaux, Transgenic expression of CD95 ligand on islet β cells induces a granulocytic infiltration, but does not confer immune privilege upon islet allografts, Proc. Natl. Acad. Sci. 94 (1997) 3943-3947.

[7] S.M. Kang, D.B. Schneider, Z. Lin, D. Hanahan, D.A. Dichek, P.G. Strock, S. Baekkeskov, Fas ligand expression in islets of Langerhans does not confer immune privilege and instead targets them for rapid destruction, Nature Med. 3 (1997) 738-743.

[8] Y. Kashii, R. Giorda, R.B. Herberman, T.L. Whiteside, N.L. Vujanovic, Constitutive expression and role of the TNF family ligands in apoptotic killing of tumor cells by human NK cells, J. Immunol. 163 (1999) 5358-5366.

[9] G. Bossi, G.M. Griffiths, Degranulation plays an essential part in regulating cell surface expression of Fas ligand in T cells and natural killer cells, Nature Med. 5 (1999) 90-96.

[10] M.J. Martinez-Lorenzo, A. Anel, S. Gamen, I. Monleon, P. Lasierra, L. Larrad, A. Pineiro, M.A. Alava, J. Naval, Activated human T cells release bioactive Fas Ligand and APO2 ligand in microvesicles, J. Immunol. 163 (1999) 1274-1281.

[11] S. Sieg, D. Smith, D. Kaplan, Differential activity of soluble versus cellular Fas ligand: regulation by an accessory molecule, Cellular Immunol. 195 (1999) 89-95.

[12] K. Shudo, K. Kinoshita, R. Inamura, H. Fan, K. Hasumoto, M. Tanaka, S. Nagata, T. Suda, The membrane-bound but not soluble form of human Fas ligand is responsible for its inflammatory activity, Eur. J. Immunol. 31 (2001) 2504-2511.

[13] M. Tanaka, T. Suda, K. Haze, N. Nakamura, K. Sato, F. Kimura, K. Motoyoshi, M. Mizuki, S. Tagawa, S. Ohga, K. Hatake, A.H. Drummond, S. Nagata, Fas ligand in human serum, Nature Med. 2 (1996) 317-322.

[14] M.H. Martinez-Lorenzo, M.A. Alava, A. Anel, A. Pineiro, J. Naval, Release of preformed Fas ligand in soluble form is the major factor for activation-induced death of Jurkat T cells, Immunol. 89 (1996) 511-517.

[15] I. Monleon, M.J. Martinez-Lorenzo, J. Monteagudo, P. Lasierra, M. Taules, M. Iturralde, A. Piniero, L. Larad, M.A. Alava, J. Naval, A. Anel, Differential secretion of Fas ligand- or APO2 ligand/TNF-related apoptosis-inducing ligand-carrying microvesicles during activation-induced death of human T cells, J. Immunol. 167 (2001) 6736-6744.

[16] K.L. Serrao, J.D. Fortenberry, M.L. Owens, F.L. Harris, L.A.S. Brown, Neutrophils induce apoptosis of lung epithelial cells via release of soluble Fas ligand, Am. J. Physiol. Lung Cell Mol. Physiol. 280 (2001) 298-305.

[17] T. Murayama, T. Koizumi, H. Das, Y. Dobayashi, K. Kajimoto, T. Sugimoto, S. Imoto, R. Nishimura, T. Nakagawa, Soluble Fas ligand in natural killer cell lymphoma, Am. J. Hematol. 62 (1999) 253-255.

[18] P. Kern, M. Dietrich, C. Hemmer, N. Wellinghausen, Increased levels of soluble Fas ligand in serum in Plasmodium falciparum malaria, Infec. Immun. 68 (2000) 3061-3063.

[19] M.H. Sohn, J.-W. Kin, W.K. Kin, G.C. Jang, E.-E. Kim, Staphylococcal enterotoxin B upregulates Fas-mediated apoptosis of peripheral blood mononuclear cells in childhood atopic dermatitis, Scan. J. Immunol. 57 (2003) 62-67.

[20] L. Jaso-Friedmann, J.H.III Leary, D.L. Evans, Role of nonspecific cytotoxic cells in the induction of programmed cell death of pathogenic protozoans: participation of the Fas ligand-Fas receptor system, Exptl Para. 96 (2000) 75-88.

[21] D.L. Evans, S.L. Taylor, J.H.III Leary, G.R. Bishop, L. Jaso-Friedmann, *In vivo* activation of nonspecific cytotoxic cells by *Streptococcus iniae* and amplification with apoptosis regulatory factors (s), Fish and Shellfish Immunol. 10 (2000) 419-434.

[22] L. Jaso-Friedmann, J. Ruiz, G.R. Bishop, D.L. Evans, Regulation of innate immunity in tilapia: activation of nonspecific cytotoxic cells by cytokine-like factors, Dev. Comp. Immunol. 24 (2000) 25-36.

[23] G.R. Bishop, L. Jaso-Friedmann, D.L. Evans, Activation induced programmed cell death of nonspecific cytotoxic cells and inhibition by apoptosis regulatory factors, Cell. Immunol. 199 (2000) 126-137.

[24] D.L. Evans, J.H.III Leary, L. Jaso-Friedmann, Nonspecific cytotoxic cells and innate immunity: regulation by programmed cell death, Dev. Comp. Immunol. 25 (2001) 791-805.

[25] G.R. Bishop, S. Taylor, L. Jaso-Friedmann, D.L. Evans, Mechanisms of nonspecific cytotoxic cell regulation of apoptosis: cytokine-like activity of soluble Fas Ligand, Fish and Shellfish Immunol.13 (2002) 47-67.

[26] D.L. Evans, E.E. Smith, L. Jaso-Friedmann, A. St. John, H.S. Koren, D.T. Harris, Identification of a putative antigen receptor on fish nonspecific cytotoxic cells with monoclonal antibodies, J. Immunol. 141 (1988) 324-332.

[27] H. Arase, N. Arase, T. Saito, Fas-mediated cytotoxicity by freshly isolated natural killer cells, J. Exptl. Med. 181 (1995) 1235-1238.

[28] H. Tsutsui, K. Nakanishi, K. Natsui, K. Higashino, H. Okamura, Y. Miyazawa, K. Kaneda, IFN-gamma-inducing factor up-regulates Fas ligand-mediated cytotoxic activity or murine natural killer cell clones, J. Immunol. 157 (1996) 3967-3973.

[29] R.K. Lee, J. Spielman, D.Y. Zhao, K.J. Olsen, E.R. Podack, Perforin, Fas ligand, and tumor necrosis factor are the major cytotoxic molecules used by lymphokine-activated killer cells, J. Immunol. 157 (1996) 1919-1925.

[30] Y. Oshimi, S. Oda, Y. Honda, S. Natata, S. Miyazaki, Involvement of Fas ligand and Fas-mediated pathway in the cytotoxicity of human natural killer cells, J. Immunol. 157 (1996) 2909-2915.

[31] C.M. Eischen, J.D. Schilling, D.H. Lynch, P.H. Drammer, P.J. Leibson, Fc receptor-induced expression of Fas ligand on activated NK cells facilitates cell-mediated cytotoxicity and subsequent autocrine NK cell apoptosis, J. Immunol. 156 (1996) 2693-2699.

[32] W.C. Liles, Apoptosis-role in infection and inflammation, Curr. Opin. Infectious Dis. 10 (1997) 165-170.

[33] M. Tanaka, T. Suda, T. Takahasi, S. Nagata, Expression of the functional soluble form of human Fas ligand in activated lymphocytes, EMBO J. 14 (1995) 1129-1135.

[34] P.A. Kiener, P.M. Davis, B.M. Rankin, S.J. Klebanoff, J.A. Ledbetter, G.C. Starling, W.C. Liles, Human monocytic cells contain high levels of intracellular Fas ligand: rapid release following cellular activation, J. Immunol. 159 (1997) 1594-1598.

[35] W.C. Liles, P.A. Kiener, J.A. Ledbetter, A. Aruffo, S.J. Klebanoff, Differential expression of Fas (CD95) and Fas ligand on normal human phagocytes:

implications for the regulation of apoptosis in neutrophils, J. Exptl. Med. 184 (1996) 429-440.

[36] S.B. Brown, J. Savill, Phagocytosis triggers macrophage release of Fas ligand and induces apoptosis of bystander leukocytes, J. Immunol. 162 (1999) 480-485.

[37] K. Seino, K. Iwabuchi, N. Kayagaki, R. Miyata, I. Nagaoka, A. Matsuzawa, K. Fukao, H. Yagita, K Okumura, Chemotactic activity of soluble Fas ligand against phagocytes.
J. Immunol. 161 (1998) 4484-4488.

[38] K. Seino, N. Kayagaki, K. Okumura, H. Yagita, Antitumor effect of locally produced CD95 ligand, Nat. Med. 3 (1997) 165-170.

[39] M.R. Alderson, R.J. Armitage, E. Maraskovsky, T.W. Tough, E. Roux, K. Schooley, F. Ramsdell, D.H. Lynch, Fas transduces activation signals in normal human T lymphocytes, J. Exp. Med. 178 (1993) 2231-2235.

[40] J.C. Rathmell, S.E. Townsend, J.C. Xu, R.A. Flavell, C.C. Goodnow, Expansion or elimination of B cells in vivo: dual roles for CD40- and Fas (CD95)-ligands modulated by the B cell antigen receptor, Cell 87 (1996) 319-329.

CHAPTER 6

ACTIVATION OF NK-LIKE YT-INDY CELLS BY SINGLE BASE OLIGODEOXYGUANOSINE AND BINDING BY PATTERN RECOGNITION PROTEINS[1]

[1]Kaur, H., L. Jaso-Friedmann, J. H. Leary III, Z. Brahmi and D. L. Evans. To be submitted to *Journal of Immunology*.

Abstract

Previously it was shown by others that oligodeoxynucleotides (ODNs) containing CpG-motifs activated natural killer cells. Certain ODNs that also contained guanosine runs augmented the activation of T cells and macrophages. The present study was designed to examine the effects of single base phosphodiester (Po) ODNs on the activation of the NK-like cell line (YT-INDY). Single base Po ODNs composed of 20-mers of guanosine (dG20), adenosine (dA20), cytosine (dC20) or thymidine (dT20) and "conventional" Po CpG ODN were examined for their ability to bind and activate YT-INDY cells. To determine whether ODN binding was receptor mediated, saturation and competitive binding experiments were conducted. Binding by dG20 and CpG ODN was saturable and specific. dG20 binding was competitively inhibited by homologous dG20 and heterologous CpG ODN but not by dC20 and dA20. The dG20 and CpG binding proteins on YT-INDY cells were identified by ligand (Southwestern) blotting. Two different YT-INDY proteins (p29 and P18 kDa) in membrane lysates bound to both these ODNs. The specificity of dG20 and CpG binding was determined using recombinant P18 (RP18). Immobilized RP18 bound to soluble dG20 and CpG to compete for binding by YT-INDY cells and YT-INDY membrane proteins. Cell proliferation and activation studies showed that dG20 and CpG treatment induced cellular DNA synthesis (i.e. G_1 to S-phase conversion) and dG20 increased cell numbers by approximately 10% at 10 hours post- treatment. Both dG20 and CpG ODN binding induced a calcium flux in YT-INDY cells within seconds of treatment. These experiments demonstrated that phosphodiester single base dG20 and CpG ODNs bind to a (potential) new class of cell-surface proteins that mediate activation of YT-INDY cells.

Introduction

Bacterial DNA is a complex macromolecule whose immunological properties for activation of innate immunity are attributed to the presence of unmethylated CpG motifs (1). Synthetic oligodeoxynucleotides (ODNs) containing CpG-motifs mimic the effects of bacterial DNA (2) for activation of cells of immune system by binding to Toll like receptor-9 (TLR9) (3) and Mac-1 (CD11b/CD18) (4). CpG ODNs activated human and mouse NK cells resulting in enhanced cytotoxicity and IFN-γ production (5,6). The optimum CpG ODN for human and mouse NK cells differed in the requirements for the composition of the central hexameric CpG motif (5, 6). In both cases, however, addition of polyguanosine [poly G] runs at 5' or 3' ends of the activatory CpG ODNs resulted in enhancement of the activation responses. These studies demonstrated that the addition of guanosine "runs" to CpG motifs almost always enhanced the immunostimulatory potential of the ODNs.

Non-CpG phosphodiester ODNs composed of single base polyguanosine also activated certain cells of the immune system in the absence of any CpG motifs (7). The other strategy consisted of tethering of polyguanosine runs at 5' and3' ends of -CpG-motifs. This conferred diverse immunological properties to these ODNs (8). Depending on the localization of polyguanosine runs and the backbone modifications (phosphodiester /Po or phosphothioate/Ps), these CpG-ODNs had enhanced (Ps) or diminished (Po) immunostimulatory properties following binding to different cell types (8). The presence of three or more guanosines at the 3' end of a fully Ps modified optimum CpG-ODN strongly enhanced the uptake and immunostimulatory activity of ODNs by macrophages (9). Partial Ps modification of the 3' end by polyguanylation was

required for an enhanced induction of ODN mediated anti-tumor activity of NK cells (10). These immunostimulatory properties were thought to be associated with the unique tetrad conformation attained by polyguanosines in solution.

Single base polyguanosine ODNs of different nucleotide lengths bind to macrophages (11), NK cells (12) and B cells (13). Competition experiments indicated that Ps 30-mers of single base oligodeoxyguanosine downregulated bacterial DNA and CpG stimulation of NO production (14) and IL-2 secretion (15) by murine macrophages. These data suggested that dG30 and CpG/bacterial DNA shared crossreactive membrane binding proteins and that immunoregulation by both single base and CpG ODNs occurred at a membrane proximal and signaling level.

Although a number of cell surface receptors like TLR-9, Mac-1 and Scavenger receptors are involved in binding and uptake of natural and synthetic DNA into cells, the identity of the DNA binding proteins critical for facilitated endocytosis of single base ODNs remains uncertain. Studies have not identified these receptors, however we suggest that a redundancy in expression of the single base versus dinucleotide motif DNA-binding cell surface proteins may exist. In order to address this question and to identify the membrane binding proteins on human NK-like cells (YT-INDY) we examined the binding and activation functions of single base oligodeoxynucleotides (e.g. dG20). These results were compared with similar studies of the stimulatory activity of Po CpG and they indicated that dG20 induced YT-INDY cellular DNA synthesis and proliferation. That this ligand induced a signaling effect was suggested by data showing calcium mobilization in these cells within a few hours of treatment. Binding data showed

that dG20 binding was saturable and competition occurred in the presence of homologous ODN and CpG. These data suggested receptor crossreactivity.

The molecular properties of these ODN binding proteins were also identified in the present study. We demonstrated that two different molecular weight species of membrane proteins bind dG20 and CpG. These are not identical in molecular characteristics to TLR9 and Mac-1.

Material and Methods

Oligonucleotides

ODNs were purchased from MWG-Biotech (High Point, NC). All ODNs were synthesised as a phosphodiester using standard methods. Prior to use, ODNs were resuspended in PBS prepared in endotoxin free water (# 210-7, Sigma). 3'end biotinylated ODNs and rhodamine conjugated ODNs were purchased from MWG-Biotech. Table 6.1 shows the ODNs used in this study. All ODNs used in this study contained <0.015 EU/ml endotoxin.

Cells and culture conditions

YT-INDY cell line was a gift from Dr. Zacharie Brahmi (Indiana Univ. School of Medicine). YT-INDY is a subline of the YT cell line, originally established from a child with acute lymphoblastic lymphoma and thymoma (Yodoi *et al*., 1985). Cells were cultured in RPMI-1640 (Cellgro, Media Tech, Washington, DC) supplemented with L-glutamine, sodium pyruvate, MEM vitamin solution, MEM essential [amino acid] solution, non-essential [amino acid] solution (Cellgro), 50 mg/ml gentamicin (Schering-

Plough Animal health Corp., Kenilworth, NJ) and 10% fetal bovine serum (FBS, Atlanta biologicals, Norcross, GA).

Oligodeoxynucleotide (ODN) binding and inhibition

For binding experiments, 5 x 10^4 cells were first incubated with PAB (1X phosphate buffer saline (PBS), 0.1% sodium azide (Sigma Chemical Co., St. Louis, MO) and 1% bovine serum albumin (Sigma Chemical Co.) for 30 min on ice. Cells were incubated with 100 µl of different concentrations of ODNs for 1 h on ice, washed and the biotinylated (dG20, dC20 and dA20) ODNs stained cells were further incubated with Streptavidin-Phycoerythrin (PE) (# 189737, Calbiochem, San Diego, CA) for 30 min on ice. Cells were washed with PAB to remove unbound ODN and resuspended in 200 µl of PAB and analyzed by flow cytometer.

For binding inhibition experiments, 5 x 10^4 cells were incubated with different concentrations of unlabeled ODNs for 1 h. Cells were washed to remove excess of unlabeled ODN and stained with 50% saturating concentration of dG20-biotin followed by Streptavidin-PE for 30 min on ice. Cells were washed and analyzed by flow cytometer. Cells were kept on ice during all the incubations. Controls for nonspecific staining included cells stained with the appropriate conjugates.

Cell proliferation and cell cycle analysis

Cell proliferation was determined by trypan blue exclusion before and after treatment with ODNs. For cell viability and cell proliferation, 1 x 10^6/ml were incubated with different concentrations of ODNs for 10 h at 37 °C. Live cells were counted at 0 h and

10 h by trypan blue exclusion. For cell cycle analysis, 1 x 10^6/ml were incubated with different concentrations of ODNs. At different time periods, 1 x 10^5 cells were incubated with 400 µl of nuclear isolation media (NIM: 0.1% Triton X-100, 5 mg propidium iodide and 100 mg RNase A in 100 ml of 1X PBS) for 5 min on ice and analyzed by flow cytometry. Change in cellular DNA content (i.e. cell proliferation) was calculated as S+G2M phase/ total DNA content. Percent increase in DNA was determined by comparisons with media control.

Preparation of cell membranes

Cells were washed three times with ice cold TBS (25 mM Tris-HCl, pH 7.5, 0.15 M NaCl). Cells were resuspended in Dounce homogenization buffer (10 mM Tris-Cl, pH 7.6, 0.5 mM $MgCl_2$, 10 µg/ml leupeptin, 10 µg/ml pepstatin and 1 mM PMSF) @ 20 x 10^6 cells/ml and incubated on ice for 15 min. Cells were subjected to 20 strokes. 333 µl of tonicity restoration buffer (10 mM Tris-cl, pH 7.6, 0.5 mM $MgCl_2$ and 0.6 M NaCl) was added per ml of homogenization buffer and cells were spun at 500 g for 5min. Supernatant was collected and EDTA was added to final concentration of 5 mM. Supernatant was spun at 13000 rpm for 10 min. After discarding the supernatant, pellet was washed twice with cold TBS containing 10 µg/ml leupeptin, 10 µg/ml pepstatin and 1 mM PMSF and finally resuspended in 100 µl of hot 1X SDS-sample buffer.

Ligand (Southwestern) blotting

Cell membrane proteins were resolved on 12.5 % SDS-PAGE gel and transferred onto nitrocellulose membrane at 100 V for 1 h. Equal amount of protein (determined by

Bradford Assay) was loaded in each lane. Nitrocellulose membrane was incubated with blocking buffer (Super-bloc, PIERCE, Rockford, IL) containing 0.1% Tween for 30 min, incubated with primary ligand (10 ng/ml of biotinylated dG20, dC20 or dA20 or 200 ng/ml of CpG) for 1 h, washed with TBS-T (0.1% Tween-20) for 20 min (four times, 5 min each) and finally incubated with secondary conjugate (ExtrAvidin-Peroxidase conjugate diluted 1:200000 in blocking buffer, # E2886, Sigma) for 1 h, washed with TBS-T for 20 min and detected with chemiluminiscent substrate (SuperSignal® West Pico Chemiluminescent, Pierce, Rockford, IL).

Production of recombinant P18 (RP18)

Recombinant P18 (RP18) was produced as previously described (submitted for publication). Briefly, the primary teleost P18 sequence was obtained by using teleost P18 specific primers and the cDNA library prepared using mRNA extracted from catfish nonspecific cytotoxic cells (NCC). To generate the recombinant protein, the P18 insert DNA containing 6 x Histidines (His-tag) was cloned into pET-21a by standard techniques and the resulting plasmid (pET-21a-P18) was transformed into *E.coli* strain BL21(DE3)pLysE. Bacteria was grown till 0.5 OD and induced with 1mM isopropyl-beta-D-thiogalactopyranoside (IPTG, # BP1620-1, Fisher, Fair Lawn, NJ) for 3 h at 30°C. Lysates were prepared from IPTG induced cultures followed by sequential incubations in lysozyme (1mg/ml), Triton X-100 (0.5%), DNaseI (5 µg/ml) and RNase A (10 µg/ml). RP18 was purified from cleared lysates using Ni-NTA-agarose (#30210, Qiagen, Valencia, CA) according to manufacturers instructions.

Depletion of ODNs with recombinant P18

RP18 was immobilized on Ni-NTA agarose beads (#30210, Qiagen,Valencia, CA) by incubating 1 ml of purified RP18 (100µg/ml) with Ni-NTA agarose beads (500µl of slurry) for 1 h at 4°C. Beads were pelleted, washed three times with wash buffer (300mM NaCl, 5mM imidazole, 50mM NaH_2PO_4, pH 8.0), washed three times with PBS and resuspended in 500µl PBS. Controls for non-specific binding included beads incubated with buffer alone or equal amount of proteins obtained from empty vector containing bacterial lysate. 100 µl of beads were used for each reaction. Beads were pelleted and 100µl of biotinylated ODNs (dG20: 10 µg/ml and 5 µg/ml and CpG: 100 µg/ml) was added. This was incubated for 5 h at 4°C. Supernatant was collected after pelleting the beads.

ODN depletion in the supernatants was determined by flow cytometry (dG20) and by ligand blotting (dG20 and CpG). For flow cytometry, 5 x 10^4 cells were incubated for 1 h on ice with following: saturating concentrations of dG20-biotin and supernatants (obtained following depletion with RP18, empty vector control and buffer control) containing equivalent amount of dG20-biotin prior to depletion. Cells were washed and incubated with Streptavidin-PE and analyzed by flow cytometry. To do ligand blotting, YT-INDY cell membrane proteins were resolved on 12.5% gel and transferred to nitrocellulose membrane. Equal amount of protein was loaded in each lane. Nitrocellulose membrane was incubated with blocking buffer for 30 min, incubated for 1 h with 10 ng/ml of biotinylated dG20 or 200 ng/ml of CpG and supernatants containing equivalent amount of ODNs prior to depletion, washed with TBS-T for 20 min (four times, 5 min each) and incubated with ExtrAvidin-Peroxidase (1:200000) for 1 h, washed

with TBS-T for 20 min and detected with chemiluminiscent substrate (SuperSignal® West Pico Chemiluminescent, Pierce, Rockford, IL).

Calcium mobilization assay

Cells were washed three times with RPMI and finally resuspended in RPMI @ 5 x 10^6 / ml. Cells were loaded with Ca^{2+}-sensitive dye Fluo-3 AM (# 343242, Calbiochem, La Jolla, CA) in the presence of Pluronic F-127 (# F-127, Molecular Probes, Eugene, OR). Briefly, equal volumes of 1mM Fluo-3 AM and 20% Pluoronic acid were mixed. 24µl of this was added to 3ml of cell suspension and incubated at 37°C for 50 min and mixed at every 10 min interval. Cells were washed three times with RPMI, resuspended at 1 x 10^6 / ml and stored at room temperature in dark. Dye loaded cells were examined by flow cytometry for 300s to generate a baseline. The Ca^{2+} moblization induced by various stimuli (PMA/A23187: 0.5 µg/ml/2.5 µg/ml; ODNs: 50 µg/ml) was then determined by flow cytometry immediately after addition of stimulus. Changes in fluorescence emission were recorded for 300s.

Flow cytometry

Analysis was performed using an EPICS XL-MCL four color analyser (Coulter Electronics Corp, Hileah, FL), equipped with 15 mW air cooled argon-ion laser operating at 488 nm wavelength. 10,000 to 15,000 events were collected per sample. Conjugate controls were included to set the baseline fluorescence and to determine nonspecific binding of ODNs. PE and rhodamine signals were detected with 575nm bandpass filter by photomultiplier tube 2 (PMT2). Fluo-3 emission was detected with 525nm bandpass filter by PMT1. Data was analysed using Coulter's System II software, version 3.0.

Results

Single base and "conventional" ODNs bind YT-INDY cells

Others previously demonstrated that CpG ODNs activated human NK cell cytotoxicity (6). Experiments however, did not examine the cellular membrane binding capacity by either phosphodiester (Po) CpG or single base ODNs. The Po 20-mers composed of oligodeoxyguanosine (dG20), oligodeoxycytosine (dC20) and oligodeoxyadenosine (dA20) were first tested for binding to YT-INDY cells. Fig. 6.1A shows saturable binding by dG20 but not dC20 or dA20 by flow cytometry. Fifty percent saturation occurred at approximately 0.62 μg/ ml for dG20. Fig. 6.1B demonstrates that "conventional" CpG ODN likewise binds YT-INDY cells. Fifty percent saturation occurred at approximately 100 times higher concentration compared to dG20.

Specificity of dG20 binding

We previous reported (16) that single base ODNs (i.e. dG20) produced 100% inhibition of CpG binding to nonspecific cytotoxic cells from teleosts, however CpG only partially inhibited dG20 binding to these cells. In other experiments (17) we demonstrated that antibody to Scavenger receptors (SR) also failed to completely block dG20 binding to NCC. These studies indicated that (potentially) several different species (in addition to SR) of crossreactive membrane binding proteins existed on NCC. To examine the possibility of receptor heterogeneity for ODNs on YT-INDY cells, competition binding experiments were done. Two types of unlabeled ODNs (CpG and single base) were added to YT cells for competition analysis. Because previous competition experiments demonstrated aggregation of dG20 (probably due to Hoogsten base pairing/G-quartet formation) under conditions of relatively high dG20 concentrations, sequential cold

competition experiments were done. Unlabeled ODNs were first added to the target cells followed by washing and addition of labeled dG20. Fig. 6.2 demonstrates by flow cytometry that both cold dG20 and CpG competitively inhibited binding by dG20 although CpG only inhibited about 58% of dG20 binding compared to 75% inhibition seen with dG20. dA20 and dC20 did not block dG20 binding even at 1000x fold levels of cold competitor.

Southwestern blot detection of ODN binding proteins and use of recombinant P18 to determine specificity

To determine the molecular weight and the composition of YT-INDY ODN binding proteins, membrane preparations were examined by Southwestern blots. Fig. 6.3A and 6.3B shows that dG20-biotin and CpG-biotin (respectively) bind 18 (i.e. P18) and 29 (i.e. P29) kDa proteins.

To further characterize the relationship between dG20 binding and these membrane proteins, peptide sequences for teleost P18 (unpublished data) were obtained and full-length P18 was sequenced from catfish cDNA and a recombinant protein was expressed (see accompanying manuscript). Recombinant P18 (RP18) was expressed in a PET21a vector and soluble RP18 was immobilized on Ni-NTA beads. Neutralization/ depletion experiments were done by first incubating of dG20-biotin with RP18-NTA-Ni beads producing "activated beads". Activated beads were centrifuged to remove bound dG20-biotin and the supernatants were examined to determine the residual binding activity by dG20. Fig. 6.4A shows that the "activated beads" specifically removed essentially all dG20-biotin. Some non-specific binding was seen with control beads at

lower dG20-biotin concentration. Fig. 6.4B lane 2 demonstrates that both P18 and P29 were removed by the activated beads. These data suggested that both P18 and P29 contain a common ligand binding motif/sequence that may be responsible for ODN binding or that P18 is a truncated expressed form of P29 (both forms retaining the receptor motif). These data also indicated that the binding of ODNs to P18 was specific.

Binding of dG20 to YT-INDY cells induces cellular proliferation

To evaluate the effects of dG20 on cell division, YT-INDY cells were incubated with different concentrations of four different single base sODNs for 10 h and total and viable cell counts were done using trypan blue dye exclusion. Fig. 6.5 shows that at each dG20 concentration, treatment appeared to not only protect the cells from death, but also to cause cellular division. This cellular protection/stimulation effect appeared to be associated with an increase in the percentage of S-phase to G_2M conversion in DNA synthesis. Fig. 6.6A and 6.6B gives the results of the DNA analysis of YT-INDY cells following treatments with dG20 (Fig. 6.6A) and CpG (Fig. 6.6B). Both the concentration and kinetics of the cellular responses are shown. The effects of CpG on YT-INDY cellular DNA synthesis is shown in Fig. 6.6C. Compared to media controls, CpG initiated a shift from S-phase to G_2M at 48 h followed by new cellular (2N) DNA synthesis at 72 h. A relatively large 4N (G_2M) population was observed at both 72 h and 96 h post-treatment.

Effects of dG20 and CpG on calcium mobilization

The above experiments examine proliferation and DNA synthesis. These are relatively late events in the cell activation process. To determine the effects of ODNs on the early responses of cells, YT-INDY cells were loaded with the calcium-specific fluoreophore, Fluo-3 AM. Fluorescence from free cytosolic Ca^{2+} binding to Fluo-3 AM was measured immediately after addition of ODNs or after addition of the positive control PMA/A23187. Flow cytometric measurement of Fluo-3 fluorescence from non-stimulated YT-INDY cells indicated homogeneous dye loading in the cells (Fig. 6.7A). A shift of 0.5 log to 1 log occurred in fluorescence following addition of dG20 and PMA/A23187 respectively (Fig. 6.7A). Compared to non-stimulated cells (media control), both PMA/A23187 and ODNs dG20 and CpG produced a higher and sustained calcium flux as indicated by the increase in positive events for fluorescence in treated cells over a period of 300s (Fig. 6.7B). ODN dA20 did not produce any increase over the media control.

Table 6.1. Sequence of the oligodeoxynucleotides

5'-Oligodeoxynucleotides-3'	Name
CCCCCCCCCCCCCCCCCCCC	dC20
AAAAAAAAAAAAAAAAAAAA	dA20
TTTTTTTTTTTTTTTTTTTT	dT20
GGGGGGGGGGGGGGGGGGGG	dG20
TCGTCGTTGTCGTTGTCGTT	CpG

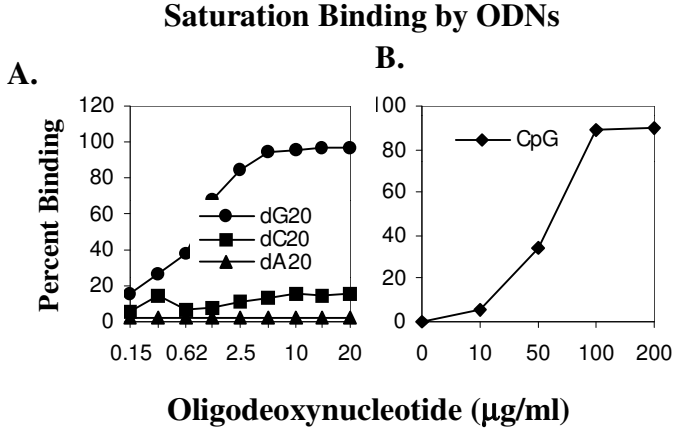


FIGURE 6.1. Polyoligodeoxynucleotides bind YT-INDY cells. 5 x 10^4 cells were incubated with 100 ml of different concentrations of ODNs (A) dG20-biotin, dC20-biotin and dA20-biotin (B) Rhodamine conjugated CpG ODN for 1 h/ice. Biotinylated ODN stained cells were washed and incubated with Streptavidin-PE for 30 min on ice. Cells were washed twice and analyzed by flow cytometry. Percent positive cells are shown for one out of three experiments.

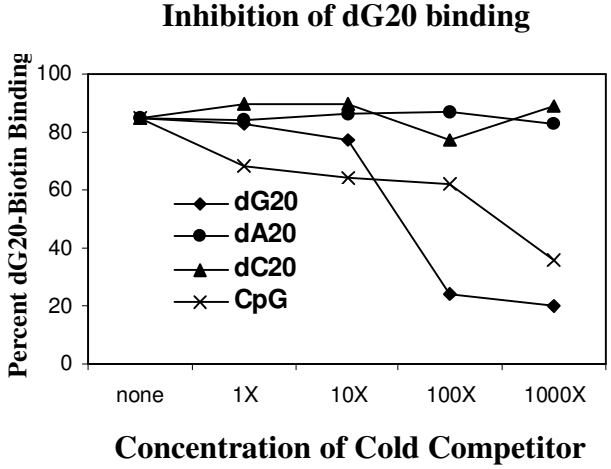


FIGURE 6.2. ODN dG20 and CpG but not dC20 and dA20 competitively inhibit dG20 binding on YT-INDY cells. 5 x 10^4 cells were incubated with different concentrations of unlabeled ODNs (dG20, dC20, dA20 and CpG) for 1 h on ice. Cells were washed and stained with 50% saturating concentration of dG20-biotin followed by Streptavidin-PE for 30 min on ice. Cells were washed twice and analyzed by flow cytometry. Percent dG20-biotin positive cells are shown. Representative experiment of three independent experiments is shown.

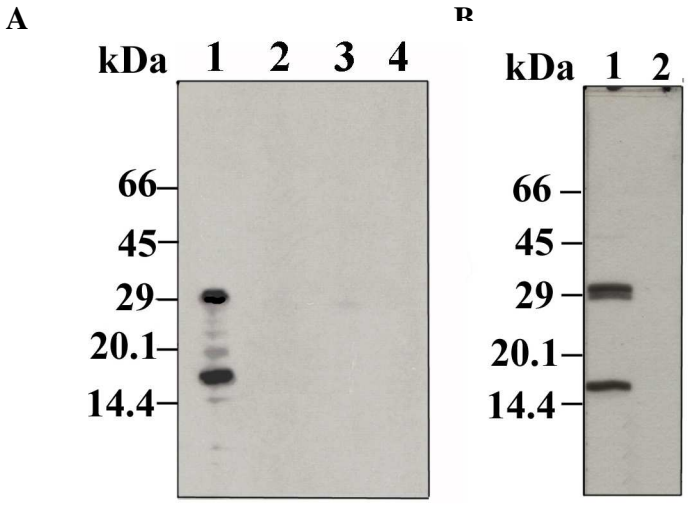
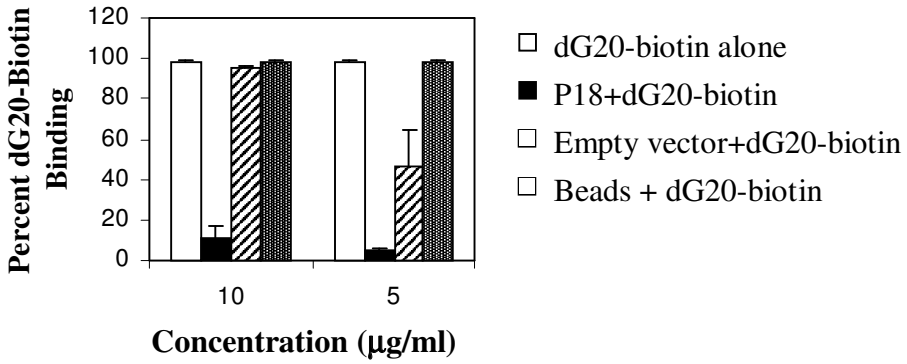


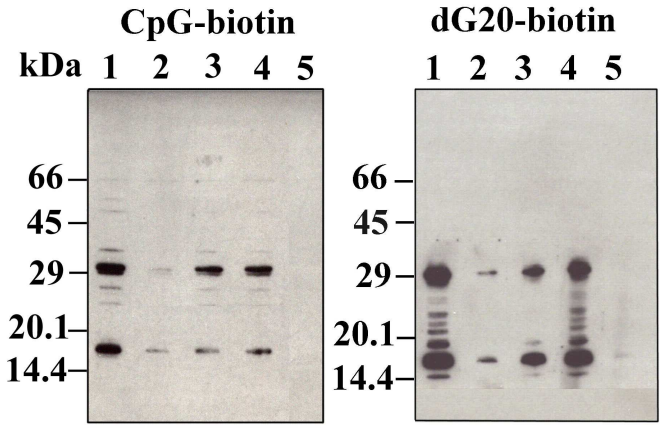
FIGURE 6.3. ODNs dG20 and CpG bind to low molecular weight proteins. Membrane preparations of YT-INDY cells were resolved by reducing SDS-PAGE on 12.5% gel and transferred onto nitrocellulose and ligand blotting was done. (A) Biotinylated ODNs (50 ng/ml): dG20-biotin (lane 1), dC20-biotin (lane 2) and dA20-biotin (lane 3) plus streptavidin-peroxidase conjugate to lanes 1,2 and 3 and streptavidin-peroxidase conjugate alone to lane 4. (B) Biotinylated CpG ODN (lane 1) and streptavidin-peroxidase conjugate alone to lane 2.

Depletion of dG20-biotin by recombinant p18

A



B



1 Positive control
2 Rp18 depleted ODN
3 Empty vector depleted ODN
4 Ni-NTA beads depleted ODN
5 Conjugate control

FIGURE 6.4. Specificity of dG20 binding to recombinant 18kD protein. Recombinant P18 (Rp18) was produced as described in material and methods and immobilized on NTA-Ni beads. (A) dG20-Biotin (10 mg/ml or 5 mg/ml) was incubated with immobilized Rp18 or control beads for 5 h/ 4°C. dG20-biotin depleted supernatants were added to 5 x 104 YT-INDY cells for 1h/ice. Cells were washed, incubated with Streptavidin-PE for 30 min/ice. Cells were washed twice and analyzed by flow cytometry. Controls were non-depleted dG20-biotin, empty vector and beads alone depleted dG0-biotin. Percent dG20-biotin positive YT-INDY cells is shown following incubation with the various "depleted" supernatants. Mean ± standard deviation from two different experiments. (B) Depletion of CpG and dG20 binding was accomplished by incubation of CpG-biotin (100 mg/ml) and dG20-biotin (10 mg/ml) with immobilized Rp18 on NTA-Ni beads (5 h/ 4°C). ODN depleted supernatants were tested by ligand blotting of YT-INDY membrane proteins (as described in material and methods). Controls were non-depleted biotinylated ODNs, empty vector and beads alone depleted ODNs.

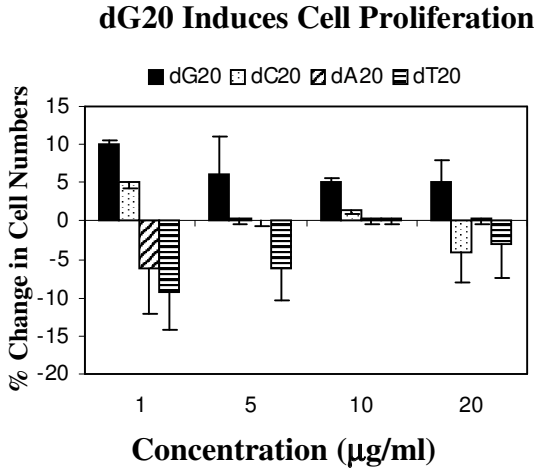


FIGURE 6.5. ODNs dG20 augments YT-INDY cell division. 1 x 106/ml were incubated with the indicated concentrations of ODNs dG20, dC20, dA20 and dT20. Cells were counted at 10 h post-treatment by trypan blue exclusion. Percent change in cell numbers is shown. Percent change= [(viable ODN treated cell number - media control cell number) / media control cell number] * 100. Mean ± standard deviation from three different experiments is shown.

Cell Cycle Analysis of dG20 and CpG treated YT-INDY Cells

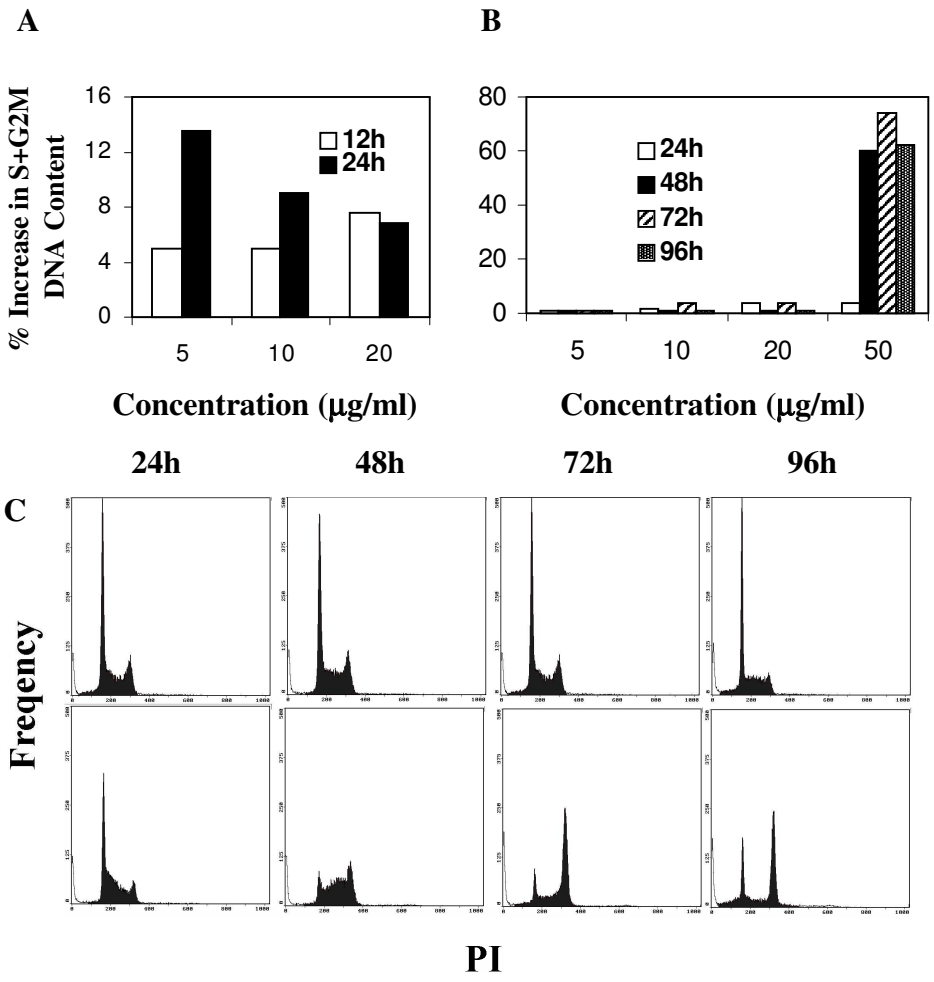


FIGURE 6.6. dG20 and CpG ODNs induce DNA synthesis in YT-INDY cells. 1 x 106 cells/ml were incubated with the indicated concentrations of (A) dG20 and (B) CpG for the time periods shown. Cell cycle analysis was done following the addition of nuclear isolation media (PI, RNAse, detergent). Change in cellular DNA content (i.e. cell proliferation) was calculated as S+G2M phase/ total DNA content. Percent increase in DNA was determined by comparisons with media control. One of three independent flow cytometry experiments is shown. (C) Cell cycle profile of CpG ODN (50 mg/ml) treated cells is shown and compared to media treated cells for same time periods.

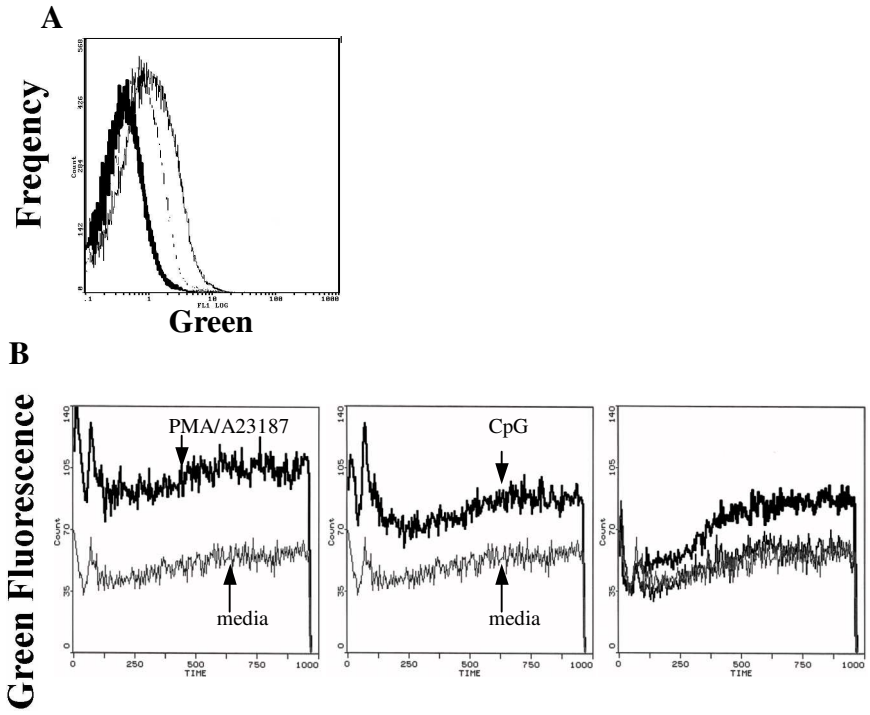


FIGURE 6.7. dG20 and CpG ODNs induce calcium mobilization in YT-INDY cells. Cells were washed (3X) and resuspended in RPMI @ 5 million/ ml. Cells were loaded with Ca2+-sensitive dye Fluo-3 in the presence of Pluronic F-127 (0.08%). Cells loaded with dye were examined by flow cytometry for 300s to generate a baseline (media control). Calcium moblilization by PMA/A23187, dG20, CpG and dA20 was determined immediately after addition of the stimuli. Events were collected for 300s. (A) Fluorescence emission in non-stimulated (thick line) and PMA/A23187 (thin line), dG20 (dotted line) treated cells is shown. (B) Fluorescent events vs time: overlays of media control and treatments are shown.

Discussion

Studies of regulation of mammalian NK cell activity by ODNs showed that human NK cell cytotoxicity was increased by CpG ODNs containing flanking 10-mers of guanosine (g10GACGA) (6). Single base ODNs composed of 30-mers of guanosine (g30) had no effects. In this same study g10GACGA increased the expression of CD69 and increased the production of IFN-γ, however g30 had no effects on these responses.

NK cells isolated from mouse spleen cells produced increased cytotoxicity following stimulation by ODNs (referred to as oligo-1) composed of -AACGTT- flanked by 5'- as well as 3' 12-mers of guanosine (12). Binding of acetyl-LDL (a Scavenger receptor ligand) to membrane preparations of J774A.1 and mouse splenocytes was inhibited by oligo-1. These data suggested that the extrapalindromic nucleotide sequence of oligo-1 bound to Scavenger receptors on NK cells. Oligodeoxynucleotides composed of -TCAACGTTGA- flanked 5' and 3' by four or six –mers of guanosine (respectively) completely protected mice from B16.F1 melanomas. NK cells were necessary for this anti-tumor effect (10) as treatment with anti-NK1.1 abrogated this protective effect.

Experiments have also been conducted to determine the effects of ODN motif (backbone) sequence, Ps modification and flanking base composition on mouse and human NK cell function (5). ODNs containing the palindrome GACGTC stimulated mouse NK cytotoxicity (18). Po backbones composed of CATGACGTTC with Ps modified 4 and 6 polyguanosine sequences at the 5' and 3' ends (respectively) were compared with the same sequence that had been entirely Ps modified. The ODN composed of the Po backbone stimulated NK cell lysis of target cells whereas the Ps modified ODN was negative. In that study the presence of poly(G) flanking sequences

had variable influences on NK cell activity that suggested only a minor role in the regulation of cellular function.

The nomenclature for CpG ODNs has apparently evolved into one based on several different functional criteria. CpG ODNs composed of Ps-modification and that caused splenic B cell proliferation, dendritic cell maturation and cytokine production are referred to as CpG-B/K type. This CpG DNA structure is referred to as K/B-type. This CpG is Ps modified through the entire length (19). The other type of CpG DNA contains a Po modified "backbone" structure with flanking Ps modified polyguanosine at both 5' and 3'ends and is referred to as D/A-type. A study by Klinman and colleagues (20) demonstrated that B-type but not A-type ODNs bound to TLR9. Later it was shown that both these ODN types utilized TLR9 to induce cell activation (19). It is important to note that in these studies both B- and A-type ODNs were composed of Ps modified flanking polyguanosine runs.

Other studies (13) examined the different cell types that expressed DNA membrane binding proteins. They demonstrated that freshly isolated human B-cells bound Ps modified ODNs preferably compared to their Po counterparts. Binding was specific and saturable and the B-cell binding protein(s) for all tested ODNs appeared to be the same including an ODN composed of -ACCGGT- flanked by 12-guanosines at both 5' and 3' ends. Although no suggestions were made regarding the identity of the ODN receptors, it was shown (13) that negatively charged molecules (fucoidan, poly I and polyvinyl sulfate) inhibited binding. This indicated that Scavenger receptors may participate as B-cell membrane ODN binding proteins. An important observation was made comparing Ps versus Po modification of ODNs containing guanosine "runs" at

either 5' or 3'ends (8). Mouse dendritic cells were treated with various ODNs and supernatants were examined for the presence of IL-12 and TNF-alpha. A six-mer polyguanosine run at the 3' end of a Po (not Ps) CpG ODN produced the greatest immunostimulatory effects. This suggested that single base guanosine runs facilitated binding and uptake of Po modified ODNs. The mechanism proposed was that the guanosine 6-mer formed a G-tetrad (Hoogsteen base pairing). This enabled binding to Scavenger receptor-A on the dendritic cells (8).

Another important issue regarding ODN binding to cells relates to receptor binding versus pinocytosis versus receptor facilitated uptake. These mechanisms of cellular associations were investigated using immobilized ODNs. The two conclusions were that uptake of ODN was not necessary in order to activate human B-cells and that the putative B-cell receptor(s) were yet to be identified (21). It was also suggested that B-cell activation was sequence-specific and not merely dependent on the Po backbone structure of ODNs.

Although certain reports have shown that guanosine-quartet or guanosine rich ODNs inhibited the cell growth of various lines (22-25), most studies demonstrated immunostimulatory effects by either guanosine containing ODNs or by single base ODNs of varying lengths. Guanosine rich non-CpG ODNs ((TTGGAGGGGGTGGTGGGG) caused macrophage-like (mouse) cells to proliferate (26). Polyguanosine runs were added to Po ODNs and tested against murine spleen cells for secretion of IL-12p40, IL-6 and NO production. This substitution produced almost equivalent effects to those produced following stimulation with CpG-Ps ODNs. Both of the ODNs increased the *in vivo* survival of BALB/c mice to *leishmania* infections. These reports pointed out several

disadvantages of Ps modification of ODNs: potential for autoimmune reactions; long-lasting Th-1 bias; sustained IFN-γ production; lymph node swelling; and Ps modification may alter the recognition process thought to be necessary for cellular activation (27). The advantages of addition of guanosine runs onto CpG motifs were stated to include enhancement of cellular uptake of ODNs and increased nuclease resistance (8-9). Ps modified single base containing ODNs were next compared with Ps modified CpG ODNs for binding to the same membrane proteins and for immunoregulatory effects. Pretreatment of mouse DC with 30-mers of single base dA, dT, dG and dC had inhibitory effects on the ability of Ps CpG to activate these cells. The read-outs were IL-12 and NO production. Single base Po modified ODNs were not examined (28). In another study, single base dG30 bound to mouse splenic B-cells and stimulated cellular proliferation. The binding protein was not identified and it was stated that B-cells do not express Scavenger receptors (7). Mouse spleen cells were stimulated to secrete IL12 (IL12p40 detection) by phosphodiester 5G-CpG. RAW264.7 cells secreted IL-6 and produced NO following treatment with the 5G-CpG. *In vivo* studies were also carried out with a *leishmania* model. The Po-5G-CpG augmented the mouse resistance to this parasite (29).

The YT-INDY cell line is a variant of the YT cell line that was originally described as an IL-2-independent NK-like cell line. Although YT cells are cytolytic for several different human target cells, YT-INDY cells do not lyse K562 but instead show a preference for Daudi, Raji and Jurkat targets. YT-INDY cells have been used by many laboratories as *in vitro* models to examine granzyme exocytosis (30), perforin functions

(31), apoptosis (32-33) as well as study mechanisms of NK killing (34-35) and NK signaling (36).

Although these studies have demonstrated that YT and YT-INDY cells are relevant models to investigate aspects of NK cell cytotoxicity and signaling, they have not been used to study activation by bacterial DNA. The effector pathways utilized by NK cells in anti-bacterial resistance have not been described beyond secretion of proinflammatory cytokines (e.g. gamma interferon). In the present study we have identified what may comprise an important new species of DNA binding proteins. These proteins specifically recognize both single base and dinucleotide motif containing oligodeoxynucleotides (Fig. 6.1 and 6.2). Southwestern blot experiments were used to identify the molecular weight and binding specificities of these ODNs and to support previous competition experiments conducted with teleost NCC (16). The present study extended these observations demonstrating that YT-INDY cells contained 18 and 29 kDa ODN binding proteins (Fig. 6.3). Indirect evidence was presented that the YT-INDY P18 protein may be either closely related to or identical to the teleost orthologue that has been sequenced and from which a recombinant protein was expressed (data not shown, see accompanying manuscript). Select functional characteristics of this (recombinant) protein include inhibition of bacterial cell growth (data not shown). In the present study we suggest that the dG20/CpG membrane binding proteins on YT-INDY cells are also associated with cellular signaling activities manifest by demonstration of their effects on cell growth and division and also on calcium mobilization. CpG or dG20 binding caused increased synthesis of YT-INDY DNA and cell proliferation (Fig. 6.5 and 6.6) and calcium mobilization (Fig. 6.7) in these cells.

The mechanism(s) of single base ODNs induced activation of NK cells is not yet known. These ODNs may induce cell activation using same molecular machinery as CpG ODNs. It should be noted that in the present study both CpG and dG20 induced similar responses in YT-INDY cells over short periods of time. Moreover, because the presence of guanosine runs are known to confer nuclease resistance to ODNs, these ODNs may be stably present for longer periods of time. Single base oligodeoxyguanosine may thus provide an alternative approach to study the innate immunity and mechanisms of anti-bacterial resistance both *in vitro* and *in vivo* yet without producing the negative effects of Th1 activation and initiation of autoimmune responses.

References

1. Krieg A. M. 2000. The role of CpG motifs in innate immunity. *Curr. Opin . Immunol*. 12:35.

2. Krieg A. M., A. K. Yi, S. Matson, T. J. Waldschmidt, G. A. Bishop, R. Teasdale, G. A. Koretzky, and D. M. Klinman. 1995. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 374:546.

3. Wagner H. 2002. Interactions between bacterial CpG-DNA and TLR9 bridge innate and adaptive immunity. *Curr. Opin. Microbiol*. 5:62.

4. Benimetskaya L., J. D. Loike, Z. Khaled, G. Loike, S. C. Silverstein, L. Cao, J. el Khoury, T. Q. Cai, and C. A. Stein. 1997. Mac-1 (CD11b/CD18) is an oligodeoxynucleotide-binding protein. *Nat. Med.* 3:414.

5. Ballas Z. K., W. L. Rasmussen, and A. M. Krieg. 1996. Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. *J. Immunol.* 157:1840.

6. Iho S., T. Yamamoto, T. Takahashi, and S. Yamamoto. 1999. Oligodeoxynucleotides containing palindrome sequences with internal 5'-CpG-3' act directly on human NK and activated T cells to induce IFN-gamma production in vitro. *J. Immunol.* 163:3642

7. Pisetsky D. S., and C. F. Reich. 1998. The influence of base sequence on the immunostimulatory properties of defined oligonucleotides. *Immunopharmacology* 40:199-208.

8. Lee S. W., M. K. Song, K. H. Baek, Y. Park, J. K. Kim, C. H. Lee, H. K. Cheong, C. Cheong, and Y. C. Sung. 2000. Effects of a hexameric deoxyriboguanosine run conjugation into CpG oligodeoxynucleotides on their immunostimulatory potentials. *J. Immunol*. 165:3631.

9. Dalpke A. H, S. Zimmermann, I. Albrecht, and K. Heeg. 2002. Phosphodiester CpG oligonucleotides as adjuvants: polyguanosine runs enhance cellular uptake and improve immunostimulative activity of phosphodiester CpG oligonucleotides in vitro and in vivo. *Immunology* 106:102.

10. Ballas Z. K., A. M. Krieg, T. Warren, W. Rasmussen, H. L. Davis, M. Waldschmidt, and G. J. Weiner. 2001. Divergent therapeutic and immunologic effects of oligodeoxynucleotides with distinct CpG motifs. *J. Immunol*. 167:4878.

11. Pearson A. M., A. Rich, and M. Krieger. 1993. Polynucleotide binding to macrophage scavenger receptors depends on the formation of base-quartet-stabilized four-stranded helices. *J. Biol. Chem*. 268:3546.

12. Kimura Y., K. Sonehara, E. Kuramoto, T. Makino, S. Yamamoto, T. Yamamoto, T. Kataoka, and T. Tokunaga. 1994. Binding of oligoguanylate to scavenger receptors is required for oligonucleotides to augment NK cell activity and induce IFN. *J. Biochem. (Tokyo)* 116:991.

13. Liang H., C. F. Reich, D. S. Pisetsky, and P. E. LiPsky. 2000. The role of cell surface receptors in the activation of human B cells by phosphorothioate oligonucleotides. *J. Immunol.* 165:1438.

14. Zhu F. G., C. F. Reich, and D. S. Pisetsky. 2002. Inhibition of murine macrophage nitric oxide production by synthetic oligonucleotides. *J. Leukoc. Biol.* 71:686.

15. Pisetsky D. S, and C. F. Reich. 2000. Inhibition of murine macrophage IL-12 production by natural and synthetic DNA. *Clin. Immunol*. 96:198.

16. Oumouna M., L. Jaso-Friedmann, and D. L. Evans. 2002. Activation of nonspecific cytotoxic cells (NCC) with synthetic oligodeoxynucleotides and bacterial genomic DNA: binding, specificity and identification of unique immunostimulatory motifs. *Dev. Comp. Immunol*. 26:257.

17. Kaur H., L. Jaso-Friedmann, and D. L. Evans. 2003. Identification of a scavenger receptor homologue on nonspecific cytotoxic cells and evidence for binding to oligodeoxyguanosine. *Fish and Shellfish Immunol*. In press.

18. Yamamoto S., T. Yamamoto, T. Kataoka, E. Kuramoto, O. Yano, and T. Tokunaga. 1992. Unique palindromic sequences in synthetic oligohucleotides are required to induce INF and augment INF-mediated natural killer activity. *J. Immunol*. 148:4072.

19. Hemmi H., T. Kaisho, K. Takeda, and S. Akira. 2003. The roles of Toll-like receptor 9, My88, and DNA-dependent protein kinase catalytic subunit in the effects of two distinct CpG DNAs on dendritic cell subsets. *J. Immunol.* 170:3059.

20. Klinman D. M., F. Takeshita, I. Gursel, C. Leifer, K. J. Ishii, D. Verthelyi, and M. Gursel. 2002. CpG DNA: recognition by and activation of monocytes. *Microbes Infect*. 4:897.

21. Liang, H., Y. Nishioka, C. F. Reich, D. S. Pisetsky, and P. E. Lipsky. 1996. Activation of human B cells by phosphorothioate oligodeoxynucleotides. *J. Clin. Invest*. 98:1119.

22. Wang, W., H. J. Chen, J. Sun, L. Benimetskaya, A. Schwarz, P. Cannon, C. A. Stein, and L. E. Rabbani. 1998. A comparison of guanosine-quartet inhibitory effects versus cytidine homopolymer inhibitory effects on rat neointimal formation. *Antisense Nucleic Acid Drug Dev*. 8:227.

23. Saijo, Y., B. Uchiyama, T. Abe, K. Satoh, and T. Nukiwa. 1997. Contiguous four-guanosine sequence in c-myc antisense phosphorothioate oligonucleotides inhibits cell growth on human lun cncer cells: possible involvement of cell adhesion inhibition. *Jpn. J. Canc. Res*. 88:26.

24. Castier, Y., E. Chemla, J. Nierat, D. Heudes, M. A. Vasseur, C. Rajnoch, P. Bruneval, A. Carpentier, and J. N. Faviani. 1998. The activity of c-myb antisense oligonucleotide to prevent intimal hyperplasia is nonspecific. *J. Cardiovasc. Surg. (Torino)* 39:1.

25. White, J. R., E. C. Gordon-Smith, and T. R. Rutherford. 1996. Phosphorothioate-capped antisense oligonucleotides to Ras GAP inhibit cell proliferation and trigger apoptosis but fail to downregulate GAP gene expression. *Biochem. Biophys. Res. Commun.* 227:118.

26. Lang R., L. Hultner, G. B. Lipford, H. Wagner, and K. Heeg. 1999. guanosine-rich oligodeoxynucleotides induce proliferation of macrophage progenitors in cultures of murine bone marrow cells. *Eur. J. Immunol*. 29:3496.

27. Lipford, G. B., T. Sparwasser, S. Zimmermann, K. Heeg, and H. Wagner. 2000. CpG-DNA-mediated transient lymphadenopathy is associated with a state of Th1 predisiposition to antigen-driven responses. *J. Immunol*. 165:1228.

28. Zhu, F. G., C. F. Reich, and D. S. Pisetsky. 2002. Inhibition of murine dendritic cell activation by synthetic phosphorothioate oligodeoxynucleotides. *J. Leuk. Biol.* 72:1154.

29. Zimmermann S., K. Heeg, and A. Dalpke. 2003. Immunostimulatory DNA as adjuvant: efficacy of phosphodiester CpG oligonucleotides is enhanced by 3' sequence modifications. *Vaccine* 21:990.

30. Su, B., M. R. Bochan, W. L. Hanna, C. J. Froelich, and Z. Brahmi. 1994. Human granzyme B is essential for DNA fragmentation of susceptible targets cells. Eur. *J. Immunol*. 24:2073.

31. Bochan, M. R., W. S. Goebel, and Z. Brahmi. 1995. Stably transfected antisense granzyme B and perforin constructs inhibit human granule-mediated lytic ability. *Cell. Immunol*. 164:234.

32. Froelich, C. J., J. Turbov, and W. Hanna, 1996. Human perforin: rapid enrichment by immobilized metal affinity chromatography (IMAC) for whole cell cytotoxicity assays. *Biochem. Biophy. Res. Comm*. 229:44.

33. Montel, A. H., M. R. Bochan, W. S. Goebel, and Z. Brahmi. 1995. Fas-mediated cytotoxicity remains intact in perforin and granzyme B antisense transfectants of a human NK-like cell line. *Cell. Immunol*. 165:312.

34. Montel, A. H., M. R. Bochan, J. A. Hobbs, D. H. Lynch, and Z. Brahmi. 1995. Fas involvement in cytotoxicity mediated by human NK cells. *Cell. Immunol*. 166:236

35. Montel, A. H., P. A. Morse, and Z. Brahmi. 1995. Upregulation of B7 molecules by the Epstein-Barr virus enhances susceptibility to lysis by a human NK-like cell line. *Cell. Immunol*. 160:104

36. Tarazona, R., G. Borrego, M. D. Galiani, J. Pena, J. E. Coligan, and R. Solana. 2001. Inhibition of CD28-mediated natural cytotoxicity by KIR2DL2 does not require $p56^{lck}$ in the NK cell line YT-INDY. *Mol. Immunol*. 38:495.

CHAPTER 7

SUMMARY AND CONCLUSIONS

Bacterial DNA activates cells of both the innate and adaptive branches of the immune system. These effects are attributed to the presence of non-methylated CpG motifs within bacterial DNA. Synthetic oligodeoxynucleotides containing these CpG motifs are used to study the effects of bacterial DNA. Although CpG motifs were the major immunostimulatory component of these ODNs, the sequences flanking these CpG motifs (for example, poly G runs) were shown to have additional synergistic or inhibitory effects on the immunostimulatory activity of the ODNs. These findings raised questions regarding the effects of the non-CpG sequences on cells of the immune system. In the present study the effects of single base ODNs on teleost nonspecific cytotoxic cells (NCC) and human natural killer (NK) cells were studied. Previous work conducted in our laboratory identified the motifs that were active for fish NCC. These motifs included the conventional "CpG motif" and the inverse "GpC motif" (non-stimulatory for mammalian cells). ODNs consisting of a CpG motif(s) flanked by guanosines or a 20-mer of guanosine were also found to enhance the cytotoxic activity of NCC against select tumor target cells. Research in this dissertation further evaluated the mechanism of activation of fish NCC and human NK cells (YT-INDY cell) by the single base oligodeoxyguanosine by identification of the ODN/DNA binding proteins. Certain of these experiments were extended to examine the binding of these unique ODNs to mouse (RAW264.7) and human (THP-1) monocyte-macrophage cell lines.

The tissue distribution (in fish) of ODN binding cells was determined. All single base ODNs were tested. Oligodeoxyguanosine had the highest levels of binding on NCC obtained from anterior kidney, spleen and liver. dC20 had comparatively lower binding levels and dA20 did not bind. The pattern of dG20 binding to YT-INDY, RAW264.7 and

THP-1 cells was similar in that all three cell lines were greater than 80% positive for dG20 binding. Evidence that these cells expressed receptors for dG20 binding was determined by saturation binding studies. Binding to NCC by dG20 was both saturable and specific as determined using flow cytometry and cold competition binding experiments. Binding was inhibited by unlabeled homologous ODN and CpG ODN but not by dC20, dA20 and dT20.

Experiments demonstrated that NCC express several different species of DNA binding proteins. One such dG20 binding protein is Scavenger receptor-A (SR-A). We were the first to report that an NK like cell (i.e. NCC) expressed SR-A. Evidence that this receptor contributed only partially to the total dG20 binding capacity of NCC was confirmed by competition studies with anti-SR antibody. This antibody inhibited only 40% of total dG20 binding in these cells.

Southwestern (ligand) blot experiments were conducted to determine the molecular weight of the ODN binding proteins on NCC, YT-INDY, RAW264.7 and THP-1 cells. Southwestern blots of cell membrane lysates and immunoprecipitation of biotin labeled cell membranes from NCC, RAW 264.7 and THP-1 cells demonstrated that two different mw species (14-18 kDa and 29-34 kDa) of binding proteins existed on these cells. These were crossreactive by Western blot examination with a polyclonal anti-histone-1 antibody. Similarly, two different YT-INDY proteins (29 kDA and 18 kDa) in membrane lysates bound to dG20 and CpG. The 18 kDa protein (P18) was sequenced and a recombinant protein (RP18) was produced. The specificity of dG20 and CpG binding was determined using recombinant P18 (RP18). Immobilized RP18 bound to

soluble dG20 and CpG and competed for their binding to YT-INDY cells (by flow cytometry) and to YT-INDY solubilized membrane proteins (Southwestern blots).

Experiments were next done to determine the activatory potential of the ODNs in fish NCC and human NK cells (YT-INDY cells). NCCRP-1 is the activation marker for NCC. Levels of NCCRP-1 expression on NCC were determined by flow cytometry following treatment with ODNs. ODN dG20 induced the highest increase in the expression of NCCRP-1 by NCC. The other three single base ODNs produced some increase in NCCRP-1 expression level over media control but these levels were much lower than that seen with dG20 treatment. The expression of homologous ODN receptor was examined following treatment with dG20 for 24 h, 36h 48h and 72 h. Treatment of cells with dG20 induced an increase in the number of cells expressing the ODN receptor and at the same time, an increase in the levels of ODN receptor expression (per cell) was seen compared to control cells.

Cell proliferation and activation studies using ODNs were done using YT-INDY cells. dG20 and CpG treatment induced cellular DNA synthesis (i.e. G_1 to S-phase conversion) and dG20 treatment increased cell numbers by approximately 10% at 10 hours post- treatment. The effects of ODNs on the early signaling responses during cell activation were studied by determining calcium mobilization in these cells immediately following ODN treatment. Both dG20 and CpG ODN induced a higher and sustained calcium flux in YT-INDY cells within seconds of treatment compared to media control and dA20 treated cells.

Studies were also carried out to investigate any relationship between ODN binding and regulation of cellular apoptotic functions. Binding of dG20 to NCC

activated increased expression of cytosolic FasL as detected by staining of permeabilized NCC with an anti-human FasL monoclonal. Treatment of FasR bearing HL-60 cells with supernatants from dG20-activated NCC produced hypoploidy and annexin-V binding. That this effect was dependent on FasL was determined by removal of the apoptosis inducing activity from the supernatants with immobilized anti-FasL monoclonal. These results demonstrated that one of the mechanisms by which ODNs up-regulated the cytotoxic activity of cytotoxic cells might be through induction of FasL expression in NCC.

Finally, experiments were conducted to determine possible mechanisms that would enhance the in vivo actions of dG20 compared to other ODNs. Specifically, the DNase sensitivities of these ODNs were evaluated. Each single base ODN was treated with DNase I followed by analysis in agarose gels to determine the degree of ODN fragmentation. dG20 appeared to be more resistant to DNase treatment compared to dC20, dA20 and dT20. These data demonstrated that single base oligodeoxyguanosine may have attained the unique conformation of a G-tetrad or G-quadraplex and as such assume a structure that is more resistant to DNase treatment compared to other ODNs.

In summary, the results of these findings suggest that single base oligodeoxyguanosine is an immunoregulatory molecule. CpG and dG20 bind to an overlapping set of DNA binding proteins. These proteins include low molecular weight proteins (18kDa and 29kDa) in addition to Scavenger receptors. The ability of dG20 to bind and activate these cells may be associated with the unique conformations attained by guanosine runs. dG20 has similar cellular effects to those seen following treatment with

conventional CpG ODNs. These results indicate that dG20 may induce cell activation using the same molecular machinery as CpG ODNs.