Preface

The observation that bacterial DNA, or its synthetic immunostimulatory oligonucleotide (ISS-ODN) analogs containing unmethylated CpG dinucleotide, are potent activators of innate immunity has attracted a wide spectrum of scientists with interest in basic and/or translational research. Like other microbial products, e.g., peptidoglycans, lipopeptides, lipopolysaccharides, or dsRNA, bacterial DNA triggers its immune properties via a member of the TLR family (i.e., TLR9). These immune properties are aimed mainly at providing an immediate defense mechanism in the mammalian host. Bacterial DNA stimulates the production of type-1 cytokines such as IL-12 and IFNs, and enhances the expression of various co-stimulatory ligands such as B7, CD40, and ICAM-1, as well as class I and class II MHC molecules, mainly by and on antigen presenting cells. This wide range of activities contributes to the use of ISS-ODN as a unique adjuvant that induces both Th1 and CTL responses to experimental and relevant clinical antigens. To date, ISS-ODN has been used as an adjuvant in a variety of clinical trials in the fields of infectious disease, allergy, and cancer. Recent data also identified unique immunomodulating properties and antiinflammatory activities induced by ISS-ODN in an antigen-independent fashion. These inhibit allergic inflammation and colitis in various animal models, respectively.

Based on the progress made to date in uncovering the basic biological principles of immune activation by immunostimulatory DNA and the initial encouraging data emerging from related clinical trials, it is predicted that more efforts will be invested in this field by both academia and industry. It is anticipated that in next few years, our knowledge of this area will be further expanded and that potentially important applications derived from this understanding will find their way to various aspects of clinical medicine.

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Historical Perspectives

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1. INTRODUCTION

The concept of immunostimulatory DNA sequence was borne in a long series of studies on BCG-mediated tumor resistance. DNA purified from BCG inhibited the growth of various guinea pig and mouse tumors, and augmented natural killer (NK) cell activity and induced interferons (IFNs) from mouse spleen cells *(1,2)*. Further, we found two remarkable facts that

1. DNAs from bacteria, but not animals and plants, showed the above-mentioned immunogical activity *(3)*.
2. The activity was completely dependent on particular base sequences having CpG motifs *(4)*.

Research interests of immunostimulatory DNA sequences were galvanized in 1995 by the report of Krieg showing murine B-cell activation with bacterial DNA containing CpG motifs *(5)*. Within a short period of time, a huge number of papers have been published in this field, and the study has expanded rapidly and extensively. Now, it includes a number of research fields, for example, host-defense mechanisms against infection, allergy, autoimmune diseases, cytokine networks, plasmid vaccination, and therapeutic application of certain diseases *(6–7,9–11)*. The response of higher animals against immunostimulatory DNA must be the most primitive but important mechanism for self-nonself discrimination against foreign DNA.

2. THE DISCOVERY OF ANTITUMOR ACTIVITY OF DNA FROM BCG

Trials of cancer immunotherapy with BCG were carried out on a worldwide scale in the 1970s and contributed much information to the fields of basic and clinical immunology. During this decade, efforts were made to isolate components of BCG possessing antitumor activity and diminished

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adverse effects. While trying to obtain water-soluble components of BCG, we found that BCG cytoplasm precipitated by streptomycin sulfate contained substances strongly active against guinea pig hepatoma *(1)*.

Streptomycin sulfate precipitate of BCG was a complex of various components—protein, nucleic acid, lipid, and sugar. Repeated injections of this fraction into guinea pigs caused severe anaphylactic shock. To avoid this phenomenon, we further extracted the fraction of streptomycin sulfate precipitate with hot water and found that the heat extract kept a strong ability to inhibit tumor growth and did not cause any adverse effect. This fraction was further purified with multi-step procedures, and finally a fraction designated MY-1 was obtained.

MY-1 composed of 98% nucleic acid (70% DNA, 28.0% RNA) and its protein and sugar content were only 1.3 and 0.2%, respectively. The DNA contained in MY-1 was single-stranded as judged by the results of an ultracentrifuge analysis, a hydroxy apatite column chromatography, and a measurement of temperature-absorbance.

MY-1 showed stronger antitumor activity than the streptomycin sulfate precipitate. No macroscopic inflammatory change was observed at the injection sites of MY-1, although a typical delayed-type inflammatory reaction was seen at the site of BCG injection. DNA contained in MY-1 was essential for the antitumor activity because the fraction of MY-1 digested with RNase showed higher antitumor activity than MY-1, although MY-1 after digestion with DNase that contained mostly RNA had reduced activity. Until recently, DNA had only been considered as the blueprint of life and was thought to be immunologically uniform and essentially inert. MY-1 is unique because its component is mostly nucleic acid and its activity is ascribed to DNA.

3. OLIGODNA SEQUENCES CONTAINED IN MY-1

A gel filtration column chromatography indicated that MY-1 was distributed over a broad range of molecular size, and its elution peak corresponded to 45 bases. To determine whether the immunostimulatory activity of MY-1 was dependent on base sequence, 13 different 45-mer single-stranded oligoDNAs were synthesized and evaluated their immunostimulatory activity to augment natural killer (NK) cell of normal mouse spleen cells *(12)*. Six of the 13 oligoDNAs, i.e., A3, A4, A6, A7, M3, and alpha-1, showed strong activity, whereas the others did not. Two oligoDNAs, A4 and A2, were selected as the representative of active and inactive oligoDNA, respectively. The cytotoxicity of the spleen cells was elevated remarkably by A4 in a dose-dependent manner, although the cells incubated with A2 showed

no significant change in the activity at any concentration. The palindromic sequence (5'-GACGTC-3') was replaced with each of the 63 theoretically possible 6-mer palindromes in the sequence of A4a, 5'-accgatGACGTCgcc ggtgacggcaccacg-3', and the resulting A4a analog were assayed for the ability to enhance NK cell activity *(13)*. Only 8 oligoDNAs including one of the following palindromic sequences: AACGTT, AGCGCT, ATCGAT, CGATCG, CGTACG, CGCGCG, GCGCGC, and TCGCGA, showed the stronger activity than that of A4a. All the potent palindromes included one or more 5'-CpG-3' motif(s).

4. IMMUNOSTIMULATORY ACTIVITY OF THE DNA PREPARED FROM VARIOUS SOURCES

The DNA-rich fraction from six species of bacteria, namely *Streptomyces aureofaciens*, *Mycobacterium bovis BCG*, *Pseudomonas putida*, *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus*, exhibited the immunostimulatory activity similar to MY-1, but DNA from calf thymus and salmon testis did not *(3)*. In addition to these eight DNA fractions tested, 23 kinds of DNA samples, all of which were extracted from various sources by the Marmur's method, were examined for augmentation of NK cells and induction of IFN in vitro. Each of the DNA samples from *Mycrococcus lysodeikticus*, *Mycobacterium bovis BCG*, *Escherichia coli*, and *Mycoplasma pneumoniae* strongly augmented NK activity and induced interferon (IFN). Biological activities of the DNA sample from *Clostridium perfringens* were relatively low, but were statistically significant. The DNA sample from X 174 phage showed strong activities, and that from adenovirus exhibited less but significant activities. In the DNA samples of the four species of invertebrate, the sample from silkworm showed strong activities, and those from sea urchin, lobster, and mussel showed less but significant activities. In contrast, all of the DNA samples from 10 different species of vertebrate, including five of mammal, and from two species of plant exhibited no activity (Fig. 1).

The activity of the bacterial DNA fractions were not influenced by the presence of polymixin B, an inhibitor of lipopolysaccharide (LPS), and were observed even in the spleen cells from LPS-insensitive C3H/HeJ mice, indicating that the activity could not be attributable to possibly contaminating LPS. The profiles of agarose-gel electrophoresis were essentially the same in all of the DNA fractions. UV absorbance at 260nm of MY-1 and the DNA from calf thymus decreased to the same proportion by DNase treatment. The (G + C) content of the bacterial DNAs used varied from more than 70% to less than 30%, all of which were active, and those of calf and salmon DNA

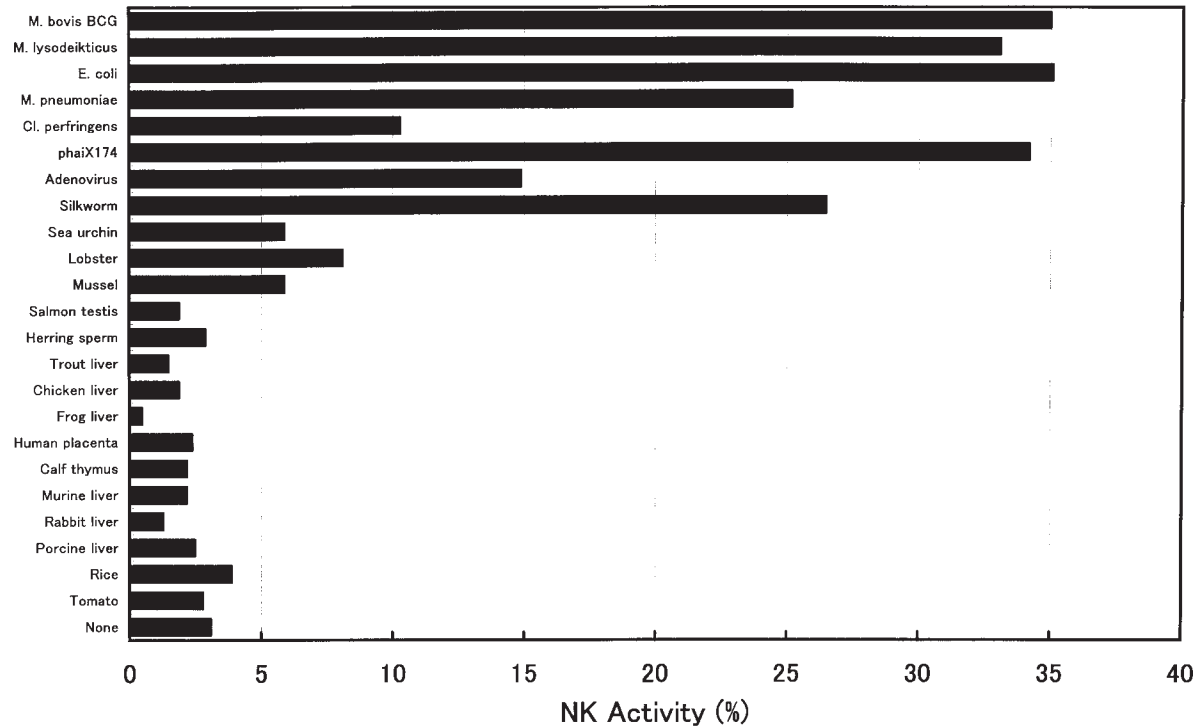

Fig. 1. Augmentation of NK activity by the DNA sample from various sources. BALB/c mouse spleen cells (1×10^7/mL) were incubated with 10 μcg/mL of each of the DNA samples for 20 h, and centrifuged. The cell fractions were assayed for NK activity to measure by a 4 h 51Cr-release assay against YAC-1 lymphoma cells as target cells. M.bovis: *Mycobacterium bovis*; M. lysodeikticus: *Micrococcus lysodeikticus*; E. coli: *Esherichia coli*; M. pneumoniae: *Mycoplasma pneumoniae*; Cl. perfringens: *Clostridium perfringens.*

were 50.2% and 40.2%, respectively. No correlation between (G + C) content and immunostimulatory activity of DNA was found. Methylation of the cytosine of AACGTT resulted in a significant decrease of its activity *(14)*. We also found that the incubation of *Escherichia coli* DNA with CpG methylase reduced the IFN-inducing activity with a lapse of incubation time. We surveyed the incidence frequency of the nine potent palindromic sequences in some of the cDNA sequences in the GenBank DNA Data Base; we chose one or more sequences at will from the cDNAs of 17 species. The summed incidences of the potent palindromic sequences in all of the cDNA sequences from vertebrates and plants were less than 1.0 in 1000 base-pairs, whereas those from most of the bacterial, viruses, and silkworm were larger than 1.0. There were some exceptions; the incidence, for instance, in the cDNA sequences from *Mycoplasma pneumoniae* and *Clostridium perfringens* was very low (0.4–0.2), but their activities were high. These discrepancies may be due to our limited analysis of only the tiny parts of the huge genomic DNA. The incidence of particular 8-, 10- or 12-mer palindromic sequences, which show stronger immunostimulatory activity than particular hexamer *(14)*, was not taken into account, either. Bird described that CpG in bulk vertebrate DNA occurs at about one-fifth of the expected frequency *(15)*. We think that the different frequency of potent sequences in DNA between vertebrate and invertebrate DNA must be another reason for the difference in activities.

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