Jpn. J. Infect. Dis., 55, 37-44, 2002

Invited Review

Discovery of Immunostimulatory CpG-DNA and Its Application to Tuberculosis Vaccine Development

Saburo Yamamoto[1]*, Toshiko Yamamoto[1,2], Yasuhiro Nojima[1], Kiyoko Umemori[1], Susan Phalen[2], David N. McMurray[2], Etsuro Kuramoto[3], Sumiko Iho[4], Rumiko Takauji[4], Yukio Sato[5], Takeshi Yamada[6], Naoya Ohara[6], Sohkichi Matsumoto[7], Yoshitaka Goto[8], Kazuhiro Matsuo[9] and Tohru Tokunaga[10]

[1]*National Institute of Infectious Diseases, Gakuen 4-7-1, Musashimurayama, Tokyo 208-0011,*
[2]*Texas A & M University Health Science Center, College Station, Texas, USA,*
[3]*Mitsui Pharmaceuticals, Inc., Togo, Mobara, Chiba 297-0017,*
[4]*Fukui Medical University School of Medicine, Matsuoka-cho, Yoshida, Fukui 910-1193,*
[5]*Fukushima Medical University School of Medicine, Hikarigaoka, Fukushima 960-1295,*
[6]*Nagasaki University School of Dentistry, Sakamoto, Nagasaki 852-8523,*
[7]*Osaka City University School of Medicine, Asahi, Abeno-ku, Osaka 545-8585,*
[8]*Miyazaki University, Faculty of Agriculture, Gakuen Kibanadai Nishi, Miyazaki 889-2192,*
[9]*Japan Science and Technology Cooperation, AIDS Vaccine Project Office, Nonthaburi 11000, Thailand and*
[10]*Fukuoka Jo-Gakuin University, Osa, Minami-ku, Fukuoka 811-1313, Japan*

(Received April 22, 2002)

CONTENTS:

SUMMARY: DNA containing an unmethylated CpG motif has a potent immunostimulatory effect on the vertebrate immune system. Because such CpG motifs are relatively common in bacterial DNA, but rare in mammalian animal and plant DNA, they may be an evolutionary adaptation augmenting innate immunity, most likely in response to pathogens that replicate within the host cells, such as viruses and intracellular bacteria. Microbial infection induces innate immunity by triggering pattern-recognition systems. The infected cells produce proinflammatory cytokines that directly combat microbial invaders and express costimulating surface molecules, which develop adaptive immunity by inducing distinct T cell differentiation. Bacterial DNA with unmethylated CpG-DNA stimulates vertebrate immature immune cells to induce maturation and to produce TNF-α as well as Th1-type cytokines, IL-12 and IFN-γ. Therefore, CpG-DNA functions as an adjuvant for regulating the initiation of Th1 differentiation. The roles of immunostimulatory CpG motifs in DNA vaccine developments and in therapeutic applications have been discussed.

*Corresponding author: Tel: +81-42-561-0771, Fax: +81-42-565-3315, E-mail: saburo@nih.go.jp

This article is an Invited Review based on a lecture presented at the 11th Symposium of the National Institute of Infectious Diseases, Tokyo, 21 May 2001.

1. Introduction

Mycobacterium bovis BCG (BCG) has been used as a viable bacterial vaccine against tuberculosis since the 1920s. In addition, BCG appears to elicit protective cell-mediated immune responses and has greatly contributed to tumor immunotherapy (1-6). Viable BCG has been thought to be effective in inducing tumor regression, and the trials have

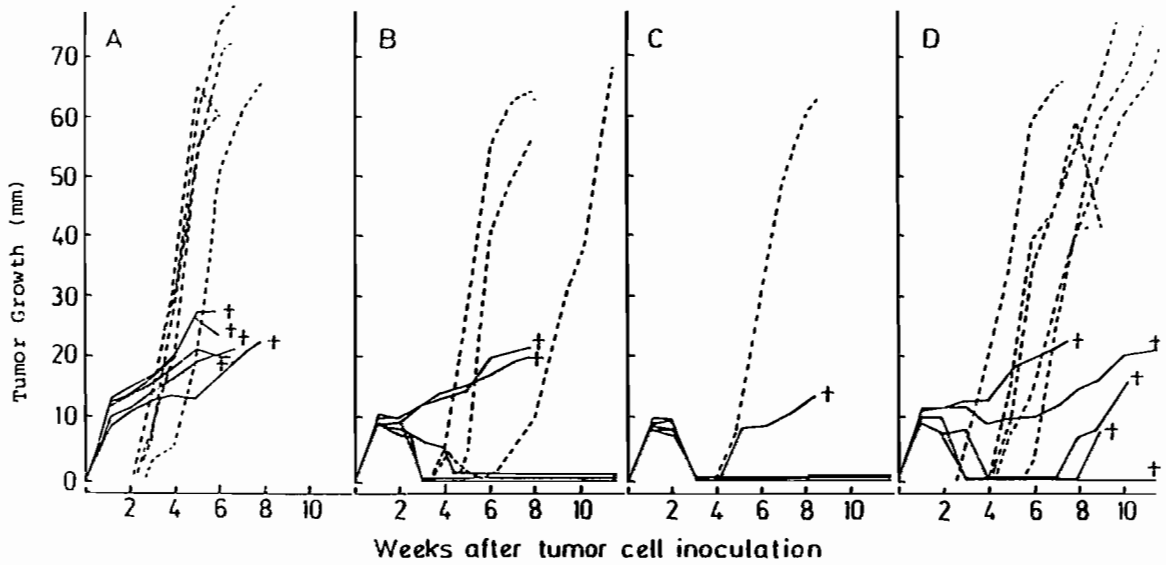

Fig. 1. Antitumor effects of MY-1, RNase digest of MY-1, and DNase digest of MY-1 on Line-10 guinea pig hepatoma. Strain-2 guinea pigs were intradermally inoculated with 10^6 Line-10 hepatoma cells in the left flanks. Seven days after cell implantation, tumors approximately 10 mm in diameter were palpable; and B; MY-1, C; RNase-digest of MY-1, D; DNase-digest of MY-1 at 500 μg in PBS or A; PBS alone was repeatedly injected into the tumor nodules every other day. The animals were examined for tumor growth (solid lines) and metastasis (dotted lines) twice a week as shown on the vertical axis.

resulted in the development of BCG therapy against bladder cancer (7-8). Many investigators have also attempted to isolate components of BCG possessing antitumor activity and producing few side effects. Tokunaga et al. first demonstrated that BCG cytoplasm precipitated by streptomycin (SM-ppt) contains a substance that strongly inhibits mouse and guinea pig syngeneic tumors. Through further purification, a nucleic acid-rich fraction possessing strong antitumor activity but producing few adverse effects in experimental animals has been obtained (9-10). This fraction is designated as MY-1 and is 98% nucleic acids (70% DNA, 28.0% RNA). DNA contained in MY-1 appears to be essential to the antitumor activity, because the RNase-digest of MY-1 shows higher antitumor activity than MY-1, while the DNase-digest of MY-1 has reduced activity (Fig. 1). MY-1 is unique because its component is mostly nucleic acids and its activity is ascribed to DNA. No direct cytotoxicity of MY-1 has been observed, and it is suggested that the antitumor activity of MY-1 is due to its immunostimulatory activity.

2. Immunostimulatory sequences

MY-1 is distributed over a broad range of molecular size, and its elution peak corresponds to 45 bases on gel filtration column chromatography (11). To determine whether the immunostimulatory activity of MY-1 is sequence-dependent, 13 kinds of 45-mer single-stranded oligodeoxynucleotide (ODN) were synthesized based on having sequence randomly selected from the known cDNA-encoding BCG proteins. Interestingly, six of the 13 ODNs were found to induce strong immunostimulatory activity to augment murine NK cell activity, while the others were not (11). The activity of spleen cells is elevated remarkably by BCG-A4, one of the active ODNs, while the cells incubated with BCG-A2, one of the inactive ODNs, show no significant change in activity. BCG-A4a, a 30-mer ODN with a sequence beginning from the 5′-terminus of BCG-A4, also activates NK cells as well as BCG-A4 (Table 1). The activity of BCG-A4b, which is a 30-mer fragment of the 3′-terminus of BCG-A4, is much less than that of BCG-A4a. These results suggest that BCG-A4 and BCG-A4a contain an active sequence. We pointed out that hexamer palindromic sequence is the case and is essential for immunostimulation of the cells augmenting the NK cells (10). In order to determine the minimal and essential sequences responsible for the immunostimulatory activity of ODNs, the palindromic sequence, GACGTC, in an active 30-mer ODN, 5′-accgat GACGTC gccggt gacggc accacg-3′ (BCG-A4a) was replaced with each of the 6-mer palindromic sequences. More than 100% relative activity was observed in the eight ODNs with one of the following palindromic sequences: AACGTT, AGCGCT, ATCGAT, CGATCG, CGTACG, CGCGCG, GCGCGC, and TCGCGA. All the potent palindromes included one or more CpG motif(s). In contrast, palindromes composed entirely of adenine (A) and thymine (T) and those with a sequence of either Pu-Pu-Pu or Py-Py-Py were generally unfavorable for the activity (12,13). Krieg et al. also noted the importance of CpG motif as the basic structure required for B cell activation, and stated that CpG dunucleotide flanked by two 5′ purines (preferably a GpA dunucleotide) and two 3′ pyrimidines (preferably a TpT) is optional (14). Ballas et al. pointed out that palindromic sequences are not always necessary for NK activation (15).

Sequences flanking hexamer palindromes are also meaningful. The NK-augmenting activity of ODNs was compared among four types of single-stranded 30-mer homo-oligomers with a potent palindromic sequence, such as AACGTT, CGATCG, GACGTC, or ATCGAT at the center position (13). No activity was found in the homo-oligomers without a palindromic sequence. Oligo-guanylate (oligo-G) showed the highest activity irrespective of the palindromic sequence included, but oligo-adenylate (oligo-A) and oligo-cytidylate (oligo-C) with the palindrome produced only marginal activity. This was also true of the homo-oligomers including the GACGTC palindrome. In contrast, no activity was found in a 30-mer oligo-G containing an impotent palindrome. These results indicate the independent and cooperative

Table 1. The sequence of ODNs used

Names	Proteins	Nucleotide residue	Base sequence					Immunostimulatory activity
BCG-A1		327-371	AACGAGGGGC	ATGACCCGGT	GCGGGGCTTC	TTGCACTCGG	CATAG	–
BCG-A2		694-738	AAAAGAAGTG	GGGTGCCCCC	ACGATCACCA	ACGATGGTGT	GTCCA	–
BCG-A3		735-779	TCCATCGCCA	AGGAGATCGA	GCTGGAGGAT	CCGTACGAGA	AGATC	+
BCG-A4	64KDa[1]	813-857	ACCGATGACG	TCGCCGGTGA	CGGCACCACG	ACGGCCACCG	TGCTG	+
BCG-A4a	(Antigen-A)	813-842	ACCGATGACG	TCGCCGGTGA	CGGCACCACG			+
BCG-A4b		828-857		GGTGA	CGGCACCACG	ACGGCCACCG	TGCTG	–
BCG-A5		1145-1189	TATGCGGTTC	GACAAGGGCT	ACATCTCGGG	GTACTTCGTG	ACCGA	–
BCG-A6		1552-1596	ACGAGACCAC	CATCGTCGAG	GGCGCCGGTG	ACACCGACGC	CATCG	+
BCG-A7		1962-2006	GCCGAGAAGG	TGCGCAACCT	GCCGGCTGGC	CACGGACTGA	ACGCT	+
BCG-A8		2371-2415	ACCGAGAACA	GCCACGCAGT	CGTGTAGGCA	ACCTTTGGCC	GCTGT	–
BCG-M1	MPB70[2]	1-45	GGCGATCTGG	TGGGCCCGGG	CTGCGCGGAA	TACGCGGCAG	CCAAT	–
BCG-M3		410-455	ACGCCGACGT	CGTCTGTGGT	GGGGTGTCTA	CCGCCAACGC	GACGG	+
BCG-α 1	α-Antigen[3]	348-392	CGACTACAAC	GGCTGGGATA	TCAACACCCC	GGCGTTCGAG	TGGTA	+

[1] 64KDa: 64KDa heat shock protein (Antigen-A), 2431bp
[2] MPB70: MPB70 protein, 492bp
[3] α-Antigen, 1165bp

effects of the palindromic sequence and the extra-palindromic sequence on the activity of ODNs.

The effects of the numbers and locations of the palindromic sequences were also investigated. Among the 30-mer oligo-G nucleotides each containing a varying number of AACGTT palindromes, an ODN with one palindrome showed the strongest activity. The ODN with AACGTT at the 5′-end or the 3′-end showed slightly stronger activity than those with it in the center, although the activity was more influenced by the number of palindromes rather than by the locations.

The ability of immunostimulatory ODNs to induce IFN is also associated with their whole base length. Ten kinds of 12- to 30-mer ODNs having an AACGTT palindromic sequence were examined, and the ODNs of 18 or more bases in length induced the immunostimulatory activity proportionally to the base length, with a maximum at 22-30 bases (16).

3. Bacterial DNA, but not animal or plant DNA, possesses immunostimulatory activity

In 1984, we found that DNA-rich fraction from various species of bacteria exhibit the immunostimulatory activity similar to that of MY-1, but that DNA extracted from calf thymus and salmon testis does not (17). These results were difficult to explain at that time. In addition to the DNA fractions tested thus far, 19 kinds of DNA samples were found to stimulate NK activity and to induce IFN production in mouse spleen cells. Each of the DNA samples from *Mycrococcus lysodeikticus*, *Mycobacterium bovis* BCG, *Escherichia coli*, and *Mycoplasma pneumoniae* strongly augmented NK activity and induced IFN. The biological activity of the DNA sample from *Clostridium perfringens* was relatively low, but was statistically significant. In contrast, all of the DNA samples from 10 different species of vertebrate, salmon testis, herring sperm, trout liver, chicken liver, frog liver, human placenta, calf thymus, murine liver, rabbit liver, and porcine liver, and from four species of plant, rice, tomato, spinach, and parsley exhibited no activity (Fig. 2). Although the data is not shown here, we tested several other DNA samples. All of the DNA from viruses and vertebrates as well as bacterium exhibited immunostimulatory activity, but that from vertebrates, including fish and mammals, showing no activity, and the same conclusion was drawn.

Bird described that between 60 and 90% of CpG in vertebrate genes is methylated at the 5-position on the cytosine ring, and that this accounts for most of the methylcytosine in the vertebrate genome (18). His hypothesis may in part explain the differences in the immunostimulatory activities among DNAs from different sources.

In 1994, we found that certain hexamer ODNs alone can induce IFN when encapsulated in liposome (19). Using this liposome system, we learned that methylation of the cytosine of AACGTT sequence resulted in a significant decrease in its activity (20). We also found that the treatment of *E. coli* DNA with CpG methylase reduced the IFN-inducing activity with a lapse in incubation time, while the *E. coli* DNA treated with buffer alone for the same period and under the same conditions retained the immunostimulatory activity (S. Yamamoto, unpublished data). Next, we synthesized a 30-mer ODN, Oligo-B, 5′-gggggg gggggg AACGTT gggggg gggggg-3′ sequence, and its analogue in which cytosine was replaced with methylated cytosine; the methylated Oligo-B gave only marginal activity in vitro, while the Oligo-B showed strong activity. Further, antitumor effects of ODNs was examined. Oligo-B completely inhibited tumor growth after up to six repeated inoculations, while methylated Oligo-B did not. We also surveyed the incidence frequency of the potent palindromic sequences in some of the cDNA sequences 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 1,000 base-pairs, while those from most of the bacterium, viruses, and silkworm were larger than 1.0. The incidence of particular 8-, 10- or 12-mer palindromic sequences, showing stronger immunostimulatory activity than particular 6-mer palindrome, however, was not taken into account. These sequences of palindrome having an unmethylated CpG core are widely observed in DNAs not only from BCG, but also from bacterium and invertebrate animals, and are rarely present in vertebrate and plant DNAs. Therefore, palindromic sequences with CpG motifs are foreign DNAs for mammalian immunocompetent cells, and this may be one of the reasons why ODNs with these sequences exhibit immunogenicity in murine and human immune systems.

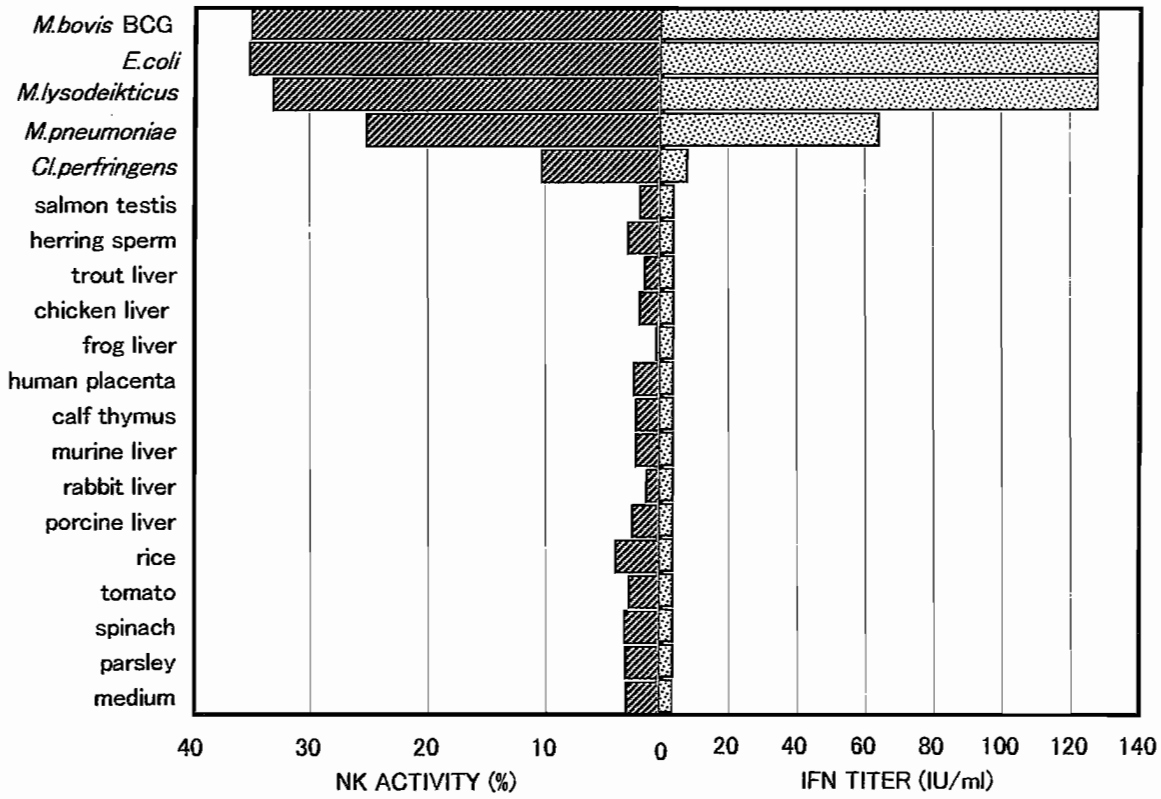

Fig. 2. Augmentation of NK activity and IFN induction by the DNA samples from various sources. Mouse spleen cells (1×10^7/ml) were incubated with 10 μg/ml of each of the DNA samples for 20 h, and centrifuged. The cell fractions were used for NK assay, and the supernatants for IFN assay.

4. Antitumor activity of immunostimulatory CpG-DNA

The antitumor activity of each ODN was examined. A cell suspension of IMC tumor cells was mixed, with each test sample containing 100 μg of the ODN, and the mixtures were intradermally inoculated into (BALB/c x DBA/2)F1 (CDF1) mice. As a control, saline alone instead of test sample was used. At 35 days after inoculation, all mice were killed and the tumors were resected for weighing. When tumor cells were mixed with BCG-A4, tumor growth was markedly suppressed, but BCG-A2 did not significantly affect the tumor growth (21). Next, the effects of repeated injections of the ODNs into established tumors were examined. IMC tumor cells (5×10^5) were inoculated intradermally into CDF1 mice. The ODN test samples (100 μg each) dissolved in 0.1 ml of saline were injected into the tumor lesion twice a week from the 5th day of tumor inoculation. At 35 days after the tumor inoculation, all the mice were killed and the tumors were resected for weighing. It was found that six of the 11 ODNs tested, i.e., BCG-A3, BCG-A4, BCG-A6, BCG-A7, BCG-M3, and BCG-α1, significantly inhibited tumor growth, while the others did not (Fig. 3). The antitumor activity of the active ODNs correlated well with the NK-augmenting and IFN-inducing activity (21). It is noteworthy that the inactive ODNs, BCG-A1, BCG-A2, BCG-A5, and BCG-A8, do not contain a hexameric palindrome, while the active ODNs do possess such a sequence, i.e., GACGTC, GGCGCC, or TGCGCA. The exception is that an inactive ODN BCG-M1, contains an overlapping palindromic sequence (GGGCCCGGG). To test the in vivo effects of the bacterial DNA fraction, mice bearing intradermal IMC tumors were treated with intralesional injections of the DNA fraction. In the group given

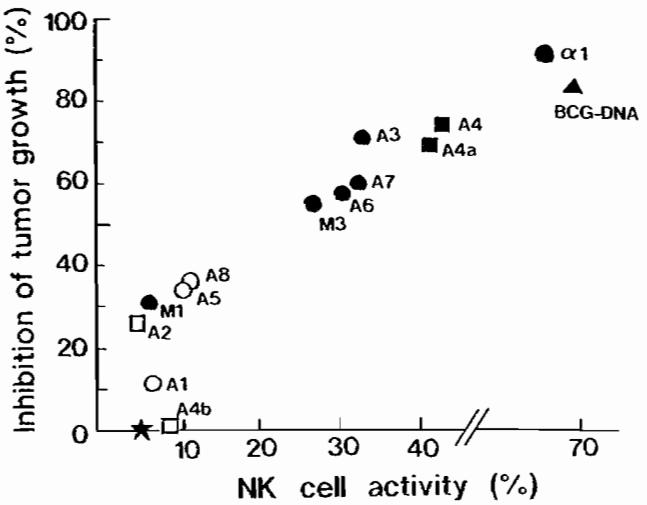

Fig. 3. Correlation between antitumor activity and NK-augmenting activity induced by ODNs. Antitumor activity of ODNs was assessed as the growth inhibition of IMC tumors in CDF1 mice. Mouse spleen cells were incubated with or without ODN (50 μg/ml) for 20 h, and the cells were assayed for NK activity.

phosphate-buffered saline (PBS) alone, tumors grew progressively; on the 35th day, the mean tumor weight was 3.82 ± 1.55 g. In the groups given a DNA fraction from either of the bacteria species, tumor growth was significantly inhibited. For instance, the mean tumor weight in the mice given the DNA fraction from *Streptomyces aureofaciens* was 0.39 ± 0.45 g, with 90% growth inhibition. Seven of eight mice were cured in this group. The DNA fraction from all of the other

bacteria was also effective and showed more than 85% tumor-growth inhibition. On the other hand, little effect was observed in the DNA fraction from the vertebrate cells. The DNA fraction from salmon testis showed moderate tumor-growth inhibition, but no mouse was cured. Although data are not shown here, the bacterial DNA fraction digested with DNase completely lost the antitumor activity; digestion with RNase had no influence.

5. Immunostimulatory activity of CpG-DNA

Profile of cytokine production by mouse BM cells stimulated with CpG-DNA was studied. BALB/c BM cells were incubated with 50 μg/ml CpG-DNA at a concentration of 1×10^7 cells/ml for 20-24 h in a 24-well plate. The culture supernatants were harvested, and the cytokines were measured by the ELISA technique. Both AAC-30 and Oligo-B induced IL-1α, IFN-γ, IL-6, and TNF-α to a similar extent, however, methylation of cytosine in the CpG motif partially reduced production of them. To confirm which cytokines contribute to augmentation of NK activity in mouse BM cells after stimulation with ODNs, the anti-cytokine antibodies were added, respectively, to the cultures of BM cells with ODNs. Monoclonal antibodies against mouse IL-6, IL-12, TNF-α, and IFN-γ and polyclonal antibodies against mouse IFN-α and IL-18 were tested. The treatment of cultures with anti-TNF-α, anti-IL-18 or anti-IFN-γ antibody significantly reduced the NK activity, while that with anti-IL-12 or anti-IFN-α did not. The type of IFN produced by mouse BM cells stimulated with ODNs was determined by the neutralizing effect of anti-IFN antibodies on the bioassay of IFN, and by ELISA technique. As a result, a large amount of IFN-α and a small amount of IFN-β were found in the culture fluid of mouse BM cells. Anti-IFN-γ antibody had no influence on IFN levels; significant levels of IFN-γ were, however, also detected in the culture fluid. In order to clarify the cell type targeted by immunostimulatory CpG-DNA for IFN-α production, we separated human PBC by the magnetic cell-sorting method, and stimulated each subset with potent ODNs, showing that the CD3-CD64-CD4dim cells produce a large amount of IFN-α. A fraction of the blood dendritic cells, the CD3-CD11b-CD16-CD4+ cells, also were found to produce a large amount of IFN-α in response to the ODNs, suggesting that immature cells in the monocyte/dendritic cell lineage of human peripheral blood mononuclear cell (PBMC) produce IFN-α in response to immunostimulatory CpG-DNA (Yamamoto, T. et al., unpublished data). More recently, the CD4+CD3-CD11c-type2 dendritic cell precursors (pre-DC2) known as plasmacytoid DC precursors (PDC) (22) were identified as natural IFN-producing cells in response to viruses, bacteria, and tumor cells (23). Kadowaki et al. also reported that immunostimulatory CpG-DNA produces IFN-α (24). Bauer et al. demonstrated that CpG-DNA promotes the survival and maturation of CD11c-CD123+ PDC (25), but the mechanisms by which CpG-DNA induces IFN-α and stimulates the immune system remain unknown.

6. Intracellular mechanisms of immunostimulatory CpG-DNA

It is well known that DNA binds to the mammalian cell surface in a manner consistent with a ligand-receptor relationship. However, it has not been known whether the nucleotide receptor complexes on the cell surface mediate the signal for IFN or other cytokine induction, or whether the ODNs are just trapped with the receptor and then taken up by endocytosis mechanisms to react with the cytoplasmic or nuclear apparatus. Several studies have demonstrated that modified low density lipoprotein (LDL) receptors, also called scavenger receptors, are expressed on macrophages (26). Oxidized LDL is rapidly taken up by cultured macrophages via scavenger receptor-mediated endocytosis, resulting in foam-cell formation. Scavenger receptors bind not only modified LDL, but also various negatively charged compounds. We have recently found that the binding of some ODNs to macrophages is inhibited by the presence of antagonists for the scavenger receptor, and also that acetyl-LDL binding to the scavenger receptor on mouse macrophages is inhibited by this ODN (27). We also observed that mouse bone marrow cells from scavenger-receptor knockout animals can be stimulated with CpG-ODNs. These results suggest that scavenger receptors are required for the binding of the ODN to cells, but not for the immunostimulatory activity of the ODN. The immunostimulatory effects of CpG ODN do not appear to be mediated through a cell-surface receptor but rather with specific, as yet unidentified, intracellular binding proteins. Thereafter, a number of intracellular signaling pathways are activated, resulting in sequence-specific generation of intracellular reactive oxygen species (ROS), the activation of NF-κB, and the induction of MAP kinases. There is a rapid enhancement of nuclear transcription (15 to 30 min) after exposure to CpG ODN, with the subsequent expression of a large number of early-response genes, proto-oncogenes, and predominantly Th1-promoting cytokines. More recently, it has been reported that the Toll-like receptor (TLR) family is responsible for recognition of microbial cell wall components. The involvement of TLR9 in the recognition of unmethylated CpG-DNA has been shown through the generation of TLR9 knockout mice (28). We have also confirmed that the immunostimulatory ODNs fail to induce IFN and augment NK cells after incubation with mouse BM cells or spleen cells from TLR9 knockout mice. However, it remains unknown how TLRs recognize invading pathogens under physiological conditions. Takeshita et al. reported that TLR9 and CpG ODN are colocalized in the same endosomes (29). Takauji, R. et al. demonstrated that unmethylated CpG-DNA induced IFN-α production in PDC is associated with the enhanced expression of IFN regulatory factor (IRF)-7, and also mentioned that CpG-DNA induces p38 MAPK-dependent phosphorylation of STAT1 in a manner independent of IFN-α/β, which may result in ISGF3 formation increasing the transcription of the IRF-7 gene, leading to IFN-α production in human PDC (30).

7. Immunostimulatory CpG-DNA as immuno-adjuvants for vaccine development

It is generally known that vaccines consisting of viable, attenuated bacterium or virus can induce strong and persistent immunity, but evoke relatively severe adverse effects. In contrast, killed vaccines are relatively safe and induce antibody responses, but hardly ever induce cell-mediated immunity. Efforts have been made to devise effective and safe immuno-adjuvants for vaccines, including microbial components, recombinant proteins thereof, and synthetic peptides. However, only aluminum salts and liposomes have been approved for use in humans. Recently, immunization with antigen-encoding plasmid DNA has been found to effectively induce immune responses in the encoded foreign antigens. The numbers of

plasmids encoding immunogens of bacteria, viruses, parasites, and tumors are now under investigation (31-36). Many of these can induce immunity against infection, and clinical trials for protection or treatment against infection of HIV, herpes, influenza, or hepatitis B viruses and against carcinoma are in progress.

In 1996, Sato et al. reported that plasmid vectors expressing large amounts of gene product do not necessarily induce immune responses in encoded antigens, but those containing short immunostimulatory ODN do (37); an effective plasmid was found to contain two repeats of the palindromic CpG hexamer 5′-AACGTT-3′. Immunostimulatory ODN was also reported to stimulate Th1 responses (38). Many subsequent studies showed that immunostimulatory ODN within the plasmid backbones contributes by stimulating an immune response in the antigens encoded by the plasmids (39-44).

We also examined DNA vaccines including genes encoding mycobacterial protein antigens for their ability to prevent the growth of bacilli in the lungs and spleens of guinea pigs after pulmonary challenge of virulent *M. tuberculosis*. Each gene was inserted into the pACB plasmid from cytomegalovirus and expressed under the control of the promoter, followed by a polyadenylation site from the bovine growth hormone gene (BGH) (Fig. 4). Incorporation of the intron A and polyadenylation site into the plasmid allowed for posttranscriptional processing within the eukaryotic host. Guinea pigs (5 animals/group) were immunized intramuscularly three times in both quadriceps, at 3-week intervals. At each vaccination, guinea pigs were given 100 μg/quadriceps of each DNA vaccine. Negative control animals were injected with saline or control plasmid vector DNA which lacked a gene insert in saline. The positive control group was injected once, intradermally, with *M. bovis* BCG Tokyo at a concentration of 10^3 bacilli on the first day of the immunization. Six weeks after the third immunization with the plasmids, guinea pigs were infected with *M. tuberculosis* by the pulmonary route with a low dose (5-10 CFU) of *M. tuberculosis* H37Rv in a special aerosol chamber originally designed by Dr. Donald W. Smith and colleagues at the University of Wisconsin, Wis., USA, to deliver droplet nuclei directly to the alveolar spaces (45). Five weeks after infection, the animals were euthanized and their spleens and lungs were obtained for enumeration of *M. tuberculosis*. The levels of protection were revealed as the mean $\log_{10}$ CFU of *M. tuberculosis* recovered from the right lower lung lobes or spleens of guinea pigs vaccinated with either plasmid or BCG. In the case of the DNA vaccines, CpG motifs in the plasmids may play an important role in immunostimulatory activity as an intramolecular adjuvant. Immunization with the DNA vaccines containing CpG motifs and encoding the mycobacterial protein antigens elicited protective responses in comparison with a vector control or saline. BCG resulted in a much higher level of protection against mycobacterial infection than the DNA vaccines. BCG has recently been questioned regarding its ability to protect against adult pulmonary tuberculosis. However, immunization with DNA vaccines following BCG vaccination may be useful for a booster of anti-tuberculosis immunity.

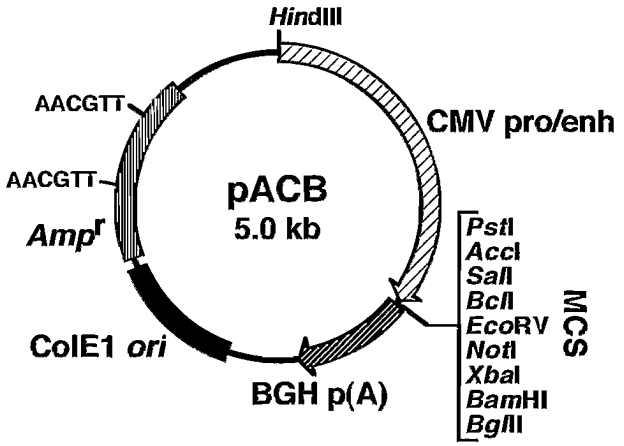

Fig. 4. Map of plasmid pACB. DNA-encoding mycobacterial secreted protein antigens were cloned into a eukaryotic expression vector. Each gene was inserted into the pACB plasmid from cytomegalovirus (CMV) and expressed under the control of the promoter, followed by polyadenylation by bovine growth hormone gene (BGH) (25).

8. Therapeutic applications of immunostimulatory CpG-DNA

Immunostimulatory ODN stimulates immune responses to coadministered antigens, for instance, β-galactosidase and HBs antigen (46), the idiotype of the surface IgM of a B lymphoma (47), intracellular pathogens such as *Listeria monocytogenesis* (48), or formalin-inactivated influenza virus (49). One of the greatest advantages of immunostimulatory ODN as an immunoadjuvant is that immunostimulatory ODN stimulates generation of a Th1-biased immune response to the coadministered antigen or to that encoded by the plasmid. It promotes IFN-α/β, IFN-γ, IL-12, and IL-18, all of which foster Th1 responses and enhance cell-mediated immunity. It is well known that a Th1 immune response conveys protection against infection with mycobacterial infection (50-51). It was reported that immunostimulatory CpG-DNAs protect against infection either with *M. tuberculosis* (52) or *M. avium* (53). Because immunostimulatory ODN is known to induce IL-12 and TNF-α, attempts were made to find oligonucleotide sequences that differentially activate IL-12 versus TNF-α production in antigen-presenting cells, and a single-stranded 18-mer ODN containing a hexamer of GACGTT was proposed as a prototype for a useful ODN without harmful side effects (54).

The dominance of the Th1 response generated by immunostimulatory CpG-DNA may be useful in treating Th2-type allergic diseases (48,55). Kline et al. observed that coadministration of immunostimulatory CpG-DNA and Th2-type response inducing antigen can redirect the immune response to Th1-type and abolish allergic reactions on subsequent antigen challenge (56). MY-1 was also examined as a treatment of allergic disease due to its a downregulation of IgE production (57).

We also reviewed the discovery of immunostimulatory DNA and its clinical studies (58).

ACKNOWLEDGMENTS

This work was partly supported by grants from the Ministry of Health, Labour and Welfare and from the Japan Health Sciences Foundation.

We express our deepest sorrow in the passing of Dr. Tetsuro Kataoka, who had been a collaborator engaged in CpG-DNA studies and who died on December 6, 2000.

The authors would also like to thank S. Toyoo for her secretarial assistance.

REFERENCES

1. Old, L. J., Benacerraf, B., Clarke, D. A., Carswell, E. A. and Stockert, E. (1961): The role of the reticuloendothelial system in the host reaction to neoplasia. Cancer Res., 21, 1281-1300.
2. Mathé, G., Amiel, J. L., Schwarzenberg, L., Schneider, M., Cattan, A., Schlumberger, J. R., Hyyat, M. and Vassal, F. (1969): Active immunotherapy for acute lymphoblastic leukemia. Lancet, I, 697-699.
3. Zbar, B., Bernsterin, I., Tanaka, T. and Rapp, H. J. (1970): Tumor immunity produced by the intradermal inoculation of living tumor cells and living *Mycobacterium bovis* (strain BCG). Science, 170, 1217-1218.
4. Morton, D. L., Eilber, F. R., Malmgren, R. A. and Wood, W. C. (1970): Immunological factors which influence response to immunotherapy in malignant melanoma. Surgery, 68, 158-164.
5. Comstock, G. W., Livesay, V. T. and Webster, R. G. (1971): Leukemia and B.C.G. A controlled trial. Lancet, II, 1062-1063.
6. Tokunaga, T., Yamamoto, S., Nakamura, R. M. and Kataoka, T. (1974): Immunotherapeutic and immunoprophylactic effects of BCG on 3-methyl-cholanthrene-induced autochthonous tumors in Swiss mice. J. Natl. Cancer Inst., 53, 459-463.
7. Morales, A., Eidinger, D. and Bruce, A. W. (1976): Intracavitary Bacillus Calmette-Guerin in the treatment of superficial bladder tumors. J. Urol., 116, 180-183.
8. Lamm, D. L., Thor, D. E., Harris, S. C., Reyna, J. A., Stogdill, V. D. and Radwin, H. M. (1980): Bacillus Calmette-Guerin immunotherapy of superficial bladder cancer. J. Urol., 124, 38-42.
9. Tokunaga, T. et al. (1984): Antitumor activity of deoxyribonucleic acid fraction from *Mycobacterium bovis* BCG. 1. Isolation, physicochemical characterization, and antitumor activity. J. Natl. Cancer Inst., 72, 955-962.
10. Shimada, S., Yano, O., Inoue, H., Kuramoto, E., Fukuda, T., Yamamoto, H., Kataoka, T. and Tokunaga, T. (1985): Antitumor activity of the DNA fraction from *Mycobacterium bovis* BCG. II. Effects on various syngeneic mouse tumors. J. Natl. Cancer Inst., 74, 681-688.
11. Tokunaga, T., Yano, O., Kuramoto, E., Kimura, Y., Yamamoto, T., Kataoka, T. and Yamamoto, S. (1992): Synthetic oligonucleotides with particular base sequences from the cCNA encoding proteins of *Mycobacterium bovis* BCG induce interferons and activate natural killer cells. Microbiol. Immunol., 36, 55-66.
12. Yamamoto, S., Yamamoto, T., Kataoka, T., Kuramoto, E., Yano, O. and Tokunaga, T. (1992): Unique palindromic sequences in synthetic oligonucleotides are required to induce IFN and augment IFN-mediated natural killer activity. J. Immunol., 148, 4072-4076.
13. Kuramoto, E., Yano, O., Kimura, Y., Baba, M., Makino, T., Yamamoto, S., Yamamoto, T., Kataoka, T. and Tokunaga, T. (1992): Oligonucleotide sequences required for natural killer cell activation. Jpn. J. Cancer Res., 83, 1128-1131.
14. Krieg, A. M., Yi, A-K., Matson, S., Waldschmidt, T. J., Bishop, G. A., Teasdale, R., Koretzkey, G. A. and Klinman, D. M. (1995): CpG motifs in bacterial DNA trigger direct B-cell activation. Nature, 374, 546-549.
15. Ballas, Z. K., Rasmussen, W. L. and Krieg, A. M. (1996): Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. J. Immunol., 157, 1840-1845.
16. Yamamoto, T., Yamamoto, S., Katoka, T. and Tokunaga, T. (1994): 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.
17. Yamamoto, S., Yamamoto, T., Shimada, S., Kuramoto, E., Yano, O., Kataoka, T. and Tokunaga, T. (1992): DNA from bacteria, but not from vertebrates, induces interferons, activates natural killer cells and inhibits tumor growth. Microbiol. Immunol., 36, 983-997.
18. Bird, A. P. (1986): CpG-rich islands and the function of DNA methylation. Nature, 321, 209.
19. Yamamoto, T., Yamamoto, S., Kataoka, T. and Tokunaga, T. (1994): Lipofection of synthetic oligodeoxyribonucleotide having a palindromic sequence of AACGTT to murine splenocytes enhances interferon production and natural killer activity. Microbiol. Immunol., 38, 831-836.
20. Sonehara, K., Saito, H., Kuramoto, E., Yamamoto, S., Yamamoto, T. and Tokunaga, T. (1996): Hexamer palindromic oligonucleotides with 5′-CG-3′ motif(s) induce production of interferon. J. Interferon Cytokine Res., 16, 799-803.
21. Kataoka, T, Yamamoto, S., Yamamoto, T., Kuramoto, E., Kimura, Y. and Tokunaga, T. (1992): Antitumor activity of synthetic oligonucleotides with sequences from cDNA encoding proteins of *Mycobacterium bovis* BCG. Jpn. J. Cancer Res., 83, 244-247.
22. Cella, M., Jarrossay, D., Facchetti, F., Alebardi, O., Nakajima, H., Lanzavecchia, A. and Colonna, M. (1999): Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amount of type I interferon. Nature Med., 5, 919-923.
23. Siegal, F. P., Kadowaki, N., Schodell, M., Fitzgerald-Bocarsly, P. A., Shah, K., Ho, S., Antonenko, S. and Liu, Y.-J. (1999): The nature of the principal type I interferon-producing cells in human blood. Science, 284, 1835-1837.
24. Kadowaki, N., Antonenko, S. and Liu, Y.-J. (2001): Distinct CpG DNA and poly I:C double-stranded RNA respectively stimulate CD11c- type 2 dendritic cell precursors and CD11c+ dendritic cells to produce type I interferon. J. Immunol., 166, 2291-2295.
25. Bauer, M., Redecke, V., Ellwart, J. W., Scherer, B., Kremer, J.-P., Wagner, H. and Lipford, G. B. (2001): Bacterial CpG-DNA triggers activation and maturation of human CD11c-, CD123+ dendritic cells. J. Immunol., 166, 5000-5007.
26. Goldstein, J. L., Ho, Y. K., Basu, S. K. and Brown, M. S. (1979): Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol depostion. Proc. Natl. Acad. Sci. USA, 76, 333-337.
27. Kimura, Y., Sonehara, K., Kuramoto, E., Makino, T., Yamamoto, S., Yamamoto, T., Kataoka, T. and Tokunaga, T. (1994): Binding of oligoguanylate to scavenger receptors is required for oligonucleotides to augment NK cell activity and induce IFN. J. Biochem., 116, 991-994.
28. Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K. and Akira, S. (2000): A toll-like receptor recognizes bacterial DNA. Nature, 408, 740-745.
29. Takeshita, F., Leifer, C. A., Gursel, M. and Klinman, D. M.

(2001): Cutting edge: Role of Toll-like receptor 9 in CpG-DNA induced activation of human cells. J. Immunol., 167, 3555-3558.
30. Takauji, R., Iho, S., Yamamoto, S., Takahashi, T., Iwasaki, M., Iida, R., Yokochi, T. and Matsuki, T. (2002): Mechanism of CpG DNA-induced IFN-α production in human dendritic cell precursor representing type I interferon-producing cells. J. Leukocyte. Immunol., (in press).
31. Ulmer, J. B. et al. (1993): Heterologous protection against influenza by injection of DNA encoding a viral protein. Science, 259, 1745-1749.
32. Barry, M. A., Lai, W. C. and Johnston, S. A. (1995): Protection against mycoplasma infection using expression-library immunization. Nature, 377, 632-635.
33. Condon, C., Watkins, S. C., Celluzzi, C. M., Thompson, K. and Falo, L. D., Jr. (1996): DNA-based immunization by *in vivo* transfection of dendritic cells. Nat. Med., 2(10), 1122-1128.
34. Gardner, M. J., Doolan, D. L., Hedstrom, R. C., Wang, R., Sedegah, M., Gramzinski, R. A., Aguiar, J. C., Wang, H., Margalith, M., Hobart, P. and Hoffman, S. L. (1996): DNA vaccines against malaria: immunogenicity and protection in a rodent model. J. Pharm. Sci., 85(12), 1294-1300.
35. Doolan, D. L., Hedstrom, R. C., Wang, R., Sedegah, M., Scheller, L. F., Hobart, P., Norman, J. A. and Hoffman, S. L. (1997): DNA vaccines for malaria: the past, the present, & the future. Indian J. Med. Res., 106, 109-119.
36. Robinson, H. L. et al. (1999): Neutralizing antibody-independent containment of immunodeficiency virus challenges by DNA priming and recombinant pox virus booster immunizations. Nat. Med., 5, 526-534.
37. Sato, Y., Roman, M., Tighe, H., Lee, D., Corr, M., Nguyen, Minh-Duc., Silverman, G. J., Lotz, M., Carson, D. A. and Raz, E. (1996): Immunostimulatory DNA sequences necessary for effective intradermal gene immunonization. Science, 273, 352-354.
38. Roman, M., Orozco, E. M., Goodman, J. S., Nguyen, M. D., Sato, Y., Ronaghy, A., Kornbluth, R. S., Richman, D., Carson, D. A. and Raz, E. (1997): Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. Nat. Med., 3, 849-854.
39. Klinman, D. M., Yamshchikov, G. and Ishigatsubo, Y. (1997): Contribution of CpG motifs to the immunogenicity of DNA vaccines. J. Immunol., 158, 3635-3639.
40. Klinman, D. M. (1998): Therapeutic applications of CpG-containing oligodeoxynucleotides. Antisense Nucleic Acid Drug Dev., 8, 181-184.
41. Lipford, G. B., Beaure, M., Blank, C., Reiter, R., Wagner, H. and Heeg, K. (1997): CpG-containing synthetic oligonucleotides promote B and cytotoxic T cell responses to protein antigen: a new class of vaccine adjuvants. Eur. J. Immunol., 27, 2340-2344.
42. Chu, R. A., Targori, O., Krieg, A. M., Lehmann, P. V. and Harding, C. V. (1997): CpG oligodeoxynucleotide act as adjuvants that switch on T helper 1 (Th1) immunity. J. Exp. Med., 186, 1623-1631.
43. Carson, D. A. and Raz, E. (1997): Oligonucleotide adjuvants for T helper 1 (Th1)-specific vaccination. J. Exp. Med., 186, 1621-1622.
44. Krieg, A. M., Yi, Ae-K., Schorr, J. and Davis, H. L. (1998): The role of CpG dinucleotides in DNA vaccines. Trends Microbiol., 6, 23-27.
45. Wiegeshaus, E. H., McMurray, D. N., Grover, A. A., Harding, G. E. and Smith, D. W. (1970): Host-parasite relationships in experimental airborne tuberculosis. III. Relevance of microbial enumeration to acquired resistance in guinea pigs. Am. Rev. Resp. Dis., 102, 422-429.
46. Leclerc, C., Deriaud, E., Rojas, M. and Whalen, R. G. (1997): The preferential induction of a Th1 immune response by DNA-based immunization is mediated by the immunostimulatory effect of plasmid DNA. Cell. Immunol., 179, 97-106.
47. Weiner, G. J., Liu, H. M., Wooldrodge, E. J., Dahle, C. E. and Krieg, A. M. (1997): Immunostimulatory oligonucleotides containing the CpG motif are effective as immune adjuvants in tumor antigen immunization. Proc. Natl. Acad. Sci. USA, 94. 10833-10837.
48. Zimmermann, S., Egeter, O., Hausmann, S., Lipford, G. B., Rocken, M., Wagner, H. and Heeg, K. (1998): CpG oligodeoxynucleotides trigger protective and curative Th1 responses in lethal murine leishmaniasis. J. Immunol., 160, 3627-3630.
49. Moldoveanu, Z., Love-Homan, L., Huang, W. Q. and Krieg, A. M. (1998): CpG DNA, a novel immune enhancer for systemic and mucosal immunization with influenza virus. Vaccine, 16, 1216-1224.
50. Cooper, A. M., Magram, J., Ferrante, J. and Orme, I. M. (1997): Interleukin 12 (IL-12) is crucial to the development of protective immunity in mice intraveneous infected with *Mycobacterium tuberculosis*. J. Exp. Med., 186, 39-45.
51. Flynn, J. L., Chan, J., Triebold, K. J., Dalton, D. K., Stewart, T. A. and Bloom, B. R. (1993): An essential role for interferon gamma in resistance to *Mycobacterium tuberculosis* infection. J. Exp. Med., 178, 2249-2254.
52. Juffermans, N. P., Leemans, J. C., Florquin, S., Verbon, A., Kolk, A. H., Speelman, P., van Deventer, S. J. H. and van der Poll, T. (2002): CpG oligodeoxynucleotides enhance host defense during murine tuberculosis. Infect. Immun., 70, 147-152.
53. Hayashi, T., Rao, S. P., Takabayashi, K., van Uden, J. H., Kornbluth, R. S., Baird, S. M., Taylor, M. W., Carson, D. A., Catanzaro, A. and Raz, E. (2001): Enhancement of innate immunity against *Mycobacterium avium* infection by immunostimulatory DNA is mediated by indoleamine 2,3-dioxygenase. Infect. Immun., 69, 6156-6164.
54. Lipford, G. B., Sparwasser, T., Bauer, M., Zimmermann, S., Koch, E. S., Heeg, K. K. and Wagner, H. (1997): Immunostimulatory DNA: sequence-dependent production of potentially harmful or useful cytokines. Eur. J. Immunol., 27, 3420-3426.
55. Pisetsky, D. S. (1996): Immune activation by bacterial DNA: A new genetic code. Immunity, 5, 303-310.
56. Kline, J. N., Waldschmidt, T. J., Businga, T. R., Lemish, J. E., Weinstock, J. V., Thorne, P. S. and Krieg, A. M. (1998): Modulation of airway inflammation by CpG oligodeoxynucleotides in a murine model of asthma. J. Immunol., 160, 2555-2559.
57. Fujieda, S., Iho, S., Kimura, Y., Sunaga, H., Igawa, H., Sugimoto, C., Yamamoto, S. and Saito, H. (1999): DNA from *Mycobacterium bovis* Bacillus Calmette-Guerin (MY-1) inhibits immunoglobulin E production by human lymphocytes. Am. J. Respir. Crit. Care Med., 160, 2056-2061.
58. Tokunaga, T., Yamamoto, T. and Yamamoto, S. (1999): How BCG led to the discovery of immunostimulatory DNA. Jpn. J. Infect. Dis., 52, 1-11.