of optimization to mammalian cells, but encoding an identical CTL epitope derived from *L. monocytogenes*, LLO 91-99 or a murine malaria parasite, *Plasmodium yoelii*, PyCSP 281-290 and showed that the codon optimization level of the genes is not precisely proportional to, but does correlate well with the translational efficiency in mammalian cells. The results also correlated with the induction level of specific CTL response in the mouse [43].

The similar effect of codon optimization was noted in murine DNA immunization model against human immunodeficiency virus type 1 (HIV-1) infection, by using the HIV-1 gp120 [44], gp160 [45] or gag [46]-encoding genes, and also in murine malaria DNA vaccine encoding *Plasmodium falciparum* merozoite protein [47].

(4) Targeting DNA to APC and enhancing antigen presentation ability of APC

Induction of specific T cells requires antigen presentation by professional APC. Therefore, introduction of target genes specifically into professional APC by DNA immunization may be useful for inducing T cells efficiently. From this intention, gene gun immunization method would be of choice, as the method allows the genes go into dermal dendritic cells, namely Langerhans cells directly. Boyle et al. [48] reported that DNA vaccines encoding human immunoglobulin attached to CTLA-4 molecule, which allows targeting the antigen to APC, enhanced antibody production against the immunoglobulin, although the same approach did not enhance CTL responses [49]. In another approach, You et al. [50] evaluated a DNA vaccine for expression of a model hepatitis B virus *e* antigen fused to an IgG Fc fragment. They showed that the secreted fusion proteins are efficiently captured and processed by dendritic cells via receptor-mediated endocytosis and then presented to the MHC class I and class II molecules.

Efficient antigen presentation requires accessory molecules expressed in APC. Latouche and Sadelain [51] showed that B7.1, ICAM-1, and LFA-3 molecules are most important for antigen presenting capacity. Co-administration of these accessory molecules will improve immunization effects of DNA immunization. In an analogous manner, the activation of T cells was facilitated by co-administration of plasmids encoding CD40 ligand and/or cytokines [52].

(5) Facilitating antigen processing and presentation

I. CTL-Oriented DNA vaccines

(1) CTL-Epitope minigene DNA vaccines and the effect of addition of the leader sequence on CTL induction

A variety of studies has been performed for the efficient induction of CTL of a particular specificity and is summarized in Figure 4. As mentioned before, we demonstrated that the minigene DNA vaccine encoding only a dominant CTL epitope of *L. monocytogenes*, LLO 91-99, was effective for inducing CTL in vivo by gene gun-mediated DNA immunization [42, 43]. This result suggests that the DNA vaccine plasmids are directly incorporated in APC and that the APC present target peptides/molecules to T cells by DNA immunization. Cho et al., however, suggested that cross-priming is a predominant mechanism for inducing $CD8^+$ T cell responses in gene gun DNA immunization [53].

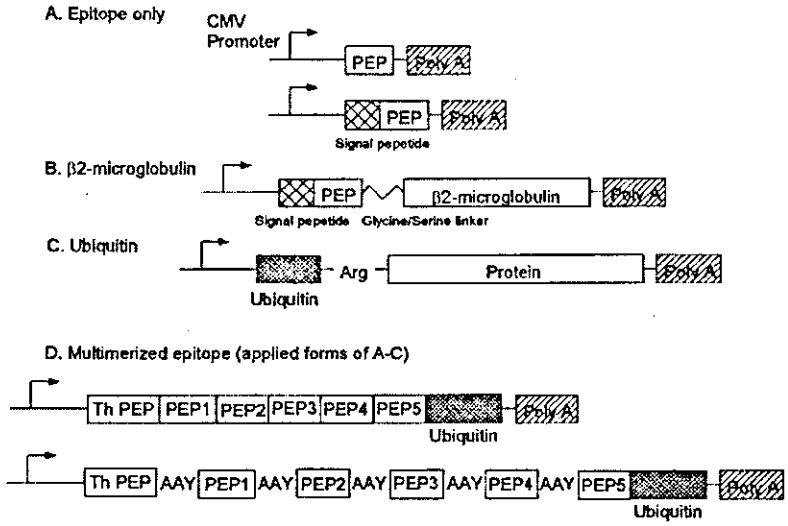

Figure 4. Schematic presentation of several CTL-oriented DNA vaccines. Gene structures of CTL-oriented DNA vaccines are shown. See following references, Uchijima et al. [42], Ciernik et al. [54] for A, Uger and Barber [56] for B, Wu and Kipps [60] for C, and Velders et al. [72] for D.

Ciernik et al. [54] added a DNA sequence encoding adenovirus E3 leader sequence to mutant p53 minigene (not the strict CTL epitope alone, but CTL epitope-covering portion) and constructed the minigene plasmid and showed that the plasmid DNA vaccination is capable of eliciting CTL against mutant p53 efficiently. Addition of the leader sequence will let the target polypeptides to the ER and subsequently, the CTL epitope of the mutant p53 peptide may bind to MHC class I molecules there. They reported addition of the leader sequence dramatically induce the specific CTL activity. They examined mutant p53 peptide whose length is 18 amino acids. The peptide has to be trimmed before binding to MHC class I molecule in the ER. In such a case, accumulation of the peptide in the ER by virtue of the leader sequence facilitate the binding of the peptide and MHC class I molecules in the ER. On the contrary, LLO 91-99 peptide used in our study [42, 43] is a 9mer peptide that further trimming is not necessary. The difference would explain why CTL minigene DNA vaccine induce specific CTL efficiently without addition of the leader sequence in our studies, although Iwasaki et al. [55] reported that addition of the leader sequence still enhances the magnitude of the CTL responses by a minimal CTL epitope DNA vaccine with both intramuscular and gene gun-mediated administration.

(2) Fusion with β2-microglobulin

Uger and Barber [56] prepared an expression plasmid of influenza virus NP 366-374 ($H2\text{-}D^b$ restricted CTL epitope) fused with β2-microglobulin molecule by a glycine/serine short linker. They introduced the plasmid into a murine cultured cell line and showed the efficient loading of the CTL epitope on MHC class I molecules. Their study is not an in vivo study, but the plasmid may be usable as DNA vaccine for CTL induction.

(3) Ubiquitination of target genes

In general, intracellular protein antigens are processed into the peptides for antigen presentation via MHC class I molecules as mentioned before. The peptides have been reported to be generated mainly via the ubiquitin-proteasome pathway although the ubiquitin-independent pathways also have been suggested [57, 58]. Ubiquitin is a 76-amino-acid peptide involved in controlling the normal protein intracellular turnover in the cytoplasm of eukaryotic cells. Ubiquitination of cellular proteins occurs in a lysine residue of the proteins in an ATP-dependent manner. And the ubiquitinated proteins have been reported to be sensitive to degradation by proteasomes. The identity of the N-terminal residues of proteins is a determinant whether the proteins are sensitive or resistant for degradation by proteasomes (N-end rule; 59). For example, if the N-terminal residue is methionine, the protein is stable. And if that is arginine, the protein is destabilizing.

So far, DNA vaccines that enhance the ubiquitin-proteasome degradation of target antigens have been reported. Wu and Kipps [60] prepared an expression plasmid for chimeric β-gal-*LacI* partial segment containing two lysine residues (served as a substrate of ubiquitination) and also an arginine residue were added in the N-terminal region (pUb-Arg-*lacZ*). They used this fusion plasmid as a DNA vaccine and showed efficient induction of β-gal-specific CTL. Interestingly, pUb-Arg-*lacZ*-transfected P815 cells (a murine mastocytoma cell line) did not have β-gal enzymatic activity, probably due to the degradation by proteasomes. Whitton's group [61, 62] also showed similar results by using expression plasmids for ubiqitinated NP derived from lymphocytic choriomeningitis virus (LCMV). These results indicate the ubiquitinated strategy is useful to generate the enhanced CTL activity and to reduce the production of the antigen-specific antibodies. However, Fu et al. [63] reported that the modifications of NP DNA vaccine with ubiquitin conjugation did not affect their ability to induce a specific CTL response, indicating ubiquitin conjugation alone does not guarantee the improved targeting of endogenously synthesized antigens for MHC class I antigen processing pathway.

Injection of a single CTL epitope minigene DNA will generate a single CTL epitope peptide, which is supposed to enter the ER through TAP transporter. Our results [42, 43] indicated such a single minigene DNA immunization without any modification induced specific CTL efficiently. Interestingly, Rodriguez et al. [64] showed even such a minigene DNA immunization improved by ubiquitination in terms of induction of high frequency of memory CTL, suggesting that ubiquitination allows more effective delivery of the minigene to the proteasome. They used a somewhat longer minigene encoding 32 amino acid residues covering two MHC class I binding peptide-encoding regions. In such a case, further trimming of peptides is necessary for the binding on MHC class I molecules as in the previously-mentioned minigene DNA vaccine construct described in Ciernick et al. [54]. This is a reason why ubiquitination improved CTL induction by their minigene DNA vaccination.

(4) Fusion with a carrier protein

As one of other approaches for efficient induction of $CD8^+$ T-cell subset, Wolkers et al. [65] recommended a carboxyl-terminal fusion of CTL epitope to a carrier protein of foreign origin. They constructed the DNA vaccines encoding a carboxy-terminal fusion

of either H2-D^b-restricted CTL epitope, NP 366-374 derived from influenza virus, or H2-D^b-restricted E7 49-57 derived from human papilloma virus to green fluorescent protein (GFP) and showed the DNA vaccines induced much larger clonal size of antigen-specific $CD8^+$ CTL by intramuscular immunization of these DNA vaccines compared with the clonal size induced by these epitope minigene DNA vaccination. The purpose of the GFP fusion strategy was the provision of $CD4^+$ T cell help through recognition of GFP-encoded $CD4^+$ T cell epitopes. Maecker et al. [66] also showed that CTL induction by both intramuscular and intradermal DNA administration is dependent upon the generation of $CD4^+$ T cell help via a class II MHC-dependent pathway. Our results showed CTL minigene DNA vaccination with gene gun induced specific CTL without any $CD4^+$ T cell help [67]. Potential explanations for this discrepancy include the following: (a) the polyepitope vaccines could lead to the assembly of neoepitopes that served to generate MHC class-II help; (b) CpG sequences can potently activate DCs in a nonspecific manner and prime $CD8^+$ T cells in the absence of CD4 help; and (c) CpG motifs induce IFN-α, a cytokine shown to be important in expansion of $CD8^+$ T cells. We reasoned that the route of naked DNA immunization is critical for necessity of CD4 T cell help. So far, intramuscular or intradermal DNA administration was carried out in many reports which show that T-cell help is important for CTL induction by DNA immunization. On the other hand, gene gun-based DNA immunization was carried out in many reports which show that CTL induction occurred independent of CD4 T-cell help.

(5) Multimerized CTL epitope DNA vaccines

In the corollary of a single CTL epitope minigene DNA vaccination, several reports tried multimerized CTL epitope DNA vaccines (polyepitope DNA vaccines). Polyepitope vaccine was first evaluated by Whitton et al. [68]. They generated a recombinant vaccinia virus system for expression of CTL epitope minigenes tandemly fused in a "string-of-beads" manner and showed that this "string-of-beads" vaccine can induce CTL specific to each different epitope and protect on different MHC backgrounds. Subsequently, Thomson et al. [69] constructed a DNA vaccine plasmid containing 10 contiguous minimal CTL epitopes, which were restricted by five MHC alleles and derived from five viruses (influenza virus, adenovirus, murine cytomegalovirus, Sendai virus, and lymphocytic choriomeningitis virus), a murine malaria parasite (*Plasmodium berghei*), and a tumor model antigen (ovalbumin). And they injected mice with the plasmid by intramuscular injection or gene gun-mediated intradermal injection. They showed that the DNA vaccination successfully induced each epitope-specific CTL activity. Results of our single CTL epitope DNA vaccine indicated that a single dominant CTL epitope is sufficient for the induction of protective immunity [70], suggesting selection of the most dominant CTL epitope for each pathogen is critical for the efficacy of DNA vaccines.

Although some reports suggest that flanking sequences of CTL epitopes are important for the precise processing of CTL epitope in vivo and that some CTL epitopes will interfere with other epitope function [71], a majority of reports showed that immunization with multimerized CTL epitope DNA without any spacer successfully induce CTL specific to each CTL epitope. But, some reports (e.g., Velders et al. [72]) suggest the importance of defined flaking sequences around epitopes and addition of ubiquitin. Ishioka et al. [73] evaluated minigene DNA vaccines encoding multiple HLA-

restricted CTL epitopes employing HLA class I transgenic mice. Such a study is useful as a pilot experiment to evaluate DNA vaccines before human study.

II. Helper T-cell oriented DNA vaccines

(1) Th epitope/CLIP-exchanged DNA vaccines

Plasmid DNA is supposed to enter directly into APC especially, in gene gun-based vaccination. So, basically, antigen presentation system through MHC class I molecules may be a primary antigen presentation route of the DNA vaccination. However, efficient MHC class II antigen presentation is indispensable for efficient induction of $CD4^+$ helper T cells. Several groups have reported that MHC class II-positive cultured cells transfected with Ii cDNA, in which CLIP region of Ii molecule was replaced with a Th epitope of interest, efficiently stimulate specific T cell lines [74-76]. We applied the method for Th-oriented DNA vaccines. We constructed recombinant Ii DNA vaccine for OVA323-336 [77] or LLO 215-226 [78] Th epitope and evaluated the immunogenicity of the DNA vaccines. We observed the epitope-specific T-cell proliferation and IFN-γ production by spleen cells derived from the recombinant Ii DNA vaccine. Interestingly, some mice immunized with OVA323-336-Ii DNA vaccine induced OVA323-336-specific antibodies in the sera as well as the epitope-specific $CD4^+$ T cells [77]. Recently, DNA vaccines of this type have been reported from other laboratories [79, 80].

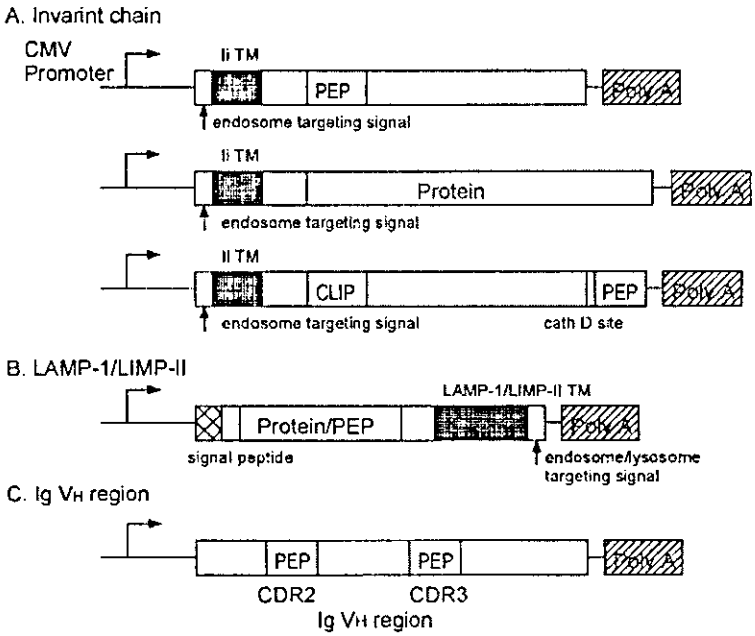

Figure 5. Schematic presentation of several Th-oriented DNA vaccines. Gene structures of reported Th-oriented DNA vaccines are shown. See following references, for example, Nagata et al. [77, 78], Toda et al. [80], van Bergen et al. [86] for A, Thomson et al. [82], Rodriguez et al. [87] for B, and Casares et al. [88, 89] for C.

(2) Utilization of endosomal/lysosomal targeting signals

Attempts for endogenous MHC class II presentation of antigens have been examined by taking advantage of lysosome associated membranous protein-1 (LAMP-1) molecule with vaccinia expression system [81]. The molecule is a type-1 transmembrane protein located predominantly in lysosomes and late endosomes involving MHC class II antigen processing pathway. The cytoplasmic domain of LAMP-1 contains the amino acid sequence, Tyr-Gln-Thr-Ile, which is important for endosomal/lysosomal protein targeting. In vitro activation of Epstein-Barr virus-, and influenza virus-specific $CD4^+$ memory CTL was successfully demonstrated by infection of cultured cells with the recombinant vaccinia virus system for the polyepitope-LAMP-1 fusion protein [82]. The similar in vivo approach with naked DNA immunization has been examined for induction of a specific $CD4^+$ T-cell subset. Ji et al. [83] showed that targeting human papillomavirus type 16 E7 to the endosomal/lysosomal compartments by gene gun immunization enhances the antitumor immunity enough to protect mice against challenge with the virus-protein-expressing tumors and to eradicate the preexisting tumor cells. We also tried a DNA vaccine for a chimeric molecule, where an antigenic peptide (LLO 215-226) was fused with the endosomal/lysosomal targeting signal of LAMP-1 molecule. We observed the LLO 215-226-LAMP-1 DNA vaccine gave induction of the epitope-specific Th induction and partial protection against *L. monocytogenes* challenge, although the magnitude of the immunogenicity was somewhat lower than that by LLO215-226/CLIP-replaced Ii DNA vaccine [84]. Vidalin et al. [85] tried a DNA vaccine of hepatitis C core protein-LAMP-1 fusion protein. Unfortunately, they failed induction of hepatitic C virus-specific T cells. They used a plasmid encoding the full-length hepatitis C virus core protein for the immunization, in which case, the amounts of the MHC class II binding peptides produced would be low, as the protein must be further subject to antigen processing to produce the peptides. DNA constructs based on Ii, LAMP-1, and MHC class II itself have been reviewed in [86].

Recently, Rodriguez et al. [87] reported successful $CD4^+$ T-cell induction by immunization of Th epitope minigene plasmid DNA by utilizing the lysosomal targeting signal located in the carboxyl terminal tail of lysosomal integral membrane protein-II (LIMP-II). They showed that the immunological consequences varied depending on the Th epitope examined. LAMP-1 or LIMP-II DNA vaccine may be useful for targeting of proteins as well as peptides to the endosomal/lysosomal compartments, although CLIP-replaced type of Ii DNA vaccine can afford to deliver only a small peptide, Th epitope, in theory. However, immunological consequences of LAMP-1 or LIMP-II DNA vaccine might be weaker than CLIP-replaced type of Ii DNA vaccine, and also will depend on target genes as suggested in Rodriguez et al. [86].

(3) Utilization of carrier protein structure-recombinant immuno-globulin molecule

Casares et al. [88, 89] reported an interesting DNA vaccine. They made a recombinant immunoglobulin molecule, where the complementarity determining region (CDR) 3 and CDR2 regions of the heavy chain variable region were exchanged with an immunodominant Th epitope (HA 110-120) and major B cell epitope (HA 150-159) of influenza virus, respectively. They showed that immunization of mice with the DNA vaccine successfully induced influenza virus-specific Th and antibodies. This interesting

approach used a structurally rigid immunoglobulin molecule as a backbone molecule for the DNA vaccine.

(4) Effect of the leader sequence on CD4 TH induction

Akbari et al. [90] showed that vaccination with a DNA construct encoding the nonsecreted form (namely without the leader sequence) of fifth component of complement (C5) protein induces strong, long-lived $CD4^+$ T cell responses. They suggest that a small number of dendritic cells taken up the transgene by DNA immunization migrated in the draining lymph nodes and induced long-lived $CD4^+$ T cell responses. This report is interesting as $CD4^+$ T-cell responses are generally thought to be induced by antigen presentation of "exogenous antigens" by dendritic cells.

Improvement of immunization regimen: Prime-boost method for DNA immunization

In addition to the design of DNA vaccine plasmids, immunization protocols also should be taken into consideration for enhancing immunological effects. Recent evaluation of DNA immunization indicates that DNA immunization alone has a limitation in terms of immunization effects and that combination of different immunization methods gives better immunization results. As a pilot study, Li et al. [91] reported that the combination of priming with recombinant influenza expressing the CTL epitope derived from *Plasmodium yoelii* circumsporozoite protein (PyCSP) and boosting with vaccinina-PySCP was more protective than immunizing with either alone or with vaccinia-PyCSP first and the recombinant influenza second. In analogy of this study, DNA heterologous prime-boost regimen, namely, primed with naked DNA vaccination and boosted with recombinant viral vectors such as vaccinia virus and adenovirus, has been shown to evoke superior levels of immunity [92, 93]. Such regimen has been evaluated in several infection models including malaria, HIV-1, tuberculosis, and herpes simplex virus (HSV) [94-101]. So far, the mechanisms of prime-boost vaccination are not clear. But, the relatively low-level, but persistent, expression of immunogenic proteins in vivo by naked DNA vaccines is suggested to be important to prime immunological responses and to induce enhanced cellular immunity [92]. Interestingly, Eo et al. [101] reported that mucosal immunological responses were optimal when animals were primed with recombinant vaccinia virus vector and boosted with naked DNA vaccine, which is an opposite regimen compared with the regimen for systemic immunological responses.

Summary

CTL and Th are key effectors in cell-mediated immunity. Here, we reviewed a variety of trials to induce specific T-cell subsets efficiently by DNA immunization method. One of the excellent features of DNA vaccines is its extraordinary flexibility. So, various modifications of DNA vaccines have been examined and reported. It is important to develop excellent vaccines capable of inducing only particular favorable immune responses and preventing adverse responses by taking advantages of the flexibility in DNA vaccine design.

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Oligodeoxynucleotides Without CpG Motifs Work as Adjuvant for the Induction of Th2 Differentiation in a Sequence-Independent Manner[1]

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The outcomes of immune responses are regulated by various parameters including how Ags are handled by APCs. In this study, we describe the intrinsic immunomodulatory characteristics of oligodeoxynucleotides (ODNs) that improve the Ag presentation by APCs. ODNs (20-mer) containing CpG motifs induced strong Th1-skewed responses. In contrast, those without CpG motifs enhanced cytokine production by effector Th cells without particular skewing toward Th1 responses or induced the differentiation of unprimed $CD4^+$ T cells toward Th2 cells. These functional features were prominently envisaged when ODNs were conjugated to the Ag, and were underlain by the facilitated binding of ODN-conjugated Ag to Ia^+ cells. Despite the functional differences between ODNs with CpG motifs and those without CpG motifs, both ODNs bound to Ia^+ cells with similar affinity and kinetics. Immunoenhancing activities of the ODNs were not sequence-dependent; the characteristics, including the facilitation of Ag capture, enhancement of effector Th cell responses, and induction of Th2 cells, were shared by randomly synthesized ODNs conjugated to Ag. This is the first study suggesting that ODNs, independent of the sequences, enhance immune responses through the promoted capture of ODN-conjugated Ag by APCs. *The Journal of Immunology,* **2003, 170: 2367–2373.**

Immunostimulatory activities of oligodeoxynucleotides (ODNs)[3] have recently attracted much interest. ODNs containing CpG motifs (CpG) induce the expression of costimulatory molecules and IL-12 in APCs, which are in turn capable of inducing Th1 cells (1–4). These steps are initiated by the binding of CpG to APCs, and appear to be mediated by surface receptors specific for ODNs (5). Although ODNs bind to several surface molecules (6–13), the identities of the receptors involved in the CpG-mediated activation have not been determined yet. Toll-like receptor (TLR)-9 is an essential intracytoplasmic mediator that colocalizes with CpG and transduces signals (14–16).

ODNs without CpG motifs (nonCpG) also bind to cells through surface receptors. CpG and nonCpG share common surface receptors and bind to them with the same affinity (5). However, binding of nonCpG failed to induce the maturation or activation of APCs (17). Failure to activate APCs was in parallel with the inability of nonCpG to colocalize with TLR-9 (15, 16). The functional involvement of nonCpG in the immune system largely remains unknown.

The first essential step for T cell immunity is the capture and presentation to T cells by APCs of Ag from the surrounding environment. Dendritic cells (DCs) are known to be specialized for Ag presentation because they are equipped with machinery to efficiently capture diverse Ags (18–20). B cells also turn into efficient APCs when Ags are captured through surface Ig (21–23). The ability of DCs and B cells to catch Ags was, however, greatly improved when the Ag was directly conjugated to ODNs (17, 24, 25). A particular consequence following the uptake of CpG-conjugated Ag by DCs is the activation of Ag-specific Th1 cells (17, 24). NonCpG also bind to cells through the same receptor as CpG (5), although little is known about the immunological effects of nonCpG or nonCpG-mediated Ag capture.

In this study, we reveal a novel feature of nonCpG as immunomodulators. NonCpG could induce enhancement of Th cell responses and Th2 skewing, which could be demonstrated when nonCpG was directly conjugated to Ag.

Materials and Methods

Animals

BALB/c mice were bred in our animal facility and were used at 7 to 12 wk of age. BALB/c mice transgenic (tg) for TCR specific for $OVA_{323-339}$ and $I\text{-}A^d$ were supplied by Dr. S. Habu (Tokai University, Kanagawa, Japan) (26), and bred in our laboratories. TLR9-deficient mice (14) were kindly provided by Dr. S. Akira (Osaka University, Osaka, Japan).

ODNs and direct conjugation to Ags

The sequences of the 20-mer CpG and nonCpG were TCCATGACGTTC CTGACGTT and TCCATGAGCTTCCTGAGTCT, respectively. CpG, nonCpG, and randomly synthesized 20-mer ODNs (RSOs) were synthesized by Nihon Gene Research Laboratories (Sendai, Japan) or Takara Shuzo (Osaka, Japan), and were fully phosphorothioated. ODNs containing a thiol residue at the 5′ end were used for the conjugation to proteins. The LPS content of ODN was <6 pg of LPS/mg of DNA, as measured by *Limulus* HS-J Single test (Wako Pure Chemical, Osaka, Japan). The ODNs were conjugated to OVA and R-PE (Molecular Probes, Eugene, OR) by mixing thiolated ODNs and maleimide-activated Ag using sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carobylate (Pierce, Rockford, IL) in our laboratory. Unconjugated ODNs were removed by extensive dialysis. The conjugates were purified by gel filtration chromatography to minimize the effects of contaminating aggregates, as described previously (17). The molar and weight ratios of the ODN-Ag are listed in Table I.

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[3] Abbreviations used in this paper: ODN, oligodeoxynucleotide; CpG, oligodeoxynucleotide containing CpG motifs; TLR, Toll-like receptor; nonCpG, oligodeoxynucleotide without CpG motifs; DC, dendritic cell; tg, transgenic; RSO, randomly synthesized oligodeoxynucleotide; LN, lymph node; MFI, mean fluorescence intensity; MDC, monodansyl cadavarine.

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Table I. *Molar and weight ratios of ODN to Ag*

	Molar Ratio	Weight Ratio
CpG-OVA	6.4	0.86
nonCpG-OVA	6.1	0.81
RSO-OVA	5.2	0.7
CpG-PE	3.7	0.092
nonCpG-PE	3.7	0.092
RSO-PE	2.8	0.07

Incubation of spleen cells with ODN-conjugate PE

BALB/c spleen cells (1×10^6/ml) depleted of RBC were incubated with 10 μg/ml PE, either alone or conjugated with CpG, nonCpG, or RSOs, at 37°C for 12 h, except in a kinetics experiment, where the incubation was conducted for varying periods of time at 4°C to minimize fluid-phase endocytosis. Then, the cells were stained with FITC-labeled anti-I-A^d mAb (MK-D6) (27) together with or without biotinylated mAb against CD40 (Caltag Laboratories, Burlingame, CA) or CD86 (Caltag Laboratories), and analyzed using FACSCaliber (BD Biosciences, Mountain View, CA). The binding of biotinylated reagents was visualized using streptavidin-conjugated allophycoerythrin (Biomeda, Foster City, CA). FITC (Sigma-Aldrich, St. Louis, MO) was conjugated to anti-I-A^d mAb in our laboratory after the partial purification of ascites by ammonium sulfate precipitation. Propidium iodide (Sigma-Aldrich)-stained dead cells were excluded from analyses.

Treatment with monodansyl cadavarine and confocal microscopy

Spleen cells were layered onto 55% Percoll (Pharmacia, Uppsala, Sweden) and centrifuged for 15 min at 2000 × *g*. Ia^+-enriched cells (>95% purity) were recovered from the top layer. After treatment with or without monodansyl cadavarine (MDC; 500 μM; Sigma-Aldrich) for 30 min at 37°C, the Ia-enriched cells were incubated with CpG-PE for 1 h at 37°C. The stained cells were examined using a confocal laser scanning microscope (MR/AG-1; Bio-Rad, Hercules, CA). PE was excited with 488-nm argon and emission spectra were collected with 570-nm long pass filters.

Competitive inhibition with free ODNs

The Ia-enriched cells were preincubated with graded doses of CpG, or 300 μg/ml CpG or nonCpG for 30 min, followed by CpG or nonCpG (1 μg/ml) conjugated with R-PE for an additional 30 min at 4°C. After washing, the cells were analyzed for the expression of R-PE using flow cytometry.

In vitro restimulation of PE-primed lymph node (LN) cell

BALB/c mice were primed s.c. with 100 μg of R-PE emulsified in CFA in the hind footpads. After 7 days, popliteal LN cells (3×10^5/well) were cultured with graded concentrations of PE, nonCpG-PE, or CpG-PE in quadruplicate in 96-well plates. After 2 days, the culture supernatants were assayed for IFN-γ.

In vitro restimulation of OVA-specific Th1 or Th2 cells

The induction of OVA-specific Th1- and Th2-enriched cells was described previously (24). $CD4^+$ Th cells (1×10^5) were cocultured with 2×10^5 APCs in the presence of OVA, CpG-OVA, or nonCpG-OVA in quadruplicate in 96-well plates. APCs were prepared by treating spleen cells from unimmunized BALB/c mice with mitomycin C (50 μg/ml; Wako Pure Chemical) for 30 min at 37°C. After 2 days, the culture supernatants were assayed for IFN-γ and IL-4.

In vitro induction of effector Th cells and in vitro restimulation

Spleen cells (5×10^6) from unimmunized anti-OVA TCR tg mice were cultured with graded doses of CpG-OVA or nonCpG-OVA in 12-well plates. For blocking with Abs, the cultures were initiated in the presence of 10 or 1 μg/ml anti-IL-4 (BD PharMingen, San Diego, CA), anti-CD86 (BD PharMingen) or isotype-matched control IgG2b mAb (BD PharMingen). After 6 days, viable lymphocytes (1×10^5) recovered from the interface by Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) density-gradient centrifugation were restimulated with 2×10^5 APCs in the presence of OVA (100 μg/ml) in quadruplicate in 96-well plates. After 2 days, the culture supernatants were assayed for IFN-γ and IL-4.

In vivo induction of effector Th cells and in vitro restimulation

BALB/c mice were primed s.c. with 5 μg of OVA, nonCpG-OVA, or CpG-OVA on days 0 and 7. On day 14, popliteal LN cells (3×10^5/well) were cultured with OVA (100 μg/ml) in quadruplicate in 96-well plates. After 2 days, the culture supernatants were assayed for IFN-γ and IL-4.

Cytokine assay

Cytokine concentrations in the culture supernatants were determined using ELISA, as described previously (28). Paired anti-IL-4 and anti-IFN-γ mAbs were purchased from BD PharMingen. Tetramethylbenzidine reagent (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was used for color development, and OD_{450} were converted to concentrations (nanograms per milliliter) according to a standard curve. Standard recombinant mouse IL-4 and IFN-γ were purchased from Genzyme (Cambridge, MA).

In vivo IgG responses

BALB/c mice were immunized i.p. with 10 μg of OVA, either alone or conjugated with CpG or nonCpG, twice at a 14-day interval. Fourteen days after the second immunization, the sera were assayed for OVA-specific IgG1 and IgG2a using ELISA after appropriate dilutions. Anti-OVA IgG1 and IgG2a Abs bound to the immobilized OVA were detected with alkaline phosphatase-conjugated anti-IgG1 (Southern Biotechnology Associates, Birmingham, AL) and IgG2a (Southern Biotechnology Associates) Abs, respectively. Colors were developed using K-Gold PNPP substrate (Neogen, Lexington, KY). Titers were compared with serial dilutions of standard sera prepared from mice hyperimmunized with OVA in alum and that in CFA for IgG1 and IgG2a detections, respectively. Results are expressed as arbitrary units per milliliter.

Statistics

Data of in vitro culture experiments and in vivo anti-OVA Ab responses were expressed as the mean ± SEM. Each experiment was repeated at least twice. The Student *t* test was used in the analysis of the results.

Results

Binding and endocytosis of CpG- and nonCpG-conjugated PE in Ia^+ cells

We first examined the binding of ODNs to splenocytes using the conjugates with fluorescent protein, PE. Spleen cells were incubated with PE (1 μg/ml), either alone or conjugated with CpG or nonCpG, and examined for fluorescence emitted from PE by flow cytometry. In contrast to PE alone that poorly bound to spleen cells, ODN-conjugated PE strongly stained spleen cells, the majority of which were positive for Ia expression (Fig. 1*A*). CpG- and nonCpG-conjugated PE stained Ia^+ cells to a similar extent. Experiments with graded doses of PE showed that unconjugated PE bound to Ia^+ cells in a dose-dependent manner over wide ranges of doses, whereas CpG- and nonCpG-conjugated PE practically reached plateau levels at lower doses of PE (Fig. 1*B*). ODN-conjugated PE was calculated to bind to Ia^+ cells ~30 times more efficiently than ODN-unconjugated PE (Fig. 1*B*). Time-course experiments revealed that the binding was rapid and reached a plateau by 30 min even at 4°C, a temperature at which endocytosis is minimized (Fig. 1*C*). Both CpG- and nonCpG-conjugated PE bound to Ia^+ cells to comparable extents at all time points examined (Fig. 1*C*).

When the Ia^+ cells were pretreated with MDC, a transglutaminase inhibitor that inhibits receptor-mediated endocytosis (29), the Ia^+ cells were stained with CpG-conjugated PE in a generalized ring type fluorescence pattern, whereas the MDC-untreated Ia^+ cells showed a granular pattern (Fig. 1*D*).

When the Ia^+ cells were preincubated with graded doses of free CpG before the incubation with CpG-conjugated PE, the binding of CpG-conjugated PE to the Ia^+ cells was inhibited in a dose-dependent manner (Fig. 2*A*). The inhibition was maximal at a dose of 1000 μg/ml CpG, where the mean fluorescence intensity (MFI) was almost equivalent to the autofluorescence level of the unstained Ia^+ cells (MFI = 17) (Fig. 2*A*). Both CpG- and nonCpG-

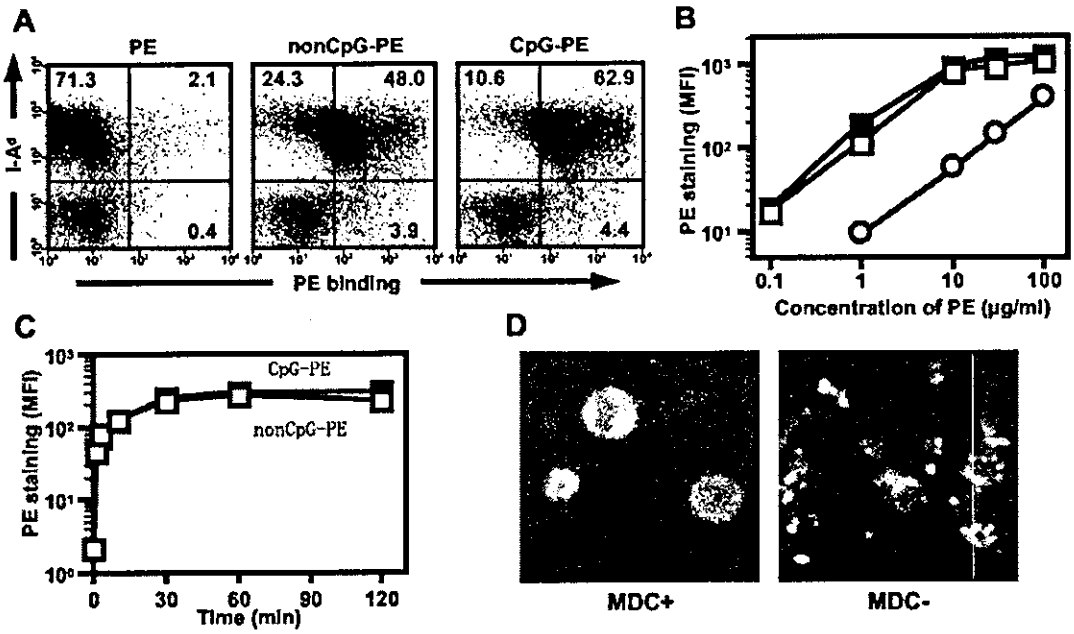

FIGURE 1. Binding and endocytosis of CpG- and nonCpG-conjugated PE in Ia$^+$ cells. Spleen cells were incubated with 10 μg/ml (*A* and *C*) or graded doses (*B*) of PE (○), CpG-conjugated PE (CpG-PE; ■), or nonCpG-conjugated PE (nonCpG-PE; □) at 37 (*A* and *B*) or 4°C (*C*). The stained cells were analyzed using flow cytometry. The relationship between PE staining and Ia expression (*A*) and MFI of PE staining in gated Ia$^+$ cells (*B* and *C*) is shown. *D*, The Ia-enriched cells were pretreated with or without MDC before the incubation with CpG-PE (10 μg/ml). Emission from PE was examined using confocal microscopy. Each experiment was repeated at least twice with similar results.

conjugated PE bound to Ia$^+$ cells to a comparable level (Fig. 2*B*). The binding of CpG- and nonCpG-conjugated PE was inhibited to a similar extent by the preincubation with excess doses of either free CpG or nonCpG (Fig. 2*B*).

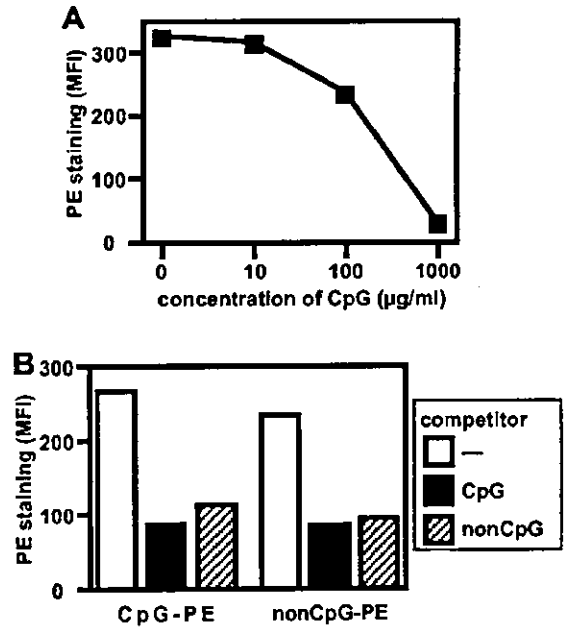

FIGURE 2. Competitive inhibition with free ODNs. The Ia-enriched cells were incubated with 1 μg/ml PE conjugated with CpG (*A* and *B*) or nonCpG (*B*) following preincubation with graded doses of free CpG (*A*), or 300 μg/ml of free CpG or nonCpG (*B*). The stained cells were analyzed using flow cytometry. Each experiment was repeated twice with similar results.

These results suggest that ODN-conjugated proteins can bind to Ia$^+$ cells through surface receptors at similar affinity and kinetics irrespective of the presence of CpG motifs in the ODNs.

No need for TLR9 in ODN-mediated Ag uptake by Ia$^+$ cells

We examined whether TLR9 is involved in the ODN-mediated uptake of Ag. Following the incubation of the Ia-enriched cells with nonCpG- or CpG-conjugated PE, either was incorporated into almost all the Ia$^+$ cells to similar extents, regardless of whether or not TLR9 was expressed in the Ia$^+$ cells (Fig. 3). In sharp contrast to the Ag uptake, CpG was essential for the activation of the Ia$^+$ cells, as revealed by the increased proportion of the CD86$^+$ cells (Fig. 3*A*) and the enhanced expression of CD40 (Fig. 3*B*). Thus, TLR9 is not required for the uptake of ODN-conjugated Ag.

Activation of Th cells by ODN-conjugated Ag

We next examined the functional difference between CpG-conjugated and nonCpG-conjugated Ag. PE-primed LN cells were stimulated with graded doses of PE, either alone or conjugated with CpG or nonCpG. In comparison to stimulation with PE alone, the IFN-γ production from PE-primed LN cells was enhanced by approximately an order of magnitude when stimulated with the same dose of PE conjugated with nonCpG (Fig. 4*A*). The enhancement of IFN-γ production by nonCpG-conjugated Ag was, however, not so high as that by CpG-conjugated Ag.

To further clarify the functional effects, we examined the activation of Th1 or Th2 cells by ODN-conjugated Ag. OVA-specific T cells enriched for Th1 and Th2 cells predominantly produced IFN-γ and IL-4 in response to OVA stimulation (100 μg/ml), respectively (Fig. 4*B*). Stimulation of Th1 or Th2 cells with 1 μg/ml OVA failed to induce the production of cytokines, whereas the same concentration of OVA conjugated with nonCpG induced the secretion of IFN-γ from Th1 cells and IL-4 from Th2 cells, and

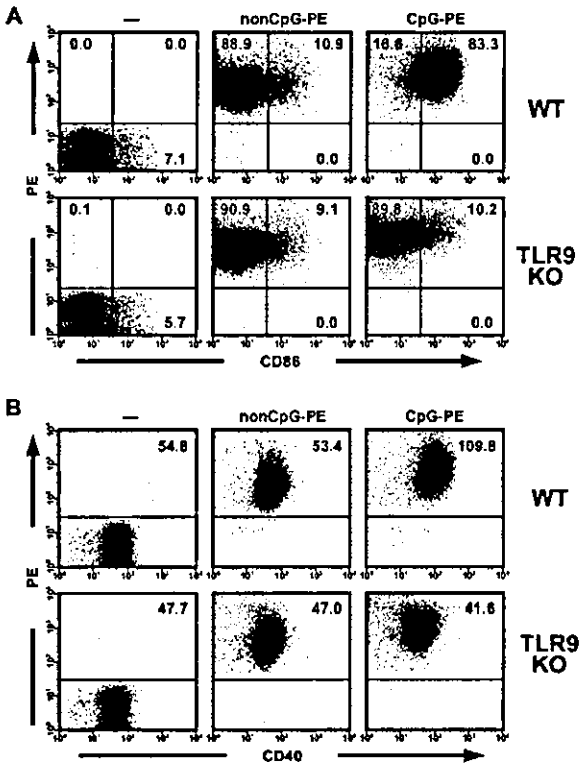

FIGURE 3. No need for TLR9 in ODN-mediated Ag uptake by Ia^+ cells. Purified Ia^+ cells from TLR9-deficient (TLR9 KO) or wild-type (WT) mice were incubated with PE (10 μg/ml) conjugated with CpG or nonCpG overnight. Then, the Ia^+ cells were examined for the expression for PE and CD86 (*A*) or PE and CD40 (*B*). Results shown are representative of three experiments with similar results. The numbers in figures indicate the percentage of cells in each quadrant (*A*) and CD40 MFI on the total acquired cells (*B*).

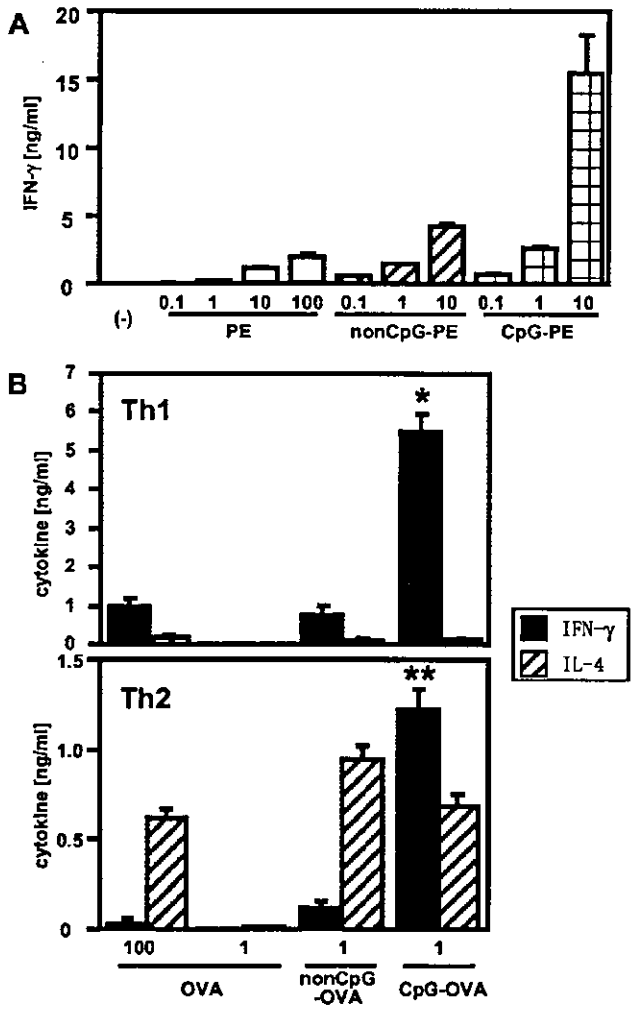

FIGURE 4. Activation of Th cells by ODN-conjugated Ag. *A*, BALB/c mice were primed with PE in CFA in the hind footpads. Regional LN cells were cultured with titrated doses of PE, nonCpG-PE, or CpG-PE for 2 days, and levels of IFN-γ in culture supernatants were determined. *B*, Th1 and Th2 cells induced in vitro from OVA-specific $CD4^+$ T cells were stimulated with indicated doses (micrograms per milliliter) of OVA, nonCpG-OVA, or CpG-OVA for 2 days, and culture supernatants were assayed for IFN-γ (■) and IL-4 (▨). Each experiment was repeated three times independently with similar results. *, $p < 10^{-8}$; **, $p < 0.001$ (compared with the nonCpG-OVA group).

the cytokine levels were comparable to those induced by a 100-fold higher dose of unconjugated OVA. In contrast, CpG-conjugated OVA enhanced the production of IFN-γ, but not IL-4, from Th1- and Th2-enriched cells as well.

Induction of effector Th cells by ODN-conjugated Ag

We next examined the effects of ODN-conjugated OVA on the differentiation of unprimed T cells into effector Th cells. OVA-specific $CD4^+$ T cells were cultured with graded concentrations of OVA conjugated with CpG or nonCpG, and restimulated with OVA. CpG-conjugated OVA induced Th1 differentiation (Fig. 5*A*), whereas nonCpG-conjugated OVA induced Th2 differentiation (Fig. 5*B*). Another difference was the antigenicity of the conjugate, since the effects of CpG-OVA reached a plateau at a dose of 0.1 μg/ml (Fig. 5*A*), whereas those of nonCpG-OVA were maximal at a 10-fold higher dose (Fig. 5*B*).

We then examined whether IL-4 and CD86 were involved in the Th2 induction by nonCpG-conjugated Ag. When T cells were cultured with nonCpG-OVA in the presence of anti-IL-4 or anti-CD86 mAb, the recovered Th cells failed to produce IL-4 in response to Ag restimulation (Fig. 5*C*), indicating that the development of nonCpG-OVA-induced Th2 cells is, like ordinal Th2 cells, dependent on IL-4 and costimulatory molecules.

We next examined the in vivo effects of ODN-conjugated OVA on the differentiation of Th cells. Regional LN cells from mice primed with OVA, either alone or conjugated with CpG or nonCpG, were stimulated in vitro with OVA, and cytokine production was assessed. Immunization with OVA alone failed to prime Th cells (Fig. 5, *D* and *E*). The same dose of OVA conjugated with nonCpG, however, induced the differentiation into Th2 cells that predominantly produced IL-4, whereas OVA conjugated with CpG induced Th1 cells (Fig. 5, *D* and *E*).

To generalize the observations obtained with nonCpG-conjugated Ag, we conducted experiments using RSOs. Spleen cells were incubated with PE, either alone, mixed or conjugated with RSOs, and analyzed by flow cytometry. Binding of PE to Ia^+ cells was enhanced when PE was conjugated with RSOs, whereas PE inefficiently bound to spleen cells even in the presence of attendant RSOs (Fig. 6*A*). Lack of the activation of Ia^+ cells by nonCpG was also substantiated, because RSO-conjugated PE failed to augment CD86 (Fig. 6*B*) or CD40 expression (Fig. 6*C*) in the gated Ia^+ cells, which was in sharp contrast to our previous observations with CpG-conjugated PE (17). Thus, the facilitated binding of

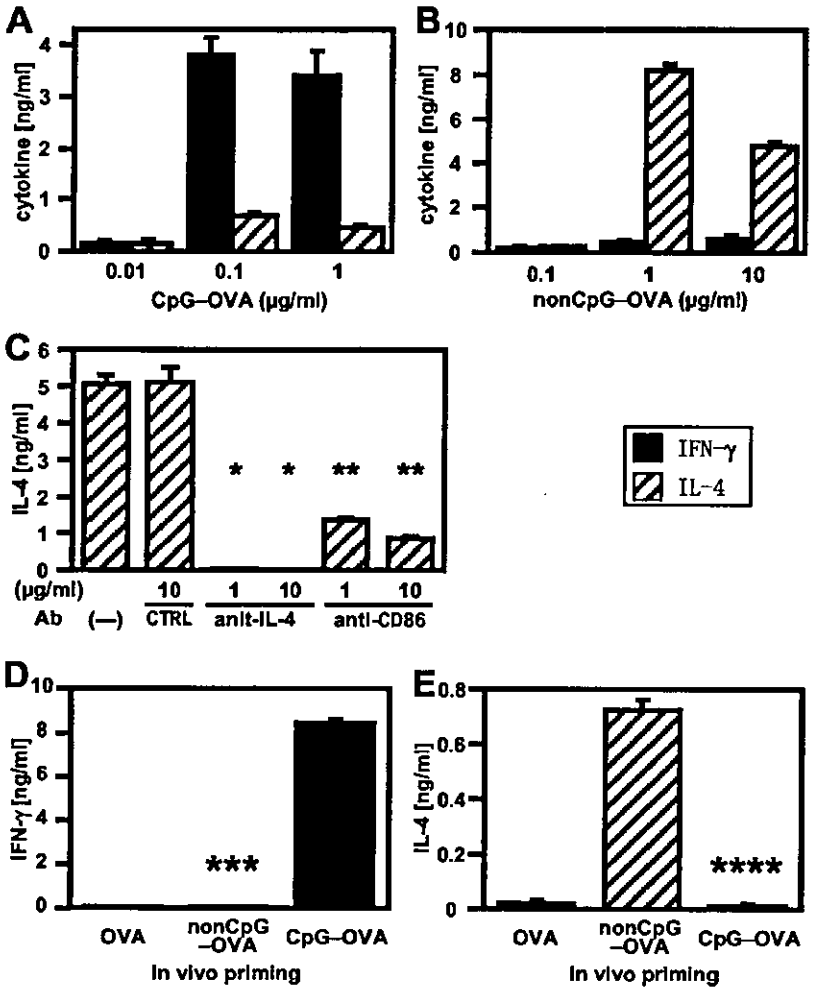

FIGURE 5. Induction of effector Th cells by ODN-conjugated Ag. *A–C*, Spleen cells from OVA-specific tg mice were cultured with graded doses of CpG-OVA (*A*) or nonCpG-OVA (*B*), or 1 μg/ml nonCpG-OVA in the presence of the indicated doses of Abs (*C*), for 6 days, and the viable cells were restimulated with 100 μg/ml OVA. *D* and *E*, BALB/c mice were primed with 5 μg of OVA, nonCpG-OVA, or CpG-OVA in the hind footpads on days 0 and 7. On day 14, regional LN cells were stimulated in vitro with 100 μg/ml OVA for 2 days, and culture supernatants were assayed for IFN-γ (*D*) and IL-4 (*E*). Data were representative of three independent experiments. *, $p < 0.0005$; **, $p < 0.001$ (compared with the control Ab group); ***, $p < 10^{-4}$; ****, $p < 0.001$ (comparison between the nonCpG-OVA and CpG-OVA groups. CTRL, Isotype-matched control Ab.

ODN-conjugated Ag to Ia^+ cells is a shared and sequence-independent characteristic of ODNs. The skewing toward Th2 cell by ODN-conjugated Ag is also a generalized feature, because the injection of OVA conjugated, but not mixed, with RSOs induced Th2 cells (Fig. 6*D*).

In vivo IgG responses

We examined the effects of nonCpG-conjugated OVA on in vivo Ig production. Immunization of mice with OVA alone failed to mount anti-OVA Ab production (Fig. 7). The same dose of OVA conjugated with nonCpG induced exclusively IgG1 production, which is a hallmark of Th2 responses. The anti-OVA IgG2a isotype was induced only by immunization with CpG-conjugated OVA.

Discussion

DNAs have been generally considered to be immunologically inert. The potent immunostimulatory activities exerted by CpG (1–4) appeared rather exceptional. In the present work, however, we demonstrated that ODNs ubiquitously have immunoenhancing activities independent of the particular sequences.

Two representative effects of ODNs we observed were the enhanced activation of Ag-primed effector Th cells (Fig. 4) and the induction of the Th2 differentiation from naive T cells (Figs. 5 and 6). The two characteristics were clearly envisaged when ODNs were directly conjugated to Ag. In the parallel experiments, the covalent conjugation of Ag to ODNs lead to the accelerated binding of Ag to Ia^+ cells (Fig. 1). In light of the fact that ODNs bind to cell membranes through specific surface receptors (5), the superiority of the direct linkage between ODNs and Ag in Th cell activation and Ag capture by APCs would reflect that the endocytosis of ODN-conjugated Ag through ODN-specific receptors was more efficient than fluid-phase endocytosis of Ag. Thus, the enhanced responses of Th cells by ODN-conjugated Ag would be accounted for by an increase in Ag capture and uptake by the Ia^+ APCs. So far as we know of, DNAs, particularly as a form conjugated to Ag, have not been examined with respect to Ag presentation and Th cell activation.

Previously, B cells were thought to be activated by ODNs either in a sequence-specific or nonspecific manner (30–32). The mechanisms of B cell activation remained unknown. It has been reported that the engagement of surface Ig by ODN-mediated charge-charge interactions leads to activation of B cells (33). More recently, DNAs were reported to induce the maturation of APCs (34). These activities are manifested by dsDNAs and abrogated by cleavage to ssDNAs.

Recently McCluskie et al. (35, 36) reported that nonCpG ODNs, when given orally together with Ag, induced Th2 cells. These effects were observed when nonCpG ODNs and Ag were delivered by mucosal but not parenteral routes. The reason remains unsolved. The nonCpG-Ag conjugates we adopted induce Th2 cells even when given parenterally or used in vitro. The mechanisms of the action include an enhancement of Ag uptake. However, the dependency of nonCpG-mediated Th2 induction on costimulatory

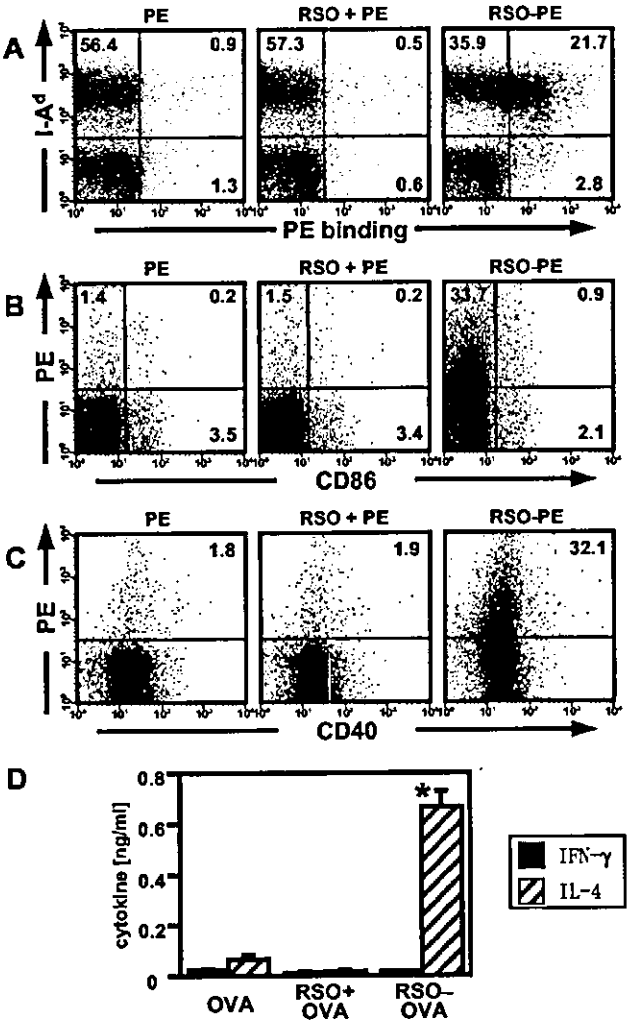

FIGURE 6. RSO-conjugated Ag enhanced Ag-binding and Th2 induction. *A–C*, Spleen cells were incubated with 10 μg/ml PE, either alone, mixed, or conjugated with RSOs for 3 h. The cells were then stained with FITC-anti-Ia mAb (*A–C*), together with anti-CD86 (*B*) or anti-CD40 (*C*) mAb, and analyzed using flow cytometry. Only gated Ia$^+$ cells were shown in *B* and *C*. *D*, BALB/c mice were primed with 5 μg of OVA, RSO + OVA, or RSO-OVA in the hind footpads on days 0 and 7. On day 14, regional LN cells were stimulated with 100 μg/ml OVA for 2 days, and culture supernatants were assayed for IFN-γ (■) and IL-4 (▨). Each experiment was repeated three or four times with similar results. *, $p < 0.005$ (compared with the RSO + OVA group)

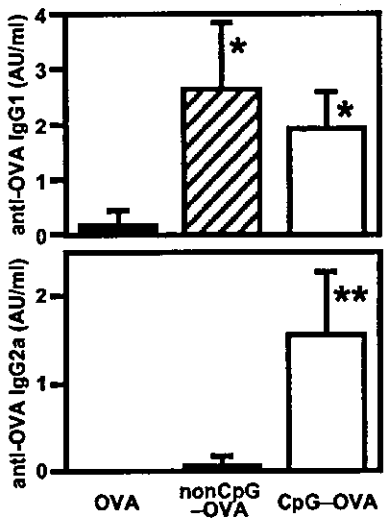

FIGURE 7. In vivo IgG production. BALB/c mice were immunized i.p. with 10 μg of OVA, nonCpG-OVA, or CpG-OVA twice at a 14-day interval. 14 days after the last immunization, sera were collected and examined for OVA-specific IgG1 and IgG2a using ELISA. Data are expressed as arbitrary units per milliliter. Each group consists of five mice, and experiments were repeated twice with similar results. *, $p < 0.005$; **, $p < 10^{-5}$ (compared with the OVA group).

molecules (Fig. 5) needs to be reconciled with the failure of nonCpG ODNs to activate APCs. DCs that had been activated by environmental stimuli and yet had lacked the IL-12 secretion might incorporate nonCpG-Ag and become competent to present Ag to Th cells for skewing toward Th2 responses. The underlying mechanisms remain unknown.

Experiments using antisense DNAs had hinted that the uptake of DNAs by cells was inefficient. It has become evident, however, that DNAs are incorporated into the cytoplasmic portion after binding to the cell surface through DNA-specific receptors (37, 38). DNAs, regardless of the presence or absence of CpG motifs in the sequences, use identical receptors despite the apparent functional difference between the two (5). Recently, the functional difference, including the expression of costimulatory molecules and IL-12, has been ascribed at least in part to the ability of CpG, but not nonCpG, to bind to TLR9 in the cytoplasm (4–6). The ODN receptors or intracellular components responsible for our current observations remain to be elucidated.

The findings obtained with the nonCpG in the present study were not exceptionally dependent on particular sequences, because RSOs had immunological effects comparable to those by the nonCpG we used (Fig. 6). For unknown reasons, the RSOs were conjugated to Ag less efficiently than the nonCpG even in repeated trials. The conjugation ratio of the RSOs to Ag was lower than that of the nonCpG, and in parallel, the RSO-conjugated Ags bound to Ia$^+$ cells less efficiently than the nonCpG-conjugated Ag.

CpG and nonCpG share identical DNA receptors, whereas they showed quantitative or qualitative differences as adjuvants. First, CpG is quantitatively superior to nonCpG in the enhancement of antigenicity. For example, the CpG-conjugated PE stimulated PE-primed T cells 10-fold more efficiently than the nonCpG-conjugated PE (Fig. 4). In another experiment, the induction of effector Th cell development by CpG-conjugated OVA reached a plateau at a 10-fold lower dose than that by nonCpG-conjugated OVA (Fig. 5). These differences could not be ascribed to the difference in uptake of ODN-conjugated Ag, because both CpG and nonCpG bind to the same receptors with the same avidity (5). More notable are the qualitative differences; CpG could initiate Th1 responses, whereas nonCpG induced Th2 differentiation (Figs. 5 and 6). CpG and nonCpG can drive uncommitted immune responses toward totally opposite directions.

How physiologically relevant would the modulation of immune responses by DNAs be? Our present study suggested two possible adjuvant effects exerted by DNAs; the induction of Th2 differentiation at the initial encounter with Ags (Figs. 5 and 6), and the amplification of the Th2-skewed response in the subsequent encounters (Fig. 4). Th2-biased responses by DNAs appear beneficial for the prevention of autoimmunities, which are largely Th1-dependent (39). However, if self Ag-specific Th1 cells had happened to be induced in advance for any reason, the situation would not be the same. The activation of the preceding Th1 cells by DNAs (Fig. 4) would make autoimmune diseases overt that would otherwise have remained concealed. The above scenario might account for

the onset or deterioration of autoimmune diseases upon viral infection that promotes the release of DNAs from infected cells. This hypothesis needs to be tested.

In summary, we demonstrated as an intrinsic immunomodulator characteristic of ODNs independent of the particular sequences.

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分子予防環境医学

生命科学研究の予防・環境医学への統合

Integration of modern life science into preventive and environmental medicine

分子予防環境医学研究会 編

本の泉社

Ⅰ-2 結　　核

国立療養所近畿中央病院　臨床研究センター　結核研究部
岡田全司

はじめに

いまだに世界の人口の1/3が結核菌の感染を受け，その中から毎年800万人の結核患者が発生し，200万人が毎年結核で死亡している，最大の感染症の一つである(図1)[1,2,3,4]．本邦でも1998年から結核罹患率の増加が認められ，1999年7月26日"結核緊急事態宣言"が厚生省より出された．1998年，米国CDCは結核に対し，政府・学術機関・企業が一体となって新世代の結核ワクチン開発の必要性を強く主張する発表をした．又，ACETは国民の健康に対する大敵である結核撲滅のためには，BCGに代わる有効なワクチンが必要であることを示した．しかしながら，BCGに代わる結核ワクチンは欧米でも臨床応用には至っていない．我々はBCGよりもはるかに強力な新しいサブユニットワクチン，DNAワクチンやリコンビナントBCGワクチンの開発に成功した[5,6,7,8]．したがって，新しい抗結核ワクチン開発の現状とまだ不明な点が多い結核免疫におけるキラーTの機能解明についても述べる[9,10]．

一方，1940年以降は分子遺伝子学的アプローチが急速に進行した．Stewart Coleらは1998年強毒結核菌株H37Rvゲノムの完全な配列を決定した[11]．H37Rvのゲノムは4,411,529塩基対から成り，約4000の遺伝子を含むものと推定されている．その後，Mycobacterium avium，BCG，Mycobacterium bovisの全塩基配列も解読された．

これらをもとに結核菌の分子疫学も進展しつつある．これらについても述べる．

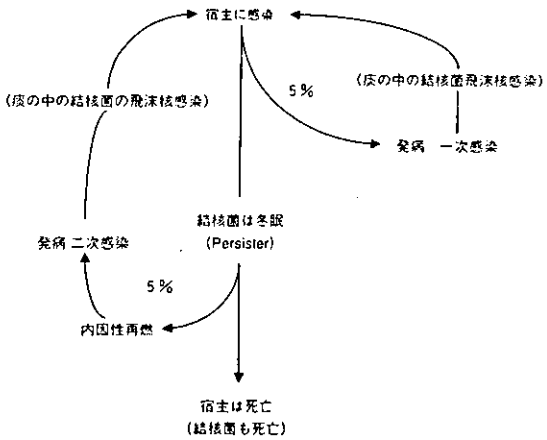

図1　結核菌のライフサイクル(ヒト結核感染)

1. 結核の疫学

1. 結核の歴史

結核が人類最古の伝染病の一つであったことは，BC7000年の(ドイツのハイデルベルグで発見された)人類の胸椎の化石にカリエス像が認められたことや，ミイラ(古代エジプトBC3000)に肺結核が認められたことより示されている．1882年Robert Kochは結核菌がヒト結核の原因であることを発見した．

地球上で広く蔓延したのは産業革命以後の産業の発達や人口増加・人の交流の活発化，都市化に平行して広がった．イギリスで1750年頃，日本