CpG Oligodeoxynucleotide is an Effective Adjuvant for Transcutaneous Immunization

ChenLu Liu,1,2 Tomomi Hashizume,1 Tomoko Kurita-Ochiai,1 and Masafumi Yamamoto1

1Department of Microbiology and Immunology, Nihon University School of Dentistry at Matsudo, Matsudo, Chiba 271-8587, Japan
2Tianjin Stomatology Hospital, Tianjin, China

Correspondence to:
Masafumi Yamamoto
E-mail: yamamoto.masafumi@nihon-u.ac.jp

Abstract
Antigen delivery systems have been designed to facilitate the development of vaccines that induce both mucosal and systemic immune responses. Transcutaneous immunization (TCI) is a new method of vaccination that can induce both mucosal and systemic immunities. However, because most protein antigens are rather weak immunogens when given transcutaneously, the development of effective adjuvants is of central importance for TCI. In this study, we assessed the potential for the use of oligodeoxynucleotides containing cytosine-phosphate-guanosine motifs (CpG), as an adjuvant for transcutaneous immunization. When female C57BL/6 mice were immunized with tetanus toxoid (TT) by direct application to shaved skin, a TT-specific serum IgG antibody response was induced; however, no response was induced in feces. When TT was given together with CpG oligonucleotide (ODN) as an adjuvant, a higher TT-specific serum IgG and IgA antibody response was induced, compared to TT alone. Furthermore, a TT-specific IgA antibody response was detected in the fecal extract of mice immunized with CpG ODN. Dose response studies established that 500 µg of CpG induced the highest fecal anti-TT antibody response. These results suggest that transcutaneous administration of CpG as an adjuvant is effective for the induction of mucosal and systemic antigen-specific antibody responses and that CpG could be used as an adjuvant for transcutaneous immunization.

Keywords: transcutaneous immunization, adjuvant, IgA antibody

Introduction
The mucosal surface is the primary site of entry for most pathogens. Gastrointestinal, respiratory, and urogenital tracts expose a large area to exogenous agents, including potentially harmful microorganisms. Therefore, the induction of an effective immune response in the mucosal surface is important to prevent the invasion of pathogens. Immunoprophylaxis by the administration of vaccines represents a feasible tool for giving targeted immune protection. Effective protection against pathogens requires both a mucosal response, including secretory IgA (S-IgA) antibodies as a first line of defense at the site of entry (1, 2), and a systemic immune response to neutralize pathogens that have penetrated this barrier (3). However, most vaccines developed to date are delivered parenterally (i.e. by intramuscular or intradermal injection), inducing a systemic but not a mucosal immune response (4). Mucosal delivery systems have been designed to facilitate the development of vaccines that induce both mucosal and systemic immune responses. At present, oral and intranasal vaccines are under development, although the rapid degradation of antigens in the gastro-intestinal tract and the possibility of host pathology induced by antigens or adjuvants in nasal and bronchial tissues are potential limitations to the broad implementation of these approaches (5, 6). Furthermore, oral administration of antigens may lead to antigen unresponsiveness (7, 8). Thus, the search continues for practical, effective strategies to introduce vaccines (5, 6). Transcutaneous immunization (TCI) is a novel delivery route for vaccination, using the topical application of vaccine antigens on intact
bare skin. It has been shown that TCI elicits both systemic and mucosal immune responses (9–12). Furthermore, transcutaneous vaccines have several advantages over traditional vaccines, including ease of administration, reduced risk of disease transmission, and the elimination of both needles and the need for specially trained healthcare specialists to administer vaccines. However, the induction of significant secretory immunity by transcutaneous vaccinations has proven difficult and requires the use of adjuvants (1, 13, 14). Recent studies have shown that cholera toxin (CT) is an effective adjuvant for TCI in animal models (9). However, its toxicity prevents its use in humans. In this regard, genetically detoxified mutants of CT have been developed using site-directed mutagenesis, which in animal models appear to be nontoxic which retaining adjuvanticity (4). Despite this progress, there is still a need for novel safe and effective adjuvants for TCI.

Bacterial DNA containing cytosine–phosphate–
guanosine (CpG) motifs with unmethylated cytokine, as well as synthetic oligodeoxynucleotides containing immunostimulatory CpG motifs (CpG ODN), has a direct immunostimulatory effect in vitro due to the presence of CpG motifs within a particular base context (4). Furthermore, it appears that the rapid immune activation in response to CpG DNA may evolve as one component of the innate immune defense mechanisms that recognize structural patterns specific to microbial molecules (4, 15). Thus, in this study, we assessed the potential of CpG ODN as an adjuvant for TCI to induce both mucosal and systemic antibody responses.

**Materials and Methods**

**Animals**

Female C57BL/6 mice purchased from Sankyo Laboratories were maintained in a pathogen-free experimental facility at Nihon University School of Dentistry at Matsudo. All mice were given sterile water, food, and bed dressing. All experiments were performed using 8- to 12-week old mice that were randomly assigned in groups of 3 or 4.

**Vaccines**

Tetanus toxoid (TT) was kindly provided by Dr. Chikateru Nozaki (The Chemo-sero-Therapeutic Research Institute, Kumamoto, Japan). Synthetic oligodeoxynucleotides containing CpG motifs (CpG ODN) (5’-TCCATGACGTTCCTGACGTT-3’) were purchased from Coley Pharmaceutical Group (Wellesley, MA, USA).

**Immunization**

Each mouse was anaesthetized with ketamine before immunization. Fur was shaved from a section of the upper back with care taken not to break the skin. The skin was swabbed with 70% ethanol. A wound plaster soaked with 150 μl phosphate-buffered saline (PBS) containing 100 μg TT with 0 μg, 100 μg, 200 μg, or 500 μg of CpG ODN was applied on days 0, 7, and 14.

**Detection of antigen-specific antibody responses**

Serum and fecal samples were collected 2 days before immunization as control samples and 6 days after the final immunization and examined for TT-specific antibody responses (16, 17). Antibody titers in serum and fecal extracts were determined by enzyme-linked immunosorbent assay (ELISA). Briefly, the plates were coated with TT (5 μg/ml) and blocked with PBS containing 1% bovine serum albumin. The starting dilution of serum was 1: 2^10 and fecal samples were 1: 2^2 by concentration. After 4 hours incubation at room temperature, the plates were washed and peroxidase-labeled goat anti-mouse γ or α heavy chain-specific antibodies (Southern Biotechnology Associates, Birmingham, AL, USA) was added to the appropriate wells. Finally, 2,2’-azino-bis (3-ethylbenzene-thiazoline-6-sulfonic acid) with H₂O₂ (Moss Inc., Pasadena, MD, USA) was added for color development. Endpoint titers were expressed as the reciprocal log₂ of the last dilution that gave an optical density at 415 nm of 0.1 greater than nonimmunized control samples after 15 minutes of incubation.
Statistical analysis
Results were expressed as the mean ± standard deviation. Statistical significance (p<0.05) was determined by unpaired Student’s t test.

Results and Discussion
Induction of TT-specific serum antibody responses by transcutaneous administration of TT and CpG
Effective protection against pathogens requires both a mucosal immune response and a systemic immune response. Transcutaneous immunization has several advantages over traditional vaccines and other mucosal vaccines (2). In this study, to evaluate the ability of TCI with TT to induce serum antibody responses, a group of mice were transcutaneously immunized with TT. When the TT-specific antibody response was analyzed by ELISA, a significant IgG antibody response was found to have been induced. However, the responses were relatively low (Fig. 1A). Furthermore, an IgA response was not detected (Fig. 1B). These results suggest that TCI with TT alone may not be an effective vaccine for the induction of protective immunity against tetanus toxin. Unmethylated CpG motifs, which are often found in bacterial but not vertebral DNA, are recognized by Toll-like receptor 9 expressed by B cells and plasmacytoid dendritic cells (18). The interaction of Toll-like receptor 9 with CpG motifs triggers an immune cascade, resulting in improved antigen uptake and presentation by antigen-presenting cells, antibodies, and chemokines, as well as cytokine secretion by B cells, NK cells, dendritic cells, and monocytes (18, 19). Synthetic CpG ODN mimics the immunostimulatory activity of bacterial DNA. This activity enables CpG ODN to act as an immune adjuvant, accelerating and boosting antigen-specific immune responses. This might be due to the close physical contact between the CpG ODN and the immunogen (18, 19). Thus, in this study, we assessed whether transcutaneous administration of CpG ODN would act as an adjuvant in the induction of TT-specific antibody responses. Mice were transcutaneously immunized with TT in the presence of different concentrations of CpG ODN. TCI with TT plus CpG ODN induced a significantly higher TT-specific IgG antibody response compared to TT alone. All doses of CpG ODN tested in this study (100–500 μg) gave comparable adjuvant activity and induced serum IgG anti-TT antibody response (Fig. 1A). Significantly, administration of 500 μg of CpG resulted in the

**Fig. 1.** Induction of TT-specific antibody responses in serum by transcutaneous immunization with TT plus CpG ODN. Groups of female C57BL/6 mice were transcutaneously immunized with 100 μg of TT combined with 0 μg (control), 100 μg, 200 μg, or 500 μg of CpG ODN on days 0, 7, and 14. After the final immunization, serum samples were collected and assayed on day 21 for TT-specific IgG (A) and IgA (B) antibody responses. The results are expressed as the mean ± standard deviation and are representative of three separate experiments containing three to four mice in each group/experiment. *p<0.05 compared with the control of mice immunized with TT alone.
highest TT-specific IgA antibody response of several doses tested (Fig. 1B). These findings clearly indicate that CpG ODN is an effective adjuvant for induction of TT-specific antibody responses when given transcutaneously.

**Antigen-specific mucosal IgA immune responses are induced by the co-administration of CpG**

It is important to note that transcutaneous administration of CpG ODN as an adjuvant induced a secretory IgA antibody response to co-administrated TT in mucosal secretions. Thus, TCI with TT plus CpG ODN resulted in the induction of a significant TT-specific IgA antibody response in fecal extracts (Fig. 2). Of all the dosages of CpG ODN tested, the administration of 500 μg of CpG produced the largest mucosal IgA anti-TT antibody response. In contrast, IgA antibodies were not induced in mice by transcutaneous immunization with TT alone (Fig. 2). These results indicate that CpG is a potent adjuvant for the induction of immune responses in both mucosal and systemic areas when administrated transcutaneously.

The amounts of CpG as adjuvant used in this study are much higher than those used in nasal immunization in previous studies (4). In this regard, it has been known that nasally administered antigen interacts directly with IgA-inductive sites, known as nasal-associated lymphoreticular tissues, while transcutaneous immunization does not. Therefore, higher doses of vaccine are required for transcutaneous immunization when compared with nasal immunization. However, previous studies have suggested that nasally administered vaccines accumulate in the olfactory nerves and epithelial regions (20, 21). On the other hand, a recent study has demonstrated that transcutaneous immunization of human volunteers elicits antigen-specific antibody responses without significant side effects (22). Taken together, these findings suggest that transcutaneous immunization represents a novel and completely independent antigen delivery system that significantly reduces the negative effects produced by nasal immunization.

The improved adjuvant effect of CpG ODN has been shown by linking of CpG ODN directly to the antigen (23, 24) and co-encapsulating CpG ODN in liposome vesicles (25). These findings suggest that optimal immune stimulation may occur when antigen and adjuvant are presented to the immune system in close spatial and temporal proximity. We are currently investigating the mucosal adjuvant effect of CpG ODN by transcutaneous immunization when the CpG ODN is directly linked to antigen.

In summary, our current study provides evidence that transcutaneous administration of CpG ODN as an adjuvant elicits TT-specific serum IgG and IgA as well as mucosal IgA antibody responses. These findings reveal a promising future for the application of CpG as a potent adjuvant for transcutaneous immunization against tetanus as well as a range of other infectious diseases in humans.

**Acknowledgments**

This work was supported by a Grant-in-Aid for Scientific Research (18592270, 19791624 and 19390537) from the Japan Society for the Promotion of Science,

References