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Research Paper

Changes in Immune Responses to Antigen Applied to Tape-Stripped Skin with CpG-Oligodeoxynucleotide in NC/Nga Mice

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Purpose. CpG-oligodeoxynucleotide (CpG-ODN) plays a critical role in immunity via the augmentation of Th_1 and the suppression of Th_2 responses. We examined here the effect of CpG-ODN on the immune response to an antigen applied to a tape-stripped skin of NC/Nga mouse, a human atopic dermatitis (AD) model, by evaluating the production of cytokines and immunoglobulin isotypes.
Methods. Model antigen, ovalbumin (OVA), and CpG-ODN were applied on to the shaved skin. The penetration of OVA and CpG-ODN was evaluated using confocal laser scanning microscopy (CLSM). Secretion of cytokine from splenocytes and changes in immunoglobulin isotype levels were determined by enzyme-linked immunosorbent assay (ELISA).
Results. Through CLSM it was revealed that the model antigen, OVA, and CpG-ODN easily penetrated the tape-stripped skin. Coadministration of CpG-ODN and OVA to the skin elicited an antigen-specific, Th_1-predominant immune response and enhanced the production of IFN-γ. On the other hand, the production of a Th_2-type cytokine, IL-4, was drastically suppressed. In terms of antigen-specific antibody production, the level of IgG2a regulated by IFN-γ was increased by CpG-ODN, but IgE production regulated by IL-4 was suppressed.
Conclusions. Administration of CpG-ODN with antigen through the skin may shift the immune response from a Th_2- to Th_1-like response. These results suggested that administration of CpG-ODN via skin is a simple strategy for patients with diseases such as AD, which is characterized by Th_2-dominated inflammation.

KEY WORDS: atopic dermatitis; CpG-ODN; NC/Nga mouse; Th_1/Th_2 balance; transdermal administration.

INTRODUCTION

Atopic disorders have a complex and chronic pathogenesis that provides many potential cellular and molecular targets for therapeutic intervention, but may also include redundant pathways mediating disease (1,2). $CD4^+$ helper T (Th) cells have been subdivided into at least two subsets, Th_1 and Th_2, on the basis of their cytokine secretion (3,4). The balance of Th_1 and Th_2 cells is relevant to the outcome of immunologically mediated clinical syndromes, and dysregulated Th_1 or Th_2 responses are thought to be central to the pathology of diseases such as asthma and atopic dermatitis (AD), which is characterized by Th_2-dominated allergic inflammation (5–7). Th_2-like immune responses mediated by the secretion of IL-4, IL-5, and IL-13 are key to the pathogenesis of atopic disorders such as allergic rhinitis, asthma, and AD (8). And it is also well known that AD is clinically characterized by cutaneous reactions resulting from barrier-disrupted skin, the skin being highly susceptible to antigen penetration. Specific immune responses against penetrated antigen in AD have been suggested to be a Th_2-dominant showing high levels of IL-4, IL-5, and IL-13 productions. The ratio of Th_2 to Th_1 cells is increased in lesional skin of AD patients. On the other hand, Th_1-like responses enhance cellular immunity associated with increased levels of Th_1-like cytokines such as IL-2 and IFN-γ, and cytotoxic T lymphocyte activity is upregulated, indicating that the suppression of Th_1 cell responses can result in the acceleration of tumor growth (9).

Unmethylated CpG dinucleotides flanked by certain bases (CpG motif), which are present in bacterial DNA, have been shown to be an immunostimulator (10). Both the bacterial CpG motif and synthetic oligodeoxynucleotides containing CpG motif (CpG-ODN) activate cells such as B cells, macrophages, and dendritic cells through Toll-like receptor-9 (TLR-9) (10,11). Signaling through TLR-9 leads to the secretion of large amounts of proinflammatory cytokines such as IL-1, IL-6, TNF-α, and IL-12 (12,13). IL-12 acts on T and NK cells inducing the production of cytokines, primarily IFN-γ, enhancing NK cell cytotoxic activity and favoring the generation of cytotoxic $CD8^+$ T lymphocytes (14). Furthermore, CpG-ODN rapidly activated B cells to proliferate and secrete IL-6 and IgM (15,16). Consequently, CpG-ODN could serve as an adjuvant for cellular and humoral immunities (17). The induction of such innate immune responses and production of Th_1-related

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cytokines is very important in controlling and determining the type of antigen-specific immune responses that are induced (17). The ability of CpG-ODN to shift the immune response from a Th_2- to Th_1-like response is exploited to develop an immunotherapy for patients with diseases such as AD that are associated with a Th_2-predominant immune response (5–7).

NC/Nga mice are an inbred strain established from Japanese fancy mice in 1957 by Kondo (Nagoya University, Nagoya, Japan). When kept under conventional conditions, they started to scratch themselves at about 8 weeks, and the skin became dry and scaly. Within the next several weeks, the mice developed lesions on the ears, back, neck, and face. Matsuda *et al.* (18) thus proposed that the NC/Nga mouse would be an excellent animal model for human AD.

In this paper, we examined the alterations in immune response to a model antigen, OVA, applied to tape-stripped skin with CpG-ODN in NC/Nga mice. CpG-oligodeoxynucleotide easily penetrated the skin, and remarkably changed immune responses from a Th_2 to a Th_1 type as evaluated from cytokine and antibody levels. These results indicated that CpG-ODN changes immune responses from a Th_2 to a Th_1 in Nc/Nga mouse skin. This is the first finding that CpG-ODN could change immune response in NC/Nga mouse, which would be an excellent model for human AD.

MATERIALS AND METHODS

Materials

The sequences for CpG-ODN and non-CpG-ODN were 5′-TCCATGACGTTCCTGATGCT-3′ and 5′-TCCATGAGCTTCCTGAGTCT-3′, respectively, and both HPLC-purified phosphorothioate ODNs were obtained from Amersham Pharmacia Biotech (Tokyo, Japan). Rhodamine (Rho)-labeled CpG-ODN and Rho-non-CpG-ODN were also obtained. OVA and fluorescein isothiocyanate (FITC)-labeled OVA were purchased from Sigma (St. Louis, MO, USA).

Animal Experiment

NC/Nga mice (male, 6–8 weeks old) were purchased from Japan SLC Inc. (Shizuoka, Japan). Animal use and relevant experimental procedures were approved by the Tokyo University of Pharmacy and Life Science Committee on the Care and Use of Laboratory Animals (permission No: 2004-003). Mice housed under specific pathogen-free (SPF) condition were anesthetized with the i.p. injection of Nembutal (1.5 mg/mouse), the abdominal skin was shaved, and then the stratum corneum was removed by stripping with adhesive tape six to ten times. After the application of FITC-labeled OVA (Sigma) and Rho-labeled CpG-ODN (Amersham Pharmacia Biotech) onto the shaved skin, the skin was excised at specific points in time, embedded in Tissue-Tek OTC compound (Miles Inc., Elkhart, IN, USA), and snap-frozen in liquid nitrogen. The skin was then cut with a cryostat into 10-μm vertical sections. To analyze the distribution of FITC-OVA and Rho-CpG-ODN, the dermal skin was fixed with 10% formalin at 4°C for 16 h, embedded, snap-frozen, and cut as described above. The sections were examined by confocal laser scanning microscopy (Micro-Radiance, Bio-Rad Laboratories Inc. Tokyo, Japan).

Preparation of Mouse Splenic Cells

Following the application of CpG-ODN and OVA to the tape-stripped skin, the spleen was removed on day 5, and splenic single cell suspensions were prepared (19). Briefly, spleen was incubated in 50-mm plastic dishes (Iwaki Glass, Chiba, Japan) for 30 min at 37°C in RPMI1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, and 1 mg/ml collagenase, and splenic single cells were obtained. To examine the production of cytokines, spleen cells (5×10^5 per well) were incubated in RPMI-1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 5×10^{-5} M 2-mercaptoethanol for 72 h in the presence of OVA (2 mg/ml).

Cytokine Determination

The levels of IL-4 and IFN-γ in the supernatant of splenic cells were routinely determined with a sandwich enzyme-linked immunosorbent assay (ELISA) using pairs of purified capture and biotinylated detection monoclonal antibodies (BD PharMingen, San Diego, CA, USA) recognizing murine IL-4, and IFN-γ, according to the manufacturer's protocols. In brief, ELISA plates (Nunc-Immune Plate MaxiSorp; Nunc, Naperville, IL USA) were coated with 50 μl of 2 μg/ml antimouse IL-4 monoclonal antibody (mAb) and antimouse IFN-γ mAb in bicarbonate buffer at 4°C for 18 h. The wells were then washed with PBS containing 0.05% Tween 20 (PBS-T) and blocked with PBS-T containing 3% BSA (BPBS-T). After a 1-h incubation, 50 μl of sample supernatants were transferred to each well, and left overnight to confirm that the immune reaction took place. After four washes with PBS-T, biotin-conjugated antimouse IL-4 or IFN-γ mAb (PharMingen), the plate was left to stand 37°C for 1 h. After further washing with PBS-T, avidin-conjugated peroxidase was added and the absorbance at 450 nm was determined as previously described (20).

Changes in OVA-Specific Antibody Levels

Blood samples from mice treated with OVA and CpG-ODN were collected on specific days from the retro-orbital plexus and sera were pooled. In brief, a doubling dilution of mouse sera (50 μl) were poured into an OVA-coated ELISA plate, and left overnight to confirm that the immune reaction took place. After four washes with PBS containing 0.05% Tween-20 (PBS-T), biotin-conjugated antimouse IgG, or IgE monoclonal antibody (PharMingen), the plate was left to stand at 4°C overnight. After further washing with PBS-T, avidin-conjugated peroxidase was added and the absorbance at 450 nm was determined as described previously (20).

Statistical Analysis

Paired Student's t test was used to compare paired groups. Analysis of variance (ANOVA) was used for multigroup analysis. Values of $P > 0.05$ were considered to indicate lack of significance.

RESULTS

Localization of OVA and CpG-ODN

Fluorescein isothiocyanate-OVA and Rho-CpG-ODN were applied to tape-stripped mouse abdominal skin, and the

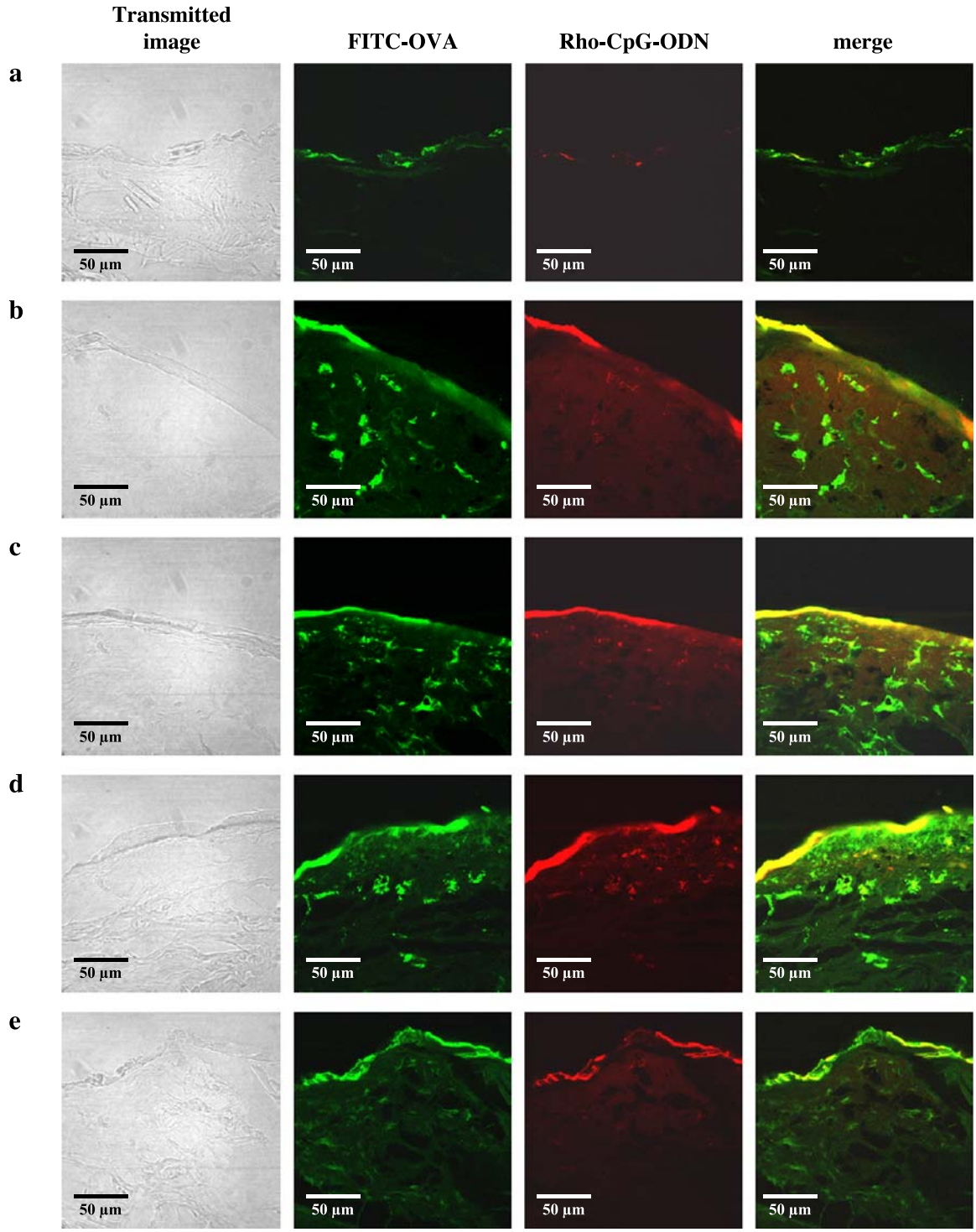

Fig. 1. Localization of FITC-labeled OVA and Rho-labeled CpG-ODN in NC/Nga mouse skin following the topical administration. FITC-OVA (250 μg) and Rho-CpG-ODN (50 μg) were applied to the tape-stripped abdominal skin. (a) Without tape stripping; (b, c, d, e) stripped ten times. Fluorescent images were obtained from the same section at 1 h (a, b), 6 h (c), 12 h (d), or 24 h (e) after application using confocal laser scanning microscopy.

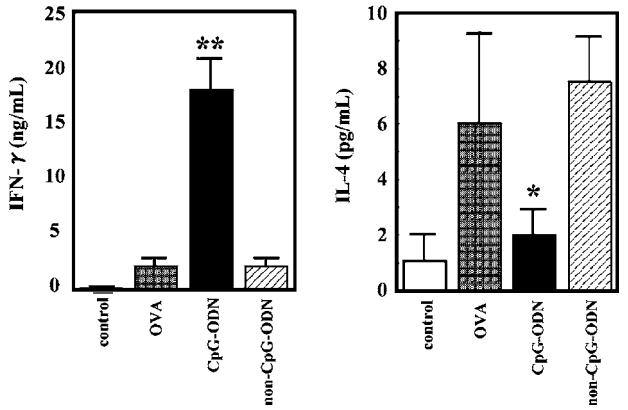

Fig. 2. Effect of CpG-ODN on the production of OVA-specific IFN-γ and IL-4 by spleen cells. NC/Nga mice were sensitized with OVA and CpG-ODN through the skin. OVA (250 μg), OVA (250 μg) plus non-CpG-ODN (50 μg), and OVA (250 μg) plus CpG-ODN (50 μg) were applied to shaved abdominal skin after barrier disruption by tape stripping (ten times). On day 5, spleen cells (5×10^5 per well) were prepared and incubated in the presence of OVA (2 mg/ml) for 72 h. Concentrations of IFN-γ and IL-4 in the culture supernatants were evaluated by ELISA. Each value represents the mean ± SE for three mice. $^{**}P < 0.01$, $^{*}P < 0.05$ compared with sensitization with OVA.

localization of OVA and CpG-ODN was examined via confocal laser scanning microscopy. In control skin (no tape stripping), the fluorescent signals for FITC and Rho were observed faintly at the stratum corneum, suggesting that neither OVA nor CpG-ODN penetrated the subcutaneous layer (Fig. 1a). In the tape-stripped NC/Nga mice, however, green fluorescence generated from FITC was observed around the corium at 1 h after the application, indicating that FITC-OVA having a molecular weight of 45,000 penetrated easily into the skin. Almost the same phenomenon was observed in Rho-CpG-ODN, and both fluorescences were overlapped partially at the epidermis. To clarify the fate of OVA and CpG-ODN in the skin, the localization of both fluorescences were explored for 12 and 24 h after the application. After 12 h, both fluorescences were overlapped partially at the corium, and migrated into the lower part of the skin (Fig. 1d), and then the intensities of green and red fluorescence from OVA and CpG-ODN, respectively, decreased at 24 h. (Fig. 1e). These findings suggest that OVA and CpG-ODN easily penetrated the tape-stripped skin, and also suggested that OVA and CpG-ODN are taken up by langerhance cells (LC) and/or skin dendritic cells (DC), and migrate to lymph node with in 24 h.

Changes in Cytokine Levels in Spleen Cells

CpG-ODN induces the secretion of large amounts of proinflammatory cytokines such as IL-1, IL-6, TNF-α, and IL-12 from macrophages, dendritic cells, and B cells (12,13) . IL-12 acts on T and NK cells, inducing the production of cytokines, primarily IFN-γ. Furthermore, CpG-ODN has been reported to shift the immune response from a Th_2- to Th_1-like response (21–23). Thus, the effect of CpG-ODN that was applied to the tape-stripped skin on the production of IFN-γ and IL-4 was investigated *in vitro*. Spleen cells were prepared from NC/Nga mice on day 5 following the transdermal administration of OVA and CpG-ODN, and were incubated in the presence of 2 mg/ml of OVA for 72 h. Supernatants were collected and concentrations of IFN-γ and IL-4 were evaluated by ELISA. As shown in Fig. 2, the production of IFN-γ increased on the coapplication of OVA and CpG-ODN, and was nine times that following treatment with OVA alone. When NC/Nga mice were treated with non-CpG-ODN and OVA, the production of IFN-γ was almost the same to that of OVA alone. On the other hand, the production of IL-4 by the spleen cells decreased drastically when the mice were treated with OVA and CpG-ODN, and was one fourth the level in spleen cells from mice treated with OVA alone (Fig. 2). Furthermore, no decrease in the production of IL-4 was observed in non-CpG-ODN.

Changes in Antibody Levels

As mentioned, CpG-ODN promotes Th_1-like immune responses and suppresses Th_2 responses *in vivo*. We therefore examined whether they redirected B cell differentiation toward a Th_1-like phenotype (24), thereby inducing

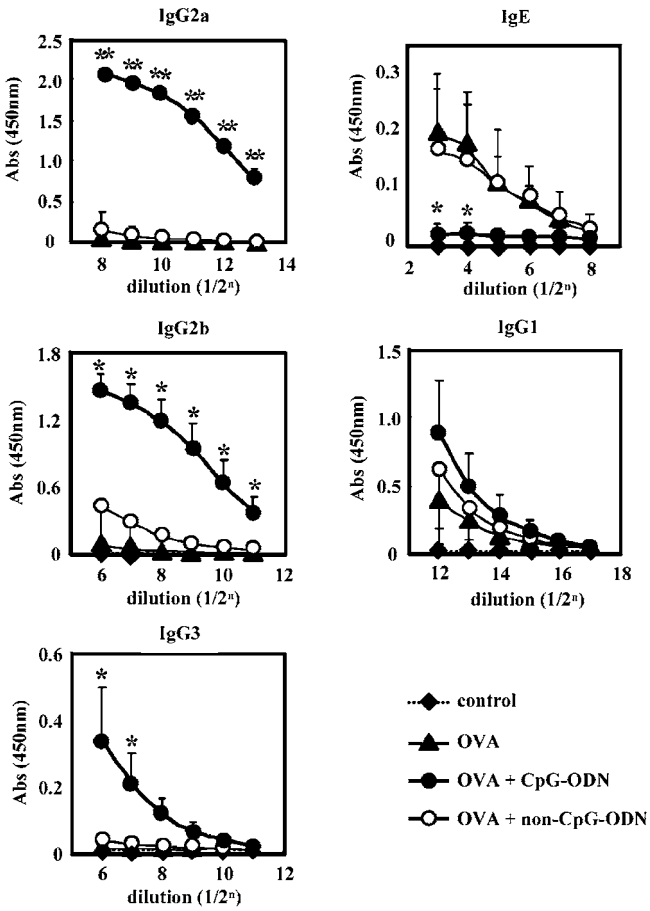

Fig 3. Effect of CpG-ODN on OVA-specific antibody responses after sensitization with OVA and CpG-ODN through barrier-disrupted skin. Control (♦), OVA 250 μg (▲), OVA 250 μg plus non-CpG-ODN 50 μg (○), and OVA 250 μg plus CpG-ODN 50 μg (●) were applied to the tape-stripped skin on day 0 and day 7. Sera were collected 2 weeks after the last application, and OVA-specific IgG1, IgG2a, IgG2b, IgG3, and IgE titers were evaluated by ELISA. Each value represents the mean ± SE for three mice. $^{**}P < 0.01$, $^{*}P < 0.05$ compared with sensitization with OVA.

multiple non-Th_2-related isotypes. Sera were collected 2 weeks after the last transdermal administration of OVA and CpG-ODN, and immunoglobulin isotypes were determined by ELISA. The changes in immunoglobulin isotypes are shown in Fig. 3. The production of OVA-specific IgG2a and IgG3, which are considered to be a Th_1-like immunoglobulin isotype, drastically increased when OVA was applied to the tape-stripped skin with CpG-ODN, but no production of IgG2a and IgG3 were observed in mice sensitized to OVA only. Although OVA-specific IgG1 is also considered to be a Th_2-like isotype, no clear change in production was observed when mice were treated with CpG-ODN. Furthermore, the production of OVA-specific IgE, a Th_2-like isotype, was strikingly suppressed by the coapplication of CpG-ODN (Fig. 3). Taking these findings into consideration, CpG-ODN coapplied with the antigen to tape-stripped skin changes the immune response from Th_2 to Th_1.

DISCUSSION

The balance of Th_1 and Th_2 cells is relevant to the outcome of immunologically mediated clinical syndromes, and asthma and AD are characterized by Th_2-dominated allergic inflammation (5–7). Because NC/Nga mouse would be an excellent animal model for human AD (18), the effects of CpG-ODN on immune response against antigen invaded from the skin was investigated in NC/Nga mice. It is considered that the stratum corneum acts as a barrier for the penetration of antigens because of their high molecular weight and high water solubility. Tape stripping is a simple method to remove the epidermal barrier of stratum corneum; it is also a low-cost and readily performed procedure that elicits minimal discomfort (25). We used this tape stripping method to disrupt the skin barrier and constructed the AD-like skin model in mice.

The system in CpG-ODN injection prevented the development of airway eosinophilia and bronchial hyperreactivity in mice sensitized to an antigen. On the other hand, parenteral administration of CpG-ODN could be associated with such side effects as hematopoieisis (26) and antigen-independent expansion of naïve T cell (27). Atopic dermatitis is a disease in which allergens such as mite or house dust would penetrate into the skin and cause a strong Th_2-type immune response. We thus investigated whether a topical application of CpG-ODN to tape-stripped skin changes the immune response from Th_2 to Th_1 by evaluating the production of cytokines and antibodies. First, we examined the localization of antigen and CpG-ODN following the application to tape-stripped skin of NC/Nga mice. In no tape-stripping mice, a faint fluorescence was only observed at stratum corneum. After stripping with adhesive tape ten times, the stratum corneum was completely removed (data not shown); the fluorescence generated from FITC and Rho was localized in the skin and both fluorescences were overlapped partially at the corium, and migrated into the lower part of the skin (Fig. 1d), and then the intensities of green and red fluorescence from OVA and CpG-ODN, respectively, decreased after 24 h. (Fig. 1e). One of the possibilities is that LC and skin DC that take up OVA and/or CpG-ODN may migrate to the lymph node. Another possibility is that OVA and ODN that penetrated into the skin were decomposed by protease or DNase.

CpG-ODN induces a Th_1-like pattern of cytokine production that is dominated by IL-12 and IFN-γ with little secretion of Th_2 cytokines, and then shifts the immune response from Th_2 to Th_1. We thus investigated whether CpG-ODN alters the immune response to the topically administered antigen, and found a change from a Th_2- to Th_1-predominant response on the basis of the cytokine secretion (Fig. 2) and changes in the immunoglobulin isotype of sera (Fig. 3). The administration route of CpG-ODN is also important for inducing antigen-specific Th_1 immune response. When mice were applied with OVA from barrier-disrupted skin and administered with CpG-ODN (i.p. or i.v.), the OVA-specific Th_1 immune response was lower than that of CpG-ODN coadministered with OVA from the skin (data not shown). Therefore CpG-ODN coadministered with OVA through the skin was important for the immune response and colocalization of CpG-ODN, and antigen in the skin may be an important factor in these responses; however, the details are still unclear.

Th_2-type immune responses mediated by the secretion of IL-4, IL-5, and IL-13 are significant in the pathogenesis of atopic disorders (28). NC/Nga mice showed Th_2-dominant immunoglobulin isotype expression against OVA administered via tape-stripped skin (Fig. 3). The regulation of IgE synthesis has been extensively investigated in recent years, and it has been reported that IFN-γ and IL-4 strongly contribute to this regulation (29–31). IFN-γ and IL-4 promote IgG2a and IgG1 class switching, respectively (32,33). Furthermore, it has been reported that IL-4 and IFN-γ strongly regulate IgE synthesis, and the Th_1-type cytokine IFN-γ acts as a counterpart of IL-4 and suppresses the production of IgE. These findings suggest that IgE synthesis is highly dependent on balance in the production of IL-4 and IFN-γ, and the difference in cytokine production following CpG-ODN administration affects the change in IgE production even in mice producing IgE.

Toll-like receptor-9 (TLR-9) is a receptor for CpG-ODN, and is known to be expressed in macrophages, B cells, and dendritic cells (DC) (10,34). Although keratinocytes have been shown to internalize ODN, they are not known to express TLR-9 (35). Thus the Th_1-predominant immune response may come from epidermal or dermal DC. On the other hand, the draining LN (lymph node) may contain a substantial number of cells that express TLR-9 compared to other tissues such as the skin and muscle, and concentrations of CpG-ODN in the draining LN may be important as CpG-ODN shows biological activities. Meanwhile, the Th_1-predominant immune response may come from the DC or T cells that were activated as they migrated into LN. Recently, Liu *et al.* (35) reported that physical stress induces the expression of TLR-9 in the skin. The mechanism of the Th_1-dominant immune response of CpG-ODN against antigen in tape-stripped skin remains still unclear.

Moreover, Klimuk *et al.* (36) reported that CpG-ODN applied onto bare skin with antigen peptide induces cytotoxic T lymphocytes (CTL). It is known that a part of CTL proliferation and survival depend on helper T cell. Therefore, peptide-specific Th_1 would be induced by CpG-ODN administration, as similarly noted in our investigation. In other

words, administration of CpG-ODN through the skin with antigen induces both antigen-specific Th_1 and CTL; consequently, that skin would be considered an excellent site for the administration of immune adjuvants. However, there are limitations in the models that Klimuk *et al.* (36) and our group have used. In human AD, antigens have been considered to be mite and house dust, and the skin status is different from that of the mouse even if NC/Nga mouse is considered an excellent animal model for human AD. Therefore more examination should be needed for AD immune therapy using CpG-ODN.

In conclusion, tape stripping is a simple way to remove the epidermal barrier of the stratum corneum, and the skin is considered an excellent site for the noninvasive administration of a vaccine. Coadministration of CpG-ODN with OVA via tape-stripped skin was found to elicit an antigen-specific Th_1-predominant immune response by the evaluation of cytokine secretion and antibody production in Nc/Nga mice. Th_2 responses are thought to be central to the pathology of diseases such as asthma and AD, which is characterized by Th_2-dominated allergic inflammation. Our results suggest that administration of CpG-ODN via the skin is a simple strategy of immunotherapy for patients with diseases such as AD.

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