Oligonucleotide containing CpG motifs enhances immune response to mucosally or systemically administered tetanus toxoid

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Abstract

Oligodeoxynucleotides (ODN) containing unmethylated CpG dinucleotides induce proliferation of B cells and activation of macrophages and thus stimulation of the immune system. We tested an oligonucleotide containing an unmethylated CpG dinucleotide flanked by two 5’ purines and two 3’ pyrimidines (GAGAACGCTCGACCTTCGAT) for the ability to affect antibody levels to tetanus toxoid (Tt). Groups of male Rowett rats (n = 5–6/group) received colloidal aluminium hydroxide (Al(OH)₃) either alone, or with Tt bound to the Al(OH)₃, or with Tt bound to Al(OH)₃ with the addition of the CpG oligonucleotide. Antigens were administered subcutaneously in the salivary gland vicinity once, or by gastric intubation on 3 consecutive days. On day 124 all animals were given a boost with the same material by the same route. Serum IgG and saliva IgA antibody to Tt was determined by ELISA. Serum antibody levels were significantly higher in ODN + Tt treated rats than in Tt-alone rats immunized by either route after primary or booster immunizations. Thus, administration of an ODN containing unmethylated CpG motifs along with an immunogen bound to Al(OH)₃ can result in enhanced specific antibody when administered by intragastric as well as subcutaneous routes. © 2001 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Unmethylated CpG dinucleotides are found more frequently in the genomes of bacteria and viruses than in vertebrates [1]. Synthetic oligodeoxynucleotides (ODN) containing CpG sequences, as well as DNA isolated from bacteria, each have been shown to induce murine B cells to proliferate and secrete immunoglobulin in vivo and in vitro [2]. This activation was shown to be enhanced if there was a simultaneous signal through the antigen receptor [2], which will induce up to 95% of splenic B cells to enter cell cycle. Macrophages and other antigen-processing and presenting cells (APC) are apparently stimulated directly, while T and NK cells are presumed to be stimulated indirectly by IL-12 produced by stimulated macrophages, and IFN-γ from stimulated NK cells [3–5]. These and other findings suggest that a part of the mammalian innate immune defense system can be based on recognition of bacterial DNA [2,6]. Therefore it was suggested that CpG ODNs could be used as adjuvants [2] and this has been successfully demonstrated in mice [7–10] and in primates [11].

When injected subcutaneously, an antigen in a finely particulate form, as in this case with Tt bound to colloidal Al(OH)₃, will most likely be taken up and processed by macrophages and dendritic cells in the loose connective tissue and presented to lymphocytes in local draining lymph nodes [12]. An intragastrically administered finely particulate antigen will most likely be taken up by M cells of the intestinal lining. M cells are found distributed among the nutrition-absorbing intestinal epithelial cells and overlying single or aggregated mucosal lymphoid follicles within the intestinal wall [13]. M cells act as antigen-sampling cells, and by transcytosis pass antigens to macrophages, dendritic...
cells and lymphocytes contained within their basolateral ‘pocket’ invagination. From there, antigen-containing cells would likely also travel to Peyer’s patches and mesenteric lymph node for further antigen processing and presentation to lymphocytes. In this situation a secretory mucosal immune response is expected to predominate. A systemic response is also likely if the particles containing the antigen are, by some mechanism, resistant to enzymic degradation, and some phagocytic cells containing antigen travel to the mesenteric lymph node. Because only a small percentage of the antigen particles administered intragastrically are likely to be ‘sampled’ by M cells the response/μg of antigen might be expected to be lower than for the same antigen preparation administered subcutaneously.

In this manuscript we show that an ODN with CpG motifs greatly enhanced systemic immune responses and demonstrated modest enhancement of mucosal immune response in rats. Protein antigen bound to Al(OH)₃ can be a more potent immunogen when given by the intragastric route with an ODN containing CpG motifs.

2. Materials and methods

2.1. Animals

Inbred male Rowett (Forsyth Institute inbred, nu/+, phenotypically normal heterozygous-nude) rats that have a restricted bacterial flora were raised in isolators then removed to sterilized microisolator cages and maintained on sterilized food and water. The rats were immunized at 13–18 weeks of age, 5–6 animals were used per group. The Forsyth animal care program is fully AAALAC accredited, and experimental procedures were carried out under Forsyth IACUC approval.

2.2. Antigen preparation and oligonucleotide

The antigen mixture was prepared as tetanus toxoid (Tt; Wyeth Ayerst, Marietta, PA) bound to aluminum hydroxide (Al(OH)₃) with or without the addition of a CpG motif synthetic ODN GAGAACGTCCCTGTCAGT (CpG dinucleotides underlined for clarity). This ODN originally described by Krieg [2], was chosen because of its potent B cell activation properties including induction of blastogenesis, IgM production in vitro and induction of proliferation in vivo. Also, while this ODN contains one classical CpG motif flanked by two 5’ purines and two 3’ pyrimidines, there are two other CpG dinucleotides which might be biologically active. This ODN was prepared, and tested for purity by polyacrylamide gel electrophoresis (Ransom Hill Bioscience, Ramona, CA). To test its activity a control scrambled ODN (GAGACCAGCACCCTGTCAGT) was also synthesized. Both ODNs were tested in an athymic rat lymph node cell stimulation assay. Only addition of the CpG ODN resulted in stimulation of B cells.

‘Alhydrogel’, a 2% finely colloidal suspension of Al(OH)₃ (Accurate/Superfos Biosector, Denmark) was mixed with Tt (3.1 mg protein/ml) and allowed to bind at room temperature overnight. Most of the (Al(OH)₃ flocculated with the protein and formed a loose sediment. After centrifugation and removal of the supernatant fluid containing unbound Tt, and resuspension in PBS, the ODN was added and a further 3 h was allowed for any binding of the ODN that might occur. The suspension was not washed again.

2.3. Immunization

For intragastric (IG) immunization, 8 mg Al(OH)₃ ± 2 mg Tt ± 200 μg ODN was used per dose, on three consecutive days, 4 h after removal of food and water. This relatively high dose of Al(OH)₃ and Tt was chosen to counteract degradation of the antigen. The mixture was given (0.5 ml) by intra-gastric intubation using a curved round-ended intragastric needle (Popper, New Hyde Park, NY). For subcutaneous injection in the salivary gland vicinity (SGV), 4 mg Al(OH)₃ ± 100 μg Tt ± 200 μg ODN per dose was injected subcutaneously once, in each of four sites above each of the major salivary glands, 50 μl/site. Immunization by this route has been shown to provide consistent and reproducible salivary mucosal antibody and systemic responses [14,15].

Both SGV and IG groups received a boost immunization at 124 days, identical to the primary immunization. Five to six rats were included in each group.

2.4. Sample collection

Animals were bled from the retroorbital sinus under ether anesthesia at 10, 17, 24, 45, 102, 145 and 165 days after the first immunization. Saliva was collected after ether anesthesia and pilocarpine injection (1 mg/100 g body weight), at 17 days after the first immunization. Following centrifugation, samples were stored at −70°C.

2.5. In vitro proliferative responses

At sacrifice, spleen and lymph node lymphocytes from selected animals were analyzed for in vitro proliferative responses to Tt. Single cell suspensions of spleen or pooled peripheral lymph nodes (cervical, axillary, brachial) from each animal were plated in 96 well plates at 2.5 × 10⁵ viable lymphocytes/well with graded doses of dialyzed Tt solution in complete RPMI (Penicillin/
Streptomycin, $5 \times 10^{-5}$ M 2ME, with 10% FCS), and cultured in 5% CO$_2$ for 5 days, with 0.5 μCi $^3$H-thymidine added 24 h before harvest. Uptake of radioactivity was assessed by liquid scintillation spectrometry and expressed as counts per minute (cpm).

2.6. Antibody analyses

Sera and salivas were tested for the presence of antibody to Tt by a previously described ELISA performed in microtiter plates [16]. The antigen used to coat the plates was 1 μg/well Tt (Wyeth Ayerst) for assay of serum IgG, or 0.5 μg/well of a column-purified Tt (4.7 mg protein/ml, Massachusetts Department of Public Health Biologic Laboratories) used for salivary IgA assay. Isotype-specific rabbit anti-rat IgA or IgG was used with goat anti-rabbit IgG-alkaline phosphatase (TAGO Inc., Burlingame, CA). The plates were developed with p-nitrophenyl-phosphate (Sigma, St. Louis, MO) and read on a photometric scanner at 405 nm. Results are expressed as optical density (O.D.).

The 45 and 165 day serum samples and the 17 day saliva samples were serially diluted and assayed by ELISA to determine the titration endpoint for each sample. For each set of samples assayed the endpoint was chosen as the level where the graphed line for the sample crossed the upper level of the experimental background, or the level for unimmunized animals. The geometric mean plus/minus standard error of the mean was calculated for each experimental group using the reciprocal of the titration endpoint. These are expressed as antilogs. Statistical analysis was performed using Instat (GraphPad Software, San Diego, CA).

3. Results

3.1. Serum IgG antibody after intragastric immunization

Rats immunized intragastrically with Tt bound on Al(OH)$_3$ along with CpG ODN developed serum IgG antibody responses to Tt by 17 days after immunization. Fig. 1A shows antibody levels tested at a single dilution (1:160) at intervals between 10 to 100 days after immunization. In the Al$_3$Tt ODN group serum antibody continued to rise significantly ($P < 0.05$ compared to other groups) through 24 to 45 days after

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**Fig. 1.** (A) Kinetics of induction of IgG antibody to tetanus toxoid in serum of rats immunized by intragastric instillation (mucosal route) with: □, Al(OH)$_3$ only; ■, Al(OH)$_3$ + Tt; ●, Al(OH)$_3$ + Tt + ODN and, X, Al(OH)$_3$ + ODN only. Each point represents the mean OD for 5–6 animals taken at the designated time. Shown is the mean ELISA O.D. at 405 nm when tested at 1/160 dilution. * Indicates statistically significant differences between Al + Tt + ODN group and the other groups ($P < 0.05$) when tested by Student–Neuman–Keul multiple comparisons test. (B) Kinetics of induction of IgG antibody to tetanus toxoid in serum of rats immunized by injection subcutaneously in the salivary gland vicinity with: □, Al(OH)$_3$ only, ■, Al(OH)$_3$ + Tt, or ●, Al(OH)$_3$ + Tt + ODN. Each point represents the mean O.D. for 5–6 animals taken at the designated time. Shown is the mean ELISA O.D. at 405 nm when tested at 1/160 dilution. Both Al(OH)$_3$ + Tt + ODN and Al + Tt groups were significantly greater than Al(OH)$_3$ alone ($P < 0.05$) when tested by Student–Neuman–Keul multiple comparisons test.
immunization. The level was still high at 100 days after immunization. The serum taken after the boost (day 165) also continued to show a significantly increased antibody level in the Al + Tt + ODN group above the other two groups (P < 0.05, Table 1). There was little or no specific antibody detectable in the group receiving Al + Tt without ODN, presumably because the dose of antigen was below the level of antigenic stimulation for this mucosal presentation. Serial dilution of the 45 and 165 day sera also confirmed the significance of the increased response in the group receiving Al + Tt + ODN, as calculated by the titer endpoint for each animal. Thus, when administered by the intragastric route, the presence of the CpG ODN significantly raised the level of serum IgG antibody to the antigen.

### 3.2. Serum IgG antibody after salivary gland vicinity injection

Animals injected subcutaneously at SGV sites with Tt bound on Al(OH)₃ developed strong serum IgG antibody responses to Tt by 17 days after immunization (Fig. 1B). Levels rose to an apparent maximum at 45 days after the injection. Fig. 1B shows antibody levels tested at a single dilution (1/160) at intervals between 10 and 100 days after immunization. There was no obvious difference in serum IgG antibody by ELISA between the groups receiving Al + Tt and Al + Tt + ODN at the dilution used for assay. Therefore, serum samples from 45 and 165 days after immunization were serially diluted, and an endpoint titer was determined from the analyses for each animal. By means of this serial dilution the two groups, Al + Tt with or without the addition of the ODN, could be clearly differentiated. These data are presented in Table 1 as the geometric mean endpoint titer of each group. At both time intervals after immunization, the addition of the ODN to the Al + Tt preparation, resulted in significantly higher antibody levels (at least P < 0.05). This result demonstrates that addition of the CpG ODN to the antigenic mixture significantly elevated the serum IgG antibody response of animals immunized subcutaneously in the SGV region.

### 3.3. Salivary IgA antibody to Tt after intragastric or salivary gland vicinity immunization

The salivary IgA antibody to Tt was assayed in the 17 day samples from intragastrically and subcutaneously immunized animals (Table 2). Considerable salivary IgA antibody developed in the Tt + ODN group injected in the salivary gland vicinity (P < 0.05 compared to the Al(OH)₃ group). Only low levels of antibody were observed in a few rats in the group injected with Al(OH)₃ + Tt. In the groups given antigen by the intragastric route only the group receiving antigen plus ODN developed specific IgA antibody as compared to controls, but this level was not statistically significantly raised above the group receiving Al(OH)₃ + Tt alone.

### 3.4. In vitro proliferative responses

In vitro proliferative responses of spleen and lymph node lymphocytes to Tt in solution were tested in these animals at termination of the experiment (day 165). Lymph node lymphocytes (which included the site-

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**Table 1**

<table>
<thead>
<tr>
<th>Groups</th>
<th>45 days after primary immunization</th>
<th>41 days after secondary immunization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Subcutaneous in SGV</td>
<td>Intragastic</td>
</tr>
<tr>
<td>Al(OH)₃ only</td>
<td>279 (198–391)</td>
<td>320 (219–468)</td>
</tr>
<tr>
<td>Al(OH)₃ + Tt</td>
<td>13 500b (10 233–17 816)</td>
<td>422 (188–947)</td>
</tr>
<tr>
<td>Al(OH)₃ + Tt + ODN</td>
<td>36 459b,c (29 464–45 115)</td>
<td>5120b,c (2862–9264)</td>
</tr>
</tbody>
</table>

*Geometric mean reciprocal endpoint at 0.05 OD units. Anti-log of geometric mean (± Standard error of the mean). Statistical analysis by Student–Newman–Keul multiple comparison test. (n = 5–6).

b Significantly elevated when compared to Al(OH)₃ only group (P < 0.05).

c Significantly elevated when compared to Al(OH)₃ + Tt group (P < 0.05).

d Significantly elevated when compared to Al(OH)₃ + Tt + ODN group (P < 0.001).
draining nodes) of the rats injected in the salivary gland vicinity, responded well to Tt. Proliferative responses in the Al(OH)₃ + Tt + ODN group (n = 5) to Tt were greater than those of the Al(OH)₃ + Tt group (n = 5) (not quite statistically significant, P = 0.1) indicating some T cell potentiation by the ODN containing CpG motifs.

4. Discussion

We have demonstrated that the addition of a previously described active ODN containing CpG was effective in enhancing serum IgG antibody levels after administration with Tt in Al(OH)₃ by local injection (s.c. in the SGV) or by intragastric intubation. Significant enhancement was observed after primary and secondary immunization. Modest enhancement with ODN was observed in salivary IgA after primary immunization by both routes.

Part of the ability of infective bacteria to induce a vigorous immune response in a mammalian host seems to be due to non-specific stimulation of host immune system cells by bacterial DNA. This would constitute a portion of the innate immune defense mechanism, and could serve to potentiate a specific adaptive immune response. The major stimulatory sequence in bacterial DNA (for mice) has been found to be an unmethylated CpG flanked by two 5′ purines and two 3′ pyrimidines [17]. This six base motif is expressed nearly 20 times more frequently in bacterial than in vertebrate DNA [17]. Immune stimulation by bacterial DNA had been described [18,19] as a stimulation of tumor rejection in mice [7]. It was later found [20,21] that this effect was attributable to stimulation of macrophages, NK and other intermediary cells of the immune system by the DNA of the bacteria, in turn leading to stimulation of specific immune responses to tumor antigens.

CpG ODNs have been shown to be stimulatory of either proliferation or cytokine production in macrophages, B cells, and NK cells. When bacterial DNA or CpG-containing ODNs are introduced in vivo, stimulation of these cell types and production of cytokines might be expected to occur and to be mutually interactive [17]. It has subsequently been shown that bacterial DNA stimulates macrophages to produce IL-12, which stimulates NK cells to produce IFN-γ, (driving the Th1 response) [3]. Thus, a cascade of immune events is set in motion by bacterial DNA via its CpG sequences [5,22]. T cell activation is indirect. CpG DNA does not induce IFN-γ production by non-adherent cells directly, but supernates from cultures of adherent cells plus DNA induce IFN-γ production in the non-adherent cell population, namely in the T cells. Macrophages are induced by CpG ODNs to produce IL-12 and TNF-α, and antibody to IL-12, or TNF-α inhibits T cell stimulation by ODNs [22].

Almost any cell will take up ODNs, after the ODNs have bound to a variety of cell surface molecules. Competition for this binding can be introduced by cationic dextrans and other unrelated molecules [23]. Immunomodulatory effects are presumably due to ODN binding to intracellular regulatory and signaling molecules, with subsequent activation of these molecules, resulting in changes in cell conformation, production of cytokines, and cell proliferation [24,25]. Yi et al. [25] showed that ODNs that are adsorbed to the cell surface, are taken in by endocytosis to become acidified in endosomes and then become active and induce reactive oxygen species, which leads to NF-κB activation and subsequent proto-oncogene and cytokine expression. There does not appear to be a specific membrane receptor for ODN, and cell uptake is required for its action. Thus, cellular uptake of DNA in both B cells and in a monocyte-like cell line [25] appears to occur via pH dependent adsorptive endocytosis into an acidified intracellular compartment.

Recently, other groups have described mucosal immunization using CpG as adjuvant via the respiratory routes [7,8] with the induction of nasal and salivary Ab. The responses observed to local and gastric intubation in the present study were not as robust as those reported results, possibly because both the protein antigen (Tt) and the non-phosphorothioated ODN were vulnerable to degradation in the environment of the gut. Also, the ODN used may have been more potent in mice [7]. Antigen sampling may also be less efficient in the gut than in the respiratory tract, including that gastrically administered antigen can be diluted in other gut contents even after 4 h without food and water. Although antigen sampling efficiency in the gut (or salivary gland locale) may differ from the respiratory tract, and the potency of the CpG ODN may have differed, it is indicated that an ODN containing CpG can function as an adjuvant when given by intragastric route.

Thus, the ODN containing CpG motifs used in this present study was an effective adjuvant in the response to Tt when given in the salivary gland vicinity or intragastrically incorporated in aluminum hydroxide for a mucosal (salivary) response. Unaltered ODNs presented in vitro and in vivo can be broken down by nuclease degradation [26]. Sun [27], found that insect DNA in oil was stimulatory as an adjuvant and therefore protected, whereas insect DNA alone was ineffective. In the present experiments the unmodified ODN may have been more effective because it was protected in, or because it was involved in, Al(OH)₃ colloidal-size particles (colloidal aggregates with protein). A synergistic effect of CpG ODN and alum together has been
demonstrated [28]. If an individual intestinal M cell was to take up one of these particles, aluminium hydroxide, Tt antigen and ODN would be present together and be able to co-stimulate the same antigen processing and presenting cell, presumably a macrophage. This effect of the several parts of a complex antigen mutually interacting in stimulation of an antigen processing/presenting cell is observable in the increased immune response induced by covalently bound complex antigens, or antigens injected together (co-immunization) as compared to the limited response to the individual components injected separately [14,29].

Theoretically, antigen injected along with a CpG ODN will benefit from the adjuvant (immunostimulatory) effect of the ODN, on NK cells and macrophages which are super-stimulated, as are B cells, and higher than usual levels of cytokines are produced that boost T cell responses. The resulting cytokines will heighten both cellular and humoral immune responses. The sets of cytokines stimulated are those that will stimulate Th1 over Th2 responses [9,10].

This study suggests a novel use for CpG ODNs in immune potentiation of orally and systemically administered particle-bound protein immunogens. Colloidal aluminium is approved as an adjuvant/carryer for protein antigens for human immunization protocols (tetanus toxoid), therefore CpG ODNs with motifs found to be active in primates [11] and in humans [30] might have a potential in clinical trials as enhancers of immune responses to aluminium-bound vaccines.

Acknowledgements

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