PyNTTTTGT prototype oligonucleotide IMT504 is a potent adjuvant for the recombinant Hepatitis B vaccine that enhances the Th1 response

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Abstract
PyNTTTTGT oligodeoxynucleotides (ODNs) cause activation, proliferation and immunoglobulin secretion on B cells, and the expression of co-stimulatory molecules on plasmacytoid dendritic cells of primates. It has now been discovered that these ODNs are also active on rat cells. This fact allowed us to investigate the adjuvant properties of PyNTTTTGT ODNs in a human Hepatitis B vaccine using this animal model. A very significant increment, as compared with the antigen alone, was observed in the antibody production induced by vaccination with the recombinant Hepatitis B surface antigen adjuvated with the PyNTTTTGT prototype IMT504 ODN. Analysis of the IgG subclass distribution in the sera of vaccinated animals indicated that, although an increase was observed in the titer of all the IgG subclasses, the increase on the Th1-associated IgG2b subclass was clearly more pronounced. Remarkably, this effect on the IgG2b titer was observed even if alum, a Th2 promoting adjuvant, was present together with IMT504 in the vaccine formulation. The increase in the Th1 response induced by IMT504 was also suggested by in vitro gamma interferon secretion assays.

Monkeys of the species Cebus apella immunized with the recombinant Hepatitis B surface antigen plus alum and IMT504 also showed titers of antibodies against the antigen several times superior to the titers observed in control animals immunized with the antigen plus alum without ODN. Since rat and monkey cells are significantly less immunostimulated “in vitro” by PyNTTTTGT ODNs than human cells, the present results reasonably predict a very good performance of these ODNs as adjuvants in human vaccination.

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1. Introduction
Hepatitis B virus (HBV) causes acute and chronic infections in humans. Immunization against HBV is recommended throughout the world [1,2]. Currently available HBV vaccines consist of recombinant Hepatitis B surface antigen adsorbed to aluminum hydroxide or aluminum phosphate. A three-dose series (0, 1, and 6 months) is usually used to reach an acceptable rate of protection. In underdeveloped countries, where Hepatitis B is a significant cause of morbidity and mortality, a single-dose vaccine would be very useful because of the high rate of failure to complete the three-dose vaccine schedule. Also, a more effective vaccine is needed to protect individuals who fail to respond to current vaccines, including elderly people, obese, heavy smokers and immuno-compromised people in general [3]. Several adjuvants have been assayed in order to improve the efficiency of the Hepatitis B vaccine [4–6]. Immunostimulatory oligonucleotides are among the most promising ones [7,8]. Immunostimulatory oligonucleotides that are active on human cells are grouped into two major classes: (a)
CpG oligodeoxynucleotides (ODNs), characterized by the presence of at least one active site bearing an unmethylated Cpg, have been described as "in vitro" immunostimulatory molecules that induce secretion of IFN alpha when phosphorothioate ODNs do not [10]. Furthermore, phosphorothioate ODNs induce expression of co-stimulatory molecules acting on plasmacytoid dendritic cells. However, phosphorothioate ODNs induce the secretion of IFN alpha [9] while phosphorothioate ODNs do not [10].

Unlike CpG ODNs, which have a widespread spectrum of animals where they are active [16], PyNTTTTGT ODNs are not genetically homogeneous. Most of these animals were born in this facility but they were not considered to be genotypically homogeneous because of their origin.

2. Materials and methods

2.1. Animals

Female Sprague–Dawley 8–12 weeks old rats were obtained from FUCAL Laboratories (Buenos Aires, Argentina) and housed in a facility at the School of Natural Sciences of the University of Buenos Aires, Buenos Aires, Argentina. Sprague–Dawley is an out-bred strain widely used to test the immune response in order to avoid the bias frequently observed in in-bred strains.

Adult monkeys of the species C. apella were maintained in the animal facility of the CEMIC (Centro de Educaci ´on y Investigaciones Clínicas), Buenos Aires, Argentina. Female Sprague–Dawley 8–12 weeks old rats were obtained from FUCAL Laboratories (Buenos Aires, Argentina) and housed in a facility at the School of Natural Sciences of the University of Buenos Aires, Buenos Aires, Argentina. Sprague–Dawley is an out-bred strain widely used to test the immune response in order to avoid the bias frequently observed in in-bred strains.

Adult monkeys of the species C. apella were maintained in the animal facility of the CEMIC (Centro de Educaci ´on y Investigaciones Clínicas), Buenos Aires, Argentina. Most of these animals were born in this facility but they were not genetically homogeneous.

Animal care and use were according to international guidelines (Guide to the Care and Use of Experimental Animals. Canadian Council on Animal Care (CCAC), 1998).

2.2. Oligodeoxynucleotides

Desalted phosphorothioate ODNs were purchased from Oligos ETC (Bethel, ME, USA). ODNs were suspended in deionized water, assayed for LPS contamination using the Limulus test, and kept at −20 °C until used. Purity was assessed by HPLC and PAGE assays. ODN preparations were used if purity was >97% and LPS levels were undetectable.

2.3. Cell culture

Blood was obtained by venipuncture from rats and monkeys using heparin as anticoagulant. Peripheral Blood Mononuclear Cells (PBMC) were isolated from Ficoll–Hypaque (Sigma–Aldrich, St. Louis, MO) density gradient centrifugation. Briefly, blood samples diluted 1/2 in RPMI 1640 medium (PAA Laboratories, Linz, Austria) and supplemented with 2.0 mM L-glutamine, 50.0 µg/ml gentamicin, and 20 mM HEPES, were centrifuged at 1000 × g for 40 min at 20 °C. All cells were cultured at 37 °C in a 5% CO2 humidified incubator and maintained in RPMI 1640 supplemented with 10% (v/v) heat-inactivated FCS, 2 mM L-glutamine, 50 µg/ml gentamicin.

2.4. Cell proliferation assays

PBMC were cultured in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FCS, 2.0 mM L-glutamine, and 50.0 µg/ml gentamicin. Cells (1 × 10⁶ cells/well) were incubated in 96-well microtiter plates (NUNC, Copenaghen, Denmark) at 37 °C in a 5% CO2 humidified atmosphere for 72 h. PBMC were stimulated with ODNs at 1.5 µg/ml. Eighteen hours before cell harvest, 1 µCi of [3H]thymidine (Amersham Pharmacia Biotech, Piscatway, NJ; sp. act., 25 Ci/mmol) was added to each well. Cells were harvested onto glass-fiber filters, and [3H]incorporation was measured by scintillation counting. Proliferation index was calculated as [3H] incorporation in treated cells/[3H] incorporation in non-treated cells.

2.5. Vaccines

Study products consisted of a recombinant surface antigen of the Hepatitis B virus (HBsAg) produced in yeast and purified through several steps including Cs gradient centrifugation (Pablo Cassarà SRL, Buenos Aires, Argentina). This antigen complies with the international norms to be used in the elaboration of commercial recombinant Hepatitis B vaccines. The antigen was used alone or in combination with alum or IMT504 (5′-TCATCATTGTTGTCATT-3′, Immunotech S.A., Buenos Aires, Argentina) or both. The antigen was stored at −70 °C and diluted with sterile buffered saline to achieve the desired concentration before addition of the adjuvant.
2.6. Immunization of rats

Immunization of rats was conducted on 8–12 weeks old female animals. Each rat received a single i.m. vaccine injection in the tibialis anterior muscles. Control groups received the vaccine alone and the treated groups the vaccine with addition of IMT504. Four weeks later, rats were bled and total IgG, IgG1, IgG2a and IgG2b specific for HBsAg were determined by ELISA using HBsAg-coated plates.

2.7. Serum antibody determinations in rats

Sera were recovered from rats 4 weeks after immunization. Specific anti-HBsAg antibody titers were determined by end-point dilution ELISA assays. Briefly, ELISA plates (Maxisorb, Nunc) were coated overnight with 3 μg/ml recombinant HBsAg. Residual protein-binding sites were blocked with carbonate-bicarbonate buffer containing 8% non-fat milk. Samples were diluted 1/200 with 0.3% Tween 20 in phosphate buffered saline (PBS). Samples were further serially diluted, and plates incubated 2 h at 37°C. Antibody subclasses were evaluated using MAbs against IgG1, IgG2a or IgG2b (Serotec, Raleigh, NC) followed by horseradish peroxidase (HRP)-conjugated goat anti mouse IgG. O-Phenylendiamine dichloride solution (1 mg/ml) was finally added to the wells. End-point titers were defined as the highest serum dilution that resulted in an absorbance value three times greater than that of non-immune serum with a cut-off value of OD490 0.15.

2.8. Immunization of monkeys and sample collection

A group of six monkeys (C. apella) were immunized i.m. and boosted 4 weeks later with a mixture containing 10 μg of the HBsAg with alum and 150 μg of ODN IMT504. A control group of six monkeys were immunized i.m. and boosted 4 weeks later with a mixture containing 10 μg of the HBsAg with alum. Blood was obtained by venipuncture every 2 weeks and serum antibody response to HBsAg was determined.

2.9. Serum antibody determinations in monkeys

Serum antibody response to HBsAg in monkeys was determined by a commercial enzyme immunoassay (AUSAB EIA, Abbott Laboratories, and Abbott Park). The seroconversion limit was considered to be 1.0 mIU/ml as in human vaccination.

2.10. Interferon gamma determination after in vitro stimulation of rat spleen-cells

Interferon gamma (IFN gamma) was assayed in supernatants of rat spleen-cells (10^7 cells/ml) previously cultured for 24 in the presence of either recombinant HBsAg or an unrelated antigen as a negative control. The cytokine was determined by sandwich ELISA using two anti-IFN gamma monoclonal antibodies (Pharmingen, San Diego, CA).

2.11. Statistical analysis

Statistical significance of differences was evaluated by the Student’s t-test. For this a logarithmic transformation of the ELISA titers was performed. Differences were considered significant for p < 0.05.

3. Results

3.1. Immunostimulation of PBMC by PyNTTTTGT ODNs in humans, monkeys and rats

In our previous study for characterization of PyNTTTTGT ODNs [10], after a screening performed in several animal species, we concluded that these ODNs were only active in primates. However, during pre-clinical trials we observed that after inoculation with large amounts of PyNTTTTGT ODNs, the spleen of rats increased its size. This fact suggested the possibility that these ODNs may be active on the immune system of rats. To investigate this possibility, the PyNTTTTGT ODNs prototype IMT504 was incubated with PBMC obtained from humans, monkeys of the species C. apella (Brown Capuchins) and rats (Sprague–Dawley strain). Fig. 1 shows that PBMC of the three species were stimulated to proliferate. However, the proliferation index for human cells was about four times higher than the proliferation index for monkeys and rats cells. These results suggested that the rats and monkeys used are, in fact, conservative models of the immunostimulatory action of IMT504.

3.2. Immunostimulation by PyNTTTTGT ODNs “in vivo” : adjuvancy in rats

The rat model was used to analyze the capacity of the PyNTTTTGT ODNs prototype IMT504 to stimulate the production of antibodies against a recombinant HBsAg. For this, groups of rats were immunized by i.m. injection with a vaccine containing 1, 3 or 9 μg of HBsAg. Animals in control
Fig. 2. Antibody titers in rats immunized with HBsAg plus ODN IMT504. Rats (20 per group) were immunized i.m. with a mixture of 1, 3 or 9 μg of HBsAg either with (B) or without alum (A). Animals in experimental groups received 50 μg per dose of IMT504 in the vaccine. Each bar represents the group mean for anti-HBsAg titers as determined by an end point dilution ELISA assay. White bars correspond to animals immunized without IMT504 and black bars to those immunized with IMT504. Asterisks indicate statistically significant differences (*p < 0.05, **p < 0.01, ***p < 0.001) compared with controls.

Fig. 3. Antibody titers in monkeys (Cebus apella) immunized with HBsAg plus ODN IMT504. Animals (six per group) were immunized i.m. with a mixture of 10 μg of HBsAg with alum. Monkeys in the experimental group received 150 μg of IMT504 per dose. Black arrows indicate the time of immunization. Serum antibodies against HBsAg were determined by an enzyme immunoassay (AUSAB EIA, Abbott Laboratories, Abbott Park). Values represent the geometric mean titer ± S.E.M. (□) Control group; (■) IMT504 group. Differences between groups remained statistically significant (p < 0.05) throughout the curves.

groups received HBsAg either with or without alum. Animals in experimental groups received HBsAg either with or without alum plus 50 μg of IMT504. Four weeks later rats were bled and total specific IgG titer in sera determined (Fig. 2). As can be observed, in all the antigen doses assayed, animals that received antigen plus IMT504 presented antibody titers higher than their respective controls. On the other hand, titers in animals that received alum plus IMT504 were the most elevated, a fact that shows that alum and the immunostimulatory ODN are complementary adjuvants. These results indicate that the immunostimulatory effect of IMT504 observed “in vitro” has its correlate “in vivo” using the rat model.

3.3. Immunostimulation by PyNTTTTGT ODNs “in vivo”: adjuvancy in monkeys

As previously described, PyNTTTTGT ODNs are mainly active on primate cells belonging to the immune system [10]. In the present study, a group of monkeys of the species C. apella were immunized and boosted with a vaccine containing HBsAg and alum plus 150 μg of IMT504. When compared with controls not receiving IMT504, anti-HBsAg titers 4 weeks post prime and two weeks post boost were 7 and 6 times higher, respectively (Fig. 3). More importantly, all the animals in the group that received the vaccine with IMT504 seroconverted 2 weeks after the first dose, whereas none of the animals in the control group that received the vaccine alone presented detectable levels of anti-HBsAg antibodies at this time. These results indicate once more that the immunostimulatory effect of IMT504 observed “in vitro” has its correlate “in vivo” using monkeys as a model.

3.4. IMT504 improvement of the Th1 component of the immune response

The rat model was also used to investigate the capacity of IMT504 to change the Th1/Th2 profile of the immune response. For this, titers of IgG subclasses in sera of the immunized animals were determined. It was observed that although there was an increase in the titer of all IgG subclasses in animals injected with the vaccine plus IMT504 as compared with controls (unshown), the effect on the IgG2b subclass was more marked (Fig. 4A). Taking into account that, in rats, the IgG2b is associated with the Th1 immune response [17,18], these results indicate that IMT504 improves the Th1 component of the immune response. It is well known that alum is a strong stimulant of the Th2 immune response [19,20]. Therefore, it was interesting to assay the Th1 stimulation capacity of IMT504 in the presence of alum. For this, rats were inoculated with a vaccine containing both alum and IMT504 as adjuvants. Fig. 4B shows that even in the presence of alum IMT504 was able to improve the Th1 immune response. On the other hand, assays performed to investigate the dose response to IMT504 in the absence of alum showed that a ten-fold increase in the total specific IgG titer could be reached with as low as 10 μg of IMT504 per dose in comparison with controls (Fig. 5). However, when the IgG subclasses were
measured, a dose-dependent response in the entire dosage range was observed only for the IgG2b subclass. This fact is remarkable because it indicates that the effect of IMT504 on the titer of total specific IgG is somehow independent of its effect on the balance in the type of immune response.

3.5. IFN gamma production in vitro

Cell-mediated immune response was also evaluated "in vitro". Spleen cells from rats immunized with HBsAg alone or with IMT504 were cultured in the presence of 100 μg of the recombinant HBsAg. After 24 h, the amount of IFN-gamma in the supernatants was determined. There was a strong positive response when cells from animals immunized with HBsAg plus IMT504 were used (Fig. 6). However, when spleen cells from animals immunized with HBsAg alone were used, the response, as expected, was weak.

4. Discussion

The present study is the first report showing that the PyNTTTTGT ODN prototype IMT504 has immunostimulatory properties "in vivo", acting as a very effective adjuvant of the recombinant HBsAg. After 24 h, the amount of IFN-gamma in the supernatants was determined. There was a strong positive response when cells from animals immunized with HBsAg plus IMT504 were used (Fig. 6). However, when spleen cells from animals immunized with HBsAg alone were used, the response, as expected, was weak.
as compared with human PBMCs. In consequence, rats and monkeys could be considered conservative models of the efficiency of IMT504-adjuvated vaccines in humans. Therefore, a very good performance of these vaccines in clinical trials could be reasonably anticipated.

The strong response elicited by the vaccine adjuvated with IMT504 could also be very important for protection of those individuals who fail to respond to current vaccines, including elderly people, obese, heavy smokers and immunocompromised people in general.

For complete vaccine efficacy, the HBV infection, a Th1-type response seems to be necessary [21–25] and alum, the currently used adjuvant in this vaccine, biases the immune response towards a Th2-type [19,20]. Thus, a vaccine designed to stimulate the clearance of the virus in chronic HBV infections should stimulate the Th1 component of the immune response. As shown in this study, the vaccine containing IMT504 was able to induce an equilibrated Th1/Th2 response even in the presence of alum. Therefore, this combined vaccine could be very useful as a therapeutic vaccine. Besides effectiveness, safety is another important consideration when developing vaccines. A strong Th2 response is associated with immunopathological complications such as allergy and asthma [26,27]. On the other hand, many of the inducers of a Th1 response do so through the induction of IFN alpha and it is worth noting that inducers of this cytokine have been implicated in the generation of some immunological disorders such as systemic lupus erythematosus [28,29].

Regarding this, it is interesting to point out that IMT504 induces a pronounced Th1 response even though it is not at all an IFN alpha inducer, and our pre-clinical studies have demonstrated that IMT504 in combination with influenza virus. A phase I study of the safety and immunogenicity of recombinant hepatitis B surface antigen co-administered with an immunostimulatory phosphodiester oligonucleotide adjuvant. Vaccine 2003;21:2461–7.


