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Biopolymer Xanthan: A New Adjuvant for DNA Vaccines

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HIGHLIGHTS

- Xanthan biopolymer improved the humoral immune response.

- Cellular immune response induced by Xanthan biopolymer

- Xanthan biopolymer as an adjuvant in DNA vaccine.

- Xanthan biopolymer induced a significant increase in IL-17 levels.

Abstract: DNA vaccines have been evaluated as an option to prevent several diseases. In this study, the capacity of the xanthan biopolymer to improve the DNA vaccines immune response, administered intramuscularly, was evaluated. The experimental vaccines consisted of genes encoding fragments of the proteins LigA and LigB of *Leptospira interrogans* serogroup Icterohaemorrhagiae serovar Copenhageni strain Fiocruz L1-130. The humoral immune response was evaluated by indirect ELISA. Cytokine expression levels were determined by RT-qPCR. Compared to the control group, the IgG antibody levels of animals immunized with pTARGET/*ligAni* and pTARGET/*ligBrep* plasmids associated with xanthan biopolymer were significantly higher than the control group. Additionally, there was a significant increase in IL-17 expression in animals vaccinated with pTARGET/*ligBrep* and xanthan.

INTRODUCTION

Vaccines have contributed significantly to the reduction of mortality and morbidity rates of many infectious diseases [1]. To obtain safer vaccines, different strategies have been evaluated, such as DNA vaccines. Compared to conventional or subunit vaccines, these have greater stability, lower production cost, high protective potential with low or no risk of reversion of virulence disease induction [2,3]. However, the results were disappointing, because of the low immunogenicity and low induction of antibody titers [2,4]. Considering this last point, the discovery of novel safe adjuvants is essential for the development of more effective DNA vaccines.

Adjuvants are natural or synthetic substances, which, when associated with antigens, can enhance the immune response [1,5]. Thus, they can reduce the number of vaccine doses required to achieve protection and the amount of antigen, thus also reducing vaccine production costs [6]. The biopolymer xanthan is an extracellular polysaccharide, produced by bacterial *Xanthomonas* spp., and was initially described as a lymphocyte activator [7]. Recently, its anti-tumor activity and ability to activate the immune system to production antibodies [5,8] and activate cytotoxic T lymphocytes were described [9]. Despite these characteristics, the xanthan biopolymer has so far not been evaluated as an adjuvant in DNA vaccines.

Leptospirosis is a neglected infectious disease of worldwide importance [10]. The vaccines currently available on the market have several flaws, such as short-term immunity and a lack of cross-protection against different serovars [1]. Currently, the most promising experimental vaccines involve the family of Leptospiral immunoglobulin-like proteins (Lig), namely LigA and LigB, which are present on the surface of pathogenic species [11]. Thus, the purpose of this study was to evaluate, in mice, the adjuvant activity of xanthan biopolymer on the humoral and cellular immune response to DNA vaccines.

MATERIAL AND METHODS

Escherichia coli TOP10 (Invitrogen) and *E. coli* BL21 (DE3) Star were grown in Luria-Bertani broth (LB) with 100 µg.ml^{-1} ampicillin at 37 °C in an orbital shaker at 200 rpm for 18 h. *Xanthomonas arboricola* pv pruni strain 106, obtained from the strain bank of the Laboratório de Biopolímeros, Centro de Desenvolvimento Tecnológico, Biotecnologia, Universidade Federal de Pelotas, was grown as previously described [8,12]. The xanthan yield was calculated in grams of dry polymer per liter of fermentation broth (g.L^{-1}) [12]. For the experiments, the xanthan was suspended in sterile pyrogen-free water at 1.0% (w/v).

The vaccine plasmids were constructed with the genes *ligAni* (pTARGET/*ligAni*) and *ligBrep* (pTARGET/*ligBrep*), and their functionality assessed as previously described [13]. Plasmids were obtained from the transformation of *E. coli* TOP10 strains by heat shock with pTARGET/*ligAni* and pTARGET/*ligBrep* as described [14]. Plasmid DNA was quantified by electrophoresis on 0.8% agarose gel, using λ DNA/HindIII marker as reference and with a Qubit Fluorometer (Invitrogen). After stored at -20 °C until use.

The cloning, expression, and purification of the proteins rLigAni and rLigBrep were obtained as previously described [11]. Proteins expression was verified by 12% SDS-PAGE and Western blot using peroxidase-conjugated monoclonal anti-6xHis-tag antibody (Sigma-Aldrich) at 1:10,000 dilution according to the manufacturer's instructions. Recombinant proteins were purified by Ni^{+2} affinity chromatography in 1 ml columns (Invitrogen), using a solubilization buffer (0.2 M NaH$_2$PO$_4$; 0.5 M NaCl; 10 mM Imidazole, 8M urea). The purified proteins were dialyzed against phosphate-buffered saline (PBS pH 7.2) at 4 °C for 24 h, quantified with BCA^{TM} Protein Assay Kit (Thermo Scientific Pierce) according to the manufacturer's instructions and stored at -20 °C until further use.

Four to six weeks old female mice were randomly divided into four groups containing 12 animals each. The immunization protocol consisted of three doses given intramuscularly on days zero, 14 and 21. Approximately 45 min before immunization, all animals received 100 µL of a solution containing 25 % sucrose at the application site of the vaccine to promote further DNA absorption. Groups 1 and 2 were inoculated with 100 µg of pTARGET/*ligAni*, while groups 3 and 4 received the same dose of pTARGET/*ligBrep*. Groups 2 and 4 received, together with the plasmid, 50 µL of 1 % xanthan biopolymer (w/v). Blood samples were collected by puncturing the retro-orbital venous plexus one day before each immunization and one week after the last (days -1, 13, 20, and 28) and centrifuged (3,000 × *g*, 5 min) for serum collection to assess the humoral immune response. Serum samples were stored at -20 °C until further use.

For cellular immune response evaluation, blood samples were collected on day 38 and stored at -70°C for RNA extraction. To increase the immune response against the targets, an intraperitoneal booster dose with recombinant protein was performed on day 35. Groups 1 and 2 received 50 µg of rLigAni, and groups 3 and 4 received 50 µg of rLigBrep. Samples for RNA extraction were stored at -70 °C. In this study, no negative

control group inoculated with empty pTARGET plasmid was added. The reason was that this had already been reported by Forster and colleagues [13], allowing a reduction in the number of animals used in the experiment.

Indirect ELISA using sera before and after immunizations was used to evaluate the IgG antibody levels as previously described [15]. Briefly, 96-well polystyrene microplates (CralPlast) were coated with 200 ng/well of rLigAni or rLigBrep diluted in carbonate-bicarbonate buffer 0.05 M (pH 9.6). The serum of each animal, collected on days -1, 13, 20, and 28, were diluted 1:30 in PBS-T and added to each well (sera from group 1 and 2 were tested against rLigAni, while group 3 and 4 against rLigBrep). Peroxidase-conjugated goat anti-mouse polyvalent antibody (Sigma-Aldrich) diluted in PBS-T (1:4,000) was used as secondary antibody. The incubation steps with antibodies (50 μL/well) occurred for 1 h at 37 °C. The plates were washed thrice with 200 μL/well of PBS-T between incubations (after the secondary antibody, the plates were washed five instead of three times). The reactions were developed by adding orthophenylenediamine solution (OPD) diluted in phosphate-citrate buffer 0.2 M (pH 4.0) with 0.01 % H_2O_2. The plates were maintained in the dark for 15 min at room temperature and read at 450 nm in a microplate spectrophotometer (Thermo Plate). The serum of each animal was evaluated in duplicate. The IgG antibody levels were expressed as ELISA units, dividing the arithmetic mean of the serum absorbance of each animal by the arithmetic mean absorbance of each animals on day -1 [16].

The cellular immune response was assessed according to the protocol as previously described [17] with modifications. Blood samples of the animals were collected three days after the intraperitoneal injection of 50 μg of recombinant proteins LigAni (groups 1 and 2) and LigBrep (groups 3 and 4). Blood samples of each group were used for three pooled samples containing 50 μL of blood of each animal. From the three pools per group, peripheral blood mononuclear cell (PBMC) total RNA was extracted by the Gene JET Whole Blood RNA Purification Mini Kit (Thermo Scientific) according to the manufacturer's instructions. The contaminating genomic DNA was removed using an RNase-Free DNase (Promega) according to the manufacturer's instructions and the total RNA was quantified with NanoVue spectrophotometer (GE Healthcare, UK). The cDNA synthesis was performed employing PBMC total RNA (0.5 μg) and the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, UK) according to the manufacturer's protocol. The expression levels of mRNA cytokines (IL-4, IL-12, IL-17, IFN-γ and TGF-β) was quantified by qPCR by using specific primers (18-20). The qPCR reactions were performed using Stratagene® Mx3005P™ Real-Time PCR System (Agilent Technologies, USA) and reactions were carried out in duplicate. The qPCR using SYBR Green PCR Master Mix (Applied Biosystems™, UK) was carried out in a 12.5 μL reaction volume (50 ng of cDNA, 6.25 μL of Master Mix, 0.1 to 0.4 μM of each primer). The reaction conditions consisted of 95°C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C for 60 s. A melting curve was included for each run in order to ensure the specificity of the amplified products. To determine the qPCR efficiencies and R^2 values for each reaction, a dilution series was made from the cDNA template for each target gene. The Ct data were analyzed by 2-ΔΔCt method with efficiencies (E) correction using software REST 2009 (21). The relative change in the gene transcription ratio (relative expression) for each target gene was calculated by normalizing gene expression to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) reference gene.

Student t test was used to determine significant differences ($P < 0.05$) between ELISA units of vaccinated animals. Tukey test was used to determine significant differences ($P < 0.05$) between the mRNA levels for cytokines. Software Prism 5 (GraphPad) was used for all statistical analyses.

Animals were maintained according to international standards and in line with the ethical principles of animal experimentation determined by the Brazilian College of Animal Experimentation (COBEA). The study outline was approved by the Committee of Ethics in Animal Experimentation (CEEA) of UFPel (registration No. 7603).

RESULTS

The plasmids pTARGET/*ligAni* and pTARGET/*ligBrep* were extracted yielding approximately 12 mg of plasmid per liter of culture. rLigAni and rLigBrep expression in *E. coli* BL21 (DE3) Star was effective, since bands of 64 and 72 kDa were detected by Western blot with anti-6xHis antibody (data not shown).

Vaccination with a three-dose strategy using xanthan biopolymer as adjuvant, enhanced the specific humoral immune response of both DNA vaccines tested (Figure 1). Antibody levels of the group vaccinated with pTARGET/*ligAni* and the xanthan biopolymer were higher than the antibody levels of the group vaccinated with the plasmid alone after the 2nd dose. The antibody levels of animals treated with pTARGET/*ligBrep* and the xanthan biopolymer were only higher than those of the group vaccinated with the plasmid alone after the 3rd dose ($P < 0.05$).

The cytokine mRNA levels involved in the T_H1 (IFN-γ, IL-12), T_H2 (IL-4), Treg (TGF-β), and T_H17 (IL-17) responses were assessed by RT-qPCR of PBMCs after *in vivo* stimulation with the recombinant antigens. Comparing the cytokine gene expression levels between the control (groups inoculated with plasmid pTARGET/*ligAni* and pTARGET/*ligBrep* without adjuvant) and those treated with the xanthan biopolymer as adjuvant, there were no differences between the cytokine levels in groups 1 and 2, which received plasmid pTARGET/*ligAni* (Figure 2A). However, between groups 3 and 4, which received the pTARGET/*ligBrep* plasmid, there was an increase in T_H17 response characterized by the induction of a significant increase in IL-17 levels in the xanthan-treated group ($P < 0.01$) (Figure 2B). The IL-17 plays a fundamental role in tissue inflammation and activation of neutrophils to combat extracellular bacteria [22,23].
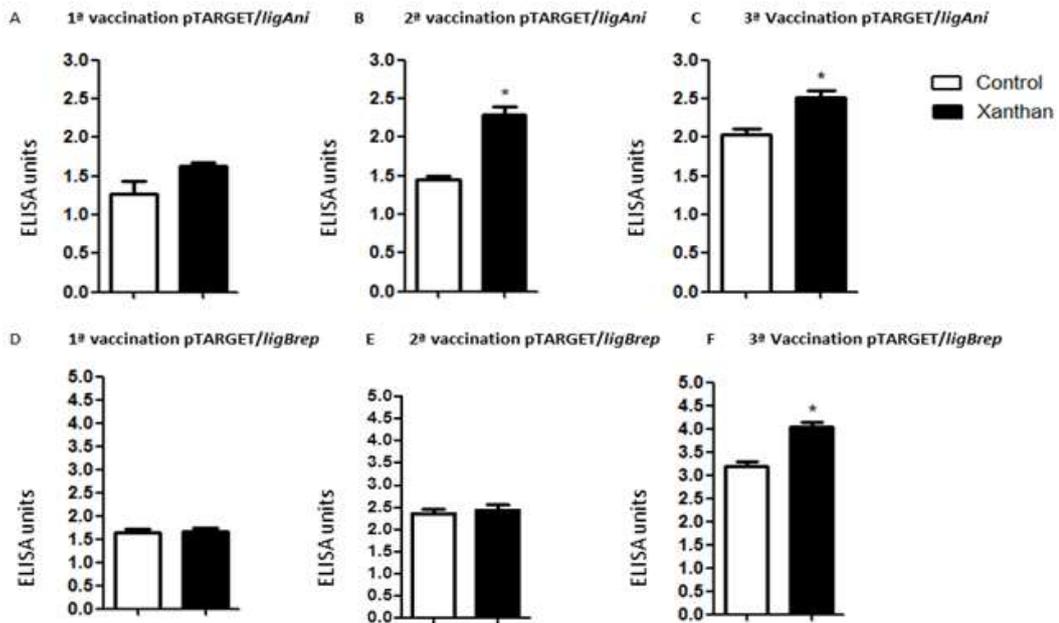
Figure 1. Evaluation of the humoral immune response induced by the DNA vaccines using xanthan biopolymer as adjuvant. Panels A, B, and C represent IgG antibody levels after the first, second, and third dose, respectively, for groups 1 and 2, treated with plasmid pTARGET/*ligAni*. Panels D, E, and F represent IgG antibody levels after the first, second, and third doses, respectively, for groups 3 and 4, which received the pTARGET/I*igBrep* plasmid. Control groups were inoculated with plasmid pTARGET/*ligAni* and pTARGET/*ligBrep* without adjuvant. Data were analysed using t Sudent test and asterisks (*) represent statistical difference to the control group (P < 0.05).
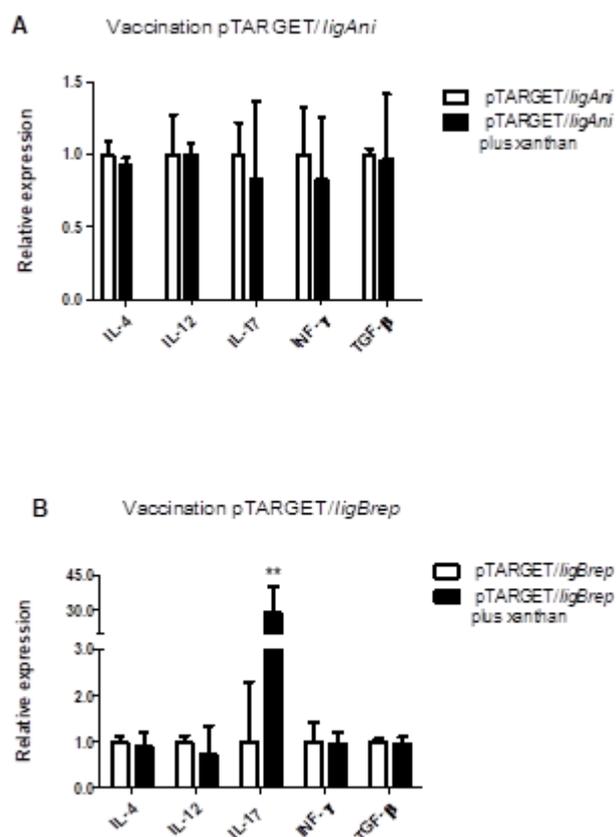
Figure 2. Relative cytokine gene expression. Gene expression of IL-4, IL-12, IL-17, IFN-γ, and TGF-β was measured in PBMCs of the mice groups 1 and 2 treated with plasmid pTARGET/*ligAni*, (A), and PBMCs of the mice groups 3 and 4, which received the pTARGET/*ligBrep* plasmid, (B). The mice groups 2 and 4 were treated with the recombinant plasmids plus biopolymer xanthan as adjuvant. Relative gene expression was determined by qPCR and calculated using the 2-ΔΔCt method with E correction, using gadph mRNA expression as reference gene. Data were analysed using Tukey test and asterisks (**) represent statistical difference to the control group (P < 0.01).

DISCUSSION

This study demonstrated that the xanthan biopolymer enhanced the humoral immune response to DNA vaccines against leptospirosis. The xanthan biopolymer adjuvant effect exact mechanism of action is not yet fully understood. However, different effects on the immune system have been reported, such as the ability to increase nasal IgA levels in an influenza vaccine [24] and liposome efficiency as delivery system for a nasal vaccine, also for the virus influenza [25]. In addition, it is able to induce the production of IL-12 and TNF-α by macrophages [9]. Studies with mice have shown that signaling through toll-like receptors (TLR) is sufficient to initiate an adaptive immune response, and since xanthan biopolymer contains mannose on its structure, it may act as an agonist to TLR-2 [25]. However, it had never been evaluated as an adjuvant for a DNA vaccine.

Previous studies have found promising results using the xanthan biopolymer as an adjuvant in a subunit vaccine for leptospirosis employing rLigAni as antigen. In this study, the use of the xanthan biopolymer increased the IgG levels and consequently improved protection hamsters against challenge. In addition, no cytotoxicity was observed in CHO cells *in vitro* [8]. A low incidence of adverse events is important for the advancement of new vaccine. The biosafety of xanthan biopolymer is evident in the fact it has been used as an FDA-approved food additive [5, 25, 26]. Moreover, previous studies have demonstrated that xanthan biopolymer preparations have a record of safety [8,27].

The major protective effect of vaccines against leptospirosis was obtained in a vaccine using the rLigAni as immunogen and Freund's complete adjuvant. However, these adjuvants can cause a number of side effects such as granuloma, pain, sensitivity, and erythema [28]. Other adjuvants evaluated in DNA vaccines were also able to enhance the humoral immune response, often leading to a reduction of symptoms and increased survival, but not preventing the infection completely [29].

Our findings represent an important contribution to the field of vaccine development. This is the first report on the capacity of xanthan biopolymer to improve mainly the humoral immune response of DNA vaccines. In addition, for being a natural polysaccharide, it is assumed that there are no adverse or side

effects to xanthan biopolymer and thus it can safely act as an adjuvant for DNA vaccines, as highlighted in this study.

Conflicts of Interest: The authors declare no conflict of interest.

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