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Generation of *Toxoplasma gondii* GRA1 protein and DNA vaccine loaded chitosan particles: preparation, characterization, and preliminary in vivo studies

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Abstract

Chitosan microparticles as carriers for GRA-1 protein vaccine were prepared and characterized with respect to loading efficiency and GRA-1 stability after short-term storage. Chitosan nanoparticles as carriers for GRA-1 pDNA vaccine were prepared and characterized with respect to size, zeta potential, and protection of the pDNA vaccine against degradation by DNase I. Both protein and pDNA vaccine preparations were tested with regard to their potential to elicit GRA-1-specific immune response after intragastric administration using different prime/boost regimen. The immune response was measured by determination of IgG2a and IgG1 antibody titers. It was shown that priming with GRA1 protein vaccine loaded chitosan particles and boosting with GRA1 pDNA vaccine resulted in high anti-GRA1 antibodies, characterized by a mixed IgG2a/IgG1 ratio. These results showed that oral delivery of vaccines using chitosan as a carrier material appears to be beneficial for inducing an immune response against *Toxoplasma gondii*. The type of immune response, however, will largely depend on the prime/boost regimen and the type of vaccine used.

Keywords: Mucosal vaccination; Toxoplasmosis; GRA-1; DNA vaccine; Chitosan

1. Introduction

Toxoplasma gondii is an obligate intracellular parasite which—after oral ingestion by the host—generally induces a mild asymptomatic infection, both in humans and animals. Control of the infection in the host occurs through the induction of strong and persistent cell mediated immunity, characterized by production of gamma-interferon (IFN-γ), immunoglobulin (Ig) IgG2a, and cytotoxic T cells (Denkers, 1999; Yap and Sher, 1999). In humans, a primary *T. gondii* infection and subsequent transplacental transmission during pregnancy can result in miscarriage or in severe disease in the infant (Holliman, 1995). On the other hand, this infection may reactivate under conditions of immunosuppression, resulting in toxoplasma encephalitis and other complications (Denkers and Gazzinelli, 1998). These pathological consequences associated with congenital toxoplasmosis not only represent a threat to humans but are also a cause of

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economic losses due to abortions in farm animals (Dubey and Beattie, 1988).

Reports on vaccination with the *T. gondii* excreted/secreted dense granule protein 1 (GRA1) have been accumulating and shown to induce protective immune responses against experimental *T. gondii* infection in different animal models (Duquesne et al., 1991; Supply et al., 1999; Vercammen et al., 2000; Scorza et al., 2003). Protection against infection with *T. gondii*, obtained after intramuscular (i.m.) GRA1 DNA vaccination, was shown to be associated with Th1 type responses, characterized by the production of IgG2a, IFN-γ, and *T. gondii*-specific cytolytic $CD8^+$ T cells (Vercammen et al., 2000; Scorza et al., 2003). However, as *T. gondii* infects the host through the gut, local immune responses may be more appropriate to reduce the risk of infection. Whereas i.m. vaccination with naked plasmid DNA can induce systemic humoral and cellular immune responses, oral delivery systems may be used to induce mucosal immune responses in the gut.

The mucosal route of vaccine administration is attractive due to the increased patient compliance and ease of application (i.e. no need of trained personnel). Furthermore, vaccination at mucosal surfaces may result in humoral and cellular responses, both systemic and local; the latter not only at the site of vaccination, but also at distant mucosal epithelia. The rationale of the work presented here was to orally immunize mice using chitosan nanoparticles as a non-viral delivery system for GRA1 encoding plasmid DNA (pDNA), and chitosan microparticles as carriers for the recombinant GRA1 protein vaccine and to compare the immune responses elicited by both systems.

Particulate mucosal delivery systems that encapsulate protein or pDNA encoding antigens have been widely explored for their ability to induce an immune response. Examples of materials used for this purpose are poly(lactide glycolide acid) (PLGA; O'Hagan, 1998; Raghuvanshi et al., 2002), starch (Wikingsson and Sjoholm, 2002), and different cationic polymers among them chitosan (McNeela et al., 2000; Illum et al., 2001; van der Lubben et al., 2001c). Chitosan is the deacetylated form of chitin that has many properties suitable for vaccine delivery. It is positively charged in acidic solutions, biodegradable, biocompatible, and is very cheap for being a waste product of the seafood industry. Chitosan is a mucoadhesive polymer that is able to open tight junctions and allow the paracellular transport of molecules across mucosal epithelium, therefore is suitable for the mucosal delivery of vaccines (Artursson et al., 1994; Luessen et al., 1996; van der Lubben et al., 2001b). Previously, properties of chitosan microparticles were explored in our lab and exhibited suitable in vitro and in vivo characteristics for oral vaccination (van der Lubben et al., 2001a; van der Lubben et al., 2001c). Microparticles loaded with diphtheria toxoid (DT) strongly enhanced local (IgA) and systemic (IgG) immune responses against DT after oral administration in mice (van der Lubben et al., 2003). When used for DNA vaccination, oral administration of chitosan nanoparticles loaded with DNA plasmid that encoded a peanut allergen gene, protected AKR mice from food allergen-induced hypersensitivity (Roy et al., 1999). Recently, intranasal immunization with chitosan nanoparticles loaded with pDNA encoding respiratory syncytial virus (RSV) proteins, was reported to induce protective Th1 type immune responses in BALB/c mice (Kumar et al., 2002).

In the present study, both GRA1 pDNA and recombinant GRA1 protein loaded chitosan formulations were generated and characterized with regard to their physico-chemical parameters such as loading efficiency, size, and zeta potential. In addition, their stability was evaluated with respect to DNA and protein degradation. The immunogenicity of these chitosan-based delivery systems was addressed in a preliminary in vivo study.

2. Materials and methods

2.1. Materials

Chitosan was purchased from Primex (Karmsund, Norway) and had a viscosity of 12 mPa s and a degree of deacetylation of 93.2%, as measured by the manufacturer. PicoGreen dsDNA quantitation kit was from Molecular Probes (Leiden, The Netherlands). Tween-80, Tween-20, lysozyme, imidazol, 4-chloro-napthol substrate tablets, 3,3′,5,5′-tetramethylbenzidine (TMB) and peroxidase-conjugated rat anti-mouse immunoglobulin (IgG) were obtained from Sigma–Aldrich (Zwijndrecht, The Netherlands). Bio-Rad

protein assay, DC protein assay, agarose gel, and loading buffer were purchased from Bio-Rad Laboratories (Veenendaal, The Netherlands). DNA 1 kb ladder was of New England Biolabs (Frankfurt, Germany). The restriction enzyme *Bam*HI and RQ1 RNase-free DNase were obtained together with the appropriate reaction buffer and stop solution from Promega (Leiden, The Netherlands). The expression vector pQE-81L PCR purification kit, EndoFree plasmid giga kit and Ni-NTA Superflow were purchased from Qiagen GmbH (Hilden, Germany). Pwo DNA polymerase, restriction enzymes (*Pst*I and *Bcl*I), and Complete Protease Inhibitor were purchased from Roche GmbH (Grenzach, Germany). Benchmark presatined protein ladder, *Escherichia coli* Top10F′ and fetal calf serum (FCS) were purchased from Invitrogen Life Technologies (Leek, The Netherlands). Isopropyl-beta-D-thiogalactopyranoside (IPTG) was purchased from Acros Organics (Geel, Belgium). CelluSep H1 dialysis membrane with a MWCO of 15 kDa were purchased from Membrane Filtration Products (Braine-l′Alleud, Belgium). Endotoxin-free phosphate-buffered saline (PBS) and QCL-1000 Chromogenic LAL Test Kit were purchased from BioWhittake Europe (Verviers, Belgium). Hybond-C nitrocellulose membranes were purchased from Amersham Biosciences (Uppsala, Sweden). Immunosorb 96-well plates were purchased from Nunc (Slangerup, Denmark). Peroxidase-conjugated rat anti-mouse IgG1 and peroxidase-conjugated rat anti-mouse IgG2a antibodies were purchased from Southern Biotechnology Associates (Birmingham, AL, USA). Bovine serum albumin (BSA) was purchased from Merck (Darmstadt, Germany). All the other reagents were of chemical grade. The DNA construct used for vaccination was based on the plasmid vector VR1020, obtained from Vical (San Diego, CA, USA).

2.2. Animals

Female inbred C3H/HeN mice, 6–8 weeks old, were purchased from Harlan (Horst, The Netherlands), and maintained at the Pasteur Institute of Brussels, according to animal house regulations. The animal study protocol was in accordance with Principles of Laboratory Animal Care (NIH publication no. 85-23, revised in 1985).

2.3. Methods

2.3.1. Cloning of recombinant GRA1

The gene encoding GRA1 was amplified with Pwo DNA polymerase from the DNA vaccine plasmid pVR1020-GRA1 (Scorza et al., 2003; Vercammen et al., 2000), using sense and antisense primers designed to contain, respectively a *Bcl*I or *Pst*I restriction site to allow in-frame cloning of the gene fragment into the pQE-81L expression vector. The amplicon was purified with the PCR purification kit, overnight incubated with restriction enzymes, and again purified. The GRA1 DNA was then ligated into the *Bam*HI and *Pst*I sites of pQE-81L using the Rapid DNA ligation kit and transformed into chemocompetent Top10F′ cells. Clones which harbored pQE81-GRA1 were selected by colony-PCR. The presence of the GRA1 gene was confirmed by restriction analysis.

2.3.2. Purification of recombinant GRA1

As the recombinant GRA1 has a histidine tag at the aminoterminal, purification could be performed by metal chelate affinity chromatography with the Ni-NTA Superflow, according to the manufacturers instructions with little modification. From a freshly plated Top10F′ pQE81-GRA1 clone (on LB-agar supplemented with 100 μl/ml ampicillin), an overnight culture was inoculated, whereafter this was used to inoculate a large volume culture. Protein expression was induced by adding 1 mM IPTG at an OD_{600} of 0.7. After 2 h of induction, the bacteria were pelleted and resuspended in 50 ml non-denaturing lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 1 mM imidazol, pH 7, supplemented with one tablet Complete Protease Inhibitor and 1 mg/ml lyzozyme) per liter bacterial culture and incubated for 30 min on ice. After four freeze–thaw cycles and sonication, the lysate was centrifuged. Per milliliter supernatant, 0.1 ml of Ni-NTA Superflow was added and incubated under light agitation for 4 h at 4 °C. Thereafter, slurry was loaded on 15 ml columns, and after flowthrough washed overnight with washbuffer 1 (50 mM NaH_2PO_4, 300 mM NaCl, 5 mM imidazol, pH 7) at 4 °C. In order to remove bacterial endotoxins an alternating wash regimen of washbuffer 1 and washbuffer 2 (washbuffer 1 in 60% isopropanol) was applied, as described elsewhere (Franken et al., 2000). GRA1 was eluated from the Ni-NTA slurry by incubating

with washbuffer 1 supplemented with 500 mM imidazol. The eluted fraction was then dialyzed in CelluSep H1 dialysis membrane against endotoxin-free PBS. After dialysis, protein concentration was determined with the DC protein assay (Bio-Rad Laboratories). Endotoxin levels were analyzed with the QCL-1000 Chromogenic LAL Test Kit, as instructed by the manufacturer (BioWhittaker), and shown to be <0.05 EU/μg protein (data not shown).

2.3.3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out on a 12% polyacrylamide gel, using the Bio-Rad minigel system (Bio-Rad Laboratories, USA). Protein bands were visualized by staining with Coomassie Brilliant Blue. In order to confirm the antigenicity of the recombinant GRA1, Western blot was performed as described elsewhere (Scorza et al., 2003). Briefly, after electrophoretic transfer on Hybond-C, the membrane was blocked by incubation with 3% BSA in Tris-buffered saline, 0.1% Tween 20 for 1 h at room temperature. A pool of sera from five *T. gondii*-infected C3H mice or a pool from sera of pVR1020-GRA1 vaccinated mice obtained from earlier studies (Scorza et al., 2003), was used as primary antibody (1:200) and incubated overnight at 4 °C. A peroxidase-labeled rat anti-mouse IgG was used as secondary antibody and incubation took place for 1 h at room temperature. The chromogenic reaction was performed with 4-chloro-napthol substrate tablets according to the manufacturer's instructions.

2.3.4. Preparation of protein loaded chitosan microparticles

Chitosan microparticles were prepared according to the procedure described by van der Lubben et al. (2001a). In short, chitosan solution was prepared as a mixture of chitosan (0.25%, w/v), acetic acid (2%, v/v), and Tween-80 (1%, w/v). After chitosan was dissolved, 2 ml of a 10% (w/v) sodium sulphate solution were added dropwise to 200 ml of chitosan mixture under constant magnetic stirring and probe sonication. After the addition of sodium sulphate, sonication and stirring were continued for 20 min. The microparticle suspension was centrifuged, particles were collected, and washed twice with Milli-Q water. Particles were freeze–dried for 24–48 h using a Christ freeze–dryer (Osterode am Harz, Germany). Dry microparticles were loaded with recombinant GRA1 by incubating 0.05% (w/v) protein with a 1% (w/v) chitosan microparticle suspension in PBS (pH 7.3) under shaking at 25 °C for 3 h. Microparticles loading efficiency was checked using the Bio-Rad protein assay. The protein stability after 5 days of storage was checked using SDS–PAGE and Coomassie Brilliant Blue staining.

2.3.5. Purification of pVR1020-GRA1 DNA

Plasmid pVR1020-GRA1, encoding the *T. gondii* dense granule protein GRA1 was produced and purified with the EndoFree plasmid giga kit as described elsewhere (Vercammen et al., 2000; Scorza et al., 2003). DNA for vaccination was dissolved in sterile endotoxin-free PBS. DNA concentration was determined photometrically at a wavelength of 260 nm.

2.3.6. Preparation of DNA loaded chitosan nanoparticles

Preparation was done using the complexation–coacervation method (Mao et al., 2001) for N/P ratios between 3 and 8. N/P ratio was calculated from the amount of DNA phosphate groups in the preparation (1 μg of DNA contain 3 nmol of phosphate) and from the chitosan nitrogen groups (calculation takes into account the chitosan molecular weight and degree of deacetylation since only deacetylated monomers can be protonated). Briefly, chitosan solution (the amount of chitosan was calculated according to the N/P ratio determined and chitosan dissolved in 5 mM sodium acetate buffer adjusted to pH 5.5) and the DNA solution (pVR1020-GRA1 100 μg/ml in 5 mM sodium sulphate solution) were separately preheated to 53–55 °C. Equal volumes of chitosan solution were added to the DNA solution and vortexed for 30 s. The final volume was less than 500 μl as required for uniform formation of chitosan nanoparticles (Mao et al., 2001). The particles were left to stand for at least 1 h and then used in the characterization studies.

2.3.7. Characterization of chitosan nanoparticles

Particle size and zeta potential measurements were performed using Zetasizer® 3000 HSA (Malvern Instruments, Bergen op Zoom, The Netherlands).

Measurements were done in a 5 mM sodium acetate buffer adjusted to pH 5.5 and 6.0.

2.3.8. Loading efficiency of chitosan nanoparticles

The amount of encapsulated DNA in the nanoparticles was calculated by measuring the difference between the amount of DNA added to the nanoparticle preparation solution and the measured non-entrapped DNA remaining in the aqueous phase after nanoparticle formation. After formation, the nanoparticle suspension was centrifuged for 15 min at 14,000 rpm and the supernatant was checked for the non-bound DNA concentration with PicoGreen dsDNA quantitation assay using a Perkin-Elmer 3000 Fluorescence Spectrometer (Gouda, The Netherlands).

2.3.9. Protection against nuclease degradation

Chitosan–DNA nanoparticles were incubated with DNase I (0.5 U/μg DNA) for up to 20 min at 37 °C. The DNase activity was stopped using the manufacturer's stop solution. Samples were analyzed in a 0.8% agarose gel containing 1 μg/ml ethidium bromide, in TBE buffer. DNA was visualized under UV light. The naked plasmid was used as control and a 1 kb DNA ladder as a reference.

2.3.10. Concentration of nanoparticle suspensions

After nanoparticles were formed they were centrifuged for 15 min at 14,000 rpm, the supernatant was discarded and the particles were concentrated 10 times, yielding a final DNA concentration of 500 μg/ml in 5 mM sodium acetate buffer or 0.15% (w/v) Tween-80 solution. Size and zeta potential were measured as described for the diluted nanoparticle suspension.

2.3.11. Vaccinations

Mice received three intragastric inoculations by feeding needle (2-day intervals) with loaded chitosan particles: one group ($n = 8$) received pVR1020-GRA1 loaded nanoparticles (N/P ratio 6:1; 50 μg DNA per 100 μl chitosan particles), and a second group ($n = 8$) received microparticles loaded with recombinant GRA1 protein (50 μg protein per 100 μl chitosan particles). A control group received a corresponding amount of empty microparticles ($n = 8$). Four weeks after priming, each group was split in two: five mice received a second round of chitosan-based formulation, and three mice were boosted i.m. with 100 μg pVR1020-GRA1 in both tibialis anterior muscles, using a 0.3 ml syringe. Control mice, which were immunized with empty microparticles, were boosted with the pVR1020 plasmid, which is devoid of GRA1 encoding sequences (Scorza et al., 2003; Vercammen et al., 2000). In two additional groups of mice, GRA1 microparticles or pVR1020-GRA1 nanoparticles were administered subcutaneously (s.c.), to examine the immune response after non-mucosal delivery. However, the s.c. administration of both formulations did not seem to have primed the immune system, as boosting with DNA elicited antibody levels obtained after priming with naked DNA (data not shown).

2.3.12. Enzyme-linked immunosorbent assay for GRA1

Sera from immunized mice were evaluated by enzyme-linked immunosorbent assay (ELISA), using Nunc Immunosorb 96-well plates, which were coated with recombinant GRA1 (5 μg/ml) in 50 mM carbonate buffer (pH 9.6) overnight at 4 °C. Following 2 h of blocking at 37 °C with 10% FCS and three times washing (10% FCS, 0.1% Tween-20), a dilution series of each serum sample was loaded on the plate and incubated overnight at 4 °C. Plates were then washed and supplemented with a peroxidase-conjugated rat anti-mouse IgG (1/1000) for 1 h at 37 °C. After washing, TMB in substrate solution (0.1 M citric acid, 0.2 M $Na_2HPO_4 \cdot 2H_20$, 0.002% H_20_2) was used for development. The reaction was stopped by addition of 1N H_2SO_4. Absorbance was read at 450/692 nm in a Titertek Multiskan MCC/340 (Labsystems, Espoo, Finland). IgG1 and IgG2a antibody determinations were performed as described for total IgG. Endpoint titers were defined as the dilution where the optical density (OD) exceeded the OD of the pre-immune serum by a factor of three. Isotype ratios were calculated as the ratio of IgG2a endpoint titer over IgG1 endpoint titer.

3. Results and discussion

3.1. Expression and purification of recombinant GRA1

After the cloning of GRA1 into pQE-81L, clones harboring pQE81-GRA1 were grown for the production of recombinant GRA1. Following protein

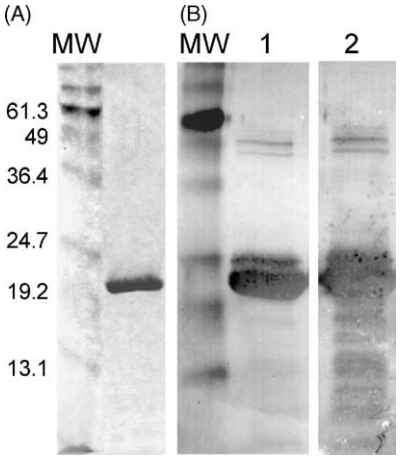

Fig. 1. SDS–PAGE Coomassie gel (panel A) and Western blot (panel B) of batch purified recombinant GRA1 recognized by serum from GRA1 vaccinated C3H mice (lane 1) and *T. gondii-infected* C3H mice (lane 2). MW: molecular weight marker.

induction and purification, the recombinant 23 kDa GRA1 was analyzed on SDS–PAGE gels, revealing that the preparation was devoid of contaminating proteins (Fig. 1, panel A). An average yield of approximately 9 mg recombinant GRA1 per liter bacterial culture was obtained. In order to confirm that the antigenic properties of the recombinant GRA1 were conserved, Western blots were performed. The recombinant GRA1 was recognized by sera from *T. gondii-infected* C3H mice and pVR1020-GRA1 vaccinated C3H mice obtained in earlier studies (Vercammen et al., 2000; Scorza et al., 2003) (Fig. 1, panel B).

3.2. Loading and stability of chitosan microparticles

The microparticles were loaded by incubation in a protein solution of a relatively low concentration (0.05%, w/v), as required for the in vivo experiment. The protein assay indicated loading efficiencies of $96.60 \pm 0.14\%$ ($n = 5$), which is in accordance with the high loading efficiencies described for low protein concentrations by van der Lubben et al. (2001a). SDS–PAGE combined with Coomassie staining showed that the GRA1 protein released from the microparticles was stable and was not degraded when stored over a period of 5 days at 4 °C in PBS (Fig. 2).

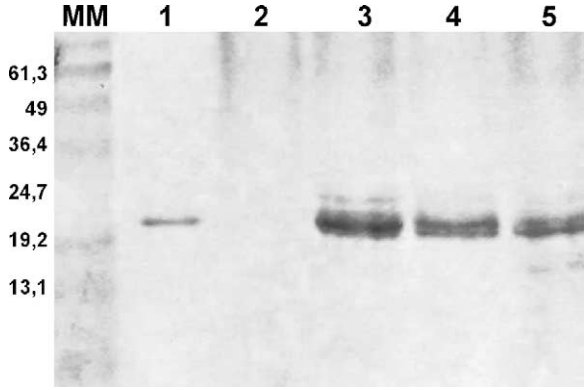

Fig. 2. SDS–PAGE Coomassie gel on GRA1 protein loaded microparticles which were stored over a period of 5 days at 4 °C, in PBS. Lane 1: purified recombinant GRA1, lane 2: empty chitosan microparticles, lane 3: GRA1 loaded microparticles 1 day at 4 °C, lane 4: GRA1 loaded microparticles 3 days at 4 °C, lane 5: GRA1 loaded microparticles 5 days at 4 °C.

Freshly prepared particles were used for every vaccination in the preliminary in vivo experiments.

3.3. Characterization of chitosan nanoparticles

The Chitosan–DNA nanoparticles were prepared at different N/P ratios, to examine whether the chitosan used in this study behaves as described in literature for the same nanoparticle preparation method. In this system, the N/P ratio represents the most accurate quantification method, although this ratio and the +/− ratio are almost similar at pH 5.5 since the pK_a of chitosan is 6.5. From the data obtained it appears that the chitosan used here (∼80 kDa, 93.2% DA) is behaving similarly to the low molecular weight (LMW, 110 kDa, 87% DA) chitosan described in literature (Mao et al., 2001). At N/P ratios lower than 2, large aggregates were formed, indicating unstable formulations (data not shown). Nevertheless, at N/P ratios between 3 and 8 the particles were sufficiently stable at a mean size range around 400 nm (Table 1). The zeta potential of all nanoparticle preparations was positive and did not differ much between the different N/P ratios. The loading procedure of the nanoparticles was very efficient, yielding encapsulation efficiencies higher than 98% for all N/P ratios investigated.

The nanoparticles were also evaluated for their ability to protect the DNA against degradation by DNase I. We found that the DNA associated to nanoparticles

Table 1
Size, zeta potential, and loading efficiency of chitosan nanoparticles loaded with pVR1020-GRA1 at different N/P ratios

N/P ratio	Mean particle size (nm)[a]	Zeta potential (mV)[a]	Loading efficiency (%)[b]
3:1	396.3 ± 70.3	27.1 ± 2.1	99.8 ± 0.0
4:1	435.8 ± 107.4	26.3 ± 1.7	98.1 ± 0.5
5:1	422.4 ± 69.4	27.2 ± 2.5	99.9 ± 0.1
6:1	419.7 ± 84.4	27.7 ± 1.8	98.2 ± 2.0
8:1	373.0 ± 42.9	29.3 ± 3.2	99.4 ± 0.3

[a] Data presented is mean ± standard deviation ($n = 5$–8).
[b] Data presented is mean ± standard deviation ($n = 3$).

was more slowly degraded by DNase I: whereas naked DNA was completely degraded after 5 min, DNA was still protected by the nanoparticles during an incubation time of up to 10 min. After 20-min incubation, DNA was almost completely degraded, although slightly better protection was seen at an N/P ratio of 6:1 (Fig. 3). We also evaluated the release of DNA from the nanoparticles in PBS (pH 7.3, 25 °C, moderate shaking), but no DNA in the supernatant was detected up to a period of 8 days (data not shown). These results indicated the strong electrostatic interactions between chitosan and DNA that cannot be destabilized even when the zeta potential is approaching zero at neutral pH. We therefore assume that the nanoparticles are stable and that most DNA is released from the nanoparticles due to enzymatic degradation of chitosan.

3.4. Concentrated chitosan nanoparticles suspensions for in vivo delivery to mice

In our study, the recommended volume for intragastric administration was 100 μl per dose. We therefore concentrated the nanoparticle suspension to a DNA concentration of 500 μg/ml, resulting in DNA concentrations of 50 μg per vaccination. We studied the effect of N/P ratios (3:1 and 6:1) and different suspending mediums at two pH values on the stability of the nanoparticles by measuring size and zeta potential after the concentrating procedure (Table 2). An N/P ratio of 6:1 resulted in a better resuspendability and preservation of particle size before concentration, while the particle size distribution at an N/P ratio of 3:1 was not preserved after concentration, even in the presence of surfactant. Further it was noted that resuspending in the presence of Tween-80 resulted in a reduction in zeta potential for both pH values at a ratio of 6:1, although this reduction was not enough to cause aggregation. In general, the concentrated nanoparticle

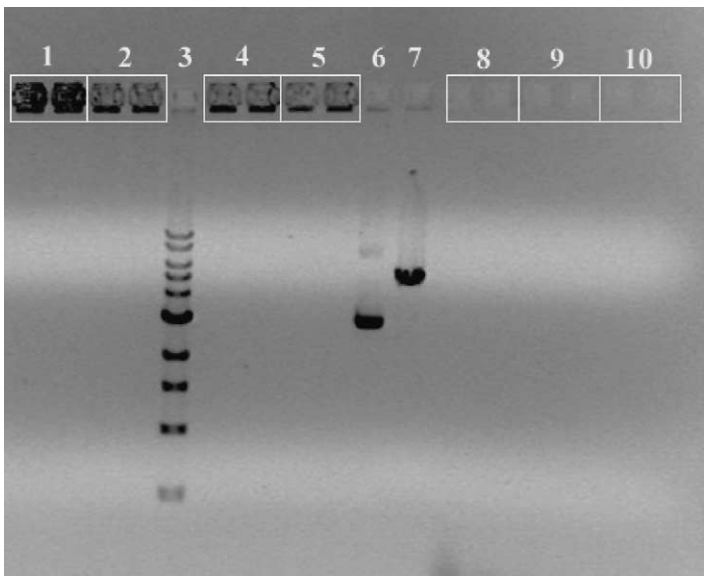

Fig. 3. Agarose gel electrophoresis of Chitosan–DNA nanoparticles: N/P ratio 6:1 incubated with DNase I for 0 (1), 5 (2), 10 (4), and 20 (5) min; naked DNA incubated for 5 (8), 10 (9), and 20 (10) min. A DNA ladder of 1 kb was used as a reference (3). The pDNA in the supercoiled form (6) and in the linear form after restriction with *Bam*HI (7) were used as controls.

Table 2
Size and zeta potential of 10 times concentrated chitosan nanoparticles loaded with pVR1020-GRA1

pH	Medium	N/P ratio (3:1)		N/P ratio (6:1)	
		Particle size (nm)	Zeta potential (mV)	Particle size (nm)	Zeta potential (mV)
5.5	Buffer[a]	586.2 ± 177.6[c]	28.4 ± 2.9[c]	546.0 ± 114.4[c]	31.5 ± 2.0[c]
	Tween[b]	Aggregates	ND	397.7 ± 56.9[c]	25.1 ± 1.1[c]
6.0	Buffer[a]	416.6 ± 3.5[d]	19.7 ± 0.8[d]	338.3 ± 36.6[c]	21.9 ± 0.8[c]
	Tween[b]	Aggregates	ND	387.7 ± 76.2[c]	18.2 ± 2.1[d]

ND: not determined.
[a] 5 mM sodium acetate buffer.
[b] 0.15% Tween®-80 in 5 mM sodium acetate buffer.
[c] Data presented is mean ± standard deviation ($n = 3$).
[d] Data presented is mean ± standard deviation ($n = 2$).

suspension of an N/P ratio of 6:1 resuspended in a 0.15% Tween-80 solution showed optimal properties and thus this formulation was chosen for successive in vivo studies.

3.5. Preliminary in vivo studies

Female C3H mice were intragastrically (i.g.) immunized with either GRA1 loaded microparticles (GRA1-mp), pVR1020-GRA1 loaded nanoparticles (pVR1020-GRA1-np) or empty microparticles (empty mp). The rationale for priming with chitosan formulations was to analyze whether oral priming could induce a Th1 type response. One month after administration, anti-GRA1 responses were determined by ELISA. Whereas the pVR1020-GRA1-np and the empty mp formulations did not elicit any significant response, the GRA1-mp elicited a weak but significant increase in antibody response ($P < 0.05$) (Fig. 4).

As strong antibody responses were not obtained, the groups were split and three mice per group were boosted by i.m. injection of pVR1020-GRA1. Control mice, who were immunized with empty chitosan microparticles were boosted with pVR1020. The other five mice received another immunization with GRA1-mp, pVR1020-GRA1-np or empty mp, respectively. Four weeks later their blood was analyzed by ELISA to determine anti-GRA1 antibody levels (Fig. 4).

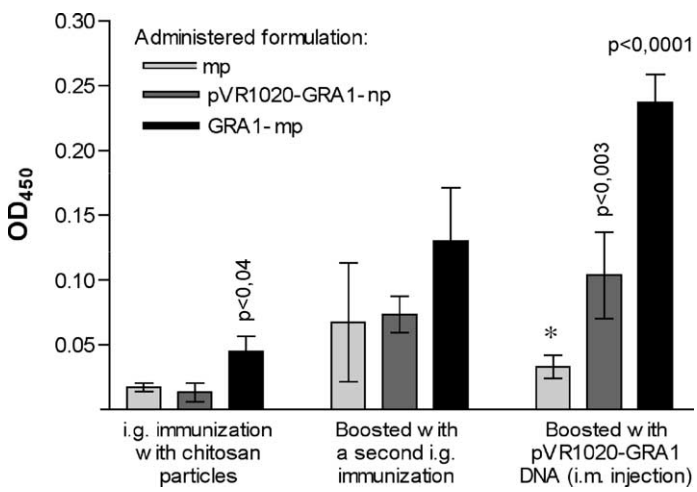

Fig. 4. Anti-GRA1 antibody levels from mice i.g. immunized with different chitosan formulations, and boosted with either a second round of chitosan formulations or i.m. injection of pVR1020-GRA1. Average OD_{450} of 1600 times diluted sera is shown with standard error (at least three mice per group). Asterisk (∗) indicates that Control mice who were immunized with empty microparticles were boosted with the pVR1020 plasmid. Mp: microparticles; np: nanoparticles.

A second i.g. immunization with the same chitosan formulation could increase the anti-GRA1 response, but as the empty chitosan elicited a high background, this increase was insignificant. In contrast, when boosting i.m. with pVR1020-GRA1, the GRA1-specific antibody levels increased significantly in mice that were primed with either pVR1020-GRA1-np or GRA1-mp, and boosted i.m. with pVR1020-GRA1 ($P < 0.003$ and $P < 0.0001$, respectively). Mice primed with GRA1 protein loaded chitosan microparticles were able to produce two-fold higher antibody levels than mice primed with pVR1020-GRA1 loaded chitosan nanoparticles, and yielded endpoint titers comparable to mice infected with *T. gondii* (data not shown).

To have an indirect indication of the type of elicited immune responses, IgG2a and IgG1 isotype ratios (IgG2a/IgG1) were determined. The isotype ratios from mice primed with GRA1-mp or pVR1020-GRA1-np, and boosted with pVR1020-GRA1 i.m. were, respectively 0.54 and 0.1. Surprisingly, in the case of priming with pVR1020-GRA1-np the predominant antibody isotype was IgG1, an isotype known to be associated with Th2 type responses. This is in sharp contrast with data from studies on intranasal administration of chitosan nanoparticles loaded with a cocktail of pDNA encoding RSV proteins, which was able to induce protective Th1 type responses, characterized by production of IFN-γ and RSV-specific cytolytic T cells (Kumar et al., 2002). However, the isotypes of the antibodies were not characterized in this study. Priming with GRA1-mp induced a mixed isotype profile, which may reflect a mixed Th1/Th2 response. Other vaccination studies with diphteria toxoid loaded chitosan particles have shown that these formulations could induce a Th2 type immune response (McNeela et al., 2000).

The obtained immune responses from our preliminary study are in contrast to protective immune responses against *T. gondii*, which are known to be of Th1 type responses, both in infection and vaccination models. For example, the protective Th1 type response obtained after DNA vaccination with GRA1 (three i.m. injections) is reflected by a high production of the IgG2a isotype (Vercammen et al., 2000). Whereas DNA vaccination against *T. gondii* has received much attention and was evaluated using various antigens, reports on mucosal vaccination strategies have focused on intranasal vaccination with *T. gondii* surface antigen 1 (SAG1). Intranasal administration of SAG1, purified from a parasite lysate, and adjuvanted with cholera toxin (Bourguin et al., 1993; Debard et al., 1996; Velge-Roussel et al., 2000) or heat-labile enterotoxins (Bonenfant et al., 2001) resulted in the induction of protective Th1 type responses.

4. Conclusions

In this study we evaluated the possibility of loading chitosan particles with *T. gondii* GRA1 protein and GRA1 encoding pDNA. These chitosan formulations were easy to prepare, shown to be stable and therefore appropriate for mucosal delivery. GRA1 protein loading efficiency was high, GRA1 protein was protected from degradation over short-term storage by loading onto chitosan microparticles. Efficiency of nanoparticle formation from chitosan and GRA1 pDNA was also very high. Nanoparticles obtained were of appropriate size for cell uptake, and protected enclosed DNA from DNase I degradation. DNA release from particles did not occur over a time period of 8 days, release is therefore assumed to take place due to chitosan degradation.

The preliminary in vivo results indicated that oral vaccination using chitosan-based formulations as particulate delivery systems can prime the immune response. Boosting with GRA1 DNA vaccine resulted in high anti-GRA1 antibody levels, but these were biased towards Th2 or mixed Th1/Th2 immune responses, whereas protective immune responses against *T. gondii* are clearly associated with Th1 type responses. Whether mucosal administration of a GRA1 chitosan formulation after GRA1 DNA priming can enhance the Th1 type elicited immune responses is currently being addressed.

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