Tetanus Toxoid Loaded Nanoparticles from Sulfobutylated Poly(Vinyl Alcohol)-Graft-Poly(Lactide-co-Glycolide): Evaluation of Antibody Response After Oral and Nasal Application in Mice

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Purpose. Aim of the study was the evaluation of the potential of novel tetanus toxoid (TT) loaded nanoparticles (NP) for electing an immune response in mice against TT.

Methods. Six week-old female Balb/c mice were immunized by oral (p.o.), nasal (i.n.) and intraperitoneal (i.p.) application of TT NP loaded by adsorption. As polymer a novel polyester, sulfobutylated poly(vinyl alcohol)-graft-poly(lactide-co-glycolide), SB(43)-PVAL-g-PLGA was used. Blood samples were collected 4 and 6 weeks after immunization and assayed for serum IgG- as well as IgA antibody titers by ELISA. NP formulations varying in size and loading were compared to alum adsorbates as well as to TT solutions.

Results. Both, p.o. and i.n. administration of TT associated NP increased serum titers up to 3×10^3 (IgG) and 2×10^3 (IgA). While small NP induced significantly higher titers then larger ones after oral administration, intermediate NP induced antibodies after nasal application. Of the mucosal routes investigated, i.n. seems to be more promising compared to p.o. immunization.

Conclusions. Antigen loaded NP prepared from surface modified polyesters combined with CT show considerable potential as a vaccine delivery system for mucosal immunization. The results warrant further experiments to explore in more detail the potential use of NP as mucosal vaccine delivery system.

KEY WORDS: nanoparticles; biodegradable branched polyester; mucosal immunization; adsorption; tetanus toxoid.

INTRODUCTION

The mucosal vaccine administration is very attractive for inducing a protective immune response, because many pathogens invade the body through mucosal surfaces. Since it is the area of first contact, mucosal surfaces have developed non-immunologic and immunologic barrier functions. Mucosa associated lymphoid tissues (MALT) contributes 80% of all immunocytes and secretes more immunoglobulins than any other organ in the human body (1). The main function of MALT is the selective uptake of antigens and the induction of local immune responses. In intestinal and airway epithelia specialized cells deliver antigens by transepithelial transport from the apical site to the gut associated lymphoid tissues (GALT) or the nasal associated lymphoid tissues (NALT), respectively (2).

Mucosal delivery of soluble antigens without adjuvant usually leads to a poor immune response and aluminum salts, currently the most widely accepted adjuvant for human use, are not very effective for mucosal immunization (3). Therefore, the development of antigen carrier systems which allow mucosal vaccine delivery is of considerable interest.

Various groups have shown the uptake of micro- (MP > 1 μm) and nanoparticles (NP < 1 μm) from gastrointestinal (4) and nasal (5) tract in the last decade. Therefore mucosal administration of antigen loaded biodegradable particles was studied with the aim to induce both mucosal and systemic immune responses against encapsulated antigen (6).

Although initial success was achieved, the exploitation of particulate carrier systems for oral vaccination remains a challenging task. Little is known about the effect of particle properties critical for absorption and the mechanisms of immune reaction against particulate antigens. Apart from NP properties (size, polymer), antigen integrity and localization after encapsulation are key factors for induction of immune responses after transport across mucosal barriers.

Since NP preparation, using commonly W/O/W (water/oil/water) double emulsion techniques, requires harsh conditions (high energy homogenizing procedures, organic solvents), destructive to many antigens, it would be desirable to associate the antigen with the NP surface by adsorption to avoid their destruction and to preserve their biological activity. Moreover, the dispersion of an antigen in a polymeric matrix may be unfavorable since the encapsulated protein is not available for direct antigen presentation after epithelial uptake.

The aim of this study was the preparation and characterization of NP, loaded with tetanus toxoid (TT) by adsorption, in mice to investigate humoral immune response. A charge modified biodegradable polymer was used to reach a high level of antigen loading by adsorption.

MATERIALS AND METHODS

Chemicals

Charge modified branched polyesters based on poly(sulfobutyl-vinyl alcohol)-g-poly(lactide-co-glycolide) (MW 221000 g/mol) with a 43% degree of substitution of total hydroxyl groups in the poly(vinyl alcohol) backbone and a 10:90 weight ratio of backbone to grafted polyester chains (SB(43)-PVAL-g-PLGA), were recently described (7).

Tetanus toxoid (TT) solution (16.6 mg protein / ml, specific activity: 3300 Lf/ ml), TT standard (TTF 6, Chiron Behring, Germany), alum-adsorbed TT (Tetanol™, Chiron Behring, Germany), purified anti-tetanus-rabbit-serum (TSH1, dilution 1:3000 in PBS, pH 7.4), purified human anti-tetanus IgG (IgGHu1, dilution 1:10000 in PBS, pH 7.4), bovine serum albumin (Boviserin™, Hoechst AG, Germany), cholera toxin (CT) adjuvant (Sigma, Germany), Goat Anti-Mouse IgG (H+L) peroxidase (POD) conjugate (Dianova, Germany), Goat Anti-Mouse IgA (H+L) POD conjugate (Calbiochem,

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LaJolla, USA), 96 well plates Immuno Module B (Dade Behring, Germany), enzyme-linked-immuno-sorbent-assay (ELISA) buffers, washing- and stopping solutions (Dade Behring, Germany) and the ELISA chromogen tetramethylbenzidine (TMB, Dade Behring, Germany) were kindly supplied by Chiron Behring, Germany. Isotonic sodium chloride solution was from Braun (Germany). All other chemicals purchased from Sigma (Germany) of analytical grade were used without further purification.

NP Preparation

NP, prepared by a modified solvent displacement technique, were described in detail elsewhere (7). Briefly, 100 mg SB(43)-PVAL-g-PLGA were dissolved in 10 of acetone (NP ~100 nm), acetone/ethylacetate 65:35 (large NP ~500 nm) or ethylacetate/dichloromethane 95:5 (MP > 1 μm). The polymer solution was subsequently added by injection (flow 10.0 /min) to 50 of a stirred isotonic sodium chloride solution containing 0.1 % (m/m) Pluronic F 68, using a solvent displacement apparatus. After injection of the organic phase the resulting colloidal suspension was stirred for 8 h at 25°C under reduced pressure to remove residual organic solvents.

Particle Size Measurement

The size distribution of the NP, were investigated by photon correlation spectroscopy (PCS) using a Zetasizer 4/AZ110 (Malvern Instruments, UK) equipped with a 4 mW laser source, a 64 channel correlator and a multiangle photomultiplier detector device. Each sample was diluted with isotonic sodium chloride solution to the appropriate concentration avoiding multiscattering events and measured with a sample time of 30 ms for 10 min in serial mode. Each measurement was performed in triplicate and the mean of the data was calculated.

Electron Microscopy

Scanning electron microscopy (SEM) was used to evaluate the morphology of NP and to confirm PCS results. A volume of 10 μL of the NP suspension was placed on a silicon waver and dried at 25°C for 12 h in the vacuum to remove residual water. The morphology and size were visualized with a field emission scanning electron microscope (S 4100, Hitachi Nissei Sangyo, Ratingen, Germany).

Measurement of ζ-Potential

The ζ-potential of the NP suspension was directly determined after solvent evaporation in isotonic sodium chloride solution by electrophoretic light scattering using a Zetasizer 4/AZ 104 (Malvern Instruments, Malvern, UK).

TT Adsorption

The NP were loaded with TT by an adsorption process. The NP were incubated with TT solutions of varying concentrations (according to Table I) in 0.9 % NaCl pH 7.4 for 24 h at 20 °C under mild stirring. The relative amount of TT was calculated indirectly by determining the amount of protein remaining in the supernatant after centrifugation. The supernatants were measured using UV spectroscopy at 280 nm or by ELISA as described below. Due to the affinity of many proteins to polymer surfaces (e.g. centrifuge tubes) and the well known potential of several process steps (e.g. centrifugation) to bring out TT aggregates control samples were run in each experiment.

TT Desorption

The amount and integrity of the desorbed protein was determined as a function of time by a TT specific ELISA as described below. Briefly, the TT loaded NP suspensions were

Table I. NP Properties and Adsorptive TT Loading

Immunization experiment	Batch code	Particle properties		TT adsorption		
		Size[a] [nm]	ζ-potential [mV]	Total TT per ml [μg (Lf)]	Adsorbed TT[b] [%]	TT loading[c] [%]
A	A/0100/w1/5.0	126 ± 54	–49.3	144.5 (25.0)	66.50	5.26
	A/0100/w2/5.0	111 ± 62	–43.7	144.5 (25.0)	67.30	5.21
	A/0100/w3/5.0	101 ± 43	–45.6	144.5 (25.0)	71.10	5.73
B	B/0100/w1/5.0			144.5 (25.0)	64.10	5.07
	B/0100/w1/1.6	96 ± 39	–51.1	46.8 (8.1)	72.20	1.85
	B/0100/w1/0.5			14.5 (2.5)	81.40	0.65
	B/0500/w1/5.0	473 ± 174	n.d.	144.5 (25.0)	31.10	2.50
	B/1500/w1/5.0	1649 ± 412	n.d.	144.5 (25.0)	6.40	0.50
	B/0100/w2/5.0			144.5 (25.0)	63.60	4.97
	B/0100/w2/1.6	98 ± 53	–52.2	46.8 (8.1)	76.30	1.93
	B/0100/w2/0.5			14.5 (2.5)	79.80	0.63
	B/0500/w2/5.0	451 ± 133	n.d.	144.5 (25.0)	29.10	2.40
	B/1500/w2/5.0	1348 ± 522	n.d.	144.5 (25.0)	6.90	0.56
	B/0100/w3/5.0			144.5 (25.0)	59.10	4.68
	B/0100/w3/1.6	103 ± 29	–45.1	46.8 (8.1)	61.10	1.57
	B/0100/w3/0.5			14.5 (2.5)	70.90	0.56
	B/0500/w3/5.0	424 ± 189	n.d.	144.5 (25.0)	32.30	2.54
	B/1500/w3/5.0	1882 ± 766	n.d.	144.5 (25.0)	6.10	0.49

[a] ± represent the standard deviations from gaussian fitted size distribution.
[b] Calculated from the amount of free TT determined by UV spectroscopy.
[c] Given as mass protein per mass polymer.

centrifuged (20,000 rpm, 25°C) for 60 min and supernatants discarded. NP were resuspended in dissolution medium (PBS pH 7.2, 37°C) and protein content monitored for 48 h using UV spectroscopy (280 nm) and ELISA as described below.

Quantitation of Adsorbed and Desorbed TT by ELISA

96-well-plates were coated with 110 μL (1:3000) purified TSH1 per well and incubated overnight at 25°C. The plates were then washed three times with PBS (pH 7.4, 0.15 molar) containing 1% (w/w) Tween® 20 between each step. 100 μL of sample or standard solutions were added in appropriate dilution to each well (standards from 0.08–2.5 μg/ TT). The plates were kept at 37°C and 100% r.h. for one hour and washed three times again. Then 100 μL/well (0.11 IU/) human antitetanus IgG were added. After incubation for one hour (37°C, 100% r.h.) and washing, 100 μL anti-human-IgG POD conjugate were added to each well. The plates were incubated at 37°C and 100% r.h. for another hour and washed again. Finally TMB in chromogen buffer containing H_2O_2 was added to the plates. After incubation for 30 minutes at 37°C and 100% r.h. the reaction was stopped by adding 100 μL stopping solution to each well. Absorbance was read at 405 nm wavelength using a multiwell plate reader. Each sample was run in triplicate with a standard curve in triplicate. The concentration of active TT was calculated as described below.

Determination of Antibody Responses by ELISA

All sera were assayed in duplicate for TT specific IgG and IgA antibody responses using an ELISA technique. Serial dilutions of sera were incubated on TTF 6 coated 96 well plates. TT specific antibodies were quantified by incubating wells with heavy chain-specific peroxidase conjugated goat anti mouse IgG or IgA. Responses were quantified by measurement of the absorbance at 405 nm following incubation of wells with TMB. The results were expressed as reciprocal end-point sera titers representing the highest sera dilutions giving an absorbance of 0.2.

Animals

Female Balb/c mice 7–9 weeks of age and a weight of 16–22 g, were obtained from Harlan-Winkelmann (Germany). The animals, used in all immunization experiments, were housed in groups of ten and had free access to food and water throughout the study, except for the night before NP application.

Immunization Protocol

Two independent consecutive experimental series were performed. In experiment A the principle possibility of mucosal immunization using TT loaded biodegradable NP was studied and an immunization protocol was developed to optimize both systemic and mucosal antibody response. Seven groups of mice were used in this experiment. TT loaded SB(43)-PVAL-g-PLGA NP were compared to conventional alum-adsorbed as well as to TT solution without adjuvant.

In experiment B, consisting of 12 animal groups, critical formulation aspects of NP for the induction of an immune response after oral and nasal administration were investigated. SB(43)-PVAL-g-PLGA NP-formulations varying in size and TT loading were administered and the induction of antibody titers was measured.

For each experiment (A and B) mice were randomized, pooled into groups of 10 animals and immunized on three consecutive weeks (week 1, 2, 3) either by peroral (p.o.), intranasal (i.n.) or intraperitoneal (i.p.) application according to Table II. Approximately 30 minutes before oral vaccination 200 μL of $NaHCO_3$ buffer (pH 8.2, 0.2 M) were administered p.o. to reduce TT degradation in the stomach. Groups of mice were inoculated p.o. with 200 μL of the appropriate preparation at week 1, 2 and 3 by gavage. The i.p. inoculations

Table II. Immunization Protocol of Experiments A and B

Immunization experiment	Type of vehicle	Batch code	Vaccination route	Dose (μg (LF))	Coadministration of adjuvant CT (μg)	Application schedule (week)	Serum sampling (week)
A	TT NP (~100 nm)	A/0100/w1-3/5.0	p.o.	28.9 (5.0)	—		
	TT NP (~100 nm)	A/0100/w1-3/5.0	p.o.	28.9 (5.0)	10		
	TT solution	—	p.o.	28.9 (5.0)	10		
	TT solution	—	p.o.	28.9 (5.0)	—	1/2/3	0/4
	TT NP (~100 nm)	A/0100/w1-3/5.0	i.n.	2.89 (0.5)	—		
	TT NP (~100 nm)	A/0100/w1-3/5.0	i.p.	28.9 (5.0)	—		
	Tetanol®	—	i.p.	28.9 (5.0)	—		
B	TT NP (~100 nm)	B/0100/w1-3/5.0	p.o.	28.9 (5.0)	10		
	TT NP (~500 nm)	B/0500/w1-3/5.0	p.o.	28.9 (5.0)	10		
	TT MP (>1 μm)	B/1000/w1-3/5.0	p.o.	28.9 (5.0)	10		
	TT NP (~100 nm)	B/0100/w1-3/1.6	p.o.	9.4 (1.6)	10		
	TT NP (~100 nm)	B/0100/w1-3/0.5	p.o.	2.89 (0.5)	10		
	TT NP (~100 nm)	B/0100/w1-3/5.0	i.n.	2.89 (0.5)	—	1/2/3	0/4/6
	TT NP (~500 nm)	B/0500/w1-3/5.0	i.n.	2.89 (0.5)	—		
	TT MP (>1 μm)	B/1000/w1-3/5.0	i.n.	2.89 (0.5)	—		
	TT NP (~100 nm)	B/0100/w1-3/5.0	i.n.	2.89 (0.5)	1		
	TT solution	—	i.n.	2.89 (0.5)	1		
	TT solution	—	i.n.	2.89 (0.5)	—		
	Tetanol®	—	i.p.	28.9 (5.0)	—		

Note: p.o. = peroral/i.n. = intranasal/i.p. = intraperitoneal.

with 200 μL of Tetanol™, TT SB(43)-PVAL-g-PLGA NP or TT solutions were performed as a positive control. For i.n. administration following procedure was used: Anaesthetized animals (approximately 10–20 sec. in a Forene ™ atmosphere) were given the vaccine i.n. by applying 20 μL (10 μL in each nostril) of the respective preparation by means of a micropipette. All animals were bled at week 0 and 4 (experiment A) or at week 0, 4 and 6 (experiment B).

Collection and Preparation of Sera

Individual blood samples were obtained from the tail vein according to the immunization protocol (Table II). Sera were prepared by centrifugation and stored at –20°C until used.

Computation and Statistics

The concentration of immuno-reactive TT was determined from standard curves after plotting absorbance versus antigen concentration, by regression analysis.

The concentrations of anti-tetanus IgG and IgA were calculated by fitting each curve to a polynom after plotting absorbance against log sample dilution. The dilution for the absorbance = 0.2 was interpolated using the ELDAN software package version 2.0 (Dade-Behring, Germany). Antibody titers were expressed as the reciprocal of the estimated serum dilution.

Results from animal studies in mice were expressed as the mean ± standard error ($n = 10$) of the reciprocal endpoint serum titers. Statistical analysis of the significance of difference between the response of groups was established by computing Student's t test.

RESULTS AND DISCUSSION

NP Characteristics

Three different batches of NP were prepared using the solvent displacement technique with SB(43)-PVAL-g-PLGA. The use of different solvents or solvent mixtures allows a control of the resulting particle sizes. General physicochemical properties of SB(43)-PVAL-g-PLGA NP has already been described in detail (7). Typical characteristics of the NP batches are summarized in Table I.

The NP batches showed different sizes (average diameters of about 100 nm, 500 nm and 1500 nm for individual batches), depending on the solvent used.

The yield, which refers the quantity of NP recovered after NP preparation and solvent evaporation to the total amount of polymer used, was at least 88.5% for all three batches.

The negative ζ-potentials are the consequence of the negatively charged SB-PVAL-g-PLGA. As outlined above the polyesters consist of biodegradable PLGA chains which are grafted onto sulfobutyl derived PVAL backbone. A special feature of this polymers is the presence of sulfobutyl groups, leading to the negatively charged NP surface, thus leading to electrostatic interaction with positively charged sequences of proteins (8).

Adsorptive NP Loading with TT

The use of NP with negatively charged surfaces allowed the adsorptive association of up to 5.2% TT (mass protein/mass polymer), equivalent to 66.5% of the initial TT amount under our experimental conditions, depending on the total surface area of the NP suspension. The ELISA method, which was also used for the control of the adsorption procedure, confirms these results from UV spectroscopy (data not shown).

In previous studies where different types of polymers were compared, we found that the amount of TT adsorbed onto the NP surface was significantly influenced by charge and hydrophobicity of the polymer. Values ranged from <1% for uncharged PVAL-g-PLGA NP up to 5.2% (w/w) using SB(43)-PVAL-g-PLGA NP. In the case of NP prepared from

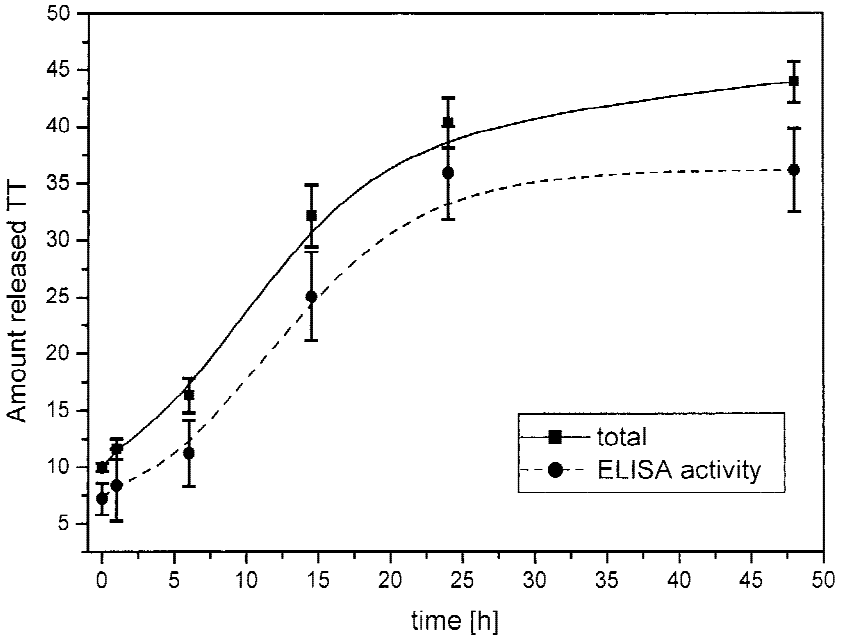

Fig. 1. Release profile of total and ELISA active TT adsorbed to SB(43)-PVAL-g-PLGA NP over 48 h.

PLGA (lactide: glycolide 50:50, MW 40000 g/mol) a loading of 3.1% (w/w) was reached.

The adsorption of proteins onto surfaces, especially charged colloid surfaces, is a complex phenomenon which can not be discussed here in detail. For an excellent review on interactions between proteins and polymeric surfaces c.f. reference (9). The release profile of TT due to desorption from NP surface over the time is shown in Figure 1.

Our results are in excellent agreement with literature. As outlined by Norde (9) and confirmed for TT (10), high molecular weight proteins with a large number of potential binding sites, do not desorb simultaneously from NP surface, yielding a continuous protein release profile. The adsorption process was only slightly influenced by the protein properties as shown by PAGE (data not shown) and ELISA. More than 75% of the released TT withstood this loading procedure in an ELISA-active form. This could be attributed to electrostatic interactions between positively charged protein residues and the negatively charged NP surface.

Immune Response to TT Loaded NP in Mice After Mucosal Application

Initially we administered TT loaded NP, suspended in isotonic saline, with and without the mucosal adjuvant CT by oral (28.9 μg) and nasal (2.89 μg) application. Anti-TT specific serum IgG and IgA antibodies induced after mucosal vaccination were compared to an i.p. application (28.9 μg) of TT NP, conventional Tetanol® as well as to TT solutions as shown in Figure 2. Four weeks after the first immunization with the NP preparations IgG as well as IgA antibody responses were observed, significantly higher than in serum samples before vaccination. The oral vaccination with TT NP induced only weak IgG titers (ca. 0.1% of those obtained for the i.p. control using Tetanol®).

Total serum IgA, composed of locally produced secretory IgA (sIgA) as a result of events initiated at NALT and MALT and serum IgA, usually not obtained after parenteral immunization, was taken as a measure for mucosal immune responses. Total serum IgA is only an indirect response parameter to monitor the complex events of mucosal immune reaction (1). Sensitive and reproducible measurement of sIgA is a difficult and time consuming undertaking. The preparation and/or washing procedures of mucosal tissues and the presence of proteolytic enzymes often leads to highly variable results. Immune cells, stimulated at one mucosal surface, can migrate to distant mucosae. Therefore, the sampling should not limited to one surface, rather several local sIgA titers have to be determined for detailed mechanistic studies. After oral vaccination IgA titers increased nearly twice as much as those induced by Tetanol™, while application of pure antigen solutions did not induce significant IgA titers at all. By converting soluble TT into a particulate species through adsorption, an adjuvant effect can be achieved. Improved uptake by M cells and an enhanced processing by antigen presenting cells of particulate materials compared to the free antigen have been discussed and could be due to the similarity of an antigen coated particle to the original pathogen; e.g. small bacteria or viruses (6,11).

Mucosal application of large doses of protein are known to result in unresponsiveness to mucosal and systemic immunization (12). CT, reported to abrogate oral tolerance to antigenically unrelated protein (13), was chosen as an mucosal adjuvant. The molecular mechanism of the exotoxin from Vibrio cholerae, a heterohexameric AB_5 class toxin, is its binding via the ring-shaped B_5 subunit to epithelial receptors, monosialoganglioside (GM_1), which are present on all nucleated cells. The interaction changes the lipid rigidity of cell membranes, leading to an increased permeability for high molecular weight proteins and NP (3). The second mechanism is the ability of CT to ADP-ribosylate the adenylate cyclase regulatory protein Gs through its catalytic A subunit. Coadministration of 10 μg of CT boosted the antibody response of orally applied TT-NP as well as free antigen distinctly. These results are in accordance with previous work of Jackson et al. (14) who studied the effect of CT and TT solutions on mucosal immune responses in detail.

Of the mucosal routes investigated, intranasal immunization seems to be more effective in inducing both, serum IgG and IgA titers. Although only 10% of the oral dose were administered i.n. without the co-application of CT, IgG and IgA titers were in the same range or even higher. The efficient induction of IgG immune responses after nasal vaccination in mice using adsorptively loaded TT PLA NP has been reported previously (10). Since L-PLA is known to degrade very slowly, the use of faster degrading SB(43)-PVAL-PLGA is more favorable.

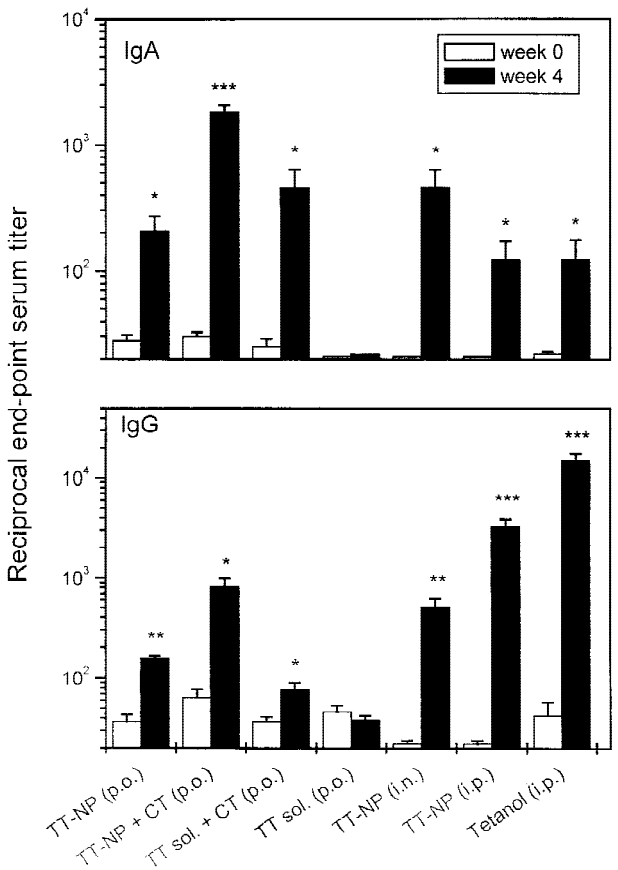

Fig. 2. Total serum IgG and IgA antibody response (mean ± standard error, $n = 10$) to TT loaded SB(43)-PVAL-g-PLGA NP and controls (experiment A). Animals were given 200 μl of the appropriate preparation p.o. and i.p. (28.9 μg TT = 5 Lf) and 20 μl (2.9 μg TT = 0.5 Lf) i.n. with or without 10 μg CT. Titers of week 4 have been compared by Student's t test to those of week 0 (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$).

Critical NP Characteristics

Among the various NP properties, the effect of particle size on the immune responses seems to be a key factor and has been intensively investigated (15–17). These studies, mostly focussing on oral application, show an influence of particle size on immune reactivity of NP. While MP are retained in the Peyer's patches and induce mucosal immunity, NP can also be taken up from the gut and induce systemic immunity as well (18). While some authors found that 4 μm particles are optimal for the induction of systemic immune responses (15,16) other showed that smaller NP sizes are more effective (17). With respect to gastrointestinal particle absorption, an inverse proportionality between absorbed amount and particle size seems to be likely (4,19,20).

Assuming that particle absorption from the gastrointestinal tract is the first step for induction of humoral immune responses, the antibody titers after mucosal vaccination, displayed in Figure 3, are in accordance with the observed high rate of absorption and adhesion of SB(43)-PVAL-g-PLGA NP by epithelial cells in culture (data not shown). The induction of IgG and IgA antibody responses after oral application is strongly influenced by the NP size distribution. While particles > 1 μm did not induce antibody titers at all (similar to pure TT in solution) significant titers were observed after application of 500 nm NP. The highest antibody titers were found in case of 100 nm NP.

A triggering of immune responses from mucosal to humoral antibody titers, reported to occur if NP size is decreased (21), was not observed in this study. This may be due to the fact, that only total serum IgA and not locally sIgA from intestinal and nasal washings was determined. There is evidence in literature about a correlation between both parameters (22).

With the nasal application of the NP a slightly different trend was found. Both, IgG and IgA titers are affected by the average particle size in a similar manner. While immune responses after oral vaccination of 100 nm NP were significantly increased compared to 500 nm NP, this difference was not found in case of nasal application. Small TT loaded NP induced immune responses equivalent to those of the medium sized NP. These results, which are in accordance with literature (10), seem to be compatible with a different mechanism of NP translocation in NALT compared to GALT. Intranasally administered TT loaded NP could also be inhaled into alveolar regions, which are not completely impermeable to the passage of antigen loaded NP and free antigen (23). NP translocation from the alveolar lumen was reported to take place by means of alveolar macrophages (24).

Oral administration of NP loaded with varying doses of TT resulted in strongly different immune responses (Figure 4). We found that different doses are necessary to induce IgG

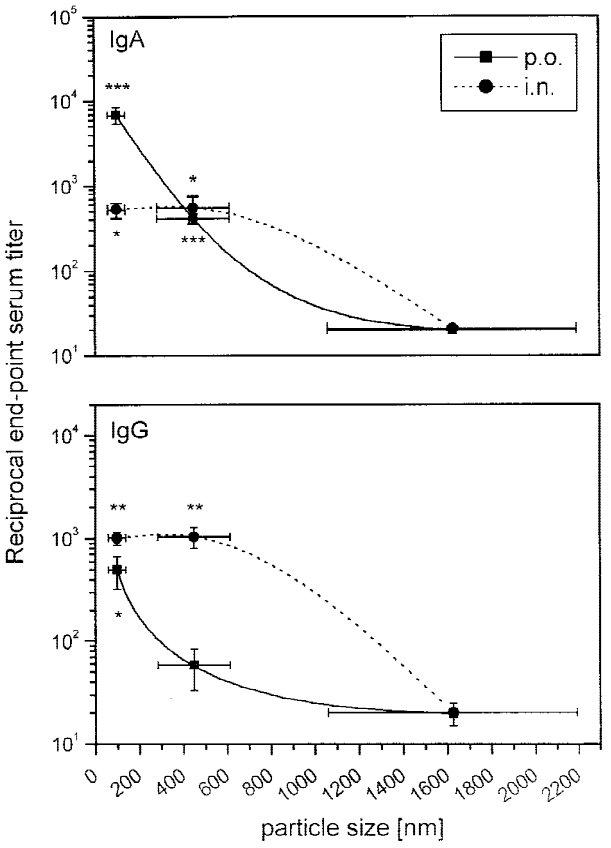

Fig. 3. Total serum IgG and IgA antibody response (mean ± standard error, $n = 10$) after 6 weeks to TT loaded SB(43)-PVAL-g-PLGA NP of varying sizes after p.o. and i.n. vaccination (mean sizes ± standard deviation of the gaussian fitted size distributions). Animals were given 200 μl of the NP suspension p.o. (28.9 μg TT = 5 Lf) with 10 μg CT and 20 μl (2.9 μg TT =0.5 Lf) i.n. without CT. Titers have been compared by Student's t test to those of untreated animals (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$).

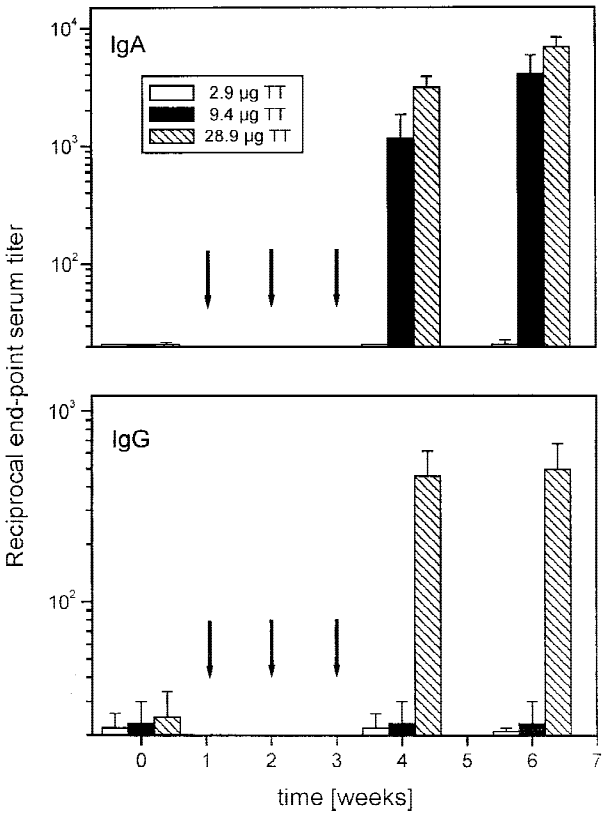

Fig. 4. Total serum IgG and IgA antibody response (mean ± standard error, $n = 10$) at week 6 to different sized SB(43)-PVAL-g-PLGA NP with varying antigen loading (mass TT/mass polymer) after p.o. vaccination. Titers have been compared by Student's t test to those of untreated animals (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$).

and IgA titers. For IgA induction 9.4 μg TT seems to be sufficient while 28.9 μg are necessary to reach significant IgG titers. For induction of systemic IgG titers a sufficient amount of TT has to be absorbed from the gut and must reach systemic lymphoid tissues, more specialized for IgG production. In the case of IgA titers the immune response is induced directly in the GALT. A characteristic feature of the adsorptive NP loading is the dependence of the protein loading from the total surface area of the NP, and therefore from the size distribution of the NP suspensions (25). Smaller NP size yield a larger total surface area and per weight of polymer consequently a larger amount of TT is adsorbed to NP. These parameters can not been adjusted easily and serological results from animal experiments should be interpreted with caution. The data showing antibody titers as a formation of TT loading need also be considered in view of NP sizes (Figure 5).

In the case of IgA responses, where 100 nm NP loaded with 1.8% TT, which are equivalent to 18 μg TT per mg polymer, induced higher titers than 500 nm NP loaded with 2.5% TT loading. The tendency seems to be mainly influenced by the NP size. Statistically significant IgG titers were only induced after application of 100 nm NP with 4.9% TT loading. It is unclear, whether the observed IgG titers in this experiment are caused by variations in NP/MP size or by the higher antigen loading.

Comparison of Oral vs. Nasal Application

Various authors studied the nasal mucosa as a potential route of vaccination against tetanus (26), where NP were demonstrated to be an useful carrier system for TT. For other clinically relevant antigens it was also found that i.n. vaccination, compared to p.o. administration, requires fewer administrations at lower dosing levels to stimulate immune responses. Similar results were obtained for the nasal vaccination with TT loaded SB(43)-PVAL-g-PLGA NP (Figure 6).

The i.n. application of TT NP leads to a distinct stimulation of humoral and mucosal immune response, resulting in significantly increased IgG and IgA serum titers compared to non-treated animals. The adjuvant CT increased antibody titers of TT NP as well as free TT, demonstrating the suitability of this route for mucosal vaccination. As mentioned above, the dose which is necessary to induce immune responses after oral NP application is at least ten times higher then that for the induction of similar responses after intranasal vaccination, probably due to less efficient particle uptake from the gut and a more destructive gastrointestinal environment for proteins. Furthermore, nasal delivery of antigens allows a direct access to the NALT, the major site of antigen uptake and immune reaction, whereas oral delivery requires diffusion of the antigen carriers to extensively widespread lymphoid aggregates. The nasal mucosa shows a relatively good permeability for protein, compared to the gastrointestinal tract and immune response in draining lymph nodes, mediated by the intraepithelial dendritic cells (2), may be more easily stimulated.

CONCLUSIONS

The association of TT with SB(43)-PVAL-g-PLGA NP through an adsorption procedure leads to an antigen delivery system which may have potential for oral and nasal vaccination, especially in combination with CT. TT loaded SB(43)-PVAL-g-PLGA NP given p.o. as well as i.n. induced serum IgG and IgA immune responses in mice in a reproducible manner. The effect was significantly increased by the coadministration of CT. The IgA titers were significantly increased compared to the control (i.p. administration).

The NP size affected the induction of antibody titers in a significant manner. For oral administration it is likely that

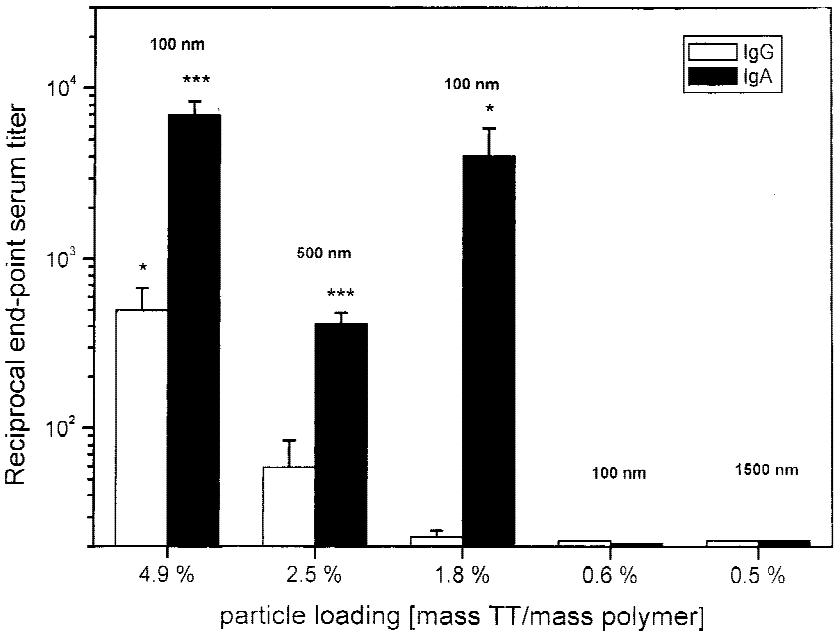

Fig. 5. Total serum IgG and IgA antibody response (mean ± standard error, $n = 10$) at week 6 to different sized SB(43)-PVAL-g-PLGA NP with varying antigen loading (mass TT/mass polymer) after p.o. vaccination. Titers have been compared by Student's t test to those of untreated animals (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$).

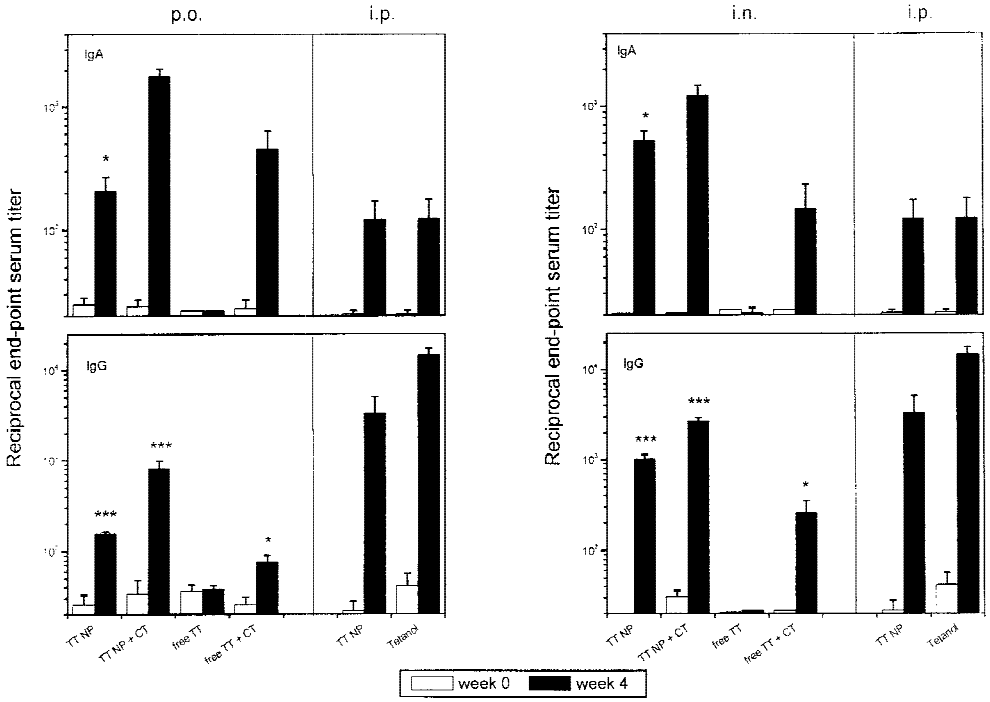

Fig. 6. Comparison of p.o. versus i.n. vaccination in mice using TT loaded SB(43)-PVAL-g-PLGA NP and controls. Total serum IgG and IgA antibody response (mean ± standard error, $n = 10$) at week 4 are displayed. Animals were given 200 μl of the appropriate preparation p.o. and i.p. (28.9 μg TT = 5 Lf) and 20 μl (2.9 μg TT = 0.5 Lf) i.n. with or without 10 μg (p.o.) or 1 μg (i.n.) CT. Titers after p.o. vaccination have been compared by Student's t test to those after i.n. vaccination ($*p < 0.05$, $**p < 0.01$, $***p < 0.005$).

smaller NP induce higher IgA and IgG titers. In the case of nasal administration 500 nm NP leads to IgG and IgA titers in the same range as NP of 100 nm size. Furthermore, different doses are needed for the induction of IgG and total serum IgA antibody titers. An oral dose of 9.4 μg TT associated to SB(43)-PVAL-g-PLGA NP is sufficient to induce an IgA response, while 28.9 μg are needed to induce significant increased serum IgG titers. In contrast, nasal dosing of 2.89 μg TT NP induces both, serum IgG as well as IgA.

Overall, the nasal route of application seems to be more promising for vaccination in mice than the oral one. Antibody titers in the same range or higher were observed although only 10% of the orally applied antigen dose was given.

Further investigations are necessary to explore the interaction of antigen loaded NP and different cell types in NALT and GALT inducing both, mucosal and humoral immunity. For example NP surface properties, hydrophobicity and charge, also may have an important role in NP absorption and induction of immune responses. Hydrophobic poly(styrene) NP, showing the highest uptake, are selectively targeted to the M cell surface of the Payers patches (27,28), while more hydrophilic NP are not only taken up via the M-cells, but also via the normal intestinal enterocytes (4).

In summary, nasal vaccine delivery systems based on biodegradable NP are useful to induce mucosal immunity and merit more detailed investigation.

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