Specific IgA subclass responses in serum and saliva: a 12-month follow-up study after parenteral booster immunization with tetanus toxoid

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Specific IgA subclass antibodies against tetanus toxoid in serum, parotid saliva, and whole saliva were quantified after booster immunization. Samples from 14 healthy individuals were collected before and 1, 6, and 12 months after subcutaneous injection with Duplex® (0.25 ml tetanus toxoid 30 Lf/mL and diphtheria 7.5 Lf/mL). Samples of whole saliva were also collected after 2 weeks. Specific IgA1 and IgA2 subclass antibodies to tetanus toxoid were quantified by enzyme-linked immunosorbent assay (ELISA). In this quantitative method, chimeric IgA1 and IgA2 antibodies directed against NP (4-hydroxy-3-nitrophenacetyl) were used as standards. Total levels of IgA1 and IgA2 were measured using a nephalometer or ELISA. Immunization with tetanus toxoid resulted in raised mean values of specific IgA1 and IgA2 antibodies against tetanus toxoid in serum after 1 month. Compared with the baseline, the mean value of specific IgA1 antibodies showed a 2.6-fold increase (mean value 10.47 μg/mL) in serum, and that of specific IgA2 antibodies a 2.7-fold increase (mean value 0.93 μg/mL). Specific IgA subclass antibody levels in parotid and whole saliva were unchanged after 1 month. The ratio of specific IgA subclass antibodies to total IgA subclass antibodies was 3 to 10 times higher in parotid saliva compared with whole saliva. In conclusion, subcutaneous booster immunization with tetanus toxoid induced immune responses of both antigen-specific IgA1 and IgA2 subclass antibodies in serum with the same increase, whereas the levels of specific IgA subclass antibodies in secretory fluids were unchanged. The ratio of specific IgA subclass antibodies to immunoglobulins was higher in parotid saliva compared with whole saliva. □ *IgA subclasses; saliva; serum; specific antibodies; tetanus toxoid*

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Specific IgA subclass distribution against tetanus toxoid in serum is of both IgA1 and IgA2 subclasses (1). After intramuscular booster immunization, toxoid-specific IgA antibodies in serum were predominantly of the IgA1 subclass, while the IgA2 response was marginal (2). Specific IgG subclass distribution response after booster immunization shows an IgG1 response followed by an IgG4 response (3).

Specific IgA and IgG subclass antibodies against tetanus toxoid in saliva after intramuscular booster immunization have been studied in patients suffering from end-stage renal diseases (2). In this group of four subjects, elevated salivary antibodies against tetanus toxoid were found for IgG antibodies and no alterations in specific IgA subclass antibody responses were detected (2).

The aims of this study were to analyze the dichotomy between the mucosal and systemic systems in IgA subclass antibody response and the interrelation of specific antibodies and total IgA subclass levels after booster immunization. For the analysis of specific antibodies, a semiquantitative method to determine IgA1 and IgA2 subclass antibodies against tetanus toxoid was used. The concentration of specific IgA subclass antibodies and total IgA subclass levels in serum, parotid saliva, and whole saliva were analyzed up to 1 year after a subcutaneous booster immunization.

Materials and methods

Parotid saliva, whole saliva, and serum from 14 healthy individuals (12 F and 2 M, age range 37–59) were included in the study. All had earlier undergone primary immunization against tetanus and diphtheria. As booster dose, all individuals were given 0.25 mL Duplex (SBL Vaccin AB, Sweden) according to the recommendations. Each dose contained tetanus toxoid 7.5 Lf and diphtheria toxoid 1.875 Lf as antigens and the vaccine contained aluminum phosphate 1.25 mg per 0.25 mL as adjuvant. Serum, parotid saliva, and whole saliva samples were collected before and 1, 6, and 12 months after immunization. In addition, whole saliva samples were collected 14 days after immunization.

Whole saliva was collected by expectoration into a plastic mug for at least 5 min. Parotid saliva was collected into Lashley cups placed directly over both parotid ducts, while the salivary flow was stimulated with 0.5 mL 3% citric acid at 0.5 min intervals for at least 5 min. The

Table 1. Tetanus toxoid-specific IgA1 and IgA2 subclass antibodies in serum (μg/mL)

	Serum							
	Baseline		1 month		6 months		12 months	
	IgA1	IgA2	IgA1	IgA2	IgA1	IgA2	IgA1	IgA2
Mean	4.05	0.34	10.47	0.93	7.84	0.53	6.05	0.32
Range	<0.02–9.76	<0.02–0.82	<0.02–24.88	0.43–1.88	<0.02–22.51	<0.02–1.09	1.8–18.01	<0.02–0.76
n	14		14		14		12	

samples were stored at −70°C. Whole saliva was clarified by centrifugation at 11,000*g* for 15 min before being analyzed.

Salivary IgA subclass quantification

IgA1 and IgA2 concentrations were quantified using an enzyme-linked immunosorbent assay (ELISA) (4). Salivary IgA subclass levels were measured in 2-fold dilution steps (1/2000 and 1/10000). All reagents were diluted in phosphate-buffered saline (PBS) with 0.05% Tween-20 (Polysorbatum 20. Apoteksbolaget, Stockholm, Sweden). The microtiter plates (Costar Corporation, Cambridge, MA, USA) were coated with monoclonal antibodies (0.5 mg/mL, Nordic Immunology, Tilburg, The Netherlands) diluted 1/200 in PBS for IgA1 and 1/100 for IgA2. All incubations were carried out at room temperature and washes were done five times with 0.15 M NaCl containing 0.05% Tween-20.

Saliva samples and IgA-subclass standards were incubated overnight and all samples and reagents were diluted in PBS with 0.05% Tween-20. Serum from an individual with homozygous IGHG gene deletions for GP-G2-G4-E-A2/GP-G2-G4-E-A2 was used as IgA1 standard, and serum from an individual with homozygous IGHG gene deletions for A1-GP-G2-G4-E/A1-GP-G2-G4-E was used as IgA2 standard (5, 6). Alkaline-phosphatase-conjugated (ALP) mouse anti-human IgA (Jackson ImmunoResearch Laboratories Inc., Baltimore, PA, USA) diluted 1/1000 was used as the second step before adding *p*-nitrophenyl phosphate (1 mg/mL) as substrate (Product No. 104, Sigma Chemical Co., St Louis, MO, USA) diluted in 10% diethanolamine buffer. The plates were read in a multi-channel spectrophotometer (BIO-TEK Instruments Inc., Winooski, VT, USA) at 405 nm.

Serum IgA subclass quantification

Serum IgA subclass levels were measured using a nephalometer (Beckman Instruments, Brea, CA, USA) or commercially available immunodiffusion plates (Nor-Partigen, Behringwerke AG, Münich, Germany).

Specific IgA subclass antibodies against tetanus toxoid

Tetanus toxoid, used at a concentration of 5 Lf/mL, and NP_2BSA [(4-hydroxy-3-nitrophenacetyl)$_2$-bovine serum albumin] used at a concentration of 20 μg/mL, were diluted in 0.1 M carbonate-bicarbonate buffer and coated on microtiter plates. Specific IgA subclass antibodies were analyzed by ELISA and as standards supernatants from mouse hybridomas producing chimeric IgA1 antibodies and IgA2 antibodies directed against NP were used (7, 8).

Antigen-coated microtiter plates were rinsed five times, after which samples or hybridoma antibodies were added. After incubation and rinsing, the plates were incubated with mouse anti-human IgA-subclass monoclonal antibodies (Nordic Laboratories, Tilburg, The Netherlands, anti-IgA diluted 1/5000 and anti-IgA2 diluted 1/2000) followed by rinsings and incubations, first with Fc-specific rabbit anti-mouse Ig (Jackson ImmunoResearch, Avondale, PA, USA, diluted 1/1000) and secondly with ALP-conjugated sheep anti-rabbit IgG F(ab′)2-fragments (Sigma Chemical Co., St Louis, MO, USA, diluted 1/1000). The plates were washed and incubated with the substrate disodium *p*-nitrophenyl phosphate (Sigma Chemical Co.),

Table 2. Tetanus toxoid-specific IgA1 and IgA2 subclass antibodies in parotid saliva (μg/mL)

	Parotid saliva							
	Baseline		1 month		6 months		12 months	
	IgA1	IgA2	IgA1	IgA2	IgA1	IgA2	IgA1	IgA2
Mean	0.29	0.05	0.18	0.06	0.18	0.06	0.17	0.06
Range	0.12–0.43	0.03–0.29	0.05–0.47	0.02–0.09	<0.02–0.42	<0.02–0.22	0.05–0.36	<0.02–0.11
n	14		14		14		13	

Table 3. Tetanus toxoid-specific IgA1 and IgA2 subclass antibodies in whole saliva (μg/mL)

	Whole saliva									
	Baseline		0.5 month		1 month		6 months		12 months	
	IgA1	IgA2	IgA1	IgA2	IgA1	IgA2	IgA1	IgA2	IgA1	IgA2
Mean	0.21	0.12	0.27	0.13	0.26	0.12	0.23	0.11	0.24	0.11
Range	<0.02–0.46	<0.02–0.27	0.07–0.57	0.04–0.44	0.04–0.53	0.03–0.42	0.04–0.45	0.02–0.34	<0.02–0.75	<0.02–0.44
n	14		13		13		14		13	

diluted to 1 mg/mL in 10% diethanolamine buffer. The plates were read in a multichannel spectrophotometer (BIO-TEK Instruments Inc., Winooski, VT, USA).

Statistical analysis

Student's paired *t* test was used to estimate the significance of differences.

Results

Specific IgA subclass antibodies against tetanus toxoid

Booster immunization with tetanus toxoid resulted in raised mean values of specific IgA1 and IgA2 antibodies against tetanus toxoid in serum after 1 month. Compared with the baseline, the mean value of specific IgA1 antibodies in serum showed a 2.6-fold increase ($P = 0.003$), mean value 10.47 μg/mL, and the mean value of specific IgA2 antibodies showed a 2.7-fold increase ($P < 0.001$), mean value 0.93 μg/mL (Table 1).

The mean levels of specific IgA1 and IgA2 antibodies against tetanus toxoid in serum after 6 months, compared with the baseline, showed a 1.9-fold increase of specific IgA1 antibodies ($P = 0.044$) and a 1.6-fold increase of specific IgA2 antibodies ($P = 0.102$) (Table 1). After 12 months, the mean values of specific IgA1 and IgA2 antibodies against tetanus toxoid in serum, compared with the baseline, showed a 1.5-fold increase of specific IgA1 antibodies ($P = 0.361$) and the mean level of specific IgA2 after 12 months was at the same level as at the baseline (Table 1).

Specific IgA subclass antibody levels in parotid saliva and whole saliva remained at the same level as at baseline level during the period of 12 months (Tables 2, 3).

Total levels of IgA1 and IgA2 and flow rate in parotid and whole saliva

The mean values of IgA1 in serum were at the baseline, 1, 6, and 12 months, between 1.8 g/L and 1.9 g/L (Table 4). The mean values of IgA2 in serum were at the baseline, 1, 6, and 12 months, between 0.33 g/L and 0.41 g/L (Table 4).

The flow rate in parotid saliva varied between 0.5 and 2.6 mL/min and the mean values of IgA1 in parotid saliva were at the baseline, 1, 6, and 12 months, between 10.3 mg/L and 14.4 mg/L (Table 5). The mean values of IgA2 in parotid saliva were at the baseline, 1, 6, and 12 months, between 10.4 mg/L and 18.7 mg/L (Table 5). The flow rate in whole saliva varied between 0.2 and 1.9 mL/min and the mean values of IgA1 in whole saliva were at the baseline, 14 days, and 1, 6, and 12 months, between 69.8 mg/L and 100.3 mg/L (Table 6). The mean values of IgA2 in whole saliva were at the baseline, 14 days, and 1, 6, and 12 months, between 86.7 mg/L and 102.7 mg/L (Table 6).

Ratios of specific IgA1 antibodies to total IgA1 and specific IgA2 antibodies to total IgA2

The ratios of specific IgA1 antibodies to total IgA1, and specific IgA2 antibodies to total IgA2 in serum were, at the baseline, 1, 6, and 12 months for IgA1; 2.3×10^{-3}, 5.8×10^{-3}, 4.4×10^{-3} and 3.2×10^{-3}, respectively, and for IgA2; 0.9×10^{-3}, 2.3×10^{-3}, 1.4×10^{-3} and 1.0×10^{-3}, respectively (Table 7).

Table 4. IgA1 and IgA2 subclass antibodies in serum (g/L)

	Serum							
	Baseline		1 month		6 months		12 months	
	IgA1	IgA2	IgA1	IgA2	IgA1	IgA2	IgA1	IgA2
Mean	1.8	0.40	1.8	0.41	1.8	0.38	1.9	0.33
Range	0.5–3.8	<0.01–0.68	0.6–3.4	<0.01–0.72	0.6–3.9	<0.01–0.6	0.8–3.4	<0.01–0.7
n	14		14		14		12	

Table 5. IgA1 and IgA2 subclass antibodies in parotid saliva (mg/L)

	Parotid saliva							
	Baseline		1 month		6 months		12 months	
	IgA1	IgA2	IgA1	IgA2	IgA1	IgA2	IgA1	IgA2
Mean	10.3	10.4	13.0	12.4	13.9	18.7	14.4	13.7
Range	2.4–24.1	1.8–29.6	5.3–23.8	5.2–26.7	4.3–20.1	7.1–41.2	4.6–22.1	3.3–26.2
n	14		14		14		13	

The ratios for IgA1 in parotid saliva were, at the baseline, 1, 6, and 12 months, 2.8×10^{-5}, 1.4×10^{-5}, 1.3×10^{-5} and 1.2×10^{-5}, respectively, and for IgA2, 0.5×10^{-5}, 0.5×10^{-5}, 0.3×10^{-5} and 0.4×10^{-5}, respectively (Table 7).

The ratios for IgA1 in whole saliva were, at the baseline, 0.5, 1, 6, and 12 months, 2.5×10^{-6}, 3.9×10^{-6}, 2.6×10^{-6}, 2.9×10^{-6} and 3.1×10^{-6}, respectively, and for IgA2, 1.2×10^{-6}, 1.5×10^{-6}, 1.4×10^{-6}, 1.2×10^{-6} and 1.2×10^{-6}, respectively (Table 7).

Discussion

Tetanus toxoid booster immunization induced by the intramuscular or subcutaneous route induces systemic immune responses primarily of IgG with the IgG-subclass distribution dominated by the IgG1 subclass, followed by IgG4 (3, 9). Intraperitoneal injection with tetanus toxoid increased the specific IgG antibodies in serum, saliva and peritoneal fluid (2). The increased salivary IgG anti-tetanus toxoid antibodies are probably due to passive transmucosal transfer of serum-derived IgG (10, 11).

Systemic IgA response to tetanus toxoid occurs in both the IgA1 and the IgA2 subclass (1). The secretory IgA in saliva originates from precursor cells from the lympho-epithelial regions in the respiratory tract and some from the gastro-intestinal tract (12). The B-cell response is demanded of T-helper cells and cytokines for the differentiation into IgA-secreting plasma cells (12, 13).

In the present study, subcutaneous booster immunization with tetanus toxoid resulted in a nearly 3-fold increase of IgA1 and IgA2 antibodies to tetanus toxoid in serum after 1 month, whereas the levels of antigen-specific IgA1 and IgA2 in parotid and whole saliva were unchanged. The increase in specific IgA subclass antibodies in serum showed the same increase for both IgA1 and IgA2 one month after the baseline. This same increase in the ratio of specific antibodies to total immunoglobulins in the two IgA subclasses suggests that the T-helper and cytokine-dependent regulations of IgA plasma cells may have the same or similar profile of mediators concerning upregulating of systemic IgA subclass antibodies to tetanus toxoid. Isotype-switching to IgA of antigen-stimulated B cells is reported to be influenced by cytokines such as interleukin-2 (IL-2), IL-5, IL-6, IL-10 and transforming growth factor-β (TGF-β) (14, 15). For the regulation of IgA responses, IL-2 is from the profile of T-helper subset Th1, IL-5, IL-6 and IL-10 from Th2 cells and TGF-β is mainly produced by Th3 cells (14, 16).

The levels of salivary IgA in unstimulated whole saliva and stimulated parotid saliva differed in concentration by an approximately 6-fold increase in unstimulated whole saliva. However, the distribution of IgA subclasses is the same in both parotid and whole saliva. In contradistinction to the findings in serum, no increase in tetanus toxoid-specific IgA subclass levels was found in whole or parotid saliva after booster immunization. The concentrations of specific IgA subclasses were found to be in the same range for both whole and parotid saliva. Subsequently, the ratios of tetanus toxoid-specific IgA subclass antibodies to total IgA subclass antibodies were higher in parotid saliva as compared with whole saliva. These findings show that the proportion of tetanus toxoid-specific IgA subclass antibodies compared with total IgA subclass antibodies is higher in parotid saliva than in whole saliva. The exposure of multiple cells and micro-organisms with their proteolytic enzymes in the oral cavity had no detectable influence

Table 6. IgA1 and IgA2 subclass antibodies in whole saliva (mg/L)

	Whole saliva									
	Baseline		0.5 month		1 month		6 months		12 months	
	IgA1	IgA2	IgA1	IgA2	IgA1	IgA2	IgA1	IgA2	IgA1	IgA2
Mean	85.7	102.7	69.8	87.2	100.3	86.7	78.0	92.4	78.6	91.7
Range	36.4–197.6	10.7–310.5	33.5–173.2	25.8–286.3	27.9–127.7	34.9–284.2	30.2–185.1	7.8–327.9	24.6–189.2	8.0–286.1
n	14		13		13		14		13	

Table 7. Ratios of specific IgA1 antibodies to total IgA1 and specific IgA2 antibodies to total IgA2 in serum, parotid and whole saliva

	Baseline	0.5 month	1 month	6 months	12 months
Serum $\times 10^{-3}$					
IgA1	2.3	n.t.*	5.8	4.4	3.2
IgA2	0.9	n.t.*	2.3	1.4	1.0
Parotid saliva $\times 10^{-5}$					
IgA1	2.8	n.t.*	1.4	1.3	1.2
IgA2	0.5	n.t.*	0.5	0.3	0.4
Whole saliva $\times 10^{-6}$					
IgA1	2.5	3.9	2.6	2.9	3.1
IgA2	1.2	1.5	1.4	1.2	1.2

* n.t. = not tested.

on the tetanus toxoid-specific IgA subclass levels in whole saliva, when comparing specific IgA subclass levels in parotid and whole saliva (17). Presence of polyreactive secretory IgA antibodies in salivary fluids may influence on the ratio of specific antibodies to total subclass antibodies (18, 19).

The unchanged levels of tetanus toxoid-specific IgA1 and IgA2 antibodies in saliva after a subcutaneous booster immunization reflects that a secretory immune system is dependent on B cells that may migrate from GALT or MALT or a regional antigen stimulation (20).

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