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Salivary IgG subclasses in individuals with and without homozygous IGHG gene deletions

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SUMMARY

In this study, the levels of salivary IgG1, IgG2, IgG3 and IgG4 from individuals with and without homozygous immunoglobulin heavy chain constant gene deletions were quantified by enzyme-linked immunosorbent assay (ELISA). To analyse the restriction of salivary IgG subclasses, we used unstimulated whole saliva and sera collected at the same time from individuals with homozygous gene deletions, two with G1 deletion, one with G4 deletion, six with both G2 and G4 deletions and from eight individuals without IGHG gene deletions and expressing all four IgG subclasses. The median values of salivary IgG from individuals with homozygous G1, or G4, or both G2 and G4 deletions, and from individuals expressing all four subclasses were 24·2 mg/l and 23·4 mg/l, respectively. The median values of serum IgG were 13·7 g/l and 15·9 g/l, respectively. Our results show that the salivary and serum IgG levels were both within the normal range in individuals with homozygous gene deletions of either G1, or G4, or both G2 and G4.

INTRODUCTION

Salivary IgG is divided into four subclasses and may be of both local and systemic origin. In serum, the proportions of IgG subclasses have been reported to be 60%, 31%, 6% and 3%, for IgG1, IgG2, IgG3 and IgG4, respectively.[1] In saliva, the IgG level is estimated to be approximately 20–30 mg/l as compared with the IgA concentration in saliva which is around 100–200 mg/l.[2,3]

The constant region of the heavy chains is encoded by one of the immunoglobulin CH localized on chromosome 14. The constant region of the γ1, γ2, γ3, γ4, α1 and α2 chains are encoded by the IGHG1, G2, G3, G4, A1 and A2 genes, respectively. The order of the human immunoglobulin CH genes is the following, 5′-M-D-G3-G1-EP1-A1-GP-G2-G4-E-A2–3.[4–7] Six different, extensive multigene deletions have been described in the immunoglobulin CH locus. The multigene deletions have been designated I to VI, in the chronological order of their being found: deletion I (del G1-EP1-A1-GP-G2-G4), deletion II (del EP1-A1-GP), deletion III (del A1-GP-G2-G4-E), deletion IV (del EP1-A1-GP-G2-G4), deletion V (del GP-G2-G4-E-A2) and deletion VI (del G1-EP1-A1-GP-G2).[5,6,8,10–13]

In an earlier study, the levels of IgA subclasses were investigated in saliva and serum in individuals with homozygous heavy chain constant A1 and A2 gene deletions.[3] The salivary IgA concentrations from two individuals homozygous for the deletion III, therefore without IgA1 and from one individual homozygous for the deletion V, and thus without IgA2, were at the same level as for the controls.[3]

In this study, the salivary IgG subclass levels from nine individuals homozygous for multigene deletions (five for deletion III, one for deletion V) or single gene deletions (two for G1 deletion, one for G4 deletion) were investigated and compared to those of eight individuals expressing all four subclasses. To study the salivary IgG subclass distribution, an IgG subclass enzyme-linked immunosorbent assay (ELISA) was developed. The precision of the subclass quantifications was evaluated against the assay to quantify the IgG class levels. The concentrations of serum IgG class and subclasses, from samples taken at the same time as the saliva samples, were also quantified.

MATERIALS AND METHODS

Saliva and serum

Unstimulated whole saliva and sera were collected in parallel and frozen from nine individuals with homozygous IGHG gene deletions, two with G1 deletion, one with IgG4 deletion and six

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Abbreviations: ELISA; enzyme-linked immunosorbent assay; Ig CH, immunoglobulin heavy chain constant genes.

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with G2 and G4 deletions (Table 1), and from eight donors expressing all four subclasses in serum. The unstimulated, whole-saliva samples were centrifuged for 15 min at 10,000 g at 4° to remove cells and debris, and the supernatant was examined. The samples were frozen until analysed.

Salivary IgG subclass quantification

To analyse the IgG subclass levels, we developed an ELISA method. Salivary IgG subclass levels were measured in twofold-dilution steps. All reagents were diluted in phosphate-buffered saline (PBS) with 0·05% Tween-20 (Polysorbatum 20, Apoteksbolaget, Stockholm, Sweden).

The microtitre plates (Costar, Costarcorporation, Cambridge, MA) were coated with monoclonal antibodies (mAb) (0·5 mg/ml, Caltag Laboratories Inc., South San Francisco, CA) diluted 1:200 in PBS. The mAb were anti-IgG1 HP 6069, anti-IgG2 HP 6014, anti-IgG3 HP 6047 and anti-IgG4 HP 6023. All incubations were made at room temperature and washes were done five times with 0·15 M NaCl and 0·05% Tween-20.

Saliva samples and IgG subclass standard (USNRP 67/97) were incubated overnight and all samples and reagents were diluted in PBS with 0·05% Tween-20. Alkaline phosphatase-conjugated, mouse anti-human IgG (Jackson Immuno-Research Laboratories Inc., Baltimore PA), 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), diluted in 10% diethanolamine buffer. The plates were read in a multichannel spectrophotometer (BIO-TEK Instruments, Inc., Winooski, VT) at 405 nm.

Salivary IgG class quantification

IgG class levels were also measured by an ELISA technique, using the USNRP 67/97 as standard for IgG.

Polystyrene microtitre plates (Costar, Costarcorporation) were coated overnight at room temperature with anti-γ immunoglobulins (Dakopatts A/S, Copenhagen, Denmark) in 0·1 M carbonate–bicarbonate buffer (pH 9·6). Saliva samples were tested in fivefold-dilution steps in single wells. All samples were diluted in PBS containing 0·05% Tween-20, incubations were made at room temperature and all washes were done five times with PBS containing 0·05% Tween-20. Diluted saliva samples were incubated overnight and the next morning, after rinsing, alkaline phosphatase-conjugated, immunoglobulin fractions of mono-specific, rabbit antisera to human immunoglobulin (γ-specific Dakopatts A/S), dilution 1/1000, were added. For the final incubation, disodium *p*-nitrophenyl phosphate, 1 mg/ml, (Sigma Chemical Co.) in 10% diethanolamine buffer was added. The plates were read in a multichannel spectrophotometer (BIO-TEK Instruments, Inc.) at 405 nm.

Serum IgG subclass and class quantifications

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

Statistical analysis

Calculations of the interassay variation for levels between the sum of salivary IgG subclasses and the salivary IgG class levels from 17 individuals were performed. The relative error between the methods for the sum of quantified IgG subclasses and the method of quantified IgG class was 17%.

The precision, *s*, i.e. the relative error between the class and the subclasses methods, was estimated according to the formula

$$s = \sqrt{\frac{1}{2n}\sum\left(\frac{d_i}{\bar{x}_i}\right)^2}$$

where d_i = the difference between the sum of quantified IgG subclasses and the quantified level of IgG for each sample and n = the number of samples.

RESULTS

Quantification of salivary IgG subclass and class levels

Salivary IgG subclass and class levels from eight individuals expressing all four subclasses are shown in Table 2. The median values of salivary IgG subclasses were IgG1 11·2 mg/l, IgG2 8·5 mg/l, IgG3 < 2 mg/l and IgG4 4·9 mg/l, whereas the median value of total salivary IgG class levels was 23·4 mg/l (Table 2).

Quantification of salivary IgG subclass and class levels was performed in individuals with homozygous IGHG gene deletions, two individuals homozygous for G1 deletion, one individual homozygous for G4 deletion, and six individuals homozygous for both G2 and G4 deletion, as shown in Table 1. Five of these six individuals are homozygous for the multigene deletion III, and one is homozygous for deletion V. These nine individuals are lacking the corresponding IgG1, IgG4, or IgG2

Table 1. Individuals (A-I) with homozygous IGHG gene deletions

		Homozygous IGHG gene deletions	Deletion	References
A	Xap	G1/G1		L.O., V.W., G.L. and M.-P.L. (unpublished)*
B	Kp 219	G1/G1		L.O., V.W., G.L. and M.-P.L. (unpublished)
C	CRU	GP-G2-G4-E-A2/GP-G2-G4-E-A2	V/V	Bottaro *et al.* 1989[10]
D	SAF	A1-GP-G2-G4-E/A1-GP-G2-G4-E	III/III	Migone *et al.* 1984[9]
E	DEM	A1-GP-G2-G4-E/A1-GP-G2-G4-E	III/III	Bottaro *et al.* 1989[10]
F	R.B.	A1-GP-G2-G4-E/A1-GP-G2-G4-E	III/III	Plebani *et al.* 1993[12]
G	D.B.	A1-GP-G2-G4-E/A1-GP-G2-G4-E	III/III	Plebani *et al.* 1993[12]
H	T 17	A1-GP-G2-G4-E/A1-GP-G2-G4-E	III/III	Wiebe *et al.* 1994[13]
I	Kp 652	G4/G4		L.O., V.W., G.L. and M.-P.L. (unpublished)

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For review on serological markers in individuals with homozygous immunoglobulin CH multigene deletions, see Wiebe *et al.* 1994.[13]

Table 2. Salivary IgG subclass and class levels (given in mg/l) in nine individuals (A-I) with homozygous IGHG gene deletions

	Homozygous IGHG gene deletions		IgG1	IgG2	IgG3	IgG4	ΣIgG1–4	IgG
A	G1		—	17·1	2·4	5·3	24·8	17·5
B	G1		—	21·3	2·9	6·2	30·4	34·0
C	G2	G4	6·9	—	<2	—	6·9	8·6
D	G2	G4	28·3	—	<2	—	28·3	31·8
E	G2	G4	25·8	—	<2	—	25·8	26·0
F	G2	G4	17·0	—	<2	—	17·0	22·1
G	G2	G4	13·7	—	<2	—	13·7	16·1
H	G2	G4	17·5	—	2·2	—	19·7	30·9
I		G4	8·4	9·5	<2	—	17·9	24·2

Salivary IgG subclass and class levels (given in mg/l) in eight individuals expressing IgG1, IgG2, IgG3 and IgG4

	IgG1	IgG2	IgG3	IgG4	ΣIgG1–4	IgG
Median	11·2	8·5	<2	4·9	24·6	23·4
Range	5·2–51·1	2·9–17·1	<2–4·1	2·5–12·3	10·8–83·2	8·2–87·3

Limits for detection 2 mg/l.

and IgG4 subclasses in both their serum and saliva, as a consequence of the IGHG gene deletions. Individuals with homozygous G1 or G4 or both G2 and G4 gene deletions had a median salivary IgG value, 24·2 mg/l (range 8·6–31·8 mg/l), at the same level as for the individuals expressing the four subclasses, median value 23·4 mg/l (range 8·2–87·3 mg/l).

Quantification of serum IgG subclass and class levels

The levels of serum IgG subclasses and class in individuals expressing all four subclasses and in individuals with immunoglobulin CH deletions are shown in Table 3. Individual B showed a normal serum IgG level (14·4 g/l), compensated by an increased IgG2 level (13·0 g/l), whereas individual A showed a decreased serum IgG level (7·5 g/l) with a normal serum IgG2 level (5·2 g/l) (Table 3). Individuals with homozygous G2 and G4 gene deletions showed a median level of serum IgG, 13·7 g/l (range 11·3–16·9 g/l) comparable with the median level of IgG for those expressing all four subclasses, 15·9 g/l (range 11·3–21·0 g/l) (Table 3).

Table 3. Serum IgG subclass and class levels (given in g/l) in nine individuals individuals (A-I) with homozygous IGHG gene deletions and median and range in eight individuals expressing IgG1, IgG2, IgG3 and IgG4

	Homozygous IGHG gene deletions		IgG1	IgG2	IgG3	IgG4	ΣIgG1–4	IgG
A	G1		—	5·2	1·7	0·17	7·07	7·5
B	G1		—	13·0	3·0	0·77	16·77	14·4
C	G2	G4	10·6	—	1·4	—	12·00	12·5
D	G2	G4	15·8	—	2·4	—	18·20	16·9
E	G2	G4	8·8	—	0·8	—	9·60	11·3
F	G2	G4	13·0	—	1·9	—	14·90	13·7
G	G2	G4	13·6	—	1·0	—	14·60	13·7
H	G2	G4	10·6	—	1·7	—	12·30	14·4
I		G4	8·5	6·1	3·0	—	17·60	19·2

Serum IgG subclass and class levels (given in g/l) in eight individuals expressing IgG1, IgG2, IgG3 and IgG4

	IgG1	IgG2	IgG3	IgG4	ΣIgG1–4	IgG
Median	8·5	5·7	1·4	0·74	15·2	15·9
Range	5·8–13·0	2·3–5·9	0·8–2·6	0·12–1·54	9·12–22·77	11·3–21·0

Limits for detection: IgG1 < 0·1 g/l, IgG2 < 0·07 g/l and IgG4 < 0·03 g/l.

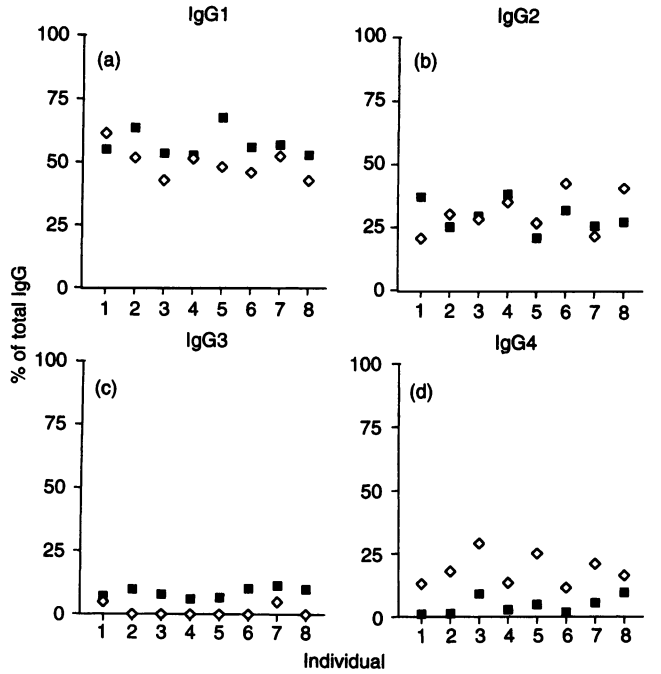

Figure 1. Percentage of IgG1 (a), IgG2 (b), IgG3 (c) and IgG4 (d) from eight individuals expressing all IgG subclasses; saliva (open diamonds) and serum (closed squares).

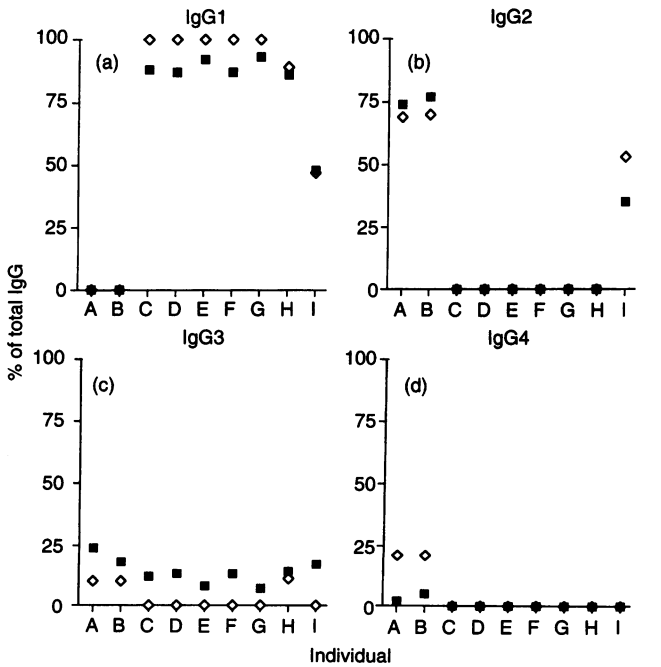

Figure 2. Percentage of IgG1 (a), IgG2 (b), IgG3 (c) and IgG4 (d) from nine individuals with homozygous IGHG gene deletions. Individuals A and B, G1 deletion; individuals C–H, G2 and G4 deletions and individual I, G4 deletion; saliva (open diamonds) and serum (closed squares).

Comparing salivary and serum IgG subclass and class levels

The percentage of salivary IgG subclasses of total IgG and the percentage of serum IgG subclasses of total IgG were compared in the individuals expressing all four subclasses (Fig. 1) and in the individuals with G1, or G4, or G2 and G4 gene deletions (Fig. 2). In individuals expressing the four subclasses, there was a decreased percentage of salivary IgG3 as compared with the serum IgG3 (Fig. 1c). In contrast, there was an increased percentage of salivary IgG4 , as compared with the serum IgG4 (Fig. 1d). Indeed, the geometric-mean percentage of salivary IgG3 was below 2%, whereas it was 8·5% for the serum IgG3. The geometric-mean percentage of salivary IgG4 (18·4%) was increased by a factor of 4, when compared to that of the serum IgG4 (4·6%).

In individuals with homozygous deletion, increased percentage of salivary IgG4, as compared with percentage of serum IgG4, were also noted in the two individuals with homozygous G1 gene deletion (Fig. 2d).

Individuals A and B displayed the highest salivary IgG2 levels (Table 2), 17·1 and 21·3 mg/l, respectively, compared with a median value of 8·5 with a range of 2·9–17·1 in individuals expressing all four subclasses.

DISCUSSION

In saliva, the IgA subclasses show a nearly equal distribution between the two subclasses, around 60% of IgA1 and around 40% of IgA2, compared with serum, in which around 90% of IgA is of subclass IgA1. An earlier study has shown that individuals with homozygous A1 or A2 gene deletions show salivary IgA levels at the same level as for individuals expressing both subclasses.[3]

We report the first analysis of salivary IgG subclasses of nine individuals with homozygous IGHG gene deletions. These individuals with different gene deletion haplotypes consist of, two individuals homozygous for G1 deletion, one individual homozygous for G4 deletion. Six individuals were homozygous for both G2 and G4 deletion, five of them being homozygous for the multigene deletion III and one individual being homozygous for the multigene deletion V. Our analysis was performed using an ELISA technique. The reactivity of the subclass ELISAs was evaluated against an ELISA measuring total IgG.

The distribution of IgG subclasses in saliva and serum were compared in individuals expressing all four subclasses. The pattern showed a lower proportion of salivary IgG3 with a correspondingly higher proportion of salivary IgG4 in the individuals expressing all four subclasses.

The IGHG4 gene is located downstream of the immunoglobulin CH locus as shown by the order of the genes on the chromosome 5′-M-D-G3-G1-EP1-A1-GP-G2-G4-E-A2-3′.[4–7] IgG4 has earlier been reported to be elevated in chronic antigenic stimulation.[14–16] The proportionally elevated, salivary IgG4 may be due to a favoured switch mechanism implicated by a prolonged antigenic stimulation from the huge number of micro-organisms in the oral cavity, around 300 species.[17] This may increase the expression of salivary IgG4, as compared with IgG3. However, the decreased IgG3 levels may also be due to the short half-life of IgG3, around 1 week, as compared with around 3 weeks for IgG1, IgG2 and IgG4.[18] Other possible mechanisms are differences in aggregations between the subclasses, and

differences in susceptibility to enzymes, as for IgA1, which may be cleaved by a variety of enzymes from oral micro-organisms.[19]

In an earlier study of IgG subclass distribution in secretions, the authors reported a higher proportional contribution of IgG2 and IgG4 in secretions, as compared with the distribution in serum.[20] In that study, they used commercial, radial immunodiffusion for the detection of IgG1, IgG2 and IgG3, and a solid-phase radioimmunoassay for the detection of IgG4. Myeloma proteins were used for the analyses of specificity, which may give erroneous results due to impurities in the myeloma protein preparations.[21] In our study, we used an ELISA for salivary quantifications and nephalometer or immunodiffusion plates for serum quantifications. Our results did not confirm the IgG subclass profile with the elevated IgG2 observed by Kim *et al.* in saliva,[20] from individuals expressing all four subclasses. The discrepancy may be explained, by the fact, first we used, for the assay accuracy, saliva from individuals lacking IgG1, IgG4 or IgG2 and IgG4 subclasses due to homozygous IGHG gene deletions, and second that, we compared for the quantifications, the sum of IgG subclasses with the quantified total salivary IgG. The proportion of salivary IgG subclasses in our study is, however, in line with the percentage of IgG subclasses in gingival crevicular fluid,[22] indicating that much of the salivary IgGs originate from oral tissues.

In conclusion, this study shows a dichotomy between the salivary and serum IgG subclass profiles, both in individuals with homozygous IGHG gene deletions and those without homozygous gene deletions. It is suggested that the increased proportion of salivary IgG4 is a result of chronic antigenic stimulation in the oral cavity.

In individuals with G1, or G4, or both G2 and G4 gene deletions, no decrease in salivary IgG was found as compared with individuals expressing all four subclasses. The data showed that the salivary IgG levels in these individuals with homozygous G1, or G4, or both G2 and G4 gene deletions remain at the normal level. The normal levels of salivary IgG in these individuals may be due to the ability of the switch mechanism to compensate for gene deletions, where the individuals with homozygous G1 deletion showed increased IgG2 and the individuals with homozygous G2 deletion showed increased IgG1.

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