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ORIGINAL ARTICLE

Assessing the levels of immunoglobulins in the saliva of diabetic individuals with periodontitis using checkerboard immunodetection

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BACKGROUND: Current methods for determining salivary antibodies are cumbersome for large-scale screenings.

OBJECTIVES: To test checkerboard immunodetection for monitoring salivary antibodies and to profile them in diabetic individuals with periodontitis.

METHODS: Salivary anti-*Porphyromonas gingivalis*, anti-*Actinobacillus actinomycetemcomitans* and total IgA levels of 10 individuals were compared using checkerboard immunoblotting and enzyme-linked immunosorbent assay (ELISA).

RESULTS: Close correlation between both methods was found in anti-*P. gingivalis* IgA and total IgA, but not in anti-*A. actinomycetemcomitans* IgA, because of high background levels in ELISA. Thereafter, checkerboard immunodetection was used to compare salivary antibodies of 20 adult type II diabetic with 32 non-diabetic individuals with ($n = 22$) or without ($n = 10$) periodontitis. Patients with periodontitis (regardless of their diabetic condition) expressed increased levels of total IgA in both whole and parotid saliva, but reduced levels of anti-*A. actinomycetemcomitans* IgA in whole saliva. Consequently, the proportion of anti-*A. actinomycetemcomitans* IgA in the total IgA was lower in saliva of patients with periodontitis compared with healthy controls.

CONCLUSIONS: Checkerboard immunodetection was reliable and economical for screening saliva samples for multiple antibody reactions. Our results support previous reports which suggested that patients with periodontitis are able to secrete high levels of salivary Ig, but are hampered in targeting their salivary response toward *A. actinomycetemcomitans*.

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Introduction

Scientific interest in saliva as a diagnostic medium has increased over the last decade. Its advantages over serum – noninvasive, simplicity of collection – are obvious (Slavkin, 1998). Therefore, it is not surprising that saliva is currently used for the diagnosis of various conditions and diseases (endocrinology, infections, oncology, etc.) and for monitoring their treatment (Bigler *et al*, 2002; Streckfus and Bigler, 2002; Sayer *et al*, 2004). Quantification of salivary antibody concentrations has been shown to be useful in screening populations for the existence of a disease or for their potential vulnerability to the disease (Lawrence, 2002; Herath, 2003).

Enzyme-linked immunosorbent assay (ELISA) is the most commonly used method to estimate antibody levels in saliva, due to its high specificity and sensitivity. Recently, the use of Western immunoblotting for detection of anti-Toxoplasma salivary antibodies has also been described (Stroehle *et al*, 2005). However, both these methods are cumbersome for screening multiple antibodies in vast populations.

The checkerboard approach enables rapid detection of numerous antigen- antibody (Kazemi and Finkelstein, 1990), or hybridization (Socransky *et al*, 1994) reactions on a single membrane. Checkerboard hybridization was applied for the detection of multiple bacterial species in a large number of dental plaque samples (Socransky *et al*, 1994). Checkerboard immunodetection was used for estimating antibody levels to oral pathogens in serum (Sakellari *et al*, 1997) and in gingival crevicular fluid (Dibart *et al*, 1998). Surprisingly, to the best of our knowledge, checkerboard immunodetection has not been previously used to estimate antibody levels in saliva.

Diabetes is an example of a systemic disease with marked oral manifestations (Sandberg *et al*, 2000). The relationship between diabetes and periodontal disease has been widely reported (Lamster and Lalla, 2001; Soskolne and Klinger, 2001). However, the role of the humoral immunity in the susceptibility to periodontitis among diabetics has been scarcely investigated. Several studies found high levels of IgA in serum of individuals with type 2 diabetes (T2D), regardless of periodontal disease (Gill *et al*, 1981; Ardawi *et al*, 1994). Others found significantly higher levels of IgA and IgG in saliva of patients with T2D and periodontitis compared with non-diabetic patients with periodontitis, or healthy controls (Anil *et al*, 1995).

In the present study we tested checkerboard immunoblotting for screening salivary antibodies, and found it to be reliable and economical. The method was then applied to compare the levels of total IgA and the specific antibodies to periodontopathogens in saliva of individuals with T2D and periodontitis, individuals with periodontitis alone, and healthy controls.

Subjects and methods

Clinical examination

Twenty-five T2D volunteers (out of 40 T2D who attended Hadassah hospital tertiary clinic), and 32 non-diabetic individuals participated in this study. All received complete periodontal examination. This examination included measurement of pocket depth (PD), clinical attachment loss (CAL) and bleeding on probing (BOP) at six sites per tooth and plaque index at four sites per tooth (excluding third molars). Subjects who had less than 11 teeth at time of examination were excluded from this study. We used more than six sites of CAL and/or PD ≥5 mm as the criterion for periodontal disease.

According to the examination, three experimental groups were formed (Table 1). The groups consisted of 20 T2D patients with periodontitis ('diabetics', mean age 63.8 ± 10.9, seven women and 13 men), 22 non-diabetic patients with periodontitis ('periodontitis-control', mean age 49.2 ± 11.3 years, 10 women and 12 men) and 10 non-diabetic individuals without periodontitis ('healthy control', mean age 48.3 ± 10.3 years, six women and four men).

All participants provided informed consent to a protocol that was approved by the institutional ethics committee.

Whole saliva samples

Unstimulated whole saliva samples were collected between 9.00 AM and 14.00 PM with the aid of test tubes used in the collection of sputum for microbial culture (Unomedical, Roskilde, Denmark). The total amount collected over a 5-min period was registered, and the salivary flow rates (ml min^{-1}) were calculated. The saliva samples were clarified by centrifugation at 12 000 *g* for 10 min at room temperature and supernatants were kept at −20°C until assayed.

Parotid saliva samples

Parotid saliva samples were collected using a parotid salivary gland cup. Salivary secretion was stimulated using 100 μl of 2% citric acid applied to the tongue every 15 s over a period of 10 min and the salivary flow rates (ml min^{-1}) were recorded. The parotid saliva samples were then treated and stored as described above.

Bacterial strains and growth conditions

Porphyromonas gingivalis ATCC 33277 was cultured in Wilkins-Chalgren Anaerobe Broth (Oxoid, Basingstoke, Hampshire, UK). *Actinobacillus actinomycetemcomitans* ATCC 29523 was grown in TSBV medium (Slots, 1982). Both strains were grown in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI, USA) under an atmosphere of 85% N_2 10% H_2 5% CO_2 at 37°C. Bacterial purity was determined by microscopy and gram staining.

Bacterial antigen preparation

Late logarithmic bacterial cultures (300 ml) were harvested by centrifugation for 10 min at 3500 *g*. Bacterial

Table 1 Clinical parameters in the three experimental groups (mean ± s.d.)

	Diabetics	*Periodontitis controls*	*Healthy controls*
n	20	22	10
% male	65	54.5	40
Age (years)	63.8 ± 10.87	49.2 ± 11.31	48.3 ± 10.3
Whole saliva flow rate (ml min^{-1})	0.52 ± 0.37	0.41 ± 0.30	0.74 ± 0.42[d]
Parotid flow rate (ml min^{-1})	0.23 ± 0.16	0.28 ± 0.18	0.51 ± 0.37[e]
No. teeth	21.6 ± 5.17	23.7 ± 4.35	27.6 ± 0.78[b]
PD (mm)	2.68 ± 0.66	3.47 ± 0.96[a]	1.79 ± 0.18[b]
No. sites PD ≥5 mm	13.45 ± 17.3	32.15 ± 25.8[a]	1.14 ± 1.46[b]
CAL (mm)	3.66 ± 1.05	3.98 ± 1.47	1.92 ± 0.25[b]
No. sites CAL ≥5 mm	36.15 ± 24.02	40.11 ± 28.12	2.28 ± 2.92[b]
% Plaque Index	68.32 ± 26.9	65.2 ± 30.08	35.17 ± 16.6[c]
% BOP	49.7 ± 33.14	37.97 ± 23.86	11.6 ± 6.16[b]

PD, pocket depth; CAL, clinical attachment loss; BOP, bleeding on probing.
[a]Difference between the diabetic and periodontitis groups ($P < 0.01$).
[b]Difference between the healthy and diabetic/periodontitis groups ($P < 0.01$).
[c]Difference between the healthy and diabetic/periodontitis groups ($P < 0.05$).
[d]Difference between the healthy and periodontitis groups ($P < 0.05$).
[e]Difference between the healthy and diabetic group ($P < 0.01$).

pellets were re-suspended in 1.2 ml 50 mM carbonate-bicarbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.02% NaN_3, pH 9.6) supplemented with antiprotease cocktail (Sigma-Aldrich, Rehovot, Israel).

Cell suspensions were transferred into 2 ml microfuge tubes, in which one-third of the volume contains glass beads (106 μ; Sigma-Aldrich). Cells were disrupted using the Fast Prep cell disruptor (Bio 101; Savant Instruments, Inc., Holbrook, NY, USA) at a speed of 6 m s^{-1} for 30 s and then incubated on ice for 1 min. This procedure was repeated five times. Suspensions were centrifuged for 10 min at 12 000 *g* and supernatants were collected. Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad Laboratories GmbH, Munich, Germany).

Quantification of salivary antibodies using ELISA

Levels of salivary anti-*P. gingivalis* IgA, anti-*A. actinomycetemcomitans* IgA and total IgA were determined by ELISA. Briefly, polystyrene plates (96-well, Nunc, Roskilde, Denmark) were coated with *P. gingivalis* (0.05 μg/well for whole saliva and 0.1 μg/well for parotid saliva), *A. actinomycetemcomitans* (1 μg/well for whole saliva and 2 μg/well for parotid saliva), or rabbit anti-human Ig antibodies (Sigma-Aldrich) diluted 1:800 in carbonate-bicarbonate buffer, for 1 h of at 37°C.

The plates were washed six times with PBST (phosphate-buffered saline, 0.05% Tween 20) and blocked with 1% PBST-BSA (PBST, 1% bovine serum albumin) at 4°C overnight. Whole saliva dilutions (1:800 for total IgA, 1:8 for specific IgA) and parotid saliva dilutions (1:160 for total IgA, 1:4 for specific IgA) were placed in duplicate wells and incubated for 2 h at 37°C. The plates were washed six times, and incubated for 1 h with 1:500 diluted alkaline phosphatase-conjugated rabbit anti-human IgA specific for α chains (Sigma-Aldrich). The plates were washed again (six times) and 100 μl p-nitrophenylphosphate (Sigma-Aldrich) dissolved in diethanolamine (pH 9.8; 1 mg ml^{-1}) was added. Color development was stopped after 15 min at 37°C by adding sodium hydroxide 3 M and optical density at 405 nm was determined by an automated ELISA reader (Dynatech, Chantilly, VA, USA). The mean value of the duplicates and the background of each saliva sample (without antigen) were determined.

Ten saliva samples, with high and low levels of antibodies, were selected after preliminary tests and used for comparison with checkerbord immunodetection. Each experiment was repeated three times with consistent results. Data (mean of duplicate) from a typical experiment are presented.

Quantification of salivary immunoglobulins using checkerboard immunoblotting

The levels of total and specific salivary Ig were assessed as previously described for serum (Sakellari *et al*, 1997; Papapanou *et al*, 2000). Briefly, 1.5 ml of bacterial antigens or rabbit anti-human Ig antibodies (Sigma-Aldrich) were loaded into a minislot^{TM} device (Immunetics, Cambridge, MA, USA) and absorbed to a nitrocellulose membrane under vacuum condition. The optimal antigen concentrations used were determined in several calibration experiments with decreasing antigen dilutions (Figure 1), as 1 μg ml^{-1} for *P. gingivalis,* 20 μg ml^{-1} for *A. actinomycetemcomitans* and 1:400 for PBS-diluted rabbit anti-human IgA antibodies, for both whole and parotid saliva samples. After 1 h of blocking in 5% skimmed milk in PBST, the membrane was transferred to a miniblotter^{TM} device (Immunetics) and 135 μl of saliva samples was loaded perpendicular

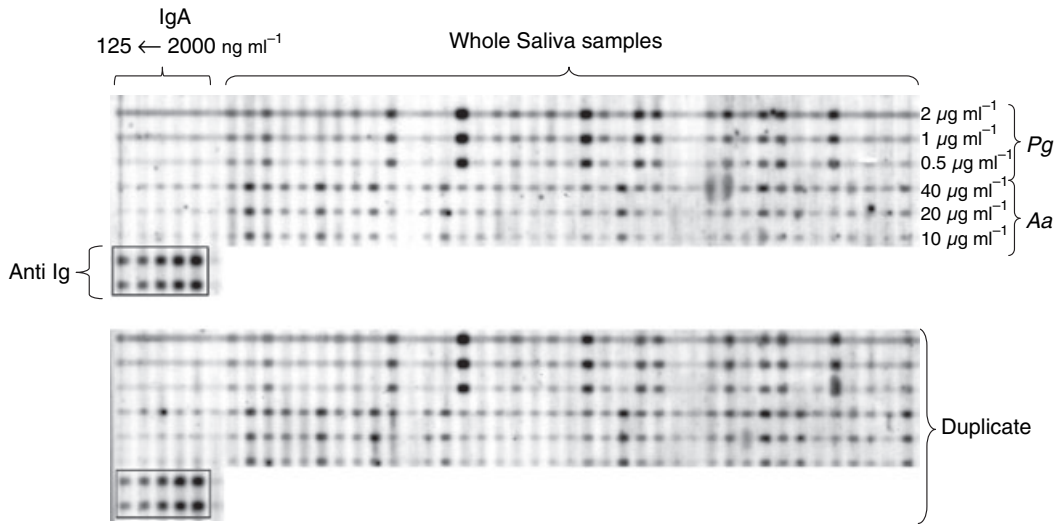

Figure 1 Calibration of salivary IgA checkerboard immunoblotting to determine optimal antigen concentration for each bacteria. The vertical lanes are the saliva samples. The horizontal lanes were loaded with anti human Ig or decreasing concentrations of bacterial extracts from *Porphyromonas gingivalis* (*Pg*) and *Actinobacillus actinomycetemcomitans* (*Aa*). A signal represents the presence of IgA antibodies. The intensity of the signal is compared with a standard curve, generated by the five most left vertical lanes loaded with decreasing concentrations of IgA (2000–125 ng ml^{-1}), shown in the rectangles, which were reacted with the two horizontal lanes containing anti human Ig. Duplicate immunoblots are presented

to the bacterial extracts/anti-Ig, and were allowed to interact in room temperature for 1 h. Whole saliva samples were diluted 1:4 for the detection of antigen-specific antibodies and 1:1000 for total IgA. Parotid saliva samples were diluted 1:2 for the specific antibodies and 1:250 for total IgA. After washing in 500 ml PBST, the membrane was incubated for 1 h with alkaline phosphatase-conjugated rabbit anti-human IgA (diluted 1:500). Membranes were then incubated with chemiluminescence substrate (CDP-Star; Roche Diagnostics, Indianapolis, IN, USA) and exposed to a radiographic film (Super RX; Fuji, Tokyo, Japan) overnight. The signal was assessed by Tina 2.07d software (Raytest, Staubenhardt, Germany).

When checkerboard immunoblotting was used for comparison of immunoglobulin levels in saliva of the three experimental groups, a standard curve was generated. Five human IgA (Sigma-Aldrich) dilutions (2000, 1000, 500, 250, and 125 ng ml^{-1} IgA) were loaded in the last five lanes of the miniblotter (Papapanou *et al*, 2004), and the specific or total IgA concentrations were calculated in micrograms per milliliter according to the standard curve, for each subject.

The secretion rates of the immunoglobulins were calculated by multiplying each subject's IgA concentration with his whole/parotid saliva secretion rate.

Statistical analysis

The correlation between the ELISA and checkerboard immunoblotting methods was evaluated using Spearman's rank correlation. The differences between the three groups were analyzed using nonparametric rank tests (Kruskal–Wallis, Mann–Whitney *U*-test, and Wilcoxon *W*-test). All *P*-values given are based on two-tailed tests and $P < 0.05$ was the criterion of significance.

Results and discussion

Estimation of salivary immunoglobulins using checkerboard immunoblotting

Checkerboard immunoblotting has been previously used as a high-throughput technique for rapid screening of multiple sera responses toward a wide range of antigens (Sakellari *et al*, 1997). As can be seen in the densitometric scan presented in Figure 1, IgA reactivity of many saliva samples could be tested to multiple oral bacterial species. The procedure is quantitative, economical in both antigen and antibody needed, rapid, and consistent. Film exposure was adjusted so that 125 ng ml^{-1} IgA standard was clearly visible and a gradation in signal intensity from 2000 to 125 ng ml^{-1} was achieved (Figure 1). The integrated densitometric scan values for the standards were computed. Integrated data values for salivary samples were transformed to $\mu g\ ml^{-1}$ using polynomial interpolation fit to standards on the same membrane (Sakellari *et al*, 1997).

Reliability of salivary checkerboard immunoblotting

The correlation between checkerboard immunodetection and ELISA was tested by comparing both methods in quantifying total IgA, and IgA-specific reactions to *P. gingivalis* and *A. actinomycetemcomitans* in saliva samples collected from 10 individuals. Close correlation between antibody levels detected using checkerboard and ELISA was observed in both whole and parotid saliva samples for anti *P. gingivalis*-specific IgA [Figure 2a (whole saliva) $r = 0.95$, $P < 0.01$ and Figure 2d (parotid saliva) $r = 0.78$, $P < 0.01$] and for total IgA [Figure 2b (whole saliva) $r = 0.84$, $P < 0.01$ and Figure 2e (parotid saliva) $r = 0.76$, $P < 0.01$]. Good correlation was also found for anti *A. actinomycetemcomitans*-specific IgA in the parotid samples (Figure 2f, $r = 0.70$ $P < 0.05$). No correlation could be found between anti-*A. actinomycetemcomitans* IgA responses measured using ELISA with those measured using checkerboard in whole saliva (Figure 2c, $r = 0.11$). This lack of correlation did not result from saturation of the checkerboard signal that was relatively weak for *A. actinomycetemcomitans*. Inconsistency between sera responses to *A. actinomycetemcomitans* measured with ELISA compared to those measured with checkerboard immunoassay has been reported previously (Sakellari *et al*, 1997). Detection of salivary antigen-specific antibodies requires low dilution of the saliva sample (up to 1:8), due to their low concentrations. When using the ELISA method for this purpose, the whole saliva background levels (saliva reaction in the absence of antigen) were high and masked the anti-*A. actinomycetemcomitans*-specific signals, which were relatively low. The anti-*P. gingivalis* IgA signals were significantly higher than those of the anti-*A. actinomycetemcomitans* and the background, allowing easy detection of this antibody with ELISA. In contrast, the background recorded in the checkerboard immunoblotting technique was negligible. Therefore, the anti-*A. actinomycetemcomitans* IgA-specific signals were easily distinguishable, suggesting that membrane-based immunoblotting is the method of choice for measuring anti-*A. actinomycetemcomitans* antibodies.

Alterations in salivary responses of individuals with periodontitis or diabetes compared to those of healthy controls.

Table 1 provides a summary of the clinical characteristics of the three experimental groups. Consistent with previous reports (Shlossman *et al*, 1990), we found that individuals with diabetes had a high prevalence of periodontitis. Only two out of the 25 diabetic individuals examined were found to be periodontitis free (data not shown).

No significant differences were found between the diabetic and the periodontitis control groups in most of the examined periodontal parameters except for the mean PD and number of sites with PD ≥5 mm, which were significantly higher in the periodontitis control group (Table 1, $P < 0.01$).

The diabetics and the periodontitis control groups showed significant differences from the healthy control group in all the examined periodontal parameters (Table 1, $P < 0.01$). The healthy control group displayed higher salivary flow rates (both whole and parotid) than the diabetic and the periodontitis control groups (Table 1).

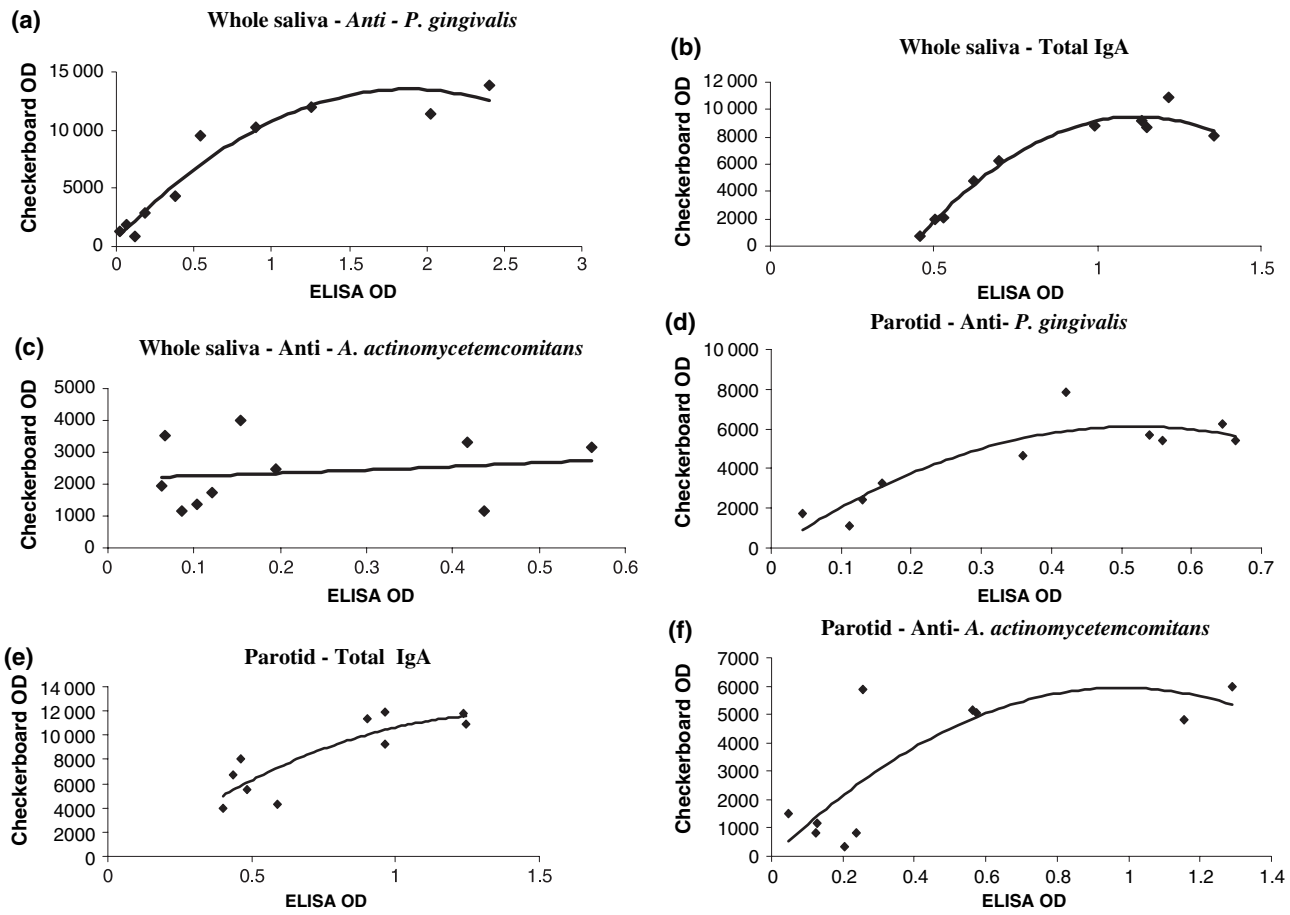

Figure 2 Correlation between antibody titers determined by ELISA and checkerboard immunoblotting. Significant correlations were found for anti-*P. gingivalis* IgA (a) and total IgA (b) in whole saliva, and for anti-*P. gingivalis* IgA (d), total IgA (e) ($P < 0.01$), and anti-*A. actinomycetemcomitans* IgA (f) ($P < 0.05$) in parotid saliva. No correlation was found for anti-*A. actinomycetemcomitans* IgA (c) measured in whole saliva by the two techniques

Whole saliva

While similar IgA responses toward *P. gingivalis* were measured in the three groups tested, healthy individuals demonstrated statistically significant ($P < 0.05$) higher anti- *A. actinomycetemcomitans* IgA antibodies concentrations (median = 2.97 μg ml^{-1} *vs* 1.28 and 1.71 μg ml^{-1}, respectively) (Figure 3a), and antibody secretion rates (median = 1.53 μg min^{-1} *vs* 0.55 and 0.51 μg min^{-1}, respectively) compared with the diabetic and periodontitis controls (Figure 3b). In contrast, the total IgA concentrations (Figure 3c) were significantly lower (median = 63.5 μg ml^{-1}) ($P < 0.05$) in healthy controls than in both groups with periodontitis (either diabetics or healthy periodontitis controls – median = 199 and 249 μg ml^{-1}, respectively). The total IgA secretion rates (Figure 3d) were also lower in the healthy group but the differences did not reach statistical significance. As a result, the proportion of the anti-*A. actinomycetemcomitans* secretion rate from the total IgA secretion rate was significantly higher in the healthy (median = 4.5%) *vs* the diabetic (median = 1.1%), and the periodontitis control group (median = 1.2%) (Figure 3e).

To validate our results with checkerboard immunodetection and to be able to compare them with the literature, the total IgA concentrations were also determined using ELISA. The median total IgA concentrations were 103 μg ml^{-1} in healthy controls, 234 μg ml^{-1} in periodontitis, and 238 μg ml^{-1} in diabetics. Thus, the concentration levels found by the two methods were closely correlated, supporting the results of the checkerboard immunodetection.

Our results are consistent with previous studies, which found higher levels of anti-*A. actinomycetemcomitans* IgA in the gingival crevicular fluid (Plombas *et al*, 2002) and in the saliva (Schenck *et al*, 1993; Hagewald *et al*, 2002; Plombas *et al*, 2002) of healthy individuals compared to those with periodontitis.

The ranges of the total IgA levels in healthy patients are also similar to those reported in the literature (Hagewald *et al*, 2002; Seemann *et al*, 2004). However, a comparison of the total IgA levels found in our group of patients with periodontitis with previous studies is more difficult, because of the conflicting results reported in the literature. Sandholm and Gronblad (1984) also found an elevation in total IgA in their juvenile periodontitis group. The range of the total IgA levels in that study is similar to that in our study. Henskens *et al* (1996) found no differences between their healthy and chronic periodontitis groups; however, the range of their results as

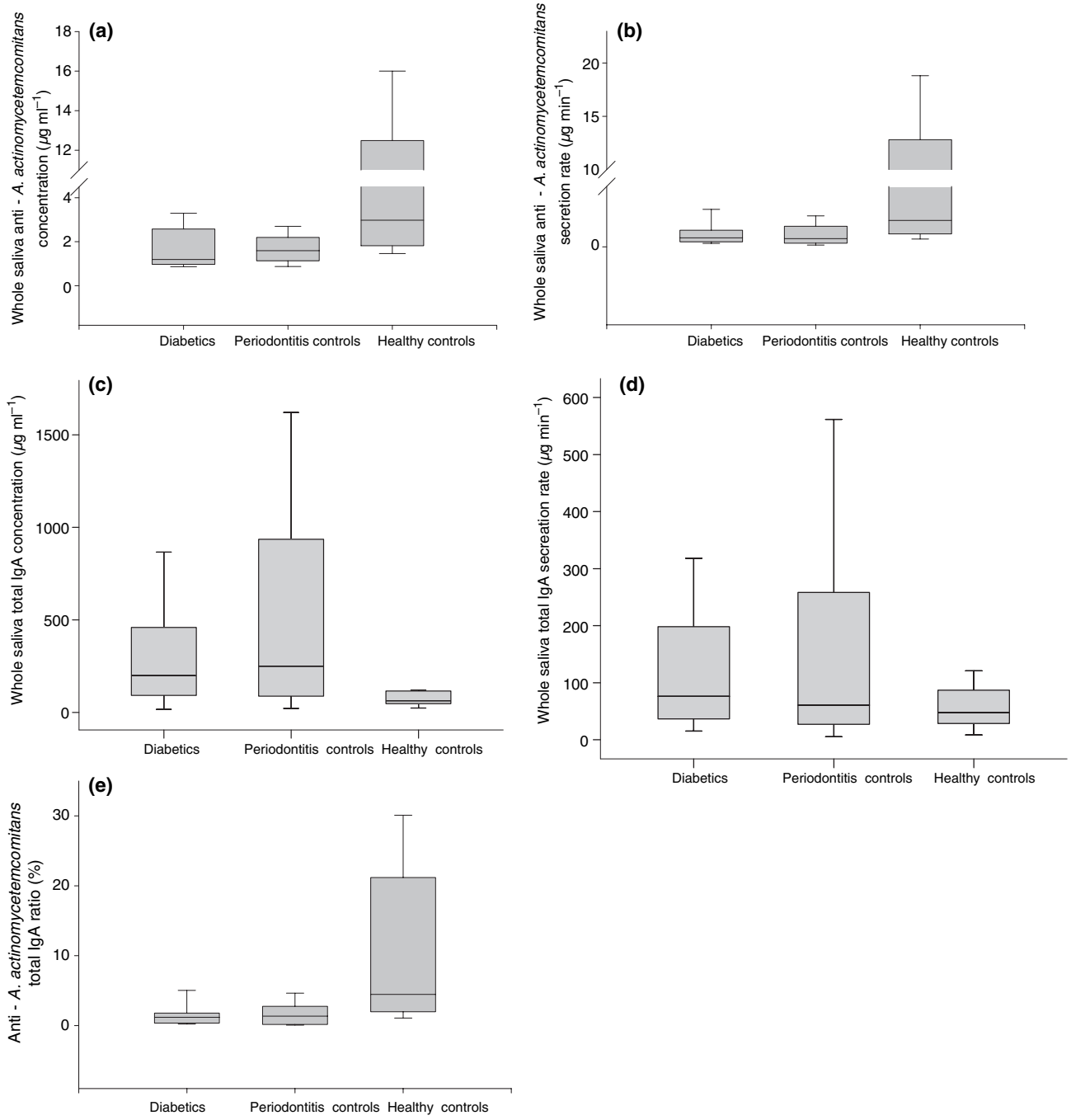

Figure 3 Comparison of specific antibodies and total IgA in whole saliva of diabetics, periodontitis, and healthy controls. Significant higher anti-*A. actinomycetemcomitans* IgA concentration (a) and secretion rate (b) were found in the healthy group along with lower total IgA concentration (c) and secretion rate (d). The ratio of anti-*A. actinomycetemcomitans* IgA to total IgA (e) was lower in diabetics and periodontitis control groups compared with healthy individuals. The boxplots show the median and the 10th, 25th, 75th, and 90th percentiles. The results were obtained using the checkerboard immunodetection method

well as age distribution of their patients was wider. In both the abovementioned studies the results are expressed as means, which might differ from median values in small groups of variables that are not distributed in a normal manner. In a more recent study, Hagewald *et al* (2002) reported a significant decrease in the median total IgA levels in periodontitis-involved patients. This result is in striking contrast to the findings of the present study. The possible explanation for this difference is the different nature and age of the experimental groups – aggressive *vs* chronic periodontitis, and a mean age of 33.3 years *vs* 49.2 years in the previous study *vs* the present study. Several previous studies reported increased age-dependent total salivary IgA levels in parotid and whole saliva (Arranz *et al*, 1992; Challacombe *et al*, 1995; Percival *et al*, 1997; Childers

et al, 2003). Therefore, the markedly increased levels of total IgA in the present study might be a result of a longer exposure to IgA-inducing antigens in older patients with chronic periodontitis *vs* younger patients with aggressive periodontitis.

The present study did not differentiate between secretory and serum-derived IgA. Therefore a possible contribution of serum IgA in the periodontitis and diabetics groups due to increased inflammation-related bleeding cannot be excluded.

However, IgA comprises only 13% of the total serum Ig (Roitt, 1994), therefore bleeding is unlikely to generate the more than twofold differences described above. Furthermore, the similar increases in total IgA levels in the periodontitis and diabetics groups found in pure parotid saliva (see below) support the assumption that the majority of measured IgA originated from local oral synthesis rather than from blood.

The decreased proportion of specific anti-*A. actinomycetemcomitans* IgA to total IgA found in the periodontitis group in the present study supports the results of Plombas *et al* (2002). However, the ratios reported in their study were 2–3 *vs* approximately 4 in the present study. This difference might evolve from several factors. First, in the previous report the results were expressed as mean and not median concentration values. Second, the periodontitis group in Plombas *et al* consisted of a mixed group of both aggressive and chronic periodontitis patients, while our group consisted only of chronic periodontitis patients. The authors also mentioned that chronic periodontitis patients displayed the greatest differences with controls, while intermediate levels were observed in patients with aggressive periodontitis. Therefore, it might be possible that the ratio of specific to total IgA could have been further decreased in the chronic periodontitis group in comparison with healthy patients, if this group had been analyzed separately.

Our results suggest that chronic periodontitis patients show an enhanced nonspecific IgA response whereas their specific IgA response is impaired.

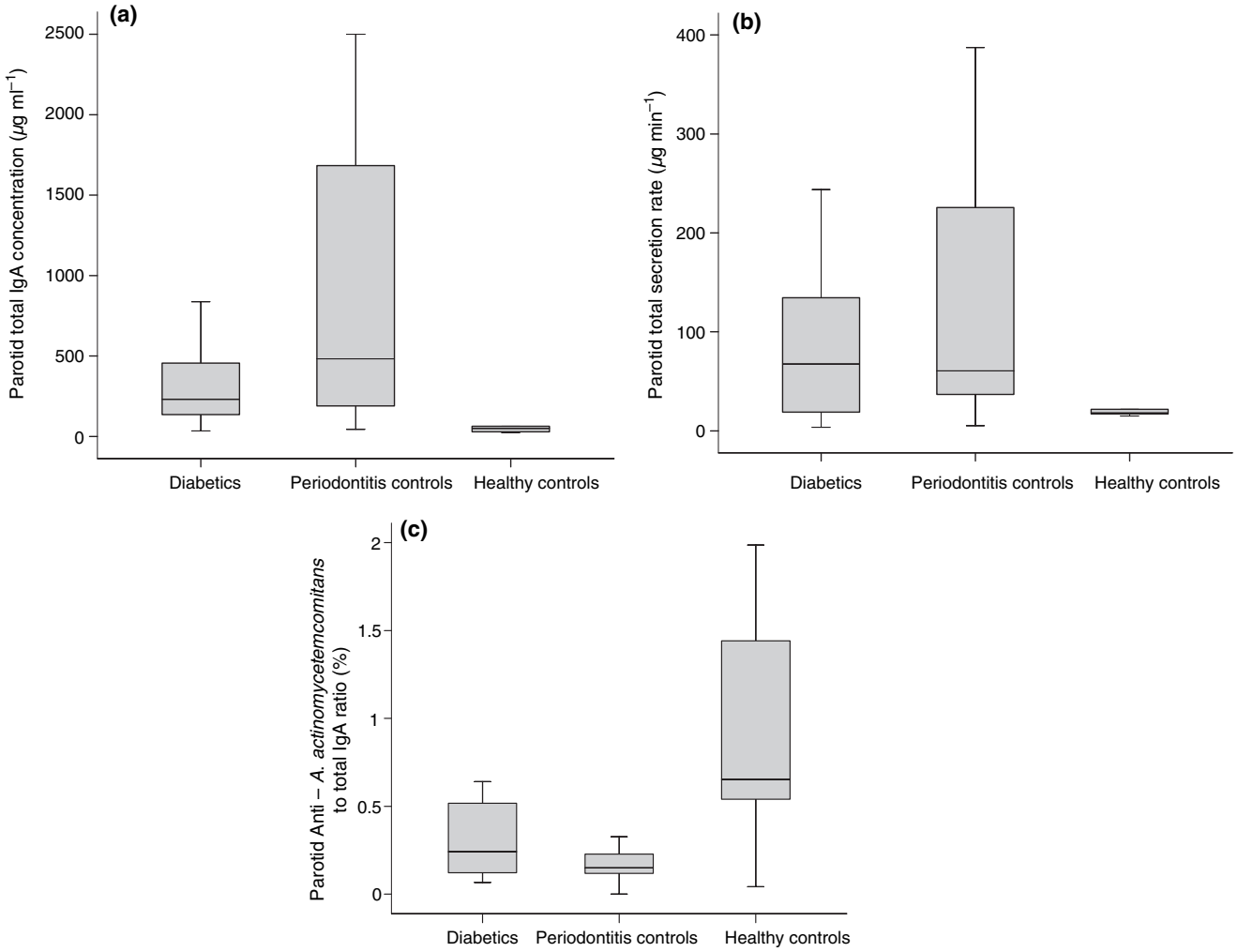

Figure 4 Comparison of total IgA in parotid saliva of diabetics, periodontitis, and healthy controls. Lower total IgA concentration (a) and secretion rate (b) were found in the healthy group. The ratio of anti-*A. actinomycetemcomitans* IgA to total IgA (c) was lower in diabetics and periodontitis control groups compared with healthy individuals (see Results and discussion section). Boxplots show the median and the 10th, 25th, 75th, and 90th percentiles. Results were obtained using the checkerboard immunodetection method

Parotid saliva

No statistically significant ($P > 0.12$) differences in the anti-*A. actinomycetemcomitans* and anti-*P. gingivalis* IgA levels were found between the three groups. Similar to our findings in whole saliva, the total IgA concentration was significantly lower in the healthy group (median = 49.6 μg ml^{-1}, $P < 0.01$, Figure 4a) compared with the diabetics (median = 230.4 μg ml^{-1}) and periodontitis controls (median = 482.2 μg ml^{-1}). The total IgA secretion rate was also lower among healthy individuals (median = 18.2 μg min^{-1}) compared with periodontitis controls (median = 60.6 μg min^{-1}, $P < 0.05$) and diabetics (median = 67.7 μg min^{-1}, not significant) (Figure 4b). Consequently, the ratio of anti-*A. actinomycetemcomitans* IgA from the total parotid IgA was significantly higher in healthy subjects (median = 0.65%) than in periodontitis controls (median = 0.15%,$P < 0.01$) and also higher compared with diabetics, although the difference was not statistically significant (median = 0.24%) (Figure 4c). However, these differences were less dramatic than those in whole saliva, because they derived mainly from differences in the total IgA levels, while in whole saliva, higher levels of anti-*A. actinomycetemcomitans* in the healthy group contributed as well.

In conclusion, checkerboard immunodetection was found to be a high-throughput method for screening salivary immunoglobulins. Our findings of a decreased proportion of anti-*A. actinomycetemcomitans* out of the total IgA in saliva of patients with periodontitis suggest a protective role for anti-*A. actinomycetemcomitans* IgA antibodies in the pathogenesis of periodontal disease. Furthermore, they also imply that periodontitis-prone individuals are capable of secreting high levels of salivary IgA, but their ability to target their salivary response toward periodontopathogens is impaired.

References

Anil S, Remani P, Beena VT, Nair RG, Vijayakumar T (1995). Immunoglobulins in the saliva of diabetic patients with periodontitis. *Ann Dent* **54:** 30–33.

Ardawi MS, Nasrat HA, Bahnassy AA (1994). Serum immunoglobulin concentrations in diabetic patients. *Diabet Med* **11:** 384–387.

Arranz E, O'Mahony S, Barton JR, Ferguson A (1992). Immunosenescence. and mucosal immunity: significant effects of old age on secretory IgA concentrations and intraepithelial lymphocyte counts. *Gut* **33:** 882–886.

Bigler LR, Streckfus CF, Copeland L *et al* (2002). The potential use of saliva to detect recurrence of disease in women with breast carcinoma. *J Oral Pathol Med* **31:** 421–431.

Challacombe SJ, Percival RS, Marsh PD (1995). Age-related changes in immunoglobulin isotypes in whole and parotid saliva and serum in healthy individuals. *Oral Microbiol Immunol* **10:** 202–207.

Childers NK, Greenleaf C, Li F, Dasanayake AP, Powell WD, Michalek SM (2003). Effect of age on immunoglobulin A subclass distribution in human parotid saliva. *Oral Microbiol Immunol* **18:** 298–301.

Dibart S, Eftimiadi C, Socransky S, Taubman MA, Van Dyke TE (1998). Rapid evaluation of serum and gingival crevicular fluid immunoglobulin G subclass antibody levels in patients with early-onset periodontitis using checkerboard immunoblotting. *Oral Microbiol Immunol* **13:** 166–172.

Gill CW, Bush WS, Burleigh WM, Cooke-Gomes D (1981). Elevation of IgA levels in the non-insulin-dependent (type II) diabetic patient. *Diabetes Care* **4:** 636–639.

Hagewald S, Bernimoulin JP, Kottgen E, Kage A (2002). Salivary IgA subclasses and bacteria-reactive IgA in patients with aggressive periodontitis. *Periodontal Res* **37:** 333–339.

Henskens YM, van den Keijbus PA, Veerman EC *et al* (1996). Protein composition of whole and parotid saliva in healthy and periodontitis subjects. Determination of cystatins, albumin, amylase and IgA. *J Periodontal Res* **31:** 57–65.

Herath HM (2003). Early diagnosis of typhoid fever by the detection of salivary IgA. *J Clin Pathol* **56:** 694–698.

Kazemi M, Finkelstein RA (1990). Checkerboard immunoblotting (CBIB): an efficient, rapid, and sensitive method of assaying multiple antigen/antibody cross-reactivities. *J Immunol Methods* **128:** 143–146.

Lamster IB, Lalla E (2001). Periodontal disease and diabetes mellitus: discussion, conclusions, and recommendations. *Ann Periodontol* **6:** 146–149.

Lawrence HP (2002). Salivary markers of systemic disease: noninvasive diagnosis of disease and monitoring of general health. *J Can Dent Assoc* **68:** 170–174.

Papapanou PN, Neiderud AM, Papadimitriou A, Sandros J, Dahlen G (2000). "Checkerboard" assessments of periodontal microbiota and serum antibody responses: a case–control study. *J Periodontol* **71:** 885–897.

Papapanou PN, Neiderud AM, Disick E, Lalla E, Miller GC, Dahlen G (2004). Longitudinal stability of serum immunoglobulin G responses to periodontal bacteria. *J Clin Periodontol* **31:** 985–990.

Percival RS, Marsh PD, Challacombe SJ (1997). Age-related changes in salivary antibodies to commensal oral and gut biota. *Oral Microbiol Immunol* **12:** 57–63.

Plombas M, Gobert B, De March AK *et al* (2002). Isotypic antibody response to plaque anaerobes in periodontal disease. *J Periodontol* **73:** 1507–1511.

Roitt I (1994). Antibodies. In: ed. *Essential Immunology*, 8th edn. Blackwell Scientific Publications: Oxford, pp. 43–63.

Sakellari D, Socransky SS, Dibart S, Eftimiadi C, Taubman MA (1997). Estimation of serum antibody to subgingival species using checkerboard immunoblotting. *Oral Microbiol Immunol* **12:** 303–310.

Sandberg GE, Sundberg HE, Fjellstrom CA, Wikblad KF (2000). Type 2 diabetes and oral health: a comparison between diabetic and non-diabetic subjects. *Diabetes Res Clin Pract* **50:** 27–34.

Sandholm L, Gronblad E (1984). Salivary immunoglobulins in patients with juvenile periodontitis and their healthy siblings. *J Periodontol* **55:** 9–12.

Sayer R, Law E, Connelly PJ, Breen KC (2004). Association of a salivary acetylcholinesterase with Alzheimer's disease and response to cholinesterase inhibitors. *Clin Biochem* **37:** 98–104.

Schenck K, Poppelsdorf D, Denis C, Tollefsen T (1993). Levels of salivary IgA antibodies reactive with bacteria from dental plaque are associated with susceptibility to experimental gingivitis. *J Clin Periodontol* **20:** 411–417.

Seemann R, Hagewald SJ, Sztankay V, Drews J, Bizhang M, Kage A (2004). Levels of parotid and submandibular/sublingual salivary immunoglobulin A in response to experimental gingivitis in humans. *Clin Oral Investig* **8:** 233–237.

Shlossman M, Knowler WC, Pettitt DJ, Genco RJ (1990). Type 2 diabetes mellitus and periodontal disease. *J Am Dent Assoc* **121:** 532–536.

Slavkin HC (1998). Toward molecularly based diagnostics for the oral cavity. *J Am Dent Assoc* **129:** 1138–1143.

Slots J (1982). Selective medium for isolation of Actinobacillus actinomycetemcomitans. *J Clin Microbiol* **15:** 606–609.

Socransky SS, Smith C, Martin L, Paster BJ, Dewhirst FE, Levin AE (1994). "Checkerboard" DNA-DNA hybridization. *Biotechniques* **17:** 788–792.

Soskolne WA, Klinger A (2001). The relationship between periodontal diseases and diabetes: an overview. *Ann Periodontol* **6:** 91–98.

Streckfus CF, Bigler LR (2002). Saliva as a diagnostic fluid. *Oral Dis* **8:** 69–76.

Stroehle A, Schmid K, Heinzer I, Naguleswaran A, Hemphill A (2005). Performance of a Western immunoblot assay to detect specific anti-*Toxoplasma gondii* IgG antibodies in human saliva. *J Parasitol* **91:** 561–563.