Salivary and Serum Anti-Desmoglein 1 and 3 ELISA and Indirect Immunofluorescence in Pemphigus Vulgaris: Correlations with Serum ELISA, Indirect Immunofluorescence and Disease Severity

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ABSTRACT Anti-desmoglein (anti-Dsg) ELISA and indirect immuno-fluorescence (IIF) are used for the diagnosis of pemphigus vulgaris (PV). The value of salivary ELISA, serum ELISA, and IIF in the diagnosis of PV, and the correlation of salivary anti-Dsg1 and anti-Dsg3 ELISA with serum ELISA, serum and salivary IIF titers, and disease severity in patients with PV were evaluated. Fifty newly diagnosed patients with PV were enrolled in the study. Demographic data and disease-severity scores were recorded for each patient. Anti-Dsg1 and anti-Dsg3 ELISA and IIF were performed on both serum and salivary samples. Given the cut-off value of 20 RU/mL for Dsg1 and Dsg3, serum Dsg1 and Dsg3 ELISA were positive in 41 (82%) and 40 (80%) patients, and saliva Dsg1 and the Dsg3 ELISA were positive in 2 (4%) and 3 (6%) patients, respectively. Using the cut-off value of 13.4 RU/mL and 7.7 RU/mL for Dsg3 and Dsg1 salivary ELISA, 25 (50%) and 23 (46%) patients tested positive for Dsg3 and Dsg1, respectively. Serum IIF results were positive in 35 (70%) patients, and salivary IIF results were positive in 16 (32%) patients. Salivary anti-Dsg1 and anti-Dsg3 showed moderate correlations with the total pemphigus disease area index (PDAI) score ($r=0.466$, $P<0.001$), ($r=0.459$, $P<0.001$), respectively. A moderate correlation between serum IIF and salivary IIF was also detected ($r=0.413$, $P<0.001$). Salivary anti-Dsg1 and anti-Dsg3 ELISA could be used as a safe and noninvasive method for the diagnosis of PV under certain circumstances, especially in children or elderly patients. Salivary ELISA is superior to salivary IIF.

KEY WORDS: autoimmune blistering diseases, desmoglein 1 and 3 pemphigus vulgaris

INTRODUCTION

Pemphigus vulgaris (PV) is a potentially fatal autoimmune blistering disease that affects the skin and mucosa. Blister formation is mediated by autoantibodies against keratinocyte desmosomal proteins, which are named desmoglein (Dsg) 1 and Dsg 3 (1-3). The diagnosis of PV is based on the histopathologic evaluation of skin or mucosal biopsies showing characteristic suprabasal clefting and acantholytic cells, which are confirmed by the demonstration of immunoglobulin G (IgG) and complements deposition on the direct immunofluorescence (DIF) staining of the perilesional normal skin. Indirect immunofluorescence (IIF) quantifies the level of the patient's circulating IgG autoantibodies in 80-90 percent of patients with PV (4). The titer of circulating antibodies is based on the correlation of IIF with disease course and severity (2,5). After enzyme-linked immunosorbent assay (ELISA) was introduced in the late 1990s, the usefulness of ELISA for the diagnosis of PV was demonstrated over the past decades as a sensitive and specific quantitative diagnostic method for the detection of anti-Dsg1 and anti-Dsg3 autoantibodies (6-8). Anti-Dsg ELISA is now used for diagnosis, assessment of severity, and disease activity monitoring in PV (7,9,10).

Saliva has been extensively investigated as a potential diagnostic biological tool for the detection of autoimmune diseases, including Sjogren's syndrome, cystic fibrosis, cardiovascular diseases, diabetes mellitus, and HIV (11-15). Saliva-based diagnostic methods are noninvasive, can be easily performed, and may have acceptable diagnostic accuracy. They are efficacious, easy, and cost-effective tests for rapid office-based diagnosis or follow-up of diseases (16,17). ELISA using saliva as the biofluid substrate, instead of blood serum, has been proposed as a noninvasive, rapid, and convenient method for the serological assessment of autoimmune disorders, including PV (7,8,18-21), mucous membrane pemphigoid, and bullous pemphigoid (22-24). In one of our previous studies on the diagnostic accuracy of saliva-based ELISA for the diagnosis of PV, the specificity of salivary anti-Dsg1 and anti-Dsg3 were both 98.9 percent, which was comparable with standard serum-based ELISA (7) and is in accord with other studies (25-27). To the best of our knowledge, the correlation of salivary ELISA with disease severity and serum and salivary IIF as an indirect marker of disease severity in PV has not been assessed in earlier studies (28).

The aim of the present study was to evaluate the correlation of salivary ELISA anti-Dsg1 and anti-Dsg3 with serum ELISA anti-Dsg1 and the anti-Dsg3 levels, IIF titers (serum and salivary), and disease severity based on the pemphigus disease area index (PDAI) in patients with PV.

PATIENTS AND METHODS

This cross-sectional study was performed at Razi Hospital, Tehran University of Medical Sciences, between May 2016 and April 2017. The study protocol was reviewed and received clearance from the Ethics in Medical Research Committee of Tehran University of Medical Sciences. Fifty newly diagnosed patients with PV were enrolled in the study. All patients were recruited after written informed consent was obtained.

The diagnosis of PV was established according to the histopathologic evaluation of prelesional skin biopsy, showing suprabasal acantholysis and/or cleft formation and DIF study, demonstrating the intercellular deposition of IgG and complement 3 (C3). Demographic data, including age and sex, PV phenotype (mucosal, mucocutaneous, and cutaneous), and disease severity were recorded for each patient. Pemphigus severity was scored by one experienced dermatologist using PDAI (28).

Serum and saliva samples were simultaneously collected from each patient between 9 and 11 AM and were stored at −70 °C until the laboratory investigations were performed. For the collection of saliva samples, we followed the method proposed by Hallaji *et al.* (18); this method is as follows:

The patients were instructed to collect unstimulated whole saliva in their oral cavity for five minutes without swallowing and then told to spit it into sterile plastic pots. Then, the supernatants of the salivary samples were separated by centrifugation at 3700 g for 10 minutes and the remaining part was stored. Anti-Dsg1 and anti-Dsg3 ELISA were performed on both the serum and the salivary samples using the EUROIMMUN (Medizinische Labordiagnostika AG, Germany) kit according to previous studies and the manufacturer's instructions for conducting serum ELISA (18,29). For serum anti-Dsg1 and anti-Dsg3 ELISA, we used the standard manufacturer's recommended cut-off value of 20.0 RU/mL. Since the kit had been specifically designed for serum anti-Dsg1 and anti-Dsg3 measurements, and we used it for testing saliva samples, we adopted the standard 20.0 RU/mL cut-off value recommended for serum ELISA and we evaluated the results using the optimized cut-off values based on our previous studies (7.7 RU/mL and 13.4 RU/mL for the salivary anti-Dsg1 and anti-Dsg3 ELISA, respectively). According to these studies, different cut-off values were used to provide more reliable results (7,18).

Table 1. Description of demographic data (including gender phenotype and PDAI score of patients with pemphigus vulgaris (PV))

	N (%)	Mean±Standard Deviation (SD)
Age (years)	50 (100)	43.4±13.1
Sex		
Male (%)	40 (80)	-
Female (%)	10 (20)	-
Disease phenotype		
Mucosal	12 (24)	-
Cutaneous	4 (8)	-
Mucocutaneous	34 (68)	-
PDAI Score		
Total	-	47.1±20.6
Body	-	26.4±15.0
Head and neck	-	1.9±1.7
Mucosal	-	18.9±13.4

For serum ELISA, we used 1/100 diluted serum, and the procedure was performed according to the manufacturer's recommendation (http://www.euro-immun.us/). We diluted serum samples based on the manufacturer's instructions for the IIF method, but we did not dilute the saliva samples. In the undiluted saliva samples, the IIF test was considered negative if antibodies were not detected; if an antibody was detected, the same process for the dilution of salivary samples was repeated. The substrate that was used for the study of serum and salivary samples was human skin. For dilution of salivary and serum samples, phosphate buffered saline (PBS) was used. Salivary and serum samples were diluted according to the manufacturer's instructions (dilution 1/1, 1/10, 1/20, 1/40, 1/80, 1/160, 1/320, 1/640, and 1/1280), and the most diluted samples in which the autoantibody was detected were reported as IIF titer.

Statistical Analysis

Statistical analysis of the data was performed using the IBM SPSS Statistics software (version 21.0; IBM Corp., Armonk, NY, USA) for Microsoft Windows. For a description of the normally distributed quantitative variables, we used mean and standard deviation (SD). The Spearman's (rs) correlation coefficient was used to investigate any correlation between the variables.

RESULTS

Of the 50 studied patients with PV, 40 (80%) were men and 10 (20%) were women. The mean age ± SD of patients was 43.4±13.1 (range: 19-74) years. Mucocutaneous, mucosal, and cutaneous PV phenotypes were observed in 34 (68%), 4 (8%), and 12 (24%) patients, respectively. The mean PDAI score was 47.1±20.6 years (Table 1).

Serum ELISA and IIF

Serum anti-Dsg1 and anti-Dsg3 were positive in 41 (82%) and 40 (80%) patients, with mean values of 166.6±89.0 RU/mL and 158.7±86.9 RU/mL, respectively (Table 2). Serum anti-Dsg1 showed a moderate correlation with serum anti-Dsg3 (r=0.413, P<0.001) (Table 3). In addition, serum anti-Dsg3 showed a moderate correlation with total (r=0.459, P<0.001) and mucosal (r=0.620, P<0.001) PDAI scores. Serum anti-Dsg1 showed moderate correlations with the total (r=0.466, P<0.001), mucosal (r=0.513, P<0.001), and body PDAI scores (r=0.321, P<0.023), respectively (Table 4).

The serum IIF results were positive in 35 (70%) patients. Serum IIF showed moderate correlation with serum anti-Dsg1 (r=0.461, P=0.001), anti-Dsg3 (r=0.347, P=0.014), total (r=0.345, P=0.014) and body (r=0.298, P=0.036) PDAI scores, respectively. With the exception of moderate correlation with the mucosal PDAI score, no correlation was found between the serum IIF results and other PDAI scores (Table 4).

Salivary ELISA and IIF

Using the standard 20 RU/mL cut-off level, salivary ELISA anti-Dsg1 and anti-Dsg3 were reported as positive in only 2 (4%) and 3 (6%) patients, respectively. Using the optimal cut-off value of 13.4 RU/mL and 7.7 RU/mL for salivary anti-Dsg3 and anti-Dsg1 ELISA (7),

Table 2. Result of serum ELISA and saliva ELISA according to different cut-off values and the results of serum and saliva ELISA mean value

Cut-off Test	Cut-off value of 20 RU/Ml for both Dsg 1&3 Positive (%)	Dsg 1 7.7 RU/mL Positive (%)	Dsg 3 13.4 RU/mL Positive (%)	Mean value ± Standard Deviation RU/mL
Serum Dsg 1	41 (82%)	-	-	166.6±89.0
Serum Dsg 3	40 (80%)	-	-	158.7±86.9
Saliva Dsg 1	2 (4%)	23 (46%)	-	37.9±57.6
Saliva Dsg 3	3 (6%)	-	25 (50%)	78.8±90.6

Table 3. Correlation coefficients between different immunological tests

	Serum Dsg 1	Serum Dsg 3	Salivary Dsg 1	Salivary Dsg 3	Serum IIF	Salivary IIF
Serum Dsg 1	1.000	0.798**	0.369**	0.463**	0.595**	0.566**
Serum Dsg 3	0.798**	1.000	0.219	0.564**	0.622**	0.550**
Salivary Dsg 1	0.369**	0.219	1.000	0.413**	0.065	0.435**
Salivary Dsg 3	0.463**	0.564**	0.413**	1.000	0.170	0.356*
Serum IIF	0.595**	0.622**	0.065	0.170	1.000	0.413**
Salivary IIF	0.566**	0.550**	0.435**	0.356*	0.413**	1.000

* P<0.05, **P< 0.01.
0.00<r≤0.29: weak, 0.30≤r≤0.69: moderate, 0.70≤r≤1: strong.

salivary anti-Dsg1 and anti-Dsg3 ELISA were positive in 23 (46%) and 25 (50%) patients (Table 2). The mean salivary anti-Dsg1 and anti-Dsg3 ELISA of the patients were 37.9±57.6 RU/mL and 78.8±90.6 RU/mL, respectively (Table 2).

Both salivary anti-Dsg1 and anti-Dsg3 showed moderate correlation with the total PDAI score (r=0.336, P=0.017 and r=0.510, P<0.001, respectively). Salivary anti-Dsg3 showed moderate correlation with mucosal PDAI score (r=0.513, P<0.001). Salivary anti-Dsg1 showed moderate correlations with the body (r=0.477, P=<0.001), head, and neck (r=0.492, P=< 0.001) PDAI scores (Table 4).

Salivary anti-Dsg1 was significantly correlated with serum anti-Dsg1 (r=0.369, P=0.008), salivary anti-Dsg3 (r=0.564, P<0.001), and serum anti-Dsg3 (r=0.463, P=0.001), respectively (Table 3).

The salivary IIF results were positive in 16 (32%) patients. Salivary IIF was moderately correlated with salivary anti-Dsg1 (r=0.592, P<0.001) and anti-Dsg3 (r=0.617, P<0.001), respectively. In addition, we detected a moderate correlation between serum IIF and salivary IIF (r= .409, P=0.003). With the exception of the mucosal PDAI score, no other significant correlation was found between the PDAI scores and serum IIF; the salivary IIF was moderately correlated to the
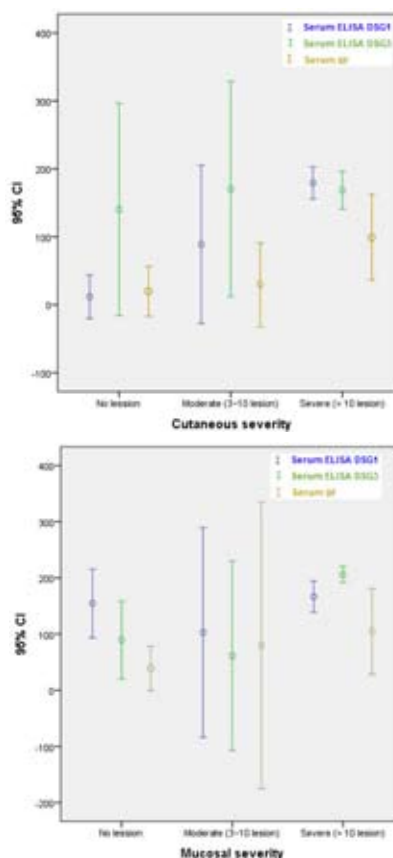

Figure 1. Mean value of serum anti-Dsg3 and anti-Dsg1 levels in the mucosal and cutaneous phenotype.

Table 4. Correlations of serum and salivary anti-Dsg 1 and 3 with PDAI scores

	Serum Dsg 1	Serum Dsg 3	Salivary Dsg 1	Salivary Dsg 3	Serum IIF	Salivary IIF
Phenotype						
Cutaneous	-0.412**	-0.543**	-0.040	-0.489**	-0.285*	-0.265
Mucocutaneous	0.508**	0.549**	0.283*	0.567**	0.195	0.362**
Mucosal	-0.225	-0.090	-0.424**	-0.205	0.114	-0.205
PDAI Score						
Total	0.466**	0.459**	0.336*	0.510**	0.175	0.345*
Body	0.321*	0.229	0.477**	0.244	0.095	0.291*
Head and Neck	0.122	0.027	0.492**	0.239	-0.029	0.074
Mucosal	0.513**	0.620**	0.119	0.585**	0.297*	0.326*

* P<0.05, **P< 0.01.
0.00<r≤0.29: weak, 0.30≤r≤0.69: moderate, 0.70≤r≤1: strong.

Figure 2. Mean value of saliva anti-Dsg3, anti-Dsg1 saliva IFF levels in the mucosal and cutaneous phenotype.
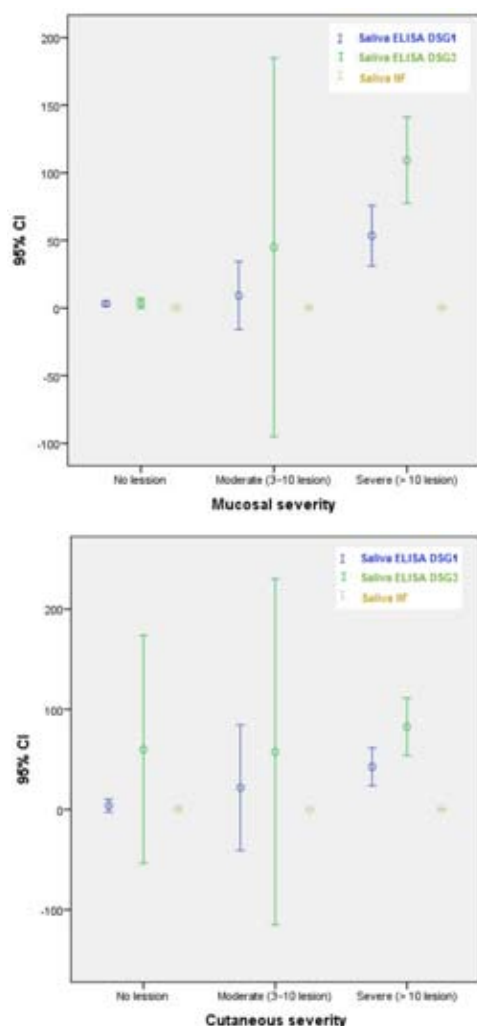

Figure 3. Mean value of saliva anti-Dsg3, anti-Dsg1 and serum IFF levels in the mucosal and cutaneous phenotype.

total and mucosal PDAI scores and weakly correlated with cutaneous PDAI scores (Table 4).

We assessed the differences between the serum anti-Dsg3 and anti-Dsg1 levels in the mucosal and cutaneous phenotype. We concluded that anti-Dsg1 was more accurate than anti-Dsg3 and IIF for monitoring the mucosal phenotype (Figure 1). This trend was observed in salivary ELISA and IIF. In the cutaneous phenotype, we found that the mean level of anti-Dsg1 and 3 and the IIF titer correlated with cutaneous severity, and this relationship was more significant in anti-Dsg1 than anti-Dsg3 and IIF.

These results were compatible with saliva finding (Figure 2). In comparison to serum IIF with salivary-ELISA anti-Dsg1 and 3, the increase in all of these values was concordant with the severity of mucosal and cutaneous lesions (Figure 3).
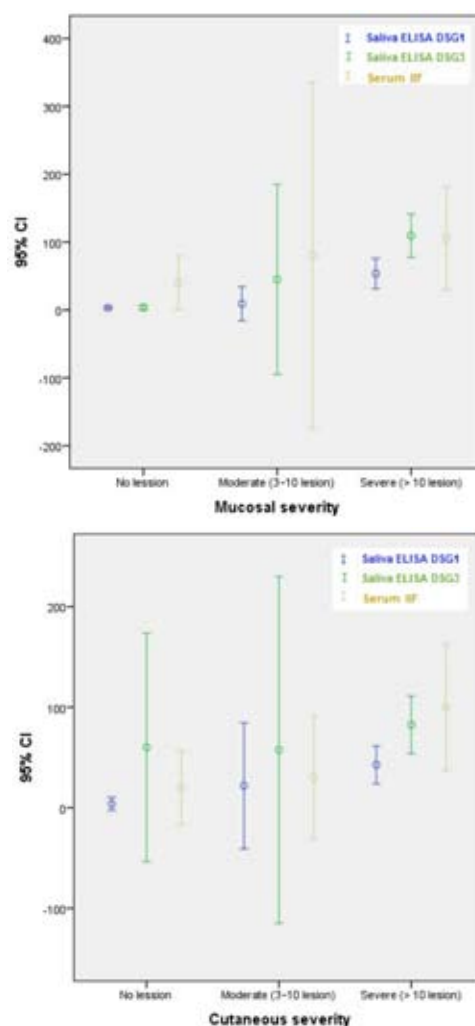
DISCUSSION

Certain studies have shown that both anti-Dsg1 and anti-Dsg3 titers had a significant correlation with IIF and the treatment status (30,31), but these studies were restricted. There is a limited amount of studies on the comparison between salivary IIF with serum IIF and the correlation between salivary IIF and disease severity (32). In addition, there is a controversy on the reliability of saliva in the detection and monitoring of PV. Certain studies indicated that disease severity in most patients with pemphigus correlates to IIF titers (7,19,33,34), but others indicate that salivary IIF and ELISA may not be an ideal substrate for the laboratory diagnosis of PV (32). A literature review is shown in Table 5.

We conducted this study to investigate the correlations of serum and saliva anti-Dsg1 and anti-Dsg3 ELISA with IIF in PV, and their correlations with

Table 5. Review of ELISA and IIF (serum and saliva) in the diagnosis of pemphigus vulgaris (PV)

	No. of. patients with PV	ELISA Serum		ELISA Salivary		IIF	
		DSG1	DSG3	DSG1	DSG3	Serum	Salivary
Zagorodniuk *et al.* 2005 (20)	33	*	*			*	
Daneshpazhooh *et al.* 2007 (27)	73	*	*				
Mortazavi *et al.* 2008 (30)	61					*	
Hallaji *et al.* 2010 (18)	37	*	*	*	*		
Anand *et al.* 2012 (25)	63	*	*				
Avgerinou *et al.* 2013 (31)	46	*	*			*	
Daneshpazhooh *et al.* 2014 (26)	46	*	*			DIF	
Gandhi *et al.* 2014 (35)	10	*	*				
Mortazavi *et al.* 2014 (36)	40	*	*	*	*	DIF	
Barnadas *et al.* 2015 (19)	35	*	*			*	
Mortazavi *et al.* 2015 (7)	86	*	*	*	*		
Weiss *et al.* 2015 (3)	47	*	*			*	
Zhou *et al.* 2016 (21)	33	*	*			* & Tzanck smear test	
Ali *et al.* 2016 (33)	23	* IgG IgA	* IgG IgA	*IgG IgA	*IgG IgA		
Ravi *et al.* 2017 (37)	50	*	*			*	
Russo *et al.* 2017 (32)	8	*	*	*	*	* BIOCHIP	* BIOCHIP
Khullar *et al.* 2017 (38)	43	*	*	*	*		
Živanović *et al.* 2017 (34)	72	*	*			*	
Present study	50	*	*	*	*	*	*

disease severity. Our study showed moderate correlations between several different immunological tests (anti-Dsg1 and anti-Dsg3 and IIF) performed on serum and saliva as well as correlations between some of the immunological tests and PDAI scores (Table 3 and Table 4).

Our findings are compatible with previous studies that have shown anti-Dsg ELISA as a valuable laboratory method for PV diagnosis, which could be used in routine practice (4,7). The assessment of saliva as an alternative biofluid substrate for ELISA is a noninvasive, rapid, and convenient method for the immunological diagnosis of autoimmune disorders, including PV (7,32,36,38).

Serum studies

According to the suggested manufacturer cut-off value of 20 RU/mL, the ELISA sensitivities for detecting serum anti-Dsg1 and anti-Dsg3 autoantibodies were 82 percent and 80 percent, respectively. In the study by Mortazavi *et al.* (7), the sensitivities of anti-Dsg1 and anti-Dsg3 were 72.1 percent and 96.5 percent, respectively. Hallaji *et al.* (18) reported that the sensitivities of serum anti-Dsg1 and anti-Dsg3 were 72 percent and 94 percent, respectively. Harman *et al.* (39) reported that the sensitivity of serum anti-Dsg3 in untreated patients with PV were 100 percent.

Tampoia *et al.* (4) also showed that serum anti-Dsg antibodies were highly sensitive and specific to the diagnosis of PV. Our serum anti-Dsg1 positivity results were similar to those of previous studies (7,18). The sensitivity of serum anti-Dsg3 in our investigation was less than what was reported in other studies (7,18,39); in certain studies, the sensitivity of serum anti-Dsg3 was less than in our study (33).

Saliva studies

Serum components, such as antibodies, could be transferred to saliva through capillary walls in the salivary glands (7). In patients with PV, serum anti-Dsg1 and anti-Dsg3 antibodies can be transmitted paracellularly to the lumen of salivary ducts via injured epithelial mucosa (18).

Using cut-off values of 7.7 RU/mL and 13.4 RU/mL, based on the study of Mortazavi *et al.* (7), the sensitivities of salivary anti-Dsg1 and anti-Dsg3 ELISA in the present study were 34 percent and 48 percent, respectively. Hallaji *et al.* reported that the sensitivities of salivary anti-Dsg1 and anti-Dsg3 were 70 percent and 96 percent, respectively (18). In the study of Mortazavi *et al.* (7), the sensitivities of salivary Dsg1 and Dsg3 were 36.1 percent and 73.3 percent, respectively. Andreadis *et al.* (40) reported sensitivities of 45 percent and 93 percent for salivary Dsg1 and Dsg3, respectively. The

sensitivities of anti-Dsg1 and anti-Dsg3 ELISA in the present study were less than the sensitivities that have been reported previously (18,40). This may be due to certain atypical cases in our study.

However, we found that serum anti-Dsg3 was significantly correlated with salivary anti-Dsg3 antibodies. Therefore, salivary samples, as well as serum samples, can be used for the assessment of anti-Dsg3 antibodies. In our study, salivary anti-Dsg1 was moderately correlated with serum anti-Dsg1.

As we discussed earlier, serum-derived IgG can be transmitted into the saliva. Although a minor fraction, the transferred antibody amount mainly depends on the integrity of the epithelial barrier in patients with PV. With severe mucosal involvement, the high titers of the serum anti-Dsg antibodies can be transmitted paracellularly to the lumen via the injured epithelial mucosa. Therefore, anti-Dsg1 is more likely to be detected in the saliva of patients with concurrent severe cutaneous and mucosal involvement (18).

IIF studies

Our findings showed that serum and salivary IIF could be potentially used in the confirmation of diagnosis as well as monitoring the activity of patients with PV, which is in accord with the work of Arbache *et al.* (41). While the salivary IIF results were positive in 16 (32%) patients, which was much lower than the serum IIF positivity of 35 (70%) patients, the salivary IIF test results were moderately correlated with salivary anti-Dsg1 and anti-Dsg3, respectively. In addition, serum IIF and salivary IIF were moderately correlated with each other. With the exception of mucosal PDAI, no other significant correlation was found between the PDAI scores and serum IIF; the salivary IIF was moderately correlated to the total and the mucosal PDAI scores, and weakly correlated to the cutaneous PDAI scores. The latter suggested a potential role of salivary IIF studies for monitoring PV severity under certain conditions. The literature on using salivary IIF in the diagnosis of or while monitoring autoimmune bullous diseases, including PV, was so scarce that it was not possible to provide an informative discussion on the issue.

Disease severity in most patients with pemphigus correlates with IIF titers, which, in turn, is determined by quantities of anti-Dsg1 and 3; according to previous studies, however, the clinical phenotype and antibody profile are not always in correlation (34). A relatively recent IIF technique named BIOCHIP mosaic-based IIF has been investigated for the screening of autoantibodies and has been found useful in the routine diagnosis of PV (42).

Correlations of Dsg values with PDAI scores

The present study showed that serum anti-Dsg3 was significantly correlated with the total skin and the mucosal PDAI scores. Serum anti-Dsg1 was also significantly correlated with the total skin, body, and mucosal PDAI scores, which were similar to the results of other studies on antibody profile and the clinical subtypes of PV (7,18,27). Salivary anti-Dsg3 was significantly correlated with the total skin and the mucosal PDAI scores. Salivary anti-Dsg1 was significantly correlated with the total skin, body, head, and neck PDAI scores, similar to what was reported in the work of Hallaji *et al.* (18). Correlation between anti-Dsg1 and the mucosal PDAI score may be due to higher titers of serum Dsg1 in patients with pemphigus with severe cutaneous and/or mucosal involvement, which can be transmitted paracellularly to the lumen via the injured epithelial mucosa (25,43).

CONCLUSION

Given the cut-off value of 20 RU/mL for both the Dsg1 and the Dsg3 serum and salivary ELISA, salivary ELISA is not a reliable test for the diagnosis of PV because of low sensitivity. With the cut-off value of 13.4 RU/mL and 7.7 RU/mL for Dsg3 and Dsg1 for salivary ELISA, respectively, salivary Dsg ELISA has a fair sensitivity to the diagnosis of PV in certain circumstances, especially in children and elderly patients. While comparing ELISA with IIF, given the cut-off values of 13.4 RU/mL and 7.7 RU/mL for Dsg3 and Dsg1 saliva ELISA, IIF has lower sensitivity than ELISA for the diagnosis of PV. In addition, serum IIF also has lower sensitivity than serum ELISA for the diagnosis of PV. Considering the IIF results, we suggest that salivary IIF has potential for the diagnosis and the monitoring of PV severity in certain and specific conditions.

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