Original Article

Utility of oral mucosa as a substrate for the serodiagnosis of pemphigus: A descriptive analysis

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Abstract

Background: The indirect immunofluorescence test is useful in the serodiagnosis of pemphigus. As indirect immunofluorescence titers correlate with disease activity in pemphigus, it is often used as a monitoring tool. The sensitivity of indirect immunofluorescence depends on the substrate used, and the preferred substrates are monkey esophagus for pemphigus vulgaris and normal human skin for pemphigus foliaceus.

Aims: We evaluated oral mucosa as a substrate for indirect immunofluorescence in pemphigus.

Methods: Fifty patients with pemphigus (40 with pemphigus vulgaris and ten with pemphigus foliaceus) and 50 controls were enrolled for study. Demographic and clinical details were recorded and indirect immunofluorescence using two substrates (oral mucosa and normal human skin) was carried out in serial dilution. Desmoglein (Dsg) 1 and 3 enzyme-linked immunosorbent assay was also evaluated simultaneously.

Results: Indirect immunofluorescence was positive in 40 patients (80%) with oral mucosa substrate and 34 patients (68%) with normal human skin substrate. Circulating antibodies were detected with oral mucosa in 33 (82.5%) of the 40 pemphigus vulgaris patients and in 26 (65%) patients using normal human skin. Antibodies were detected in eight of the ten pemphigus foliaceus patients (80%) with normal human skin and in seven (70%) patients with oral mucosa. Dsg enzyme-linked immunosorbent assay was positive in 45 (90%) patients, and 37 of these were also indirect immunofluorescence positive with oral mucosa. In the five Dsg enzyme-linked immunosorbent assay-negative patients, indirect immunofluorescence with oral mucosa was positive in three.

Limitations: A comparison of oral mucosa with monkey esophagus could not be performed.

Conclusion: Oral mucosa is a suitable and sensitive substrate for indirect immunofluorescence in pemphigus. Further studies comparing the sensitivity of indirect immunofluorescence using oral mucosa with monkey esophagus are recommended.

Key words: Indirect immunofluorescence, oral mucosa, pemphigus

Plain Language Summary

Pemphigus is a potentially life- threatening skin disease characterized by blisters and erosions in the skin with or without mucosal lesions. Early diagnosis and institution of appropriate therapy can reduce the morbidity and mortality associated with the disease. Immunofluorescence test is the most preferred test for the laboratory diagnosis of pemphigus. There are two types: direct immunofluorescence involves taking skin biopsy and indirect immunofluorescence involves the examination of blood to look for autoantibodies. The sensitivity of the latter depends on the substrate used. Authors in this study have used oral mucosa as a substrate for indirect immunofluorescence and have found that it is a sensitive and cost effective alternative to the existing substrates.

Introduction

Pemphigus is a tissue specific autoimmune blistering disorder affecting the skin and mucosa. Pemphigus vulgaris and pemphigus foliaceus are the two common forms encountered in clinical practice and they differ in their clinical features, location of blisters within the epidermis and target antigens.[1]

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Autoantibodies in pemphigus are directed against desmosomal antigens desmoglein (Dsg) 1 and 3. Detection of serum and skin bound autoantibodies is a prerequisite for the diagnosis of pemphigus.[2] Direct immunofluorescence microscopy of biopsy sections of perilesional skin demonstrating immunoglobulin G and complement fraction 3 (C3) in the intercellular space of the epidermis is considered the gold standard for the diagnosis of pemphigus.[3]

Serological tests such as indirect immunofluorescence (IIF) microscopy and Dsg 1 and 3 enzyme linked immunosorbent assay (ELISA) are useful to detect the circulating antibodies. These tests are particularly useful in the diagnosis of pemphigus in children and uncooperative adults who refuse to undergo biopsy.[4] Dsg enzyme-linked immunosorbent assay is highly sensitive and specific, and since it is a quantitative test, it is often used to monitor the immunological activity of pemphigus.[5-7] The Dsg profile has been shown to correlate with the pemphigus phenotype. Patients with Dsg1 antibodies have predominantly cutaneous disease (pemphigus foliaceus) with relative sparing of mucosal surface while patients with Dsg3 antibodies have mucosal pemphigus. A subset of patients with mucocutaneous disease has antibodies directed against both Dsg1 and 3.[7-9]

The sensitivity of indirect immunofluorescence in detecting circulating antibodies in pemphigus is between 70 and 90%.[10,11] Although it is a semi-quantitative test and the results are observer dependent,[4,12] it is less expensive than Dsg enzyme-linked immunosorbent assay and can be performed in laboratories with facilities for immunofluorescence.[13] Further, while Dsg enzyme-linked immunosorbent assay detects antibodies directed only against a specific epitope, indirect immunofluorescence can detect all cell surface antibodies.[11] Using serial dilutions, indirect immunofluorescence titers reflect disease activity and can be used for monitoring pemphigus.[14-16]

The sensitivity of indirect immunofluorescence is dependent on the substrate used. Normal human skin and monkey esophagus are the preferred substrates for demonstrating pemphigus antibodies.[17-20] Other substrates that have been evaluated for the serodiagnosis of pemphigus include the esophagus and lip of both guinea pigs and rabbits and the esophagus, foreskin, amnion and tonsil of humans.[17,21-24]

In this study, we evaluated the utility of oral mucosa as a substrate for indirect immunofluorescence in pemphigus patients.

Methods

Fifty patients with pemphigus and an equal number of controls (patients with dermatological diseases other than pemphigus) attending the dermatology outpatients' department of Kasturba Hospital, Manipal, were prospectively recruited over 18 months. The Institutional Ethics Committee (number-482/2014) approval was taken, and written informed consent was obtained from all participants before the initiation of the study.

The study group included 40 cases clinically diagnosed with pemphigus vulgaris and ten with pemphigus foliaceus. The diagnosis of pemphigus was confirmed in each case by direct immunofluorescence. Disease activity was assessed using the pemphigus disease activity index scoring system developed by International Pemphigus Committee.[25] Patients were categorized as moderate, significant and extensive in accordance with the grading recommended by Boulard *et al*.[26] However, patients were enrolled irrespective of their disease activity. Demographic and clinical details of patients were recorded in a predesigned pro forma.

Frozen sections (six micrometer) of normal human skin and oral mucosa (obtained from department of oral and faciomaxillary surgery) were taken on special adhesive slides (obtained from Hendley, UK). Serum (two milliliters) was collected from both cases and controls and stored in a deep freezer at –40°C until needed. Direct immunofluorescence was performed on frozen sections of the test substrates (both normal human skin and oral mucosa) to rule out non-specific or false-positive staining.

Serial dilutions (1:10, 1:100, 1:200, 1:400, 1:800 and 1:1600) of the stored sera from patients and controls were incubated with six micrometer frozen sections of the normal human skin and oral mucosa substrates and indirect immunofluorescence was carried out as per standard protocol. A single experienced observer recorded the results. Dsg1 and 3 enzyme-linked immunosorbent assay was also performed simultaneously in all patients. The cutoff index values used were 20 U/ml for Dsg 1 and 30 U/ml for Dsg 3.

Data were entered and analyzed using Statistical Package for the Social Sciences version 15 (Statistical Package for the Social Sciences Inc. released 2006. Statistical Package for the Social Sciences for Windows, Version 15.0., Chicago). Data were expressed as percentage and proportions.

Results

Clinical data

The study group comprised 50 patients (28 males and 22 females; mean age 44 years) including 40 patients with pemphigus vulgaris and ten with pemphigus foliaceus. Of 40 patients with pemphigus vulgaris, 35 had mucocutaneous disease while the remaining five had purely mucosal disease. Active disease was seen in 41 of the 50 patients and nine patients were in clinical remission. Of the nine patients in remission, four had mucocutaneous pemphigus vulgaris, two had mucosal pemphigus vulgaris and three had pemphigus foliaceus.

The control group comprised patients suffering from dermatoses other than pemphigus [Table 1].

Immunofluorescence data

Correlation of substrate positivity with phenotype

Overall, indirect immunofluorescence was positive in 45 of the 50 (90%) pemphigus patients using both substrates. Indirect immunofluorescence was positive in 40 patients (80%) with oral mucosa and in 34 patients (68%) with normal human skin. Indirect immunofluorescence was negative in five of the nine patients (56%) in clinical remission.

In the 35 patients with mucocutaneous pemphigus vulgaris, indirect immunofluorescence was positive with oral mucosa in 29 (82.9%) and 26 (74.3%) with normal human skin [Table 2]. Four of five patients (80%) with pure mucosal pemphigus vulgaris were indirect immunofluorescence positive with oral mucosa but all five patients were negative with normal human skin. Indirect immunofluorescence was positive in seven of the ten pemphigus foliaceus patients (70%) with the oral mucosa and in eight patients (80%) with the normal human skin. One patient with pemphigus foliaceus had positive indirect immunofluorescence with both the substrates. Indirect immunofluorescence was positive with normal human skin alone in one patient each of pemphigus foliaceus and mucocutaneous pemphigus vulgaris [Figure 1].

Oral mucosa was more sensitive than normal human skin in pemphigus vulgaris (82.5% vs. 65%), whereas normal human skin was more sensitive in pemphigus foliaceus (80% vs. 70%).

In the control group, two patients (one each with dermatophytosis and dermatitis) showed low titer (1:10) intercellular space fluorescence with oral mucosa substrate. Thus, the sensitivity of oral mucosa in detecting circulating pemphigus antibodies was 80% and the specificity, 96% [Table 3].

Table 1: Composition of the control group

Diagnosis	No. of cases
Epidermolysis bullosa acquisita	12
Bullous pemphigoid	8
Psoriasis	7
Dermatitis	7
Dermatophytosis	5
Acne	2
Chronic urticaria, SLE, lipodermatosclerosis, telogen effluvium, mucous membrane pemphigoid, lichen planus, pruritus, prurigo nodularis and Hailey-Hailey disease.	1 each

Association between disease phenotype and indirect immunofluorescence titers with oral mucosa and normal human skin

In 32 of the 40 patients with pemphigus vulgaris, the oral mucosa titers were either equal to or greater than normal human skin, and ten of these patients showed intercellular space only with oral mucosa. In pemphigus foliaceus patients, however, higher titers were recorded with normal human skin than with oral mucosa (seven vs. two) [Table 4].

Association between pemphigus disease activity index scoring with indirect immunofluorescence using oral mucosa substrate

Patients were categorized into three groups based on pemphigus disease activity index score [Table 5]. High indirect immunofluorescence titers (>1:200) with oral mucosa were seen in 31 out of 37 patients (83.8%) with significant or extensive disease, but the majority of patients (9/13; 69%) with moderate disease activity had negative or low (1:10) indirect immunofluorescence titers.

Association between indirect immunofluorescence and Dsg titers

Enzyme-linked immunosorbent assay was positive with either Dsg1 or Dsg3 antigen or both in 36 of the 40 patients with pemphigus vulgaris and nine of the ten patients with pemphigus foliaceus [Table 2]. Reactivity to both Dsg1 and 3 was seen in 24 out of 31 (77.4%) patients with active mucocutaneous disease and with Dsg 3 alone in four of five (80%) patients with mucosal pemphigus vulgaris. Six

Table 2: Serodiagnosis using IIF (with two substrates) and ELISA among pemphigus patients (*n*=50)

	Phenotype					
	Mucocutaneous PV (*n*=35)		Mucosal PV (*n*=05)		PF (*n*=10)	
	Active (*n*=31)	Remission (*n*=4)	Active (*n*=3)	Remission (*n*=2)	Active (*n*=7)	Remission (*n*=3)
IIF						
Both OM and NHS+	23	-	-	-	5	1
OM+	6	-	3	1	1	
NHS+	2	1	-	-	1	1
Both negative	-	3	-	1	-	1
ELISA						
Both Dsg1 and 3+	24	-	-	-	1	1
Dsg3+only	2	1	3	1	-	-
Dsg1+only	4	1	-		6	1
Both negative	1	2	-	1	-	1

OM: Oral mucosa, NHS: Normal human skin, PV: Pemphigus vulgaris, PF: Pemphigus foliaceus, IIF: Indirect immuno fluorescence, ELISA: Enzymelinked immunosorbent assay, Dsg: Desmoglein

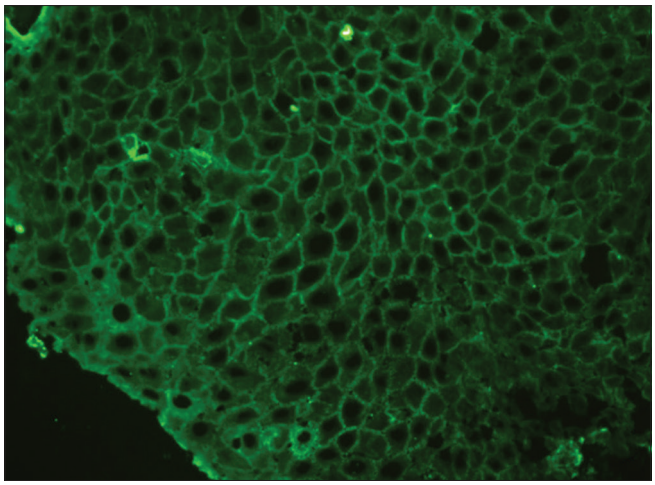

Figure 1a: Intercellular staining with immunoglobulin G in oral mucosa

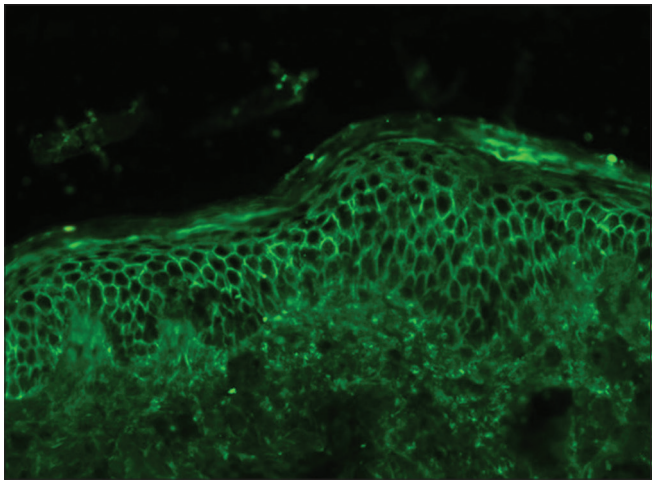

Figure 1b: Normal human skin substrate showing equal staining with both substrates in a patient with mucocutaneous pemphigus vulgaris (FITC, ×200)

patients with mucocutaneous disease had antibodies against Dsg1 (n = 4) alone or Dsg3 (n = 2) alone.

Dsg1 reactivity was seen in seven of the ten pemphigus foliaceus patients (70%). One patient with active pemphigus foliaceus and no history of mucosal lesions showed positivity to both Dsg1 and 3.

Discordance between the Dsg profile and clinical phenotype was seen in seven patients with active disease. These included six patients with mucocutaneous disease who were reactive to either Dsg1 (four patients) or Dsg3 (two patients) alone and one patient with active pemphigus foliaceus who had no history of mucosal lesions and was positive to both Dsg1 and 3.

Circulating autoantibodies were demonstrable in seven of the nine patients in clinical remission. Of these, two patients were positive by both indirect immunofluorescence and enzyme-linked immunosorbent assay, two by enzyme-linked immunosorbent assay alone and three cases only by indirect immunofluorescence.

Table 3: Sensitivity and specificity of OM and NHS among cases and controls

	Cases (n=50)		Controls (n=50)	Sensitivity (%)	Specificity (%)
	PV (n=40)	PF (n=10)			
OM+	33	7	2*	80	96
OM–	3	48			
NHS+	26	8	0	68	100
NHS–	14	2	50		

*One patient each of dermatophytosis and dermatitis. OM: Oral mucosa, NHS: Normal human skin, PV: Pemphigus vulgaris, PF: Pemphigus foliaceus

Table 4: Comparison of IIF titers using OM and NHS as substrates among pemphigus patients

IIF titers in NHS and OM	Mucocutaneous PV (n=35)	Mucosal PV (n=5)	PF (n=10)
OM=NHS	11	0	0
OM>NHS	11	0	1
OM<NHS	1	0	5
OM+NHS–	6	4	1
OM–NHS+	3	0	2
Both –ve’	3	1	1

OM: Oral mucosa, NHS: Normal human skin, PV: Pemphigus vulgaris, PF: Pemphigus foliaceus

Table 5: Correlation of PDAI score with OM titers among pemphigus patients

	PDAI score		
IIF titer	Moderate (n=13)	Significant (n=21)	Extensive (n=16)
Negative	7	1	2
1:10	2	1	
1:100	1	1	1
1:200	2	2	2
1:400	-	9	2
1:800	1	3	4
1:1600	-	4	5

PDAI: Pemphigus disease activity index, OM: Oral mucosa, IIF: Indirect immuno fluorescence

Analysis of the results of the Dsg profile and substrate specificity [Table 6] showed that oral mucosa was the superior substrate in patients with both Dsg 1 and 3 positivity.

Discussion

Serological tests play an important role in the management of pemphigus. The decision to stop treatment may be based on a negative enzyme-linked immunosorbent assay or indirect immunofluorescence, while rising antibody titers during clinical remission may signal an impending relapse.[2] Although Dsg enzyme-linked immunosorbent assay is becoming increasingly popular, many laboratories around the world still use indirect immunofluorescence to detect pemphigus autoantibodies.[11,27]

Selection of a suitable substrate is crucial in obtaining an optimal indirect immunofluorescence result. Since

Table 6: Comparison of Dsg ELISA and IIF results with two substrates in pemphigus

IIF	ELISA				Total
	Both Dsg1 and 3+	Only Dsg1+	Only Dsg3+	Negative ELISA	
Both OM and NHS+	22	6	-	1	29
Only OM+	2	2	5	2	11
Only NHS+	1	3	1	-	5
Negative IIF	1	1	1	2	5
Total	26	12	7	5	50

OM: Oral mucosa, NHS: Normal human skin, IIF: Indirect immuno fluorescence, ELISA: Enzymelinked immunosorbent assay, Dsg: Desmoglein

studies have shown that pemphigus foliaceus (Dsg1) and pemphigus vulgaris (Dsg3) antigens are maximally expressed in the skin of upper trunk and buccal mucosa, respectively, both normal human skin and monkey esophagus are used for routine serodiagnosis of pemphigus patients by indirect immunofluorescence.[28,29]

The sensitivity of indirect immunofluorescence using normal human skin and monkey esophagus individually has been reported as 83% and 90%, respectively, but pooling these results increases the sensitivity to 100%.[29] Unfortunately, monkey esophagus is difficult to procure due to ethical reasons and its high cost. Other substrates such as the human cervix have been evaluated for indirect immunofluorescence in pemphigus and have been shown to be comparable in sensitivity and specificity to monkey esophagus.[13] We explored the utility of oral mucosa for indirect immunofluorescence as it is easily obtained in the dental wing of tertiary care centers. We also found that the larger surface area of epithelium in oral mucosa allows easier recognition of intercellular space staining as compared to normal human skin.

The sensitivity of oral mucosa in our study was 70% in pemphigus foliaceus and 82.5% in pemphigus vulgaris as compared to normal human skin with a sensitivity of 80% and 65%, respectively. In an earlier study, it was observed that the sensitivity of indirect immunofluorescence was greatest with normal human skin in pemphigus foliaceus patients, while in pemphigus vulgaris patients, the sensitivity was greatest on monkey esophagus.[29] However, other workers have found monkey esophagus to be superior to normal human skin irrespective of the disease phenotype.[30]

Although Harman *et al*. observed that normal human skin titers were well correlated with Dsg 1 levels and vice versa (Dsg3 levels with monkey esophagus titers),[29] Ng *et al*. noted that monkey esophagus was a better substrate than normal human skin irrespective of Dsg profile.[30] In the present study, oral mucosa proved to be better than normal human skin irrespective of the Dsg profile. Recently, Kamaguchi *et al* demonstrated the utility of OM to detect the circulating antibodies in 20 patients with mucous membrane pemphigoid (MMP). They observed that IIF using OM was positive in all patients while with NHS it was positive among only 8 patients.[31]

There was good correlation between disease activity (as defined by the pemphigus disease activity index) and the indirect immunofluorescence titer with oral mucosa. High titers were seen in patients with significant or extensive disease activity and patients with moderate disease activity had negative indirect immunofluorescence or low titer positivity. Thus, indirect immunofluorescence with oral mucosa can be used to monitor the disease activity of pemphigus patients. Indirect immunofluorescence was positive in all patients with active disease in the present study, but Dsg enzyme-linked immunosorbent assay was negative in one patient.

Among patients in clinical remission, circulating autoantibodies were identified in seven patients. Though two patients tested showed reactivity with both indirect immunofluorescence and Dsg enzyme linked immunosorbent assay techniques, in five patients autoantibodies could be detected using only one technique. Thus, indirect immunofluorescence and enzyme-linked immunosorbent assay are complementary and ideally both should be done. In the control arm, two patients were low titer indirect immunofluorescence positive with oral mucosa but we were unable to perform direct immunofluorescence in these patients to identify the tissue bound antibodies. False-positive reactions with indirect immunofluorescence have been reported earlier too and may occur in variety of conditions.[32,33]

Limitations

A comparison between oral mucosa and monkey esophagus was not possible due to the non-availability of monkey esophagus.

Conclusion

This is the first report of the use of oral mucosa as a substrate for indirect immunofluorescence in pemphigus. We observed oral mucosa to be more sensitive than normal human skin in pemphigus vulgaris patients, especially in those with lesions confined to the oral mucosa. The good sensitivity of oral mucosa substantiates its utility as an alternate substrate for the serodiagnosis of pemphigus. In addition, usage of two substrates for indirect immunofluorescence is strongly recommended. Indirect immunofluorescence and enzyme-linked immunosorbent assay serve as complimentary tests for the diagnosis and monitoring of disease activity in pemphigus.

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Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent.

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Conflicts of interest
There are no conflicts of interest.

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