Application of Indirect Immunofluorescence on the Diagnosis of Pemphigus

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ABSTRACT Pemphigus is an autoimmune bullous disease, and although several diagnostic methods are now in use indirect immunofluorescence (IIF) is still considered an important tool for diagnosing pemphigus because of its convenience, repeatability, and reduced pain for patients. The goal of the present study was to evaluate the diagnostic value of IIF on normal human skin (NS), monkey esophagus (ME), and salt-split skin (SS) for better diagnosis of pemphigus. Clinical data of 70 patients with pemphigus and 56 control were collected. IIF on NS, ME, and SS were assessed separately by observing fluorescein deposition and comparing its differentiation to different kinds of pemphigus and its sensitivities and specificities to different substrates. Intercellular deposition of IgG was visible when IIF on NS, ME, and SS were positive in patients with pemphigus. Their corresponding sensitivities and specificities were 30.0%, 84.3%, and 70.0% and 96.4%, 96.4%, and 94.6%, respectively. The differences in sensitivity were statistically significant between NS and ME and between NS and SS ($P<0.001$) and the specificities among the three substrates were not statistically significantly different ($P>0.05$). As for different types of pemphigus, the sensitivities between NS and ME and between NS and SS were statistically significantly different in both Dsg1- and Dsg3-positive and only Dsg1-positive patients with pemphigus ($P<0.01$); the sensitivities between NS and ME were statistically significantly different only in Dsg3-positive patients with pemphigus ($P<0.001$); there were no statistically significant differences between ME and SS. We therefore propose that ME is a good substrate for pemphigus diagnosis with higher sensitivity and superior to NS, particularly for patients with anti-Dsg3 antibodies. SS is a good alternative substrate to ME with almost identical higher sensitivities and specificities for diagnosis of pemphigus.

KEY WORDS: indirect immunofluorescence, pemphigus, bullous diseases, diagnosis

INTRODUCTION

Pemphigus is a potentially life-threatening autoimmune bullous disease, characterized by autoantibodies to the desmosomal proteins (desmoglein 1 and 3) that connect keratinocytes. Pemphigus can be divided into different types According to clinical manifestations and autoantibodies, pemphigus can be divided into different groups. Pemphigus vulgaris (PV) and pemphigus foliaceus (PF) are the two main

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Table 1. The sensitivities of indirect immunofluorescence (IIF) on different substrates

	Group	No.	NS	ME	SS	χ^2	*P* value
Pemphigus	Dsg1 and Dsg3 both positive	25	9 (36.0%)	23 (92.0%)	19 (76.0%)	19.1	<0.001
	Only Dsg1 positive	33	9 (27.3%)	24 (72.7%)	22 (66.7%)	16.3	<0.001
	Only Dsg3 positive	12	3 (25.0%)	12 (100%)	8 (66.7%)	15.3	<0.001
	All	70	21 (30.0%)	59 (84.3%)	49 (70.0%)	46.8	<0.001
Control	Subepidermal bullous diseases	26	1	2	2		
	Eczema	15	1	0	1		
	Healthy people	15	0	0	0		
	All	56	2	2	3		

NS: normal human skin; ME: monkey esophagus; SS: salt-split skin

types, and less common variants include paraneoplastic pemphigus and IgA pemphigus. Although the gold standard for diagnosing pemphigus is the detection of autoantibodies by direct immunofluorescence (DIF) and enzyme-linked immunosorbent assay (ELISA) systems (1), indirect immunofluorescence (IIF) is still considered an important tool for diagnosing pemphigus because of its convenience, repeatability, and reduced pain for patients.

Previous studies have suggested that normal human skin (NS) and monkey esophagus (ME) can be good substrate to detect pemphigus antibodies with IIF, and monkey esophagus has been largely accepted as the optimal substrate. But monkey esophagus is not easy to acquire, although commercial kits of ME substrate are already available. It is however expensive and cannot by widely used. Herein we evaluated the diagnostic value of IIF on three different kinds of substrates including normal human skin, monkey esophagus, and salt-split skin (SS) for diagnosing pemphigus.

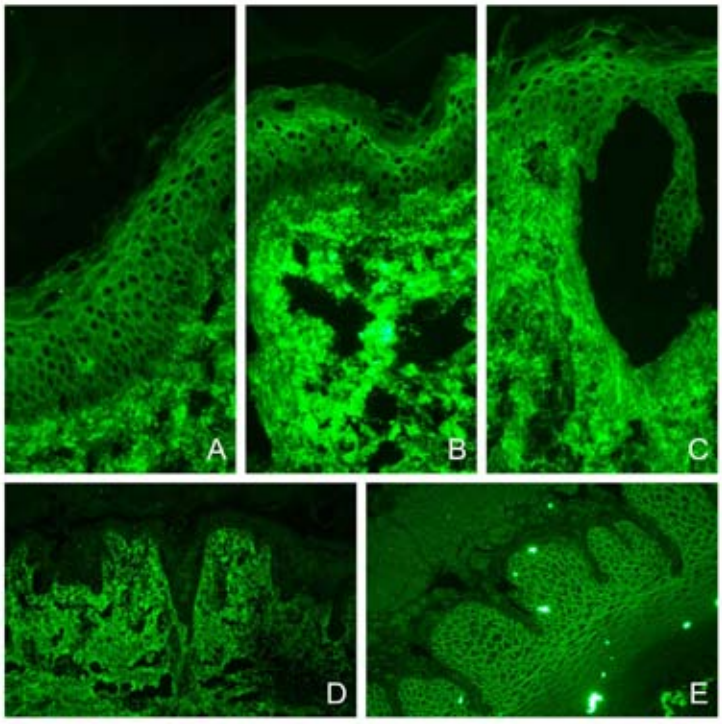

Figure 1. Different patterns of fluorescein deposition with indirect immunofluorescence (IIF) on different substrates. (A) A whole layer of intercellular deposition of IgG on stratum spinosum was visible with IIF on normal human skin (NS) in pemphigus vulgaris. (B) Intercellular deposition of IgG on the upper stratum spinosum was visible with IIF on NS in pemphigus foliaceus. (C) Intercellular deposition of IgG on stratum spinosum was visible with IIF on salt-split skin (SS) in pemphigus foliaceus. (D) No fluorescein deposition of IgG on the stratum spinosum and the basement membrane zone was visible with IIF on NS in controls. (E) Intercellular deposition of IgG with IIF on monkey esophagus (ME) in pemphigus vulgaris.

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Table 2. Statistical results of sensitivities of indirect immunofluorescence (IIF) on different substrates

Statistical results	Pemphigus		Dsg1 and Dsg3 both positive		Only Dsg1 positive		Only Dsg3 positive	
	χ^2	*P* value	χ^2	*P* value	χ^2	*P* value	χ^2	*P* value
NS IIF vs. ME IIF	42.1	<0.001	14.7	<0.001	13.6	<0.001		<0.001
NS IIF vs. SS IIF	22.4	<0.001	8.1	0.004	10.3	0.001		0.1
ME IIF vs. SS IIF	4.05	0.04	1.3	0.25	0.29	0.6		0.09

NS: normal human skin; ME: monkey esophagus; SS: salt-split skin

PATIENTS AND METHODS

This study was performed in adherence with the Declaration of Helsinki and guidelines of local ethics committees of the Institute of Dermatology, Chinese Academy of Medical Sciences, and Peking Union Medical College. Informed consent was provided by all patients and controls.

The sera from 70 newly-diagnosed patients with pemphigus and 56 control individuals (without pemphigus) were tested. The pemphigus group consisted of 43 men and 27 women aged 15-97 years (mean: 48.4 years). Pemphigus was diagnosed based on the clinical, histologic, DIF, and ELISA results for anti-Dsg1/anti-Dsg3. The patients were classified into three groups according to the results of ELISA for anti-Dsg1/anti-Dsg3 antibodies. Out of the 70 patients with pemphigus, 37 had PV (25 cases positive for both Dsg1 and Dsg3 and 12 cases positive for only Dsg3) and 33 patients had PF (only Dsg1 positive). The 56 control cases without pemphigus included 26 patients with autoimmune subepidermal bullous diseases, 15 patients with chronic eczema, and 15 healthy persons. ELISA was performed to detect anti-Dsg1 and anti-Dsg3 antibodies, and all control cases were negative.

Desmoglein 1 and 3 ELISAs were performed in accordance with the manufacturer's instructions (MBL, Nagoya, Japan). The sera were diluted to 1:100 and the cutoff index value was 20.0 as recommended by the instructions on the kit. SS was made with normal human skin through incubation in 1.0 M sodium chloride, mounted in Tissue-Tek O.C.T (Sakura Finetek, USA), and then cut into frozen sections, as previously described (2). IIF was conducted on NS and SS using routine methods. The skin samples were collected from normal skin remaining from plastic surgery and were embedded, cut into frozen sections, and then stained with fluorescein isothiocyanate-labled polyclonal goat anti-human IgG antibodies (ZSbio, Beijing, China). IIF was performed on ME with kits utilizing the standard technique (EUROIMMUN AG, Lübeck, Germany). The sera of IIF were diluted to 1:10. The results were examined independently by two of the authors to determine whether the intercellular deposition of IgG was positive. The data were analyzed using SPSS 23.0 software package (IBM, Chicago, USA). Descriptive statistics were summarized as number, percentage, and mean. The numerical data were assessed using the chi-square test or Fisher's exact test. *P* values less than 0.05 were considered significant, indicated by an asterisk in the figures.

RESULTS

The intercellular deposition of IgG was visible with IIF on NS, ME, and SS and was positive in patients with pemphigus (Figure 1); the corresponding sensitivities of the substrates were 30.0%, 84.3%, and 70.0%, respectively (Table 1), while the specificities were 96.4%, 96.4%, and 94.6%, respectively. The differences in sensitivity between NS and ME and between NS and SS were statistically significant ($P<0.001$) and the differences in specificity among the three substrates were not statistically significant ($P>0.05$), which means ME and SS were superior to NS as substrates for pemphigus. As for different types of pemphigus, the differences in sensitivity between NS and ME and between NS and SS were statistically significant in both Dsg1- and Dsg3-positive and only Dsg1-positive patients with pemphigus ($P<0.01$); only in Dsg3-positive pemphigus patients the difference in sensitivity between NS and ME statistically significant ($P<0.001$) was found, which showed that ME is better than NS as substrate. However, there were no statistically significant differences between ME and SS, which indicates that SS could be a good alternative substrate to ME with almost identical sensitivity in pemphigus diagnosis (Table 2).

DISCUSSION

Serological diagnostic tools with enhanced sensitivity and specificity have become available due to the development of modern diagnosis techniques for autoimmune bullous diseases. Nonetheless, IIF is still considered a well-established method for detecting circulating autoantibodies in patients with pemphigus. Finding the optimal substrate is critical to improving the diagnostic performance of this tool.

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In 1964, Beutner and Jordon (3) first applied IIF to detect the skin autoantibodies in the sera of patients with PV. In 1969, Stephen *et al*. (4) used human skin as substrate to detect pemphigus antibodies, and its sensitivity was superior to that of rabbit esophagus. Feibelman *et al*. (5) demonstrated that ME is a more sensitive substrate than guinea pig esophagus. Harman *et al*. (6) compared the sensitivity of NS and ME as IIF substrates and found that for patients with only anti-Dsg3 antibodies, the sensitivity was the highest with ME, which is in accordance with our data. However, in contrast to our results, they reported that for patients with only anti-Dsg1 antibodies the sensitivity was the highest with NS. Our study found that ME or SS were a better IIF substrate than NS and had higher sensitivity in patients regardless of whether they were Dsg1- or Dsg3-positive. When ME was used as the substrate, its sensitivity was higher in patients with only anti-Dsg3 antibodies than that in patients with only anti-Dsg1 antibodies. This difference could be attributed to the insufficient number of cases or ethnic differences. We also compared SS with ME and NS the first time, and the high sensitivity and specificity of SS indicated that SS could be a good alternative substrate for pemphigus diagnosis; potentially due to the exposure of antigenic epitopes after the cleavage of the skin.

CONCLUSION

Monkey esophagus is a good substrate for pemphigus diagnosis with higher sensitivity and superior to NS, particularly for patients with anti-Dsg3 antibodies. Moreover, SS is a good alternative substrate to ME with almost identical higher sensitivities and specificities for diagnosis of pemphigus.

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