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Abstract

Background: Mucous membrane pemphigoid (MMP) is an autoimmune disease characterized by scarring lesions at mucosal sites. Although the pathogenic role of specific IgG and/or IgA has been already demonstrated and the detection of these immunoglobulins is a criterion in the diagnosis of MMP, little is known about IgE role in this disease. Therefore, the main purpose of this study was to assess the presence of circulating and tissue-bound IgE in patients with MMP and their possible correlations with clinical presentation and disease course.

Methods: We conducted a retrospective study on 29 patients affected by MMP, recruited from a single centre. Direct and indirect immunofluorescence studies were assessed to analyse the presence of specific IgE directed against the basal membrane zone. For each patient, fluorescence data were compared to clinical features.

Results: Linear deposits of C3, IgG and IgA were present in 86.2%, 62% and 37.9% of cases respectively, while IgE linear deposits were detected in 17 out of 29 patients (58.6%) including one case with isolated IgE positivity. Circulating IgE and IgA anti-BMZ were present in 7 (24.1%) and 5 (17.2%) patients, respectively. Both the presence of circulating IgA and of tissue-bound IgE deposits correlated with disease activity index (P<0.014).

Conclusions: Our results demonstrated the presence of IgE autoantibodies in MMP, particularly in more severe cases. Thus, IgE detection may represent an additional useful diagnostic tool in this disease.

Body of manuscript

INTRODUCTION

Mucous membrane pemphigoid (MMP) is a heterogeneous group of autoimmune bullous blistering diseases with predominant mucosal involvement. Clinically, MMP is a chronic and progressive disease characterized by subepithelial blisters, erosions, and scarring of mucous membranes, skin, or both. Mucous sites mainly involved are oral cavity, conjunctivae, nasal cavity, anogenital area, pharynx, larynx and esophagus [1, 2]. Clinical severity is highly variable and can range from minimal to generalized ulcerative mucosal involvement.

Various targets have been studied and characterized in MMP patients, including laminin 332, laminin 311, bullous pemphigoid 180 and 230 (BP180 and BP230), type-VII collagen, and the β4 integrin subunit [3]. Among these, BP180 is thought to be the major autoantigen. Both c-terminal region and NC16A domain are targeted, but extracellular domain is predominantly recognized [4-6].

While the role of IgG antibodies to BP180 in mediating tissue damage has been convincingly demonstrated, the pathogenic potential of IgE anti-BP180 autoantibodies has been incompletely addressed. Only a few studies have analyzed the presence of tissue-bound IgE deposits in bullous pemphigoid (BP) [7, 8] and MMP[3, 9]. It is well known that direct immunofluorescence (DIF) analysis highlights a linear and continuous deposition of C3 and IgG, and occasionally IgA, at the basal membrane zone (BMZ) in the majority of MMP patients [10]. By contrast circulating antibodies anti-BMZ are often absent or too low to be detected by indirect immunofluorescence (IIF).[10, 11]

MATERIALS AND METHODS

Among 50 patients evaluated for a new diagnosis of MMP in the Department of Dermatology of Milan from April 2003 to September 2017, 21 patients were excluded due to the lack of samples (biopsy and/or serum) or detailed data collected. In the remaining 29 patients, 11 males and 18 females, the mean age at diagnosis was 67.6 years (range 41-86) and the mean diagnosis delay from first manifestations was a year. The mean follow-up was 72 months (range 0-278) (Table I).

For all patients we obtained written informed consent. Diagnosis of MMP in all patients was assessed in agreement with clinical, histopathological and immunofluorescence criteria established in the first international consensus on MMP [1].

Activity disease scores were calculated at time of diagnosis basing on MMP Disease Area Index (MMPDAI) defined by Murrell *et al.* [12]. In one patient, showing only esophageal involvement not included in MMPDAI, we estimated a MMPDAI of 10, considering the entire area involved.

Immunofluorescence analysis

For DIF analysis perilesional biopsies taken from skin, oral cavity, esophagus or conjunctiva were sectioned at 5 µm thick. Deposits were detected using polyclonal rabbit anti-human IgG FITC-conjugated (Dako, Glostrup, Denmark, 1:100), polyclonal rabbit anti-human C3c complement FITC-conjugated (Dako, Glostrup, Denmark 1:50), polyclonal rabbit anti-human IgA FITC-conjugated (Scimedx Corporation, Denville, USA, 1:2) with phosphate-buffered saline using standard techniques for standard routine examinations. For IgE tissue-bound detection, sections were incubated with monoclonal mouse anti-human IgE (Immunological Sciences, Rome, Italy, 1:30) followed by a polyclonal rabbit anti-mouse immunoglobulins FITC-conjugated (Dako, Glostrup, Denmark, 1:50) incubation.

Indirect immunofluorescence analysis were performed using standard techniques with normal human skin and 1 mol/L salt-split skin (SSS). Dilutions of sera of 1:10 were used to detect circulating IgA, IgG and IgE using the same antibodies as DIF. Controls included sera from patients with BP, linear IgA disease and normal human sera. Appropriate tests were performed to exclude possible cross-reactivity of human IgG by using secondary anti-mouse antibody.

Statistical analysis

We investigated possible correlations between the presence of specific immunoglobulins deposits and the clinical features such as MMPDAI, the number of affected sites, the involvement of specific mucous or cutaneous sites and the time to achieve quiescence. Fisher's exact test was used for qualitative variables,

while nonparametric Wilcoxon Mann-Whitney test was used for quantitative values. Statistical significance was set at $P < 0.05$. Statistical computations were performed using the R environment.

RESULTS

In our cohort, 23 patients had a strictly MMP while 6 presented also skin involvement. Oral mucosa was the most frequent involved area: 26 out of 29 patients (89.7%). Genital mucosa was involved in 9 patients (31%). Conjunctival lesions were present in 8 patients (27.6%) while cutaneous lesions localized at face, scalp, trunk and upper limbs were present in 6 patients (20.7%). Upper airways (nose, pharynx, larynx) were involved in 2 patients (6,9%) and esophagus in one more patient (3.4%). Demographic profiles and clinical features are reported in Table I.

Medical treatments used were oral steroids in 14 out of 29 patients (48.3%), oral steroids and methotrexate in 4 (13.8%), oral steroids and azathioprine in 4 (13.8%), topical steroids in 4 (13.8%), intravenous corticosteroids in 2 (6.9%), one of which was finally treated with Rituximab; one patient did not receive treatment because newly diagnosed.

Direct Immunofluorescence examination

DIF examinations on perilesional mucosa and/or skin biopsies detected in all patients continuous deposits of one or a combination of the following in the BMZ: IgG, C3, IgA and IgE (Fig. 1 and Table II). Linear deposits of C3, IgG and IgA were present in 86.2%, 62% and 37.9% of cases respectively, while the presence of IgE linear deposits were detected in 17 out of 29 patients (58.6%; Fig. 2A).

One patient presented only IgE deposits (3.4 %), while IgE were associated with C3 in 3 patients (10.3%), with IgA in 1 case (3.4%), with both IgG and C3 in 5 patients (17,2%), and with both IgA and C3 in 1 patient (3.4%). In 6 patients (20.7%) we found concomitantly IgE, IgG, IgA and C3.

Indirect Immunofluorescence examination

In the examined group of MMP patients IgG was the class of circulating autoantibodies detected more frequently (44.9%). Circulating IgE and IgA anti-BMZ were present in 7 (24.1%) and 5 (17.2%) patients,

respectively. In one patient specific IgE were detected alone, while in 4 patients they were associated with specific circulating IgG, and in the other 2 cases IgE were associated with both circulating IgA and IgG (Fig. 2B). IgA were associated with IgG in all but one cases (Table II). All tested immunoglobulins reacted with the epidermal side of SSS.

Correlation between specific IgE detection and clinical features

In the analyzed group of 29 patients, MMPDAI ranged from 1 to 42. Interestingly, we found a significant association between the disease score and the presence of linear IgE deposits in the BMZ detected by DIF (*P*=0.0135; Fig. 3A).

Fifteen patients had lesions at a single site, while 14 in different sites. Patients characterized by IgE-negative DIF have a single site involved in 75% of the cases, while multiple sites were involved only in 3 patients (25%). In patients with IgE-positive DIF the rate of multiple-site involvement increased until 11 out of 17 patients (64.7%), nevertheless this difference did not achieve statistical significance (P=0.06). No significant associations were found considering IgA, IgG and C3 deposits and MMPDAI. In addition, patients with IgE-positive DIF had skin lesions in 5 out of 17 cases (29.4%), while only one patient with IgE-negative DIF had skin lesions (P=0.35).

Average time-to-quiescence was 20 months (range 3-144 months); quiescence was not yet achieved at last follow-up in 5 patients. There was not a significant association between disease score and time-to-quiescence. The mean time-to-quiescence was 25.2 months (range 3-144 months) in patients with IgE-positive DIF compared to 10.1 months (range 3-19 months) in those with IgE-negative DIF (*P*=0.064).

In our group of patients, 5 out 29 cases (17.2%) showed circulating IgA. Even if this subgroup represented just a small part of the entire cohort, they had significant higher MMPDAI (*P*=0.0137, Fig. 3B). No further significant associations between IIF results and clinical features were found.

DISCUSSION

First findings reporting a possible link between IgE and BP were published in 1970s[13]. During the following decades, several authors tried to elucidate the role of these immunoglobulins in the disease and suggested

the involvement of IgE-class autoantibodies in the mechanism inducing the dermo-epidermal detachment and the subsequent development of specific lesions in autoimmune bullous diseases [14-19]. Nevertheless, only a few case series have been published about the IgE role in MMP until now [3, 9]. To our knowledge, this is the largest study that analyses the presence of autoreactive IgE both in tissues and in serum samples of MMP patients, and assess their correlation with clinical presentation and severity of disease.

Circulating autoantibodies have been investigated in many published works, even if most of them are not focused only on MMP and few have large cohorts of MMP patients [3-5, 20-22]. Data on serum IgA autoantibodies are still controversial: reported percentages of patients with detectable circulating antibodies are heterogeneous and range from 0% to 62%. In our cohort, IgA circulating antibodies were found by IIF in 17.2% of patients. Oyama *et al.* and Setterfield *et al.* suggested a more aggressive disease severity in presence of IgA autoantibodies. Our results support this hypothesis as the subgroup of patients with circulating IgA had a higher MMPDAI.

Until now, a few small case series have focused detection of circulating IgE autoantibodies in MMP [3, 20]. Only one study detected IgE antilaminin-332 in a single MMP patient by immunoblot.[3] So far, there are not reported evidence of significant nor suggestive associations between IgE circulating antibodies detection and disease severity, clinical course or outcome. No associations between circulating IgE and MMPDAI, sites of involvement or times-to-quiescence have been found also in our group of MMP patients. We cannot rule out that some factors not strictly related with the pathophysiology of the disease, such as treatment choices could affected time-to-quiescence data.

Although IIF testing of patients' serum is a sensitive method for detecting circulating antibodies in MMP, the binding patterns of these antibodies are not disease specific and they are not detectable in some patients with MMP. Therefore, the DIF test is considered the gold standard in the diagnosis of MMP [1]. Nevertheless, recent studies on large cohort of patients with MMP, reported DIF sensitivity is far less than 100%, particularly in ocular MMP [5, 23, 24]. In their analysis, authors considered only tissue-bound IgG, IgA and C3 deposits, while interestingly the presence of IgE deposits has not yet been fully characterized. In fact, to the best of our knowledge, only Yayli *et al.* analyzed IgE in a group of MMP patients, highlighting that the rate of IgE deposits in MMP appeared higher compared with that in patients with BP [9].

According to Yayli and colleagues, that analyzed 13 MMP patients and detected IgE deposits by DIF in 9 of them (69.2%), we found that more than a half of MMP patients (58.6%) have IgE deposits in BMZ. Both in our and Yayli's cohort, only one patient had isolated IgE deposits, in absence of other Ig or C3 deposits. We confirmed that percentages of patients with IgE deposits in BMZ seem to be higher in MMP than in BP, which range from 11% to 41% in reported case series [9, 25-27]. Yayli et *al.* suggested that demonstration of tissue-bound IgE deposits provides a useful additional criterion for diagnosis of BP and MMP in some cases. Nevertheless, they did not find significant associations between the presence of these deposits in MMP and distinct clinical features. On contrast, we found a significant correlation (P=0.013) between MMPDAI and *in vivo* IgE deposition at the dermal epidermal junction, analysed by DIF. IgE-positive patients had also more frequently multiple sites involvements and longer times-to-quiescence, even if these data did not quite achieve the threshold for statistical significance (P=0.06). Discrepancies could due to our larger cohort of patients as well as the different clinical features analysed as Yayli *et al.* focused only on itching and the presence of urticarial lesions.

A specific role of IgE deposits in pemphigoid is still controversial, even in BP where the first report of tissue bound IgE is dated 1974 [25]. Moriuchi *et al.* on a large cohort of patients did not find significant differences in disease severity, clinical course and outcome between IgE-positive and IgE-negative patients [27]. On contrast, van Beek *et al.* suggested a pathogenic role of IgE autoantibodies in BP [28]. In addition, in a recently published review article, Saniklidou *et al.* reported that higher serum IgE autoantibody levels are associated with more severe clinical manifestations of BP [19, 28].

CONCLUSIONS

Although not of diagnostic relevance, IgA IIF test in MMP could be useful to identify patients with a possible more aggressive course of the disease. IgE deposits detection could increase the sensitivity of the DIF test for MMP diagnosis due to the demonstration of IgE positive, but IgG- C3- IgA-negative cases. IgE autoantibodies in MMP could be related to worst clinical presentations and courses, but further studies are needed to define their possible pathogenic impact.

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Notes

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Table I: Demographic profiles, clinical features and prognoses

Patient number	Age (years)	Sex	Follow up time (months)	time to quiescence (months)	MMPDAI	Sites of involvement	Treatments used
1	74	M	154	19	11	oral, skin	OS+AZA
2	65	F	5	NR	15	oral	OS
3	67	F	133	9	6	oral	OS
4	50	F	144	7	10	oral, skin	OS
5	76	F	83	33	22	oral, genital, conjunctiva, upper airways, skin	OS+MTX
6	70	F	49	15	13	oral, conjunctiva	OS
7	81	F	101	3	2	oral	OS
8	66	M	30	19	1	oral, genital	OS
9	58	M	103	20	13	oral, conjunctiva	OS
10	41	M	107	18	7	oral, conjunctiva	OS+MTX
11	72	M	19	13	22	oral, conjunctiva	OS+MTX
12	54	F	278	144	7	oral, genital, conjunctiva, skin	OS
13	61	F	98	11	3	oral	OS
14	73	M	5	NR	1	genital	OS
15	71	F	170	24	2	oral	OS
16	60	F	14	9	3	oral	TS
17	55	F	6	NR	4	oral	TS
18	66	M	132	11	7	oral	OS+AZA
19	82	F	63	8	19	oral, conjunctiva	OS+MTX
20	64	F	88	13	7	oral	OS
21	70	F	100	5	7	oral, genital	OS+AZA
22	70	F	97	24	8	oral, genital	TS
23	52	M	11	11	2	genital	OS+AZA
24	86	F	0	NR	3	oral	/
25	72	F	20	8	2	oral	OS
26	73	F	6	NR	4	oral	TS
27	83	M	19	16	10	esophagus	OS+SS+AZA
28	71	M	42	26	6	oral, genital, skin	OS
29	76	M	10	3	42	oral mucosa, conjunctiva, genital mucosa, upper airways, skin	OS+SS+rituximab

OS = oral steroids; TS = topic steroids; SS = systemic steroids; AZA = azathioprine; MTX = methotrexate; NR = not raised

Table II: Results of immunofluorescence analysis

Patient number	DIF				IIF		
	IgA	IgG	C3c	IgE	IgG	IgA	IgE
1	-	+	+	+	+	-	+
2	-	+	+	+	-	-	-
3	-	+	+	-	-	-	-
4	+	+	+	+	-	-	+
5	+	+	+	+	+	+	+
6	+	+	+	+	-	-	-
7	-	-	+	+	-	-	-
8	-	+	+	-	-	-	-
9	+	-	+	+	-	-	-
10	+	+	+	+	+	+	-
11	-	-	+	+	+	-	-
12	-	+	+	+	-	-	-
13	-	+	+	-	+	-	+
14	-	-	+	-	-	-	-
15	+	-	-	+	-	-	-
16	+	-	+	-	-	-	-
17	-	-	+	+	+	-	+
18	-	+	+	+	+	-	+
19	-	+	+	+	+	+	+
20	+	+	+	+	-	-	-
21	-	+	+	-	+	-	-
22	+	+	+	+	-	+	-
23	+	-	+	-	-	-	-
24	-	-	+	-	+	-	-
25	-	+	+	-	+	-	-
26	-	+	+	-	-	-	-
27	-	+	-	-	-	-	-
28	-	-	-	+	+	-	-
29	+	-	-	-	+	+	-

Figure legend

Figure 1. “Venn diagram of direct immunofluorescence (DIF) results in patients with mucous membrane pemphigoid, showing all relations of tissue bound linear deposits of IgA, C3, IgE and/or IgG.”

Figure 2. “IgE immunofluorescence stainings. (A) Example of a case with linear deposits along basal membrane zone of oral mucosa by direct immunofluorescence. (B) Example of a case with linear deposits along the epidermal side of salt-split skin by indirect immunofluorescence.”

Figure 3. “(A) Box-and-whisker plot showing MMPDAI in IgE-positive and IgE-negative groups of patients. Patients with IgE tissue bound deposits show significant higher disease scores (*P*=0.0135). (B) Box-and-whisker plot showing MMPDAI in patients with and without circulating IgA autoantibodies. Patients with circulating IgA show significant higher disease scores (*P*=0.0137).”