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DEMONSTRATION OF BASEMENT MEMBRANE AUTOANTIBODIES IN PATIENTS WITH BENIGN MUCOUS MEMBRANE PEMPHIGOID[1]

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Abstract. Sera from 8 patients with benign mucous membrane pemphigoid were examined for antibasement membrane antibodies by means of an indirect immunofluorescence technique. Oral lesions from 6 of the patients were examined for in vivo bound antibodies by means of a direct immunofluorescence technique. Antibodies (IgG and/or IgA) in low titres were found in 7 patients. In vivo bound antibodies and/or complement were found in four of the oral biopsies.

Sera from patients with pemphigus contain antibodies which react with cell coats in stratified squamous epithelium, and sera from patients with bullous pemphigoid contain autoantibodies to the basement membrane of stratified squamous epithelia. These reactions have proved to be a valuable tool in the diagnosis of bullous lesions of the skin and mucous membranes (2).

Benign mucous membrane pemphigoid (BMMP) is a disease with histological features similar to those of bullous pemphigoid (BP) (13). In BMMP, bullous eruptions appear on the mucous membranes, particularly of the oral cavity and conjunctiva; the skin may also be slightly involved in 10% of the patients. Scarring, which is never seen in bullous pemphigoid, is a frequent feature of ocular lesions in BMMP (7, 13, 16).

Until recently, it was supposed that a distinction between BP and BMMP could be made by immunological methods in that the former is connected with the presence of circulating and tissue-bound antibodies, whereas such antibodies had not been detected in the latter (1, 4, 7, 8, 10, 12, 14).

However, one case of BMMP exhibiting circulating antibasement membrane antibodies (ABMA) has been demonstrated (6) and recently four cases showing in vivo basement membrane bound antibodies have been published by two independent investigators (1, 9). To our knowledge this is the only previous evidence of the presence of immunopathological reactions in patients with BMMP.

The purpose of the present paper is to describe investigations into 8 cases of BMMP for the presence of circulating ABMA, and for the presence of in vivo basement membrane bound antibodies in lesions from the same patients.

MATERIAL AND METHODS

The material consisted of 8 patients with the diagnosis BMMP. The diagnosis was based on the clinical and histological criteria proposed by Lever (13) and Shklar (16). The clinical data of the patients are given in Table 1. Histologically, all showed sub-epithelial bullae.

A control group included 8 clinically healthy individuals. All patients and normal controls were matched for age and sex. One patient with bullous pemphigoid, in whom the serum was known to contain ABMA in high titres, was included as a positive control.

Staining reaction to detect circulating ABMA

The sera were investigated by a double-layer immunofluorescence staining technique (17).

Antigen. Unfixed 4–8 μm frozen sections of guinea pig lips. They were stored at −20°C and were never older than 4 days.

Sera. Blood was collected from a cubital vein and the serum stored at −20°C.

The sera of the patients and the normal controls were titrated using the following dilutions: 1:1, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and 1:256. The serum of the positive control was titrated up to a dilution of 1:8 000.

Conjugates. In order to investigate the nature of possible ABMA all stainings were performed with heavy chain specific rabbit antihuman globulins conjugated with fluorescein isothiocyanate (Dakopatts®; A-S, Copenhagen, Denmark). Antihuman IgG, IgM and IgA were used. The average conjugation degree is approx. 2.3 moles of FITC per mole of protein as calculated from the extinction ratio E_{495}/E_{278} nm which is 0.63. The mean optical density (OD) ratio 495/278

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Table I. *Clinical data of eight females with BMMP at time of investigation*

Patient	Age	Duration of disease	Mucous membranes affected				Skin involvement	Scarring	Activity	Systemic steroids
			Eyes	Mouth	Larynx	Ano-genital				
HA	52	2 years	+	+	+	−	−	+	+	None
RY	84	18 years	+	+	−	+	+	+	+ +	None
PE	68	5 years	+	+	−	+	+	+	+ +	None
CH	74	10 years	+	+	−	−	+	+	+	+
BR	53	6 months	+	+	−	−	−	−	+ +	None
ST	54	6 months	−	+	+	−	−	−	+ +	None
FU	70	2 years	−	+	−	+	+	−	+ +	None
JE	81	22 years	−	+	−	+	−	−	+	None

nm of the conjugates was 0.63 with 0.30 as lowest and 0.95 as highest ratio. (Unconjugated immunoglobulins, OD ratio below 0.30, and conjugated immunoglobulins with an OD ratio above 0.95 were removed by ion exchange chromatography). The antibody titre of the conjugates was 100 (i.e. 1 ml conjugate absorbs 100 μg pure human immunoglobulin).

The heavy chain specificity of the conjugates was obtained by immunization with pure human immunoglobulins followed by absorption with free light chains.

The specificity was tested on monoclonal bone marrow cells (Dakopatts®: A-S). A working titre of 1:20 was found for all conjugates by chessboard titration (18).

Staining. Cryostat cut sections were air-dried for 15 min, then incubated at room temperature in a moist chamber with the various dilutions of antiserum for 30 min, washed three times for 5 min in phosphate-buffered saline (PBS) at pH 7.2, and incubated for a further 30 min with conjugate. Before mounting, the slides were washed again for three periods of 5 min and mounted in PBS (pH 7.2) with 10% glycerol.

The BP control and at least one of the normal controls were always included in the staining procedure. Only titrations in which the BP control had an IgG ABMA titre of 1:4 000 or more were accepted as successful. Controls in which the patients' sera were replaced by PBS were included as well. The sections were read blind, the investigator never knowing how many normal sera had been included.

Test for demonstrating basement-bound immunoglobulins

Punch biopsies from lesions of the oral mucosa were used for this purpose. Local infiltration anaesthesia (2% lidocain noradrenalin) was used and care was taken not to inject directly into the site of biopsy. Each biopsy was immediately frozen and stored at a temperature of −20° C. Sections were cut at 4–8 μm in a cryostat, air-dried for 15 min, incubated in a moist chamber with a drop of conjugate for 30 min, washed three times for 5 min in PBS (pH 7.2) and mounted in 10% glycerol in PBS, pH 7.2.

Three sections from each patient were stained with conjugated rabbit antihuman globulins, one with antihuman IgG/FITC, one with antihuman IgM/FITC, and finally one with antihuman IgA/FITC. The properties of these conjugates have been described above. In this part of the study, working titres were also 1:20.

To investigate whether in vivo bound antibodies are complement-fixing, one further section was stained with rabbit antihuman C'3 conjugated with FITC. The antibody titre and OD 495/278 nm ratio of this conjugate were identical with the conjugates described above.

Control reactions were made by blocking the antigen in the tissue sections with unlabelled antibody before the labelled antibody was added.

Fluorescence microscopy

All stainings were examined in a Leitz Orthoplan® fluorescence microscope modified with a Tiyoda® darkfield oil immersion condenser. A FITC-interference filter with red contrast band (15) was used as the primary filter, and a 2 mm glass filter (Schott & Gen, Germany) matched to fit the primary filter was used as the secondary filter (15). The light source was an Osram HBO 200 lamp.

RESULTS

A positive reaction was seen as bright green fluorescence in the basement membrane region of the mucosa (Fig. 1).

Detection of circulating ABMA

In 6 of 8 patients, ABMA of the IgG class were found with endpoint titres ranging from 1:8 to 1:128 (Table II). ABMA of the IgM class were not found in any patient. Six of 8 patients demonstrated ABMA of the IgA class. One of these (CH) was completely negative when tested for AMA of IgG class. The endpoint titres ranged between 1:4 and 1:32 (Table II). All control reactions were negative, and all patients' sera reacted negatively in stainings where the BP control had an endpoint titre lower than 1:4 000.

Detection of in vivo bound antibodies

In 2 patients (HA and BR) no in vivo basement membrane immunoglobulins could be demonstrated,

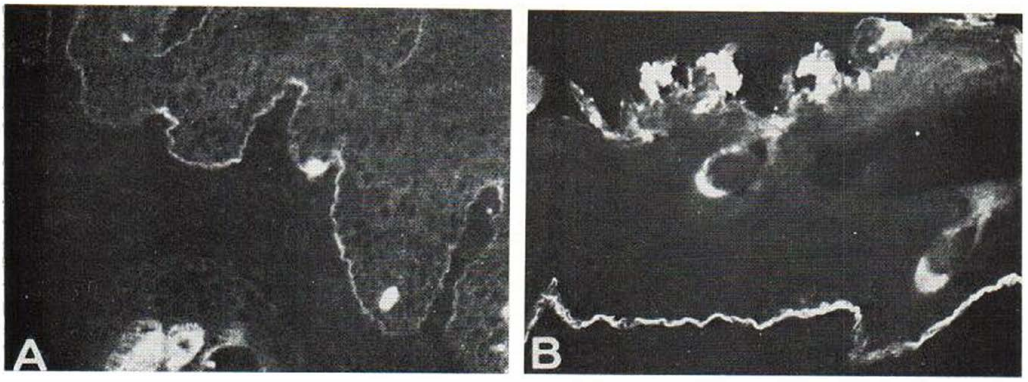

Fig. 1. Double-layer immunofluorescence staining of guinea pig lip. (A) Serum from patient (*RY*) with benign mucous membrane pemphigoid diluted 1:32. (B) Serum from patient with bullous pemphigoid diluted 1:1 000. Note the positive staining of the basement membrane zone. × 140.

while in 4 cases such immunoglobulins as well as C_3 were found. These results are summarized in Table III. All control reactions were negative.

DISCUSSION

Every patient in the present study fulfils the clinical and histological criteria of BMMP (13, 16) and may thus be distinguished from patients with BP.

The demonstration in a previous study (1) of basement membrane in vivo fixed immunoglobulins and complement in 3 patients with BMMP, suggests that immunological reactions are involved in this disease as they are in BP. The finding of circulating ABMA, as well as in vivo bound antibodies in the patients in the present study supports this concept. It should be emphasized that the ABMA from the patients exist in very low titres when compared with the titres obtained from patients with BP. This is clearly demonstrated by the fact that all patients with BMMP reacted negatively in the staining procedure when the positive control had an endpoint titre of less than 1:4 000.

It is remarkable that not only ABMA of the IgG class are found in patients with BMMP, but also antibodies of the IgA class. They seem to be present in slightly lower titres than the IgG ABMA. Whether a difference between BP and BMMP exists in this respect is not clear; most authors (5) indicate that the ABMA in patients with BP are of the IgG class but it cannot be ruled out that a sensitive IF system would reveal IgA ABMA in low titres in these patients as well.

The low titres of ABMA in BMMP illustrate the need for a sensitive IF system. In a study on circulating autoantibodies in patients with BP, Cormane & Szabó (5) concluded that the conventional IF method is not sufficiently sensitive in cases with low antibody titres. The highly sensitive IF system used

Table II. *Antibodies to basement membrane of lip in eight patients with benign mucous membrane pemphigoid*

Immunofluorescence staining

Patient	Endpoint titres of antibodies of immunoglobulin class		
	IgG	IgM	IgA
HA	1:4	—	1:4
RY	1:128	—	1:16
PE	1:8	—	1:16
CH	—	—	1:8
BR	1:64	—	1:16
ST	1:64	—	1:64
FU	1:32	—	1:8
JE	—	—	—

Table III. *Direct immunofluorescent staining for IgG, IgM, IgA and C'3 in biopsies of lesions of the oral mucosa in six patients with BMMP*

n.t. = not tested

Patient	IgG	IgM	IgA	C'3
HA	—	n.t.	n.t.	n.t.
RY	+	—	+	+
PE	+	+	+	+
BR	—	—	—	—
ST	+	+	—	+
FU	+	+	+	+

in this study could explain the positive reactions obtained in contrast to previous reports.

Although corticosteroids usually have no marked influence on the ABMA titres in patients with BP (2) it cannot be ruled out that it may be of importance for the positive reactions that 4 of the patients in this study had never been treated for their disease and that one other patient had been without treatment for 6 months.

Furthermore, one of the patients with the strongest reaction of circulating ABMA showed no tissue-bound antibodies. This is of particular interest as the previous failure to demonstrate circulating autoantibodies has been explained by the fact that they were all tissue-bound (10).

It is known that a certain number of clinically healthy patients exhibit autoimmune antibodies (3). Although all control sera investigated in the present study reacted negatively, it cannot be excluded that a certain number of healthy individuals have ABMA in low titres. In order to investigate this further, a more extensive screening of elderly women for the presence of ABMA would be necessary.

Usually, in vivo basement membrane bound immunoglobulins in patients with BP are complement-fixing IgG (5, 11). The results obtained in the present study show not only IgG and C'3 deposits but also in vivo deposits of IgA and IgM.

In conclusion, the present work has confirmed previous reports describing in vivo bound antibasement membrane antibodies in patients with BMMP and, furthermore, demonstrated the presence of circulating ABMA in some of these patients. For the moment we can only explain the positive results found by us and not found by others on the basis of the highly sensitive IF system used in this study.

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