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T Sugi, … , T Hibi, T Nishikawa

Research Article

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Production of Human Monoclonal Anti–Basement Membrane Zone (BMZ) Antibodies from a Patient with Bullous Pemphigoid (BP) by Epstein-Barr Virus Transformation

Analyses of the Heterogeneity of Anti-BMZ Antibodies in BP Sera Using Them

Toshiyuki Sugi,* Takashi Hashimoto,* Toshifumi Hibi,‡ and Takeji Nishikawa*
*Department of Dermatology, Keio University School of Medicine, Tokyo 160, Japan; and ‡Section of Internal Medicine, Kitasato Institute Hospital, Tokyo 108, Japan

Abstract

We established three lymphoblastoid cell lines from a bullous pemphigoid (BP) patient's peripheral blood by means of EBV transformation, which produced human monoclonal anti–basement membrane zone (BMZ) IgG antibodies. A blocking immunofluorescence test using these MAbs, designated 5A, 5E, and 10D, revealed that 5A and 5E recognized the same or a closely associated epitope, but the epitope for 10D was completely different. 18 of 30 BP sera blocked the reactivity of 10D MAb and 17 sera blocked 5E, while 9 sera did not block the staining of either antibody. Immunoblot analysis demonstrated that both 5A and 5E MAbs reacted exclusively with a protein band of ~ 230 kD in normal human epidermal extracts. However, 10D did not show any protein band. 22 of 30 BP sera strongly reacted with the same 230-kD protein, while none of control sera showed such reactivity.

These results clearly demonstrated the heterogeneity of anti-BMZ antibodies in terms of epitopes. These MAbs should be useful in future investigations concerning not only the immunopathology but also the biochemical and molecular analyses of the BP antigen.

Introduction

Bullous pemphigoid (BP)[1] is an autoimmune skin disease characterized by tense blister formation at the dermoepidermal junction (1). The direct immunofluorescence test reveals IgG deposits along the basement membrane zone (BMZ; 2). In most cases circulating anti-BMZ antibodies are also detected by the indirect immunofluorescence (IIF) technique (3). It has recently been reported that anti-BMZ autoantibodies in the sera from BP patients play important roles in its pathogenesis (4–8). Furthermore, some investigators have suggested the heterogeneity of the antibodies (9–15), while others have implied that BP antibody recognizes a single unique antigen (16–21).

Recently, production of human MAbs using EBV transformation (22–27) or hybridoma techniques (28–32) has made a marked advance, and it has contributed to the progress in various fields of research, although this approach has not been applied to autoimmune blistering diseases such as BP and pemphigus. In this study we have tried to elaborate human monoclonal anti-BMZ antibodies by EBV transformation and to examine the heterogeneity of the anti-BMZ antibodies in BP sera.

Methods

Patients and sera. Peripheral blood lymphocytes were obtained from a 72-yr-old male BP patient having anti-BMZ antibodies in the sera at a titer of 320. Sera for blocking immunofluorescence and Western blot experiments were obtained from 30 confirmed BP patients possessing circulating antibodies at titers of 40–2,560 by IIF test.

IIF. IIF was performed according to the previously established method (2, 3) using normal human skin section as a substrate. All of FITC-conjugated anti–human IgG (γ-chains), IgM (μ-chains), IgA (α-chains), and IgE (ϵ-chains) rabbit antisera were purchased from DAKO, Copenhagen, Denmark, and used at a dilution of 1:10–1:20.

Lymphocyte preparation. 20 ml of heparinized peripheral blood was obtained by venipuncture. Mononuclear cells were isolated by centrifugation on Lymphoprep (Nycomed AS, Oslo, Norway). Cells were washed three times with PBS and rosetted with neuraminidase-treated sheep erythrocytes. The rosetted cells were removed by centrifugation on Lymphoprep. The B lymphocyte–enriched fraction was washed three times with PBS and used for the following experiments.

EBV transformation. EBV was obtained from the culture supernatant of the B95-8 marmoset cell line. 2×10^5/ml B95-8 cells were cultured in 5% CO_2 humidified incubator at 37°C with RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS and penicillin/streptomycin (Gibco Laboratories) for 8 d without medium change (33). The supernatant containing EBV was harvested, filtered through a 0.45-μm filter (Millipore Japan, Yamagata, Japan), and stored at −70°C. Peripheral blood lymphocytes prepared as previously mentioned were incubated with 1 ml of supernatant containing EBV for 2 h at 37°C with occasional swirling. After incubation the cells were washed three times with PBS, resuspended in 20 ml RPMI 1640 with 20% FCS and penicillin/streptomycin, and 0.2 ml of suspended medium was seeded in each well of a 96-well culture plate (Falcon Plastics, Cockeysville, MD). The plate was placed in a 5% CO_2 humidified atmosphere, and half of the medium was changed every fourth day until colonies grew enough for screening assay.

Screening assay. Supernatants from wells with colony formation were screened for anti-BMZ antibodies by IIF, using FITC-conjugated anti–human IgG antiserum as a second antibody.

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1. *Abbreviations used in this paper:* BMZ, basement membrane zone; BP, bullous pemphigoid; IIF, indirect immunofluorescence; TBS, Tris (hydroxymethyl) aminomethane hydrochloride–buffered saline.

Cloning. The transformants producing anti-BMZ antibodies were cloned in 96-well plates by a limiting dilution method on feeder layers of cord blood lymphocytes that had been previously treated with 20 μg/ml mitomycin C (Kyowa Hakko, Tokyo, Japan) for 30 min. Supernatants from wells with growing colonies were again tested by IIF for the presence of anti-BMZ antibodies.

Study for IgG subclasses. IgG subclasses of the antibodies were determined by IIF using anti–human IgG subclass mouse MAbs as previously described (34).

Biotinylation of the antibodies. Cloned lymphoblastoid cell lines were cultured with serum-free medium, S-clone SF-B (Sankou Jun-yaku, Tokyo, Japan). IgG was prepared from the supernatant by salt precipitation with 50% saturated ammonium sulphate. Biotinylation of IgG was performed according to the previously reported method (35).

Blocking immunofluorescence experiments. To characterize the epitopes recognized by the human MAbs, a blocking immunofluorescence test was performed according to the previously described method with minor modifications (11, 36). Normal human skin sections were pretreated with IgG solution purified from culture supernatant containing anti-BMZ MAbs or with the diluted sera from BP patients or normal controls for 30 min at 37°C, and subsequently incubated with diluted biotin-conjugated human MAbs for 30 min at 37°C, followed by incubation with 1:1,000 diluted FITC-conjugated egg white avidin (Tago Inc., Burlingame, CA) for 30 min at room temperature. Between each treatment the sections were rinsed for 15 min with three changes of cooled PBS. The preparations were observed by epifluorescence microscope (BH2-RFL; Olympus, Tokyo, Japan).

Immunoblot analysis. Immunoblotting was performed mainly according to the method described by Labib et al. (15). Adult foreskin was obtained at circumcision surgery. Skin pieces prepared by forceps and scissors were incubated in PBS containing 2 mM EDTA (Nakarai Tesque, Kyoto, Japan) and 2 mM PMSF (Sigma Chemical Co., St. Louis, MO) for 2 d at 4°C, and epidermis was peeled off by forceps as previously reported (37). This epidermis preparation was confirmed to retain BP antigen by IIF. The pieces of epidermis were collected and homogenized by glass homogenizer in an ice-water bath with 1% SDS, 0.01 M Tris (hydroxymethyl) aminomethane hydrochloride buffer (Nakarai Tesque) supplemented with 2 mM EDTA, 2 mM PMSF, 5 mg/liter each of four proteinase inhibitors (leupeptin, antipain, chymostatin, and pepstatin A; Sigma Chemical Co.), and 5% β-mercaptoethanol (Kanto Chemical Co., Tokyo, Japan), pH 6.8. The homoge-

nized sample was boiled for 5 min and centrifuged. Supernatant was harvested and stored at −70°C as aliquots. SDS-PAGE was performed by Laemmli's method (38) using 6% separating gel. Separated proteins were electrophoretically transferred to a nitrocellulose sheet (Schleicher and Schuell, Dassel, FRG; 39). For immunostaining, strips of blotted sheet were first blocked by 3% skimmed milk (Morinaga, Tokyo, Japan) in Tris (hydroxymethyl) aminomethane hydrochloride buffered saline (TBS) for 2 h at room temperature. Blocked strips were treated with MAbs or sera from BP patients or normal individuals diluted with 3% skimmed milk overnight at 4°C. Subsequently the strips were reacted with peroxidase-conjugated anti–human IgG (γ-chains) rabbit antiserum (DAKO) diluted at 1:100 with 3% skimmed milk in TBS for 2 h. Between each treatment the strips were washed for 15 min with three changes of TBS containing 0.5% Tween 20 (Sigma Chemical Co.). Finally, color was developed by 4-chloro-1-naphthol (Bio-Rad Laboratories, Richmond, CA) in the presence of hydrogen peroxide.

Protein measurement and IgG purification from BP sera. Total protein was determined by the method of Lowry et al. (40) using BSA as a standard. IgG fractions were isolated from several BP sera by salt precipitation with 50% saturated ammonium sulphate, followed by protein A–Sepharose CL-4B (Pharmacia AB, Uppsala, Sweden) affinity chromatography (41). The eluate was dialyzed against PBS containing 0.1% NaN₃ and concentrated on a concentrator (Centriprep 10; Amicon Corp., Danvers, MA).

Results

Establishment of lymphoblastoid cell lines. B lymphocytes infected with EBV were cultured for 3 wk in a 96-well plate. Most wells showed colony formation. Screening test demonstrated that six wells were positive for anti-BMZ antibodies. Three were not stable and the production of antibody decreased rapidly. The remaining three wells (designated 5A, 5E, and 10D) were cloned by the limiting dilution method. Each clone was again confirmed by IIF to produce anti–BMZ IgG antibody (Fig. 1), but no IgM, IgA, or IgE antibodies were found. All wells with colony formation showed the presence of anti-BMZ antibodies, which suggested that monoclonality of the antibodies had been almost achieved in the initial cultures.
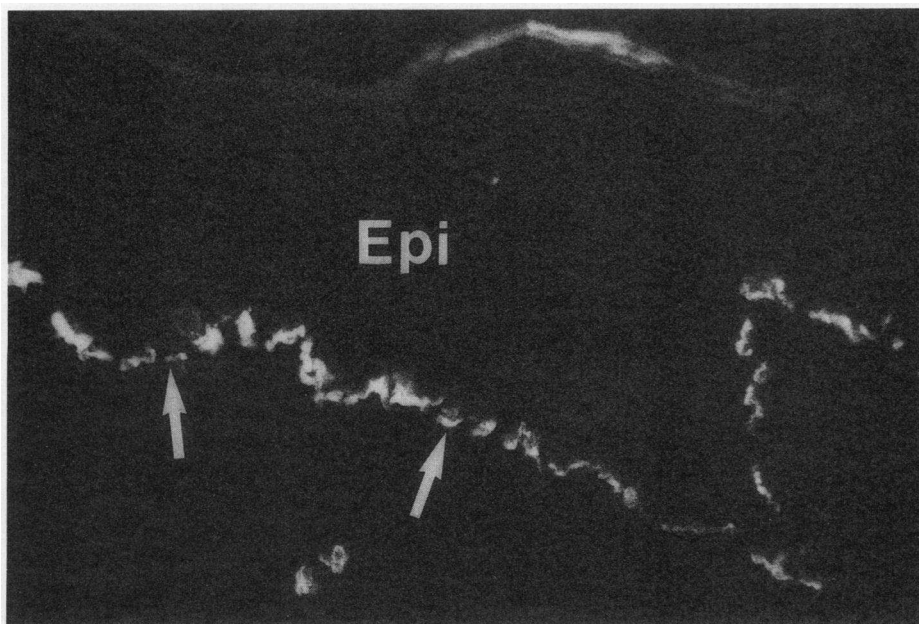

Figure 1. IIF staining by 10D human monoclonal anti-BMZ antibody using EDTA separated human epidermis as a substrate. Positive reaction is seen discontinuously along the BMZ of epidermis (×200). *Arrows,* BMZ; *Epi,* epidermis.

These cell lines continued to produce antibodies for more than 6 mo until cells were frozen to be stored. The IgG subclass was analyzed for three clones and for the serum from the donor BP patient. All three human MAbs were IgG2, while the serum of the donor contained IgG1, IgG2, and IgG4, but not IgG3, autoantibodies.

IgG isolation from the supernatant of lymphoblastoid cell cultures and biotinylation. Total protein concentration and the terminal dilution point for IIF were determined for each of the IgG fractions prepared from the culture supernatant of lymphoblastoid cells and the biotin conjugate. Total protein concentrations in undiluted solutions and the terminal dilution points, respectively, were as follows: 6 mg/ml and 1:640 for 5A IgG; 12 mg/ml and 1:2,560 for 5E IgG; 1.2 mg/ml and 1:160 for 10D IgG; 6 mg/ml and 1:160 for biotinylated 5A; 10 mg/ml and 1:640 for biotinylated 5E; and 1.5 mg/ml and 1:40 for biotinylated 10D. To correspond to the titers of BP sera and make a comparison easier, dilution instead of protein concentration was used for these solutions in the following experiments.

Blocking immunofluorescence experiments. For this test, biotin-conjugated monoclonal anti-BMZ antibodies were used at dilutions that were four times as high as the terminal dilution point of each conjugate (i.e., 1:40 for 5A, 1:160 for 5E, and 1:10 for 10D). The sera diluted in this way will be stated as fourfold concentrated sera hereafter. In all experiments both blocking test and serum titration by IIF were performed simultaneously. Serum dilutions tested for blocking effect and titration were 1:2.5–1:640 and 1:10–1:2,560, respectively. When 16-fold concentrated sera or MAbs were able to block the reactivities of biotinylated antibodies, the blocking capability of these sera or MAbs was arbitrarily designated as positive.

To determine the identity among the three MAbs, the blocking effect of each purified MAb on individual conjugate was first examined. BMZ staining of all biotinylated MAbs was blocked by pretreatment of the purified IgG of each corresponding MAb. Reactivity of biotinylated 5A was blocked by 5E but not 10D. Reaction of conjugated 5E was blocked by 5A but not 10D. Further, reaction of 10D was not blocked by 5A or 5E. Fourfold-concentrated 5A and 5E IgGs still showed blocking effects on biotinylated 5A and 5E. On the contrary, even 64-fold concentrated 10D IgG did not block the reactivities of 5A or 5E.

Since 5A and 5E were found to react with the same or a closely associated epitope, only 5E and 10D conjugates were used for further studies on blocking effect of BP sera. Results of the blocking effect of BP sera are summarized in Table I. The reactivity of biotinylated 5E was blocked by 17 of the 30 BP sera tested. BMZ staining of 10D was blocked by 18 BP sera. Reactivities of both 5E and 10D MAbs were blocked by sera from 14 BP patients, including the donor BP patient. Three sera showed a blocking effect on 5E reactivity but not on 10D, while four sera blocked only 10D. Nine BP sera did not block either 5E or 10D. Absence of the blocking effect on these sera was obvious even at 64- to 256-fold concentrations. None of the 18 normal sera showed this blocking effect.

Immunoblot analysis (Fig. 2) and its relation to blocking immunofluorescence. By immunoblotting, 5E MAb showed reactivity with a single protein band of an apparent molecular mass of 230 kD. 5A also stained the same band exclusively. However, 10D did not demonstrate any protein band even when nondiluted IgG sample was used. 30 BP sera were also

Table I. Results of Blocking Immunofluorescence and Immunoblot Labeling of 30 BP Sera

Immunoblot labeling of (230 kD, 170 kD)	Blocking effect on (5E, 10D)				Total No. of the sera
	(+, +)*	(+, −)	(−, +)	(−, −)	
(+, +)‡	5	2	1	2	10/30
(+, −)	7	0	2	3	12/30
(−, +)	0	0	0	2	2/30
(−, −)	2	1	1	2	6/30
Total No. of the sera	14/30	3/30	4/30	9/30	

This table shows the combined data of the results of blocking immunofluorescence and immunoblot analysis of 30 BP sera. The bottom line indicates the total No. for the blocking experiments, and the right column indicates the total No. for immunoblot analyses.
* The reactivities of both 5E and 10D were blocked by the 16-fold concentrated BP sera.
‡ Both the 230- and 170-kD bands were stained by the 1:40 diluted BP sera.

examined by immunoblotting as shown in Table I. 22 sera reacted strongly with the same 230-kD protein band. In addition, 12 sera specifically stained a protein band of ~ 170 kD. 10 sera yielded both 230- and 170-kD bands, 12 sera showed only a 230-kD band and 2 sera showed only a 170-kD band. Six BP sera did not react with either protein band. None of 18 normal sera showed reactivity with either band. No specific correlation between the results of blocking immunofluorescence and immunoblotting was observed in this table.

Because 5E MAb reacted with the 230-kD protein, we studied the relationship between the blocking effect on 5E reactivity and the staining of the 230-kD band on immunoblotting of individual BP serum (Table II). 14 of 30 BP sera showed both the blocking effect on 5E and staining of the 230-kD band. Three sera showed a clear blocking effect on 5E, although they did not stain the 230-kD band on immunoblotting. Furthermore, eight sera that stained the 230-kD band did not block 5E reactivity. Five sera did not show either the blocking effect or staining.

Discussion

We established three individual lymphoblastoid cell lines that continued to produce human monoclonal anti-BMZ IgG antibodies for more than 6 mo. That all cloned wells with colony formation contained anti-BMZ antibodies in the supernatant safely confirmed the monoclonality of these antibodies. This was further substantiated by the fact that immunoblot analysis showed a single positive band reacted with 5A and 5E antibodies. The IgG subclass study revealed that all of our antibodies were of the IgG2 subclass. It remains unknown why only IgG2-producing B cells were preferentially transformed, despite the fact that the patient's serum contained IgG1, IgG2, and IgG4 anti-BMZ antibodies.

There are many previous reports about the establishment of lymphoblastoid cell lines by EBV transformation, which produced a variety of human monoclonal autoantibodies (22–27). Most of them belonged to the IgM subtype. However,
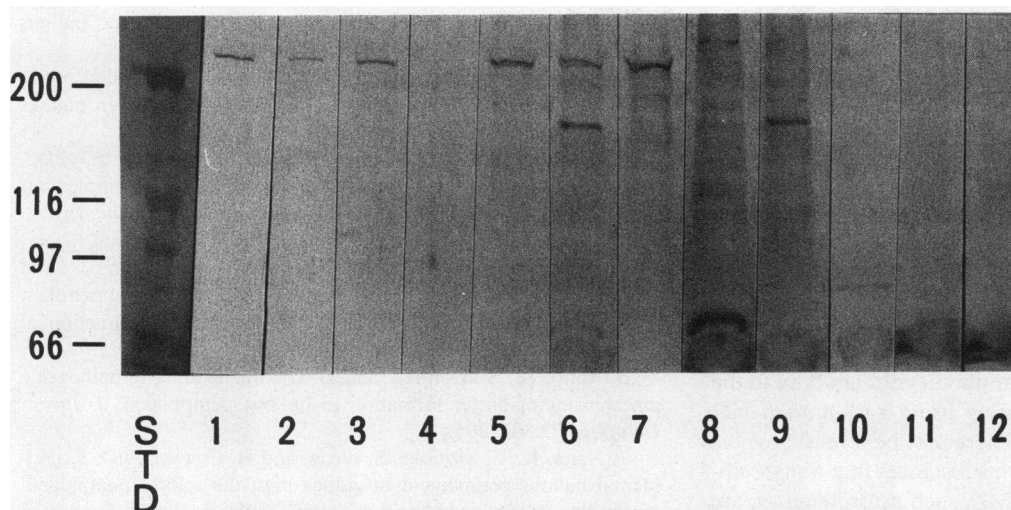
Figure 2. Immunoblot analysis. BP antigen extracted from normal human epidermis was electrophoresed and stained with human MAbs or 1:40 diluted sera from BP patients or normal controls. 5E antibody reacted with a single protein band of ~ 230 mol wt at dilutions of 1:160 and 1:640 (lanes 1 and 2, respectively). 5A showed the same band at 1:40 dilution (lane 3). 10D did not show any reactivity at 1:10 dilution (lane 4). In five representative BP sera two showed a single band of 230 kD (lanes 5 and 7), one serum showed both 230- and 170-kD bands (lane 6) (this was the serum from the donor BP patient for the lymphoblastoid cell lines), one serum showed only the 170-kD band (lane 9), and one serum showed neither band (lane 8). Normal sera did not react with either band (lanes 10–12). Left lane (STD) showed molecular weight standards, the apparent molecular weights of which are indicated in the left margin (×10³).

we have found no previous reports on human monoclonal IgG antibodies against epidermal components such as anti-BMZ antibodies in BP or anti–intercellular antibodies in pemphigus. Very recently, Rico and Hall (42) have reported the production of human monoclonal anti-BMZ antibodies that belonged to the IgM subtype. Since IgG anti-BMZ antibodies are predominant and IgM antibodies are rarely found by IIF in sera of BP patients, IgG antibodies are considered to be more relevant to pathogenesis in BP. Therefore, our human monoclonal anti-BMZ antibodies should be useful in many ways to study the role of autoantibodies in the pathogenesis in BP.

In this study we performed two sets of experiments using our MAbs: blocking immunofluorescence and immunoblotting. Using blocking immunofluorescence we demonstrated that 5A and 5E MAbs reacted with the same or a closely associated epitope of the antigen, but the epitope for 10D was completely different. Furthermore, 5E reactivity was blocked by 17 of the 30 BP sera tested, and 10D was blocked by 18 BP sera, while neither reactivity was blocked by 9 BP sera. Al-

though 14 sera blocked both 5E and 10D, 7 sera blocked either 5E or 10D. These results indicate that there should be more than three different epitopes with which antibodies in BP sera react. Further, both 5E and 10D antibodies are considered to be frequent in the sera from many BP patients.

Recently a series of studies by Stanley and others have suggested that BP sera specifically recognized a unique protein with a molecular mass of 220–240 kD by means of immunoprecipitation (16–21) and immunoblot (17, 19) techniques. Labib et al. (15) also found the similar 240-kD protein band, but in addition they found another specific antigenic protein of 180 kD using immunoblotting.

Using the same procedure of immunoblotting as Labib et al. (15), we demonstrated that 5A and 5E MAbs reacted exclusively with a ~ 230-kD protein from human epidermal extracts. However, 10D antibodies did not show any band on immunoblot analysis. This result further confirmed the different nature of 10D antibodies from those of 5A and 5E. Furthermore, we observed that 22 of 30 BP sera reacted with the identical 230-kD protein. In addition, we detected the reactivity to 170 kD in 12 BP sera, while none of 18 normal sera showed either band. Our results appeared to confirm the result reported by Labib et al. (15). However, as Stanley et al. (17, 19) used different immunoblot techniques from Labib's and ours, we are unable to completely exclude the possibility that the 170-kD band was merely a nonspecific reaction. The fact that 10D antibody failed to yield any stained band on immunoblotting may indicate the presence of additional BP antigen different from that identified as the 230-kD band by 5A and 5E. However, it is also possible that 10D antibody reacts with a different epitope of the same 230-kD protein and this epitope may be destroyed or masked during the procedure of immunoblotting.

The accuracy of the blocking immunofluorescence test used in this study is supported by three observations: (a) the reactivity of each biotinylated antibody was blocked by the corresponding IgG fraction; (b) the reactivities of 5A and 5E MAbs were blocked by each other; and (c) the majority of BP sera were able to block the reactivity of all MAbs, in contrast to

Table II. The Relationship between the Blocking Effect on 5E MAb Reactivity and the Presence of the 230-kD Protein Band on Immunoblotting in 30 BP Sera

Blocking effect on 5E	Presence of the 230-kD band	No. of BP sera
(+)*	(+)§	14/30
(+)	(−)‖	3/30
(−)‡	(+)	8/30
(−)	(−)	5/30

* The reactivity of 5E was blocked by the 16-fold concentrated BP sera, or ‡ it was not blocked by the BP sera on blocking immunofluorescence test.
§ The 230-kD band was stained by the 1:40 diluted BP sera, or ‖ it was not stained by the BP sera on immunoblot analysis.

the absence of such an effect with any of the normal sera. These results suggested that the blocking effect was due to the presence of IgG reacting with the same epitope. Nevertheless, several discrepancies existed between the results of blocking immunofluorescence and Western blot experiments for 5E MAb. Although 14 of 30 BP sera showed both blocking effect and reactivity with the 230-kD protein, and 5 BP sera did not show either, 8 BP sera that recognized the 230-kD protein on immunoblotting did not block 5E antibody. This may be because these sera contained antibodies reacting with epitope(s) of the 230-kD protein different from that recognized by 5E antibody. Another discrepancy was that three sera that were able to block the reactivity of 5E antibodies did not bind to the 230-kD antigen on immunoblotting using anti-human IgG antiserum as a second antibody. There may be several possible explanations for this: (a) unknown substances that nonspecifically destroy antigenic sites in BMZ, such as proteinases, are present in some of BP sera but not in normal sera; (b) these sera possess Ig other than IgG reacting with the same epitope for 5E antibody; and (c) these sera bind to an epitope on the 230-kD molecule near enough to the 5E epitope to block the latter by steric hindrance, but the former is destroyed by denaturation for immunoblotting, whereas the 5E epitope is not. To solve this issue, immunoblotting was performed using peroxidase-conjugated anti-human IgM, IgA, and IgE antisera as second antibodies. This study revealed that three sera possessed both IgM and IgE types of Ig, which clearly reacted with the 230-kD protein (data not shown). Furthermore, IgG fractions purified from these sera by protein A chromatography did not show blocking effects on 5E reactivity by blocking immunofluorescence. These results suggested that the blocking effect on 5E reactivity of these sera might be due to the presence of IgM and/or IgE antibodies reacting with the same epitope of the 5E antibody.

For several years there have been many arguments about the heterogeneity of anti-BMZ antibodies in BP sera (9–20). The results in the present study clearly demonstrate the diversity of epitopes that bind to anti-BMZ antibodies. However, as we have not found any molecules on immunoblotting other than 230 kD using our MAbs, the problem of the molecular heterogeneity of BP antigen must be solved by further investigation.

The human monoclonal anti-BMZ antibodies we established should be extremely valuable for various fields of investigation, such as immunopathology, cellular immunology, biochemistry, and molecular biology, and should be used to reveal the mechanisms of blister formation in BP. Further investigations using these MAbs are in progress in our laboratory.

Acknowledgments

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