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Purification of Bullous Pemphigoid IgG Subclasses and Their Capability for Complement Fixation

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The antibody reactivity and complement fixing capability of circulating IgG subclass antibodies of patients with bullous pemphigoid (BP) were investigated. Four subclasses of polyclonal IgG were purified from the sera of BP patients. The first stage of purification was a combination of chromatographies on DEAE Affi-Gel Blue and protein A-Cellulofine columns. The four polyclonal IgG subclasses were then isolated from the above-mentioned semi-purified subclass fractions using IgG subclass-specific immuno-affinity chromatographies. Using an immunohistopathological technique, IgG deposits were detected at the basement membrane zone (BMZ) in the normal skin incubated with IgG subclasses other than IgG_3. C3 deposits were detected at BMZ in the skin incubated with IgG_1. Neither IgG nor C3 deposits were in skin incubated with IgG_3. These findings suggest that the subclasses of circulating BP antibodies are IgG_1, IgG_2 and IgG_4, but not IgG_3. Moreover, only the BP IgG_1 subclass appears to fix complement. ***Key words: Autoimmune bullous disease; Anti-basement membrane zone antibody.***

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Bullous pemphigoid (BP) is an autoimmune skin disease characterized by tense blister formation at the dermo-epidermal junction (1). Direct immuno-fluorescence (IF) reveals IgG deposits along the basement membrane zone (BMZ) (2). In most cases, circulating anti-BMZ IgG antibodies are also detected by indirect IF (3). Using a complement immunofluorescent technique, Jordon et al have also shown that anti-BMZ antibodies are capable of fixing complement (4).

It is generally recognized that IgG has four main subclasses: IgG_1, IgG_2, IgG_3 and IgG_4, all of which possess different biologic characteristics. IgG_1, IgG_2, and IgG_3 fix complement, but IgG_4 does not (5). In examining the relationship between the IgG subclass and complement in BP using ion exchange chromatography, Sams & Schur first reported that anti-BMZ antibody activity in non-complement fixing BP serum was confined to IgG_4 and that complement-fixing IgG was found in the IgG_3 fraction (6). On the other hand, the IgG subclass of the patient with BP bound to protein A, as did the complement-fixing IgG (7, 8). This result suggested that the complement-fixing IgG subclass of BP antibody might be IgG_1 and/or IgG_2. Later, Bird et al. reported that IgG_4 was predominant in BP, IgG_1 was next, and IgG_3 was found only occasionally in both circulating antibodies and IgG deposition in perilesional skin, using monoclonal antibodies specific for the IgG subclasses (9, 10). This result raised the question of whether BP antibody was capable of fixing complement, whereas IgG_4, which was predominatly found, did not usually activate complement (5). They thought that other IgG subclasses without IgG_4 found in much smaller quantities and fixed complement.

As mentioned above, the relationship between the IgG subclass and complement in BP is not consistent throughout these reports (6–10). The relationship has not been clearly resolved because there has been no precise IgG subclass purification technique. We therefore performed IgG subclass purification from the sera of patients with BP using our (S. H.) method, which is a more precise IgG subclass purification technique (11). We studied the complement fixing capability of each IgG subclass in the BP sera. We think that this study may not only provide a resolution of the relationship between IgG subclass and complement in BP but may also be the first step in the study of the biological characteristics of the four IgG subclasses in BP.

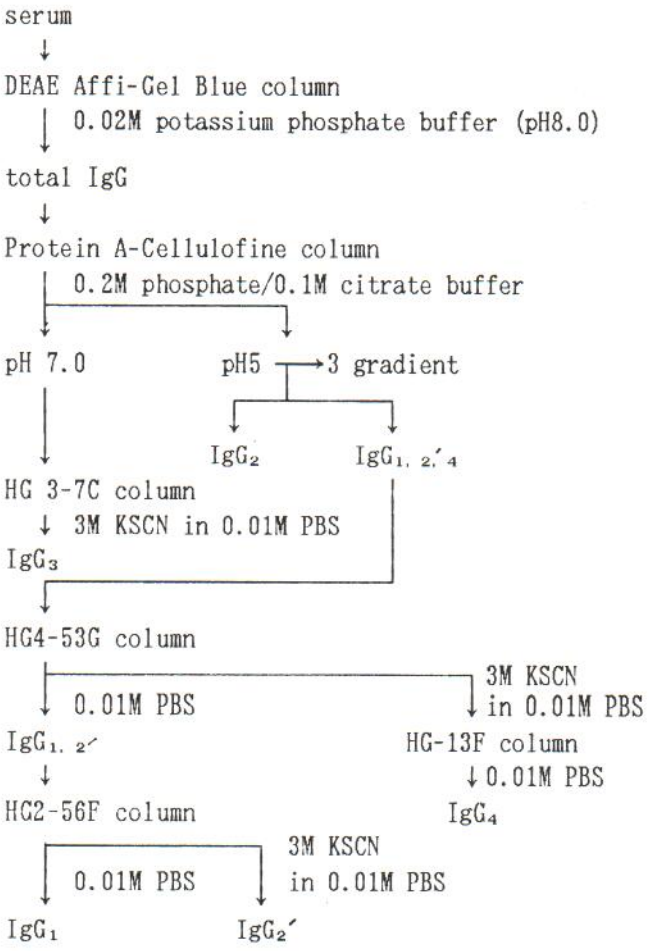

Fig. 1. Isolation of IgG subclass fractions.

Table I. *Anti-basement membrane zone (BMZ) antibody titers, and complement fixing titers of bullous pemphigoid patients' sera*

Patient	Age/Sex	Anti BMZ antibody titer	Complement fixing titer
B.N.	76, ♀	1:256	1:1
T.Y.	79, ♀	1:512	1:5
K.O.	66, ♂	1:512	1:10
S.T.	76, ♀	1:64	1:4

MATERIALS AND METHODS

1. Patient sera

The sera were obtained from four untreated patients with clinically and histopathologically typical BP (Table I). Normal human sera were obtained from five healthy volunteers (age range 23–84Y). These sera were divided into aliquots of 0.5 ml and stored at −70°C until they were used. Their anti-BMZ antibody titers were determined by indirect immunofluorescence staining methods using normal skin as a substrate.

2. Skin used as substrates

Human skin samples used as substrates were obtained from healthy volunteers. Skin specimens were embedded in OCT Embedding Media (Miles, Elkhart, Indiana). They were stored at −70°C for no longer than 7 days, cut into 4 μm sections in an HM500 cryostat (MICROM, Heiderberg West Germany), and processed, using an indirect or complement immunofluorescence staining method.

3. Monoclonal antibodies

Clone numbers of anti-human IgG subclass monoclonal antibodies were anti-G_1 (NL16), anti-G_2 (HG2-56F), anti-G_3 (HG3-7C), anti-G_4 (HG4-53G) and anti-$G_{1,2,3}$ (HG-13F). These monoclonal antibodies (other than NL16) were produced in our (S.H.) laboratory, and their specificities were also examined there (11).

4. IgG subclass purification procedure (Fig. 1)

The sera of patients and healthy human volunteers were diluted at 1:10 with 0.02M potassium phosphate buffer (K-PB), pH 8.0, applied to DEAE Affi-Gel Blue column (Bio-Rad Lab. Richmond, CA), and equilibrated with 0.02M K-PB, pH 8.0. The protein peak, monitored

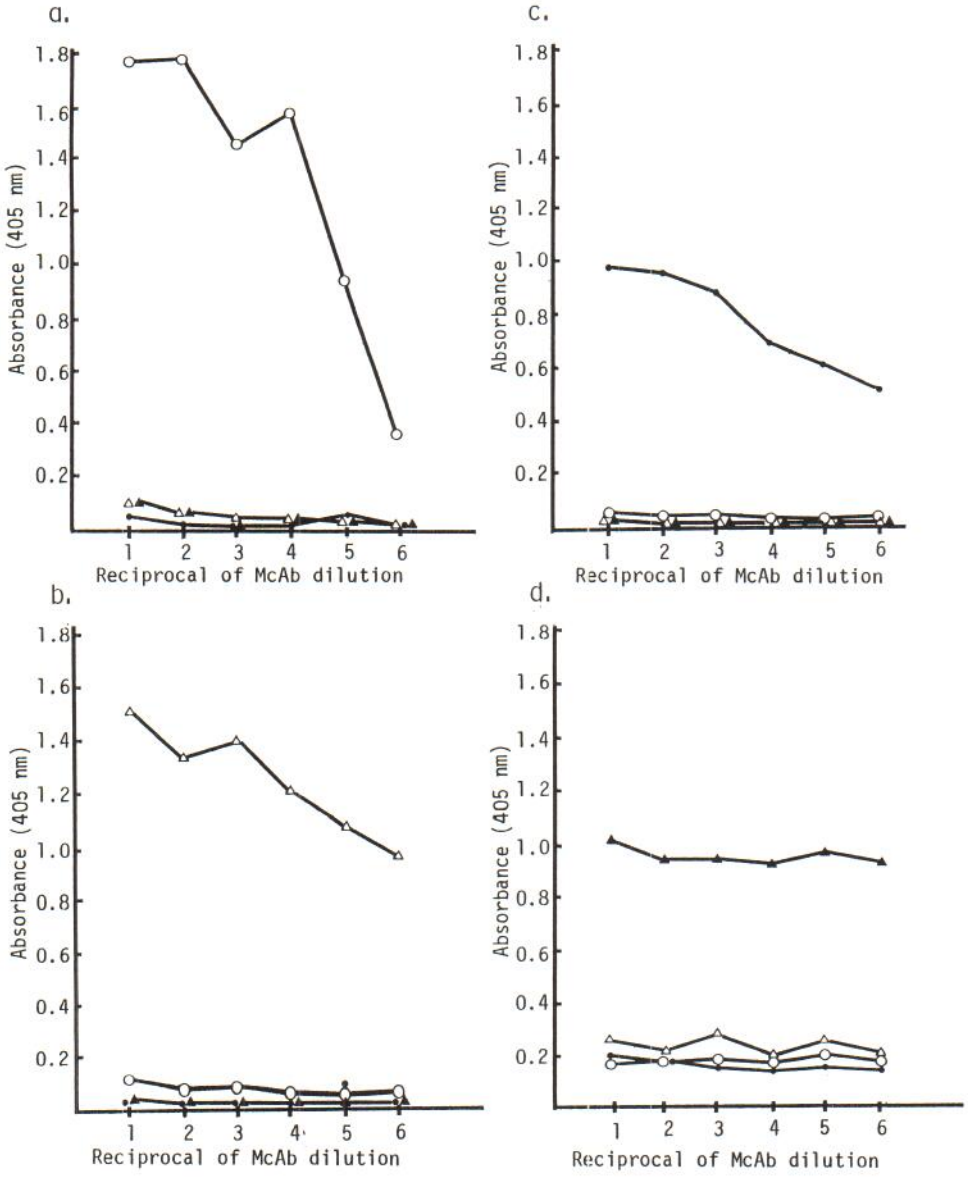

Fig. 2. Direct binding assay in ELISA for estimation of purified IgG subclass fractions (a. IgG_1, b. IgG_2, c. IgG_3, d. IgG_4) from bullous pemphigoid patients. Monoclonal Antibodies (McAbs) were ○, NL-16 (anti-G_1); △, HG2-56F (anti-G_2); ●, HG3-7 (anti-G_3); ▲, HG4-53G (anti-G_4) diluted three fold serially from 2×10^2 (1) to 4.86×10^4 (6).

Table II. *Anti-basement membrane zone (BMZ) antibody titers, complement fixing titers, and protein concentrations of IgG subclass fractions for bullous pemphigoid patients. Prot. conc.: Protein concentration*

Patient		Prot. conc. (mg/ml)	Anti-BMZ antibody titer	Prot. conc. × anti-BMZ antibody titer (μg/ml)	Complement fixing titer	Prot. conc. × complement fixing titer (μg/ml)
B N	IgG_1	6.41	1:50	128	1:10	641
	IgG_2	6.44	1:10	644	(–)	(–)
	IgG_3	1.41	(–)	(–)	(–)	(–)
	IgG_4	2.83	1:100	28.3	(–)	(–)
T Y	IgG_1	5.75	1:50	115	1:10	575
	IgG_2	9.78	1:5	1956	(–)	(–)
	IgG_3	1.60	(–)	(–)	(–)	(–)
	IgG_4	5.21	1:50	104	(–)	(–)
K O	IgG_1	5.62	1:50	112	1:10	562
	IgG_2	3.89	1:5	778	(–)	(–)
	IgG_3	1.17	(–)	(–)	(–)	(–)
	IgG_4	2.05	1:100	20.5	(–)	(–)
S T	IgG_1	9.7	1:100	97	1:50	194
	IgG_2	7.28	1:50	145.6	(–)	(–)
	IgG_3	3.45	(–)	(–)	(–)	(–)
	IgG_4	1.68	1:50	33.6	(–)	(–)

by the absorbance at 280 nm (2138 UVICORD S, LKB, Sweden), was obtained by elution with 0.02M K-PB, pH 8.0. The peak fraction contained IgG. The peak fraction was then applied to a protein A-Cellulofine column (Seikagaku Kougyo, Tokyo, Japan) equilibrated with 0.2M phosphate/0.1M citrate buffer (pH 7.0) and the linear gradient (pH 5 → 3) of the same buffer (12, 13). IgG_3 was passed through the protein A column by elution with pH 7.0. IgG_2 was obtained by elution with the same buffer at about pH 4.7, and IgG_1, IgG_2, and IgG_4 ($IgG_{1,2,4}$) were obtained by elution with the same buffer at pH 3.0. IgG_1, IgG_2, IgG_3 and IgG_4 fractions were then obtained with the combination of IgG subclass-specific immuno-affinity chromatography, as shown in Fig. 1 (11).

In this study we used four kinds of immuno-affinity columns: anti-IgG_3 (HG3-7C) column, anti-IgG_4 (HG4-53G) column, anti-IgG_2 (HG2-56F) column and anti-$IgG_{1,2,3}$ (HG-13F) column. The IgG_1, IgG_2, IgG_3 and IgG_4 fractions were concentrated by using Aquaside II (Calbiochem Co. La Jolla CA). The protein concentration of each fraction was examined by the absorbance at 280 nm (DU-50 Spectrophotometer, Beckman).

5. Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to estimate the contamination of the purified IgG subclasses, based on the reactivities of monoclonal antibodies specific to each one of the four subclasses. Ninety-six well microtiter plates (Coster Lab. Cambridge, MA) were coated with 100 μl of each purified subclass (2 μg/ml) in 0.01M PBS, pH 7.4, at 37°C for 1hr. After blocking with 150 μl of 10% fetal calf serum (FCS) (MA Bioproducts, Maryland) in 0.01M phosphate-buffered saline (PBS) at 37°C for 60 min followed by washing, 100 μl of anti-IgG subclass monoclonal antibody from ascitic fluid, appropriately diluted, was added to each well and incubated at 37°C for 60 min. After washing, 100 μl of a 1:500 dilution of peroxidase conjugated sheep $F(ab')_2$ anti-mouse IgG reagent (no crossreaction with human IgG, Cappel Lab) was added and left to stand at 37°C for 60 min. Development with ABTS solution (Bio-Rad Lab.) was conducted at room temperature for 30 min, then stopped with 2% oxalic acid and read by the absorbance at 405 nm with a microplate reader Mode 13550 (Bio-Rad Lab.).

6. Immunofluorescence (IF)

The IgG_1, IgG_2, IgG_3 and IgG_4 fractions purified from the sera of BP patients and healthy volunteers were incubated with the normal human skin described above to examine the antibody reactivity against BMZ by using the standard indirect IF procedure (14). The complement IF technique was performed according to the method described by Jordon et al. (4). Normal human skin sections were incubated with heat-inactivated (56°C 30 min) IgG subclass fractions, and then with fresh normal human serum as a complement source. They were incubated with FITC-labeled anti-human C3 antisera. FITC-labeled rabbit antisera against human IgG and C3 (Hoechst-Bering, West Germany, F/P molar ratio 2.2, 2.7) were used at 1:10 dilutions.

Direct and indirect IF were then performed using anti-human IgG subclass monoclonal antibodies. In direct IF, perilesional pemphigoid skin sections were incubated with anti-human IgG subclass monoclonal antibodies which were diluted 1:10 and 1:40. They were subsequently incubated with 1:40 diluted FITC-conjugated anti-mouse IgG antibody (Cappel, West Chester, PA, F/P molar ratio 4.76). In indirect IF, normal human skin sections were incubated with 1:10 diluted patients' sera and normal sera as a negative control. The following procedure was carried out according to the above mentioned direct IF method.

RESULTS

1. ELISA

The binding of four purified IgG subclasses on microtiter wells was sufficiently comparable to allow for comparative analysis as previously described (11). Depending on the reactivities of monoclonal anti-IgG subclasses, the coating concentration of purified subclasses and the dilution factor of monoclonal antibodies were appropriately chosen. As shown in Fig. 2, the purified IgG subclasses reacted very strongly with the corresponding monoclonal antibodies in a dose-dependent fashion. They did not react with the non-corresponding monoclonal antibodies. The same results were obtained using a human IgG subclass combi RID kit (University of Birmingham Research Institute, Birmingham, UK).

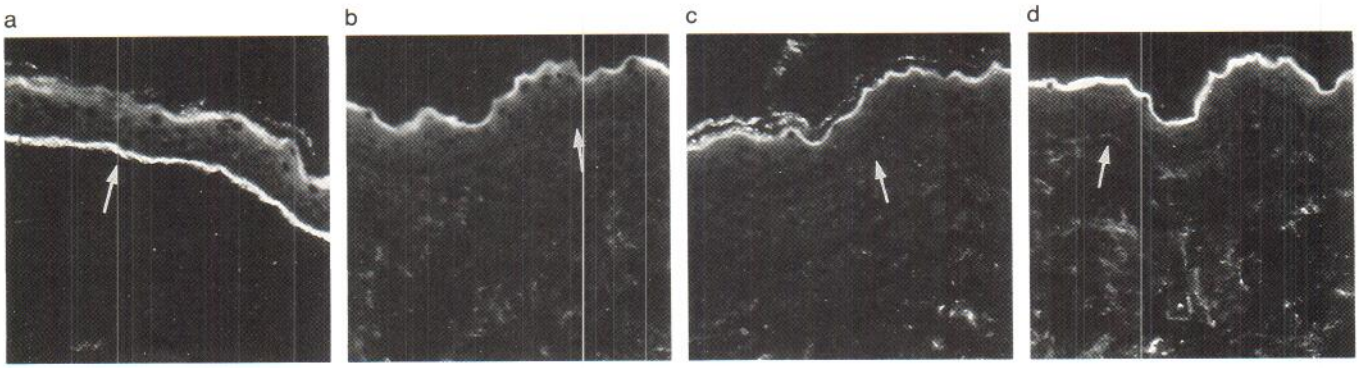

Fig. 3. Complement immunofluorescence of bullous pemphigoid (BP) IgG subclass fraction. C_3 deposit was observed along the basement membrane zone (arrow) of the skin incubated with IgG_1 (a) fraction from BP patient (case N.B.) but was not observed when incubated with IgG_2 (b), IgG_3 (c), or IgG_4 (d).

2. Immunofluorescence (IF) of IgG subclass fraction

The findings of indirect and complement IF are shown in Table II. In all cases, IgG_1, IgG_2 and IgG_4 bound along the BMZ, but IgG_3 did not. To facilitate the comparison with anti-BMZ antibody reactivity and complement fixing reactivity of the four IgG subclasses, we calculated the parameters "Prot. conc. (Protein concentration) X anti-BMZ antibody titer" and "Prot. conc. X complement fixing titer". These parameters reveal the protein concentration of which the anti-BMZ antibody titer or complement fixing titer was 1:1. The smaller the value of the parameters, the higher the anti-BMZ antibody activity and complement fixing capability. The parameter of the protein concentration × anti-BMZ antibody titer showed that even though the protein concentration of IgG_3 was the same as that of the other IgG subclasses, IgG_3 did not exhibit any anti-BMZ antibody activity, while other IgG subclasses did. Furthermore, we also examined the anti-BMZ antibody activity using the sodium chloride-separated skin technique (15). IgG_3 did not demonstrate any anti-BMZ antibody activity in this test either. Complement IF showed that C3 deposition was observed with IgG_1, but not with IgG_2, IgG_3 or IgG_4 (Fig. 3). To insure that IgG_2 and IgG_4 do not inhibit complement fixation in vitro, we examined IgG_1 complement fixation added to IgG_2 and IgG_4. No inhibitory activity of IgG_2 and IgG_4 however, was seen.

3. Direct and indirect immunofluorescence of anti-IgG subclass monoclonal antibodies

The findings for direct IF are shown in Table III. Deposits of IgG_1 were seen in two cases, IgG_2 in one case and IgG_4 in three cases, whereas IgG_3 was not seen. The findings of indirect IF are shown in Table IV. Anti-BMZ antibodies indicated the presence of IgG_1 in three cases, IgG_2 in two cases and IgG_4 in three cases, while IgG_3 was not observed.

DISCUSSION

In this study, we demonstrated that IgG_1, IgG_2 and IgG_4 but not IgG_3 were able to bind to the dermo-epidermal junction in BP. IgG_1 had the capability of fixing complement. In 1973, Sams & Schur first demonstrated that the complement fixing IgG subclass in BP was IgG_3 (6). To explain this difference, we considered the two possibilities. First, the peak fraction might contain not only IgG_3 but also IgG_1 and G_2 when the sera were applied to the DEAE column and equilibrated with 0.01M phosphate buffer of pH 8.0. Our purified IgG subclasses did not react with the non-corresponding monoclonal antibodies using ELISA. Second, the serum which they used might be that of a special case. Later, Carruthers & Ewins showed that the IgG subclasses of their seven BP patients bound to protein A and that complement fixing IgG also bound (7). We showed the same result using the sera of three BP patients (8). These two results support those of this study.

Moreover, in this study we purified IgG subclasses from the sera of four BP patients. IgG_1 was the only complement fixing IgG subclass among the protein A binding BP IgG subclasses.

On the other hand, using monoclonal antibodies to IgG subclasses, Flotte & Bird reported that BP IgG_4 was predominant in both circulating and IgG deposition in perilesional skin (9). IgG_4 does not appear to fix C_1 (5). However, BP antibody fixes complements at the BMZ. A possible explanation of these facts is that IgG_4 may activate the complement via the alternate pathway in BP (16). Complement components of the alternate pathway have been demonstrated in BP lesions

Table III. *Direct immunofluorescent staining for IgG subclass deposits in BP, ND: not done*

Patient	IgG_1	IgG_2	IgG_3	IgG_4
B.N.	N.D.	N.D.	N.D.	N.D.
T.Y.	+	+	−	+
K.O.	+	−	−	+
S.T.	−	−	−	+

Table IV. *Indirect immunofluorescent staining for circulating IgG subclass in BP, ND: not done*

Patient	IgG_1	IgG_2	IgG_3	IgG_4
B. N.	+	+	−	+
T. Y.	+	+	−	+
K.O.	+	−	−	+
S.T.	N.D.	N.D.	N.D.	N.D.

(17). Using serum devoid of properdin as the source of complement, the deposit of properdin was not seen and the intensity of C3 staining diminished. After properdin had been added to the serum, the deposit of properdin was seen and the intensity of C3 staining restored (18). Participation of the alternate pathway in vivo was also suggested by the presence of activated Factor B in BP blister fluids (19). These studies suggest that the BP antibodies will fix components of the alternative pathway.

There is strong evidence that the classical pathway plays a major role in fixing complement deposits at the BMZ (20). These results suggest that IgG_1 may have the most important role in complement activation via the classical pathway in BP. However, there is a possibility that IgG_4 may activate complement via the alternate pathway in vivo.

Complement activation is now considered to be essential in the cascade of blister formation in BP (21–23). However, the importance of the complement as a direct mediator in the early events which initiate the blister formation in BP has recently been questioned (24, 25). In our study, BP IgG_2 and IgG_4 did not fix complement. IgG_2 and IgG_4 may act in complement-independent pathogenic mechanisms, for example, directly affecting basal cells.

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