The Subclass Distribution of IgG Autoantibodies in Cicatricial Pemphigoid and Epidermolysis Bullosa Acquisita

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To study the subclass distribution of autoantibodies and their complement-fixing capacity in cicatricial pemphigoid (CP) and epidermolysis bullosa acquista (EBA) we studied the sera from 23 patients by both indirect immunofluorescence (IIF) on 4-μm cryostat sections of normal human skin and immunoblotting of epidermal or dermal extracts. Monoclonal antibodies of strict specificity for human IgG subclasses were used. Sera from 20 patients with BP served as controls. In addition, total IgG subclass levels were determined by indirect competitive ELISA in all sera. Complement binding capacity was studied by IIF using antibodies to C3 after incubation of skin section with autoantibodies and source of fresh complement.

CP autoantibodies reacting with the 230–240 kD and/or the 180-kD epidermal bands showed an IgG4/IgG1 subclass restriction, with a predominance of IgG4 in 10 cases, of IgG1 in four. In BP sera, IgG4 and IgG1 autoantibodies were detected with a similar frequency (100% and 83%, respectively). In EBA sera, autoantibodies reacting with the 290 kD and 145 kD dermal bands also showed an IgG1/IgG4 restriction. Concordant results were obtained by IIF. However, the IIF method had a lower sensitivity for the detection of IgG4 CP antibodies and IgG1 EBA antibodies than immunoblotting. Finally, when CP antibodies were analyzed for their complement-binding activity, it was found that sera containing IgG4 autoantibodies alone never fixed complement whereas all complement-fixing CP sera had IgG1 autoantibodies, suggesting that only this subclass of antibodies is capable of fixing complement. J Invest Dermatol 97:259–263, 1991

Cicatricial pemphigoid (CP) and epidermolysis bullosa acquista (EBA) are rare autoimmune subepidermal bullous diseases characterized by scar formation and by vivo-bound immunoglobulins and/or complement along the basement membrane zone (BMZ) of the epidermis [1–4]. Circulating anti-BMZ autoantibodies can be detected by indirect immunofluorescence (IIF) in both CP [3,5–7] and EBA [8,9]. Direct and indirect immunoelectron microscopic studies have shown that CP autoantibodies bind within the dermo-

Epidermal junction (DEJ) mostly to lamina densa and to the lower part of lamina lucida [10–16], whereas autoantibodies in EBA bind determinants immediately below the lamina densa [8,10,17]. However, according to the immunoelectron microscopic studies of Briggaman et al [9], the immune deposits in CP and the inflammatory type of EBA were localized both in the lamina densa. These findings raise the question of whether inflammatory EBA and CP autoantibodies bind to the same target antigen. However, immunochromic studies have demonstrated that autoantibodies in EBA and CP are distinct. The antigens of both inflammatory and chronic EBA have been identified as proteins of 145 kD and 290 kD by Western blot analysis on skin-BMZ extracts [9,18]. The EBA antigen has been shown to be identical with the globular carboxyl terminal of type VII procollagen [19]. We have recently reported that IgG autoantibodies in CP antigen detect two antigens of 230–240 kD and/or 180 kD on immunoblots from heat-separated epidermal extracts. The molecular weight of these antigens are similar to those recognized by sera from patients with bullous pemphigoid (BP) [15]. Among autoimmune subepidermal bullous diseases, IgG subclass distribution has best been studied in BP [20–23] and in pemphigoid (herpes) gestationis [24]. The purpose of the present study was 1) to examine the IgG subclass distribution of EBA and CP antibodies by using both Western blot and IIF [25] techniques and 2) to investigate the relation between complement fixation and IgG subclass distribution of the autoantibodies in EBA and CP.

Materials and Methods

Patients and Sera Sera from 14 patients with CP and nine patients with EBA were studied. All patients had subepidermal bullous...
Table I. Total Serum IgG Subclasses Levels in CP, EBA, BP and Control Sera

<table>
<thead>
<tr>
<th>Sera</th>
<th>n</th>
<th>IgG1 (mg/ml)</th>
<th>IgG2 (mg/ml)</th>
<th>IgG3 (μg/ml)</th>
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<tr>
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<td>14</td>
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<tr>
<td>BP*</td>
<td>20</td>
<td>6.13</td>
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<td>EBA</td>
<td>9</td>
<td>11.10*</td>
<td>2.49</td>
<td>340</td>
<td>712*</td>
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<tr>
<td>Normal adult</td>
<td>73</td>
<td>6.08</td>
<td>2.61</td>
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* From [23].
* p < 0.001.
* p < 0.01.
* Not significant.

disease with a chronic course, scarring, and deposits of IgG and/or C3 along the BMZ of the epidermis by direct immunofluorescence. Six patients with CP and two with EBA also had linear IgA deposits in addition to IgG and C3.

The diagnosis of CP was established by two criteria: 1) predilection for mucosal involvement (especially oral and ocular) with fewer skin lesions predominating on the trunk, neck and face [2] (three patients had the chronic scarring form of Brunstig-Perry), and 2) thick discontinuous deposits on the lamina densa and the lower part of lamina lucida by direct immunoelectron microscopy [15].

The diagnosis of EBA was based on the following criteria: 1) acral bullae, usually trauma-induced, healing with scarring and milia formation; and 2) granular deposits beneath the lamina densa by direct immunoelectron microscopy [10]. Sera from patients with typical clinical, immunologic, and immunoelectron microscopic features of BP (n = 20) that contained anti-BMZ antibodies by IIF, or from healthy subjects served as controls. Data of IgG subclasses distribution of these BP patients have been partially published [23].

All serum samples were obtained at time of diagnosis and stored at -70°C.

Total IgG Subclass Measurement: Total IgG subclass levels were measured by an indirect competitive ELISA with monoclonal antibodies (MoAb) as described previously [26]. The MoAb used in this study were NL16 (purchased from Unipath, Bedford, UK) for IgG1, a mixture of HP6014 (provided from Dr CB Reimer, Atlanta, GA, or from Dr. R Jefferis, Birmingham, UK) and GOM2 (Unipath) for IgG2, ZG4 and RJ4 (Unipath) for IgG3, and IgG4, respectively, as reported [26]. These MoAb have previously been shown to be highly specific for IgG subclasses.

Western Immunoblotting

Tissue Extracts: Surgical specimens from normal human skin were used as a source of epidermal extracts. They were prepared from heat-separated epidermis as previously described in detail [27]. To study sera from patients with EBA, skin BMZ extracts were prepared as reported by Stanley et al [28].

SDS-PAGE and Immunoblotting: Epidermal or skin BMZ extracts were subjected to a SDS-PAGE as described by Laemmli [29], with slab gels of 5% acrylamide, then electrophoretically transferred overnight to a 0.45-μm nitrocellulose, as described by Towbin et al [30]. After incubation with 3% bovine serum albumin for 2 h at room temperature, the nitrocellulose strips were incubated with a 1:10 to 1:100 dilution of the tested sera in 10 mM Tris-HCl, 0.5 M NaCl, 3% bovine serum albumin, 0.5% Tween 20 (pH 7.4) at room temperature for 2 h. The strips were washed 5 times with 10 mM Tris-HCl, 0.15 M NaCl, 0.5% bovine serum albumin, and 0.5% Tween 20 (pH 7.4) (washing buffer) and then incubated for 1 h at room temperature, with the following dilutions in washing buffer of the various IgG subclass-specific MoAb, as previously reported [23]: 1:1000 for NL16, 1:200 for GOM2 mixed with 1:3000 HP6014, 1:3000 for ZG4, and 1:9000 for RJ4. After five washes each strip was incubated for 1 h at room temperature with a 1:400 dilution of a peroxidase-conjugated sheep anti-mouse Ig (Amer sham Laboratories UK) in washing buffer. After a final wash, strips were incubated with diaminobenzidine and H2O2. Each serum studied was also tested in the absence of IgG subclass-specific MoAb to verify the specificity. Sera were tested twice, and the average intensity of staining was scored visually from 0 to +++ as reported [23].

Immunofluorescence Studies: Circulating anti-BMZ antibodies were detected by standard (minimal dilution 1:10) IIF techniques on 4-μm cryostat sections of normal human skin as described by others [31]. The IgG subclass distribution of circulating anti-BMZ antibodies was also studied by IIF on 4-μm cryostat sections of normal human skin as previously described by Bird et al [20].

Briefly, cryostat sections of tissue were sequentially incubated with i) patient’s serum at a dilution of 1:10 in phosphate-buffered saline (PBS), ii) IgG subclass-specific MoAb, using the same clones of IgG Mab as in the Western blot studies, in PBS at the dilutions 1:20 for NL16 and GOM2, 1:50 for ZG4, and 1:200 for RJ4, and iii) FITC-labeled sheep anti-mouse Ig 1:40 in PBS (Amersham Lab-

Table II. Immunoblot and IIF Analysis of Circulating CP Autoantibodies

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<th>CP Patient</th>
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* Protein bands from heat separated epidermis.
* Indirect immunofluorescence.
* Patients with localized cutaneous PC of the Brunstig-Perry type.
Figure 1. Subclass immunoblot analysis of CP serum 5 on protein extracts from heat-separated epidermis. The serum was probed for IgG1 (lane 1), IgG2 (lane 2), IgG3 (lane 3), and IgG4 (lane 4) on adjacent nitro-cellulose strips from transfers of the same gel. A negative control, by omission of the IgG subclass MoAb is shown on lane 5. This serum contained IgG2 and IgG4 subclass antibodies to the 180-kD band (arrow).

Figure 2. Subclass immunoblot analysis of EBA serum 1 on skin-BMZ protein extracts. The serum was probed for IgG1 (lane 1), IgG2 (lane 2), IgG3 (lane 3), and IgG4 (lane 4) on adjacent nitro-cellulose strips from transfers of the same gel. This serum shows IgG1, IgG2, and IgG4 subclass antibodies to the 290-kD band (arrow) and IgG4 subclass antibodies to the 145-kD band (arrowhead).

RESULTS

Total IgG Subclass Measurement Table I shows the total serum IgG subclass levels of CP patients compared with those of BP and EBA patients, or normal subjects in the same age range (50–95 years). Total serum IgG subclass concentrations of CP patients were not significantly different from those of normal controls. However, the IgG1 subclass concentration (mean, 11.10 ± 3.8 mg/ml; range, 5.1–23.3) was significantly raised in EBA patients (Wilcoxon non-parametric test, p < 0.01) when compared to normal subjects. More precisely, seven of the nine EBA patients showed an increased IgG1 concentration (i.e., higher than 7 mg/ml). The IgG4 subclass concentrations in BP patients were significantly higher than in normal controls, as previously reported [23].

Immunoblotting All 14 CP sera detected either 180-kD or 230–240 kD epidermal antigens. Ten sera from patients with CP reacted with an epidermal antigen of 180 kD, 2 CP sera detected a 230–240-kD antigen, and 2 CP sera bound to both the 180- and 230–240-kD antigens. All CP sera contained IgG4; nine, IgG1; two IgG2; and one, IgG3 autoantibodies (Table II). At a dilution of 1:20, five CP sera contained IgG4 but no other subclass antibodies. In the remaining nine CP sera, autoantibodies belonged to several IgG subclasses (Fig 1, Table II). The strongest staining within the four IgG subclasses was observed with IgG4 from ten sera, and with IgG1 from four sera. In our CP sera, there was no relation between the position of the bands by Western blotting, i.e., its molecular weight and the IgG subclass of the autoantibodies (Table II). In our BP sera, autoantibodies reacted with similar 230–240-kD and/or 180-kD bands by immunoblotting, and IgG4 and IgG3 autoantibodies were detected with a similar frequency (100% and 85%, respectively) than in CP [23]. IgG4 autoantibodies always predominated, as previously reported in detail [23]. In EBA, circulating autoantibodies reacting with the 290-kD and 145-kD dermal bands (Fig 2) were detectable in six of nine sera at a dilution of 1:10. They were of IgG1 subclass in six cases, IgG4 subclass in five cases, and IgG3 subclass in two cases (Table III). The strongest staining was observed in the IgG4 subclass in three cases, in the IgG1 subclass in two cases, and in IgG4, in one case. As in CP sera, there was no relation between the molecular weight of the bands by Western blotting and the IgG subclass distribution of the EBA autoantibodies.

Immunofluorescence Studies Results of IIF studies of IgG subclass distribution of CP and EBA antibodies are detailed in Tables II and III. They were superposable to the Western blot results except for one EBA serum (from patient 6) in which IgG1/ IgG4 antibodies were found by immunoblotting, and IgG2/IgG4 by...
Table III. Immunoblot and IIF Analysis of Circulating EBA Autoantibodies

<table>
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<tr>
<th>EBA Patient</th>
<th>Immunoblot</th>
<th>IIF*</th>
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<tr>
<td></td>
<td>290 kDa*</td>
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<tr>
<td></td>
<td>IgG₁, IgG₂, IgG₃, IgG₄</td>
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<td>Total</td>
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* Protein bands from skin-BMZ extracts.
* Indirect immunofluorescence.

IIF. The Western blot technique generally had a higher sensitivity than the IIF, particularly for the detection of IgG₄ CP autoantibodies and for the detection of IgG₁ EBA autoantibodies (Tables II and III). Six of 14 CP sera demonstrated C3-fixing anti-BMZ antibodies. All CP sera fixing C3 had autoantibodies of the IgG₄ subclass. In contrast, all CP sera that contained only IgG₄ autoantibody did not fix C3. Furthermore, IgG₄ subclass profiles of autoantibodies in CP were not relevant to the presence of C3 deposits along the BMZ by direct immunofluorescence, which were seen in 13 of 14 patients, including the four with only circulating IgG₂ antibodies. The only EBA serum (from patient 6) that fixed C3 demonstrated IgG₁ and IgG₄ antibodies by Western blot and IgG₂ and IgG₄ antibodies by IIF.

**DISCUSSION**

To date, the IgG₄ subclass distribution of autoantibodies in subepidermal bullous diseases has been studied by immunofluorescence techniques [20–22,24,25]. Using MoAb with strict specificity for IgG₄ subclasses, several authors have shown that in BP IgG₄ is the predominant autoantibody both in skin and serum [20–22]. Recently, we have confirmed these data by using Western blot analysis of 20 BP sera with MoAb highly specific for IgG₄ subclasses [23]. However, in this latter study the IgG₄ subclass restriction of pemphigoid antibodies was not absolute, because BP sera frequently contained low levels of IgG₁ and/or IgG₂ antibodies in addition to IgG₄. Our data also confirmed that IgG₄ represents by far the least important subclass in BP antibodies [20–23]. Finally, a recent study has clearly shown that pemphigoid (herpes) gestationis factor is an IgG₄ antibody with involved complement binding capacity.

The present study establishes distinct profiles of IgG₄ subclass autoantibodies in CP and EBA, with IgG₄ or IgG₂ being predominant. In BP sera IgG₄ autoantibodies always predominated. The IgG₄ subclass profiles differ from that of BP by the predominance of IgG₁ antibodies over IgG₄ in some CP (four of 14) and EBA (two of six) sera. Total IgG₄ subclass determination showed that IgG₁ was significantly elevated in EBA. Total IgG₄ subclass levels in CP were normal (Table I). Whereas no previous data were available concerning IgG₄ subclass restriction in CP, the distribution of IgG₄ subclass autoantibodies in EBA found in this study is different from that reported by Mooney and Gammon [25]. They found that EBA autoantibodies belonged to all IgG subclasses and that there was no relation between IgG subclasses and complement binding ability of autoantibodies by direct or indirect IF [25]. The differences between these and our results might be due to i) the fact that in the study of Mooney, patients were included according to criteria of inflammatory EBA [9], whereas seven of our nine patients presented the chronic form; ii) the use of different MoAb and working dilutions; and iii) the relative lack of sensitivity of the IIF technique for the detection of IgG₁ anti-BMZ antibodies (Table III). However, further studies are necessary to determine whether the IgG₄ subclass distribution of EBA or CP autoantibodies is associated with different stages of the disease, as it has been reported in pemphigus vulgaris [33] and/or with the clinical variants. It remains striking that IgG autoantibody activity resides mainly in the IgG₄ and the IgG₂ subclasses in BP [20–23,34,35], CP, and chronic EBA (present study), as in pemphigus [33,36].

Except in one EBA case, Western blot and IIF yielded concordant results of IgG₄ subclass distribution in the three groups of patients studied. The Western blot technique showed clearly a higher sensitivity, especially for the detection of IgG₁ and IgG₄ autoantibodies, and this despite the use of the same IgG₄ subclass-specific MoAb and the same lot of sheep anti-mouse Ig. Because the Western blot separates SDS-denatured antigens based on their migration in polyacrylamide gel, i.e., their molecular weight, this technique also allowed us to compare the IgG₄ subclass of autoantibodies with the molecular weight of the antigens they detect. But in contrast to our previous findings in BP sera [23], there was no relationship between the molecular weight of antigens and the subclass distribution of IgG₄ autoantibodies in both CP and EBA sera.

When the IgG₄ subclasses of CP antibodies were analyzed for their complement-binding activity, it was found that non-complement fixing CP sera contain only IgG₄ autoantibodies as reported in BP [23,34,35] and that all complement-fixing CP sera had IgG₂ autoantibodies. This suggests that the presence of IgG₂ CP antibodies is required for complement fixation. However, not all CP antibodies of the IgG₂ subclass were identified to fix complement in vitro. This may be partly due to the lack of sensitivity of the immunofluorescence technique used to study complement fixation, especially when IgG₂ antibodies were present at very low titers, as was the case in both CP and chronic EBA. Because we were unable to demonstrate in vitro complement fixation by circulating autoantibodies of the IgG₂ subclass in CP and EBA, it is possible that this antibody mediates tissue alteration by other mechanisms than complement activation.

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**REFERENCES**

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