The Major Cicatricial Pemphigoid Antigen Is a 180-kD Protein that Shows Immunologic Cross-Reactivities with the Bullous Pemphigoid Antigen

Philippe Bernard, Catherine Prost, Nathalie Durepaire, Nicole Basset-Seguin, Liliane Didierjean, and Jean-Hilaire Saurat

Department of Dermatology (PB, LD, J-HS), University Canton-Hospital, Genève, Switzerland; Department of Dermatology (PB) et Laboratory of Virology (ND), University Hospital Dupuytren, Limoges; INSERM U 312 (cP), University Hospital Henri Mondor, Créteil; and Department of Dermatology (NB-S), University Hospital St. Charles, Montpellier, France

Recent studies have shown that sera from patients with cicatricial pemphigoid (CP) contained autoantibodies against epidermal antigens of molecular weight 230 kD and/or 180 kD by immunoblotting, similar to those recognized by bullous pemphigoid (BP) sera. Previous immunoprecipitation studies have shown that BP sera only precipitated the 230-kD antigen. To characterize the CP antigen(s) we tested 10 CP sera, 10 BP sera, and four controls by both immunoprecipitation of radiolabeled cells and immunoblotting of epidermal extracts. For immunoprecipitation, we used 0.5% NP-40 extracts of both normal human keratinocytes and Pam cells. All CP sera precipitated a 180-kD protein that co-migrated with the BP180 antigen precipitated by some individual BP sera. Two of these CP sera also faintly bound a 230-kD protein of similar molecular weight as the major BP230 antigen. CP and BP sera with an immunoblotting pattern of 180 kD immunoprecipitated a co-migrating 180-kD protein. CP sera reacting by immunoblotting with the 230-kD antigen precipitated the 180-kD and/or the 230-kD antigen. In contrast, BP sera reacting with the 230-kD antigen only precipitated this antigen. In further experiments, labeled 0.5% NP-40 extracts from Pam cells were first preabsorbed with a reference BP serum and then immunoprecipitated with CP sera. Under these conditions, CP sera that immunoprecipitated both 180-kD and 230-kD proteins with the standard procedure no longer precipitated these proteins. Our results suggest that a 180-kD protein is the major CP target-antigen that demonstrated immunologic cross-reactivities with the BP180 and the BP230 antigens. *J Invest Dermatol 99:174–179, 1992*

Cicatricial pemphigoid (CP) is an autoimmune subepidermal bullous disease characterized by mucous membrane involvement and scar formation and by in vivo bound immunoglobulins and/or complement along the basement membrane (BMZ) of the epidermis and other affected tissues [1,2]. Circulating anti-BMZ autoantibodies can be detected by indirect immunofluorescence (IIF) in 25–75% of patients with CP using various substrates [1–4]. Direct immunoelectron microscopic studies have demonstrated that CP autoantibodies bind in vivo within the dermo-epidermal junction (DEJ) to the lower part of lamina lucida and to lamina densa [5–8]. A recent indirect immunoelectron microscopic study has shown that CP autoantibodies bind determinants that are localized extracellularly beneath the hemidesmosomes of basal keratinocytes and to the adjoining lamina densa within the DEJ of normal human skin [9]. This suggests that CP antigen is different from the 230-kD bullous pemphigoid (BP) antigen that is mostly localized intracellularly to the hemidesmosomes of basal keratinocytes [9–14].

So far, characterization of target-antigen(s) in CP has been carried out by immunochemical means. By immunoblotting of extracts of normal human skin, autoantibodies from patients with CP bind a 230–240 kD protein [8,15,16] and/or a 180-kD protein [8]. The molecular weights of these proteins are similar to BP230 and BP180, the major antigens recognized by BP autoantibodies [15–21]. Recently, the isolation of partial cDNA clones corresponding to the BP230 and BP180 antigens and further sequence analysis have been reported [22–25]. Such data at the molecular level are lacking concerning the nature of target-antigen(s) in CP. The purpose of the present study was 1) to characterize CP antigen(s) by immunoprecipitation using CP autoantibodies, which are restricted to Ig4 and IgG1 subclasses [26], and 2) to further study the relationship between CP and BP antigens by sequential immunoprecipitations.

MATERIALS AND METHODS

Patients and Sera Sera from 10 patients with CP were used in this study. They have been selected in this study on the basis of the

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Reprint requests to: Philippe Bernard, Service de Dermatologie, CHU Dupuytren, 2, avenue Alexis Carrel, 87042 Limoges cedex, France.

Abbreviations:
- BMZ: basement membrane zone
- BP: bullous pemphigoid
- CP: cicatricial pemphigoid
- DEJ: dermo-epidermal junction
- DMEM: Dulbecco's modified Eagle's medium
- ELISA: enzyme-linked immunosorbent assay
- FITC: fluorescein isothiocyanate
- IIF: indirect immunofluorescence
- NP-40: Nonidet P-40
- PAGE: polyacrylamide gel electrophoresis
- PBS: phosphate-buffered saline
- PV: pemphigus vulgaris
- SDS: sodium dodecyl sulfate

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Table I. Immunoprecipitation and Western Blot Data of Patients with Cicatricial Pemphigoid (CP) and Bullous Pemphigoid (BP)

CP			BP		
Patient (number)	Westernblot (kD)	Immunoprecipitation (kD)	Patient (number)	Westernblot (kD)	Immunoprecipitation (kD)
1	230	230 + 180	11	230	230
2	230	180	12	230	230
3	230 + 180	230 + 180	13	230	230
4	230 + 180	180	14	230	230
5	180	180	15	230	230
6	180	180	16	230	230
7	180	180	17	180	180
8	180	180	18	180	180
9	180	180	19	180	180
10	180	180	20	180	180

presence of circulating anti-BMZ antibodies by IIF among 26 CP patients prospectively studied in two centers from 1985 to 1990. Circulating anti-BMZ IgG antibodies either by standard IIF [27] or by IIF using monoclonal antibodies specific for human IgG subclasses [26] on normal human skin substrate. Anti-BMZ antibody titers ranged from 1/10 to 1/80. All patients had a subepidermal bullous disease with clinical features of CP and deposits of IgG and/or C3 along the BMZ of the epidermis by direct immunofluorescence.

Patients with CP had clinically a chronic and scarring disease with a predilection for mucosal involvement (especially oral and ocular) contrasting with a less important skin involvement [2]. Two patients had a chronic scarring cutaneous localized form of the Brunstig-Perry type [2,28]. Four patients with CP also had linear IgA deposits in addition to IgG and C3 by direct immunofluorescence. Direct immunoelectron microscopy was performed in all cases and showed thick and discontinuous immune deposits localized on lamina densa and to the lower part of lamina lucida, as previously reported [8].

The controls included sera of ten patients with typical clinical, immunologic, and immunoelectron microscopic features of BP that contained circulating anti-BMZ IgG antibodies by IIF ranging from 1/10 to >1/3200; sera of two patients with pemphigus vulgaris (PV) that contained IgG antibodies that bind cell surface of keratinocytes by IIF ranging from 1/50 to 1/200 on normal skin substrate; and sera of four healthy subjects.

Serum samples were obtained at time of diagnosis and stored at −70°C until they were analyzed.

Western Immunoblotting Two percent sodium dodecyl sulfate (SDS) protein extracts of normal heat-separated epidermis or cultured normal human keratinocytes subjected to SDS-polyacrylamide gel electrophoresis (PAGE) were electrophoretically transferred to nitrocellulose and stained by the peroxydase method using a peroxydase-conjugated anti-human IgG (H+L) goat antibody (Institut Pasteur, Marnes la Coquette, France) as previously reported [20].

Immunoprecipitation

Standard Technique: Pam cells, a mouse epidermal cell line [29], and normal human keratinocytes were used in the present study. Pam cells were grown in RPMI 1640 supplemented with 10% fetal calf serum. Human keratinocytes from normal human skin obtained after surgery were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum or in MCDB 153 medium. Subconfluent cultures were radiolabeled for 18 to 24 h at 37°C with 50 μCi/ml of (^{35}S) methionine (Amersham). The radiolabeled cell layer was then extracted with 0.5% Nonidet P-40 (NP-40) and the solubilized proteins immunoprecipitated using patient sera and protein A–bearing staphylococci (Pansorbin, Calbiochem, La Jolla, CA) as previously described by Stanley et al [17]. The technique included a preabsorption with a normal human serum (or a PV serum in some experiments) prior to the immunoprecipitation of protein extracts with the CP or BP sera. Antigen-antibody complexes were eluted from the protein A and dissociated in 100 μl of gel sample buffer containing 2% SDS and 0.1 M dithiotreitol. The resultant proteins were separated by SDS-PAGE on 6% or 7% polyacrylamide gels. Gels were fluorographed and autoradiographed for 4–10 d at −70°C. Each serum was tested at least twice.

Sequential Immunoprecipitation: In these experiments, 0.5% NP-40–labeled extracts were immunoabsorbed using 10 μl of a CP, BP, or PV serum (instead of a normal human serum as in the standard procedure) and 100 μl of 10% (w/v) protein A–bearing staphylococci (1 h × 2) prior to the immunoprecipitation with CP sera. In other experiments, protein extracts were immunoabsorbed using a CP serum and protein A prior to the immunoprecipitation with control BP sera. Thus, several types of control experiments were carried out, including 1) BP-CP; 2) PV-CP; 3) CP-BP; 4) CP-(autologous) CP and CP-(heterologous) CP sequential immunoprecipitations.

RESULTS

Western Immunoblotting By immunoblotting from both epidermal and keratinocytes extracts, all sera from patients with either CP or BP reacted with either 180-kD or 230-kD antigens. Six CP sera specifically reacted with an epidermal antigen of 180-kD, two CP sera bound to a 230-kD antigen, and two CP sera recognized both the 180-kD and the 230-kD antigens (Table I). Six BP sera only recognized the 230-kD antigen and four the 180-kD antigen.

Immunoprecipitation

Standard Technique: Results of immunoprecipitation experiments (including a pre-clearing with a normal human serum) performed with CP sera are summarized in Table I. Eight CP sera immunoprecipitated a 180-kD protein (Fig 1, *lanes 2* and *3*) of apparently identical molecular weight as the 180-kD protein precipitated by some BP sera (Fig 1, *lane 1*). This 180-kD protein was specific for these sera because normal human sera did not precipitate this band. Two CP sera precipitated both a 180-kD protein and a 230-kD protein (Fig 2, *lanes 3* and *4*). Six BP sera precipitated a similar 230-kD protein (Fig 2, *lane 2;* Fig 3, *lanes 1* and *2*). Four BP sera only precipitated a 180-kD protein. Similar results were obtained with different extracts from either Pam cells or normal human keratinocytes.

Immunoprecipitation Pattern Versus Western Immunoblotting: CP sera reacting by immunoblotting with the 180-kD antigen alone immunoprecipitated a similar 180-kD protein. On the contrary, a discrepancy between immunoblotting and immunoprecipitation patterns was observed in CP sera reacting by immunoblotting with the 230-kD antigen. These sera precipitated a 180-kD protein either alone or associated to the 230-kD protein (Table I). In contrast, immunoblotting and immunoprecipitation patterns of the 10 control BP sera were identical. Particularly, the four BP sera reacting by immuno-

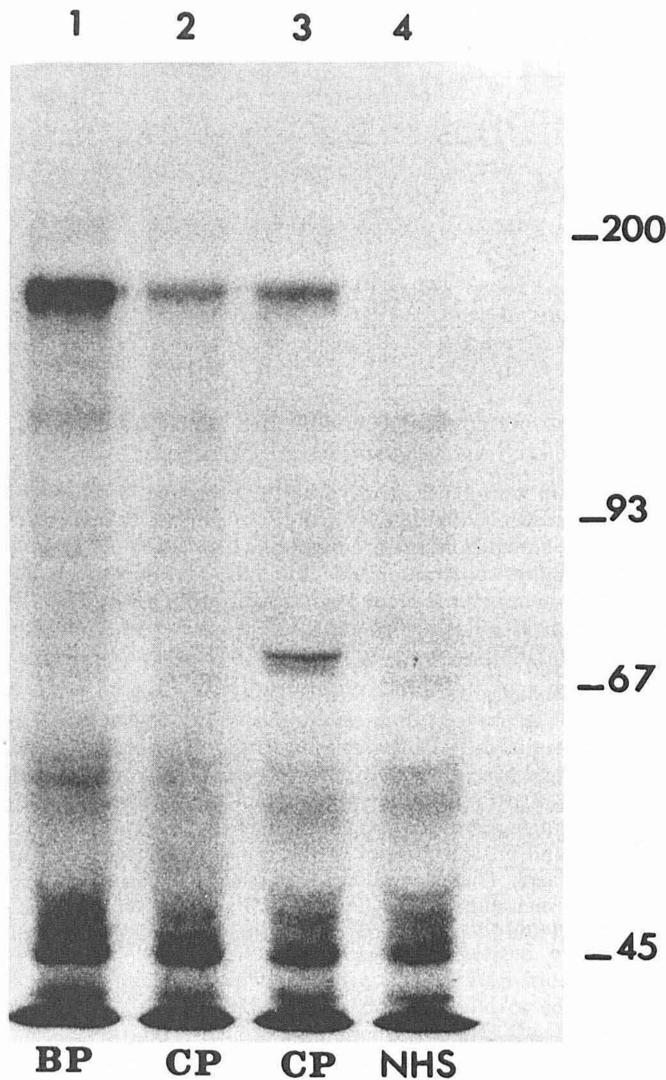

Figure 1. 180-kD antigen immunoprecipitated from 0.5% NP-40 extracts of cultured keratinocytes. Fluorography of SDS-PAGE-separated, ^{35}S labeled immunoprecipitates with BP serum number 17 *(lane 1)*, CP sera numbers 6 and 7, respectively (*lanes 2* and *3*) or normal human serum *(lane 4)*. Concurrently electrophoresed standards are indicated at right (kD). A protein of 180 kD is specifically precipitated by these CP and BP sera that had an immunoblotting pattern of 180 kD.

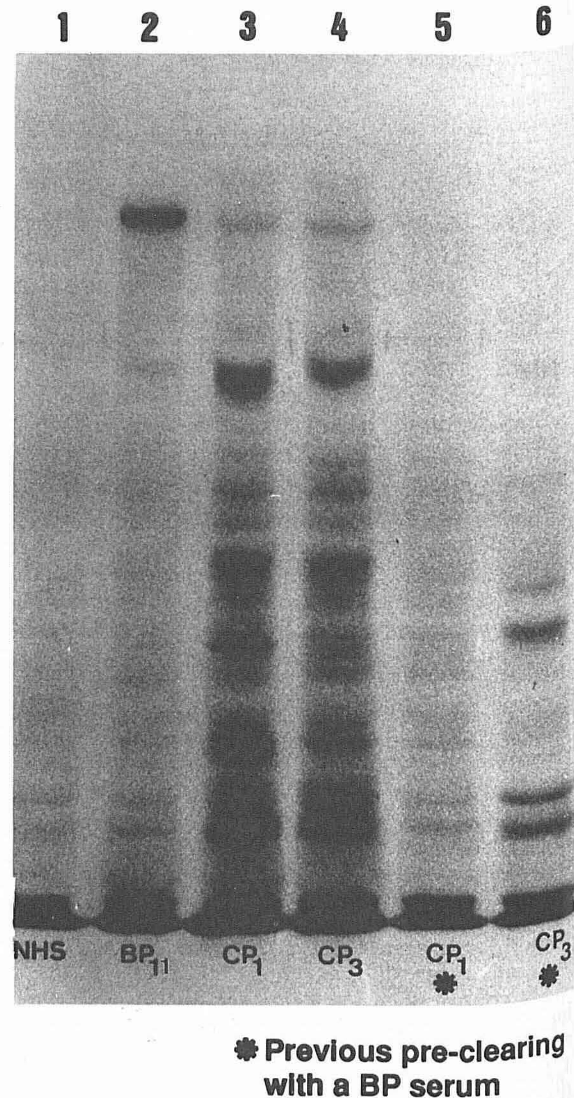

Figure 2. CP sera no longer precipitated the 180-kD and 230-kD proteins when the extracts were preabsorbed with a BP serum. Immunoprecipitation of 0.5% NP-40 extracts from ^{35}S labeled Pam cells with BP, CP, or normal human sera. *Lane 1* is a control with normal human serum used for immunoprecipitation. *Lane 2* shows the immunoprecipitation of the 230-kD antigen with BP serum number 1. *Lanes 3* and *4* show immunoprecipitation of the 180-kD and (faintly) of the 230-kD antigens with two CP sera, numbers 1 and 3, respectively (standard technique including a preabsorption with a normal human serum). *Lanes 5* and *6* show immunoprecipitation with the same CP sera including a pre-clearing of the extract with reference BP serum. Under these conditions CP sera no longer precipitate either the 180-kD or the 230-kD antigen.

blotting with the 180-kD antigen alone immunoprecipitated a similar 180-kD protein from 0.5% NP-40 protein extracts (Table I).

Sequential Immunoprecipitations: Labeled 0.5% NP-40 extracts from Pam cells were first preabsorbed with either a BP serum with an immunoblotting pattern of 230-kD or a normal human serum, and then immunoprecipitated with CP sera. Under these conditions, CP sera that immunoprecipitated both 180-kD and (faintly) 230-kD proteins with the standard procedure (Fig 2, *lanes 3* and *4*) no longer precipitated these proteins (Fig 2, *lanes 5* and *6*). On the contrary, BP sera still precipitated the 230-kD or the 180-kD proteins when the technique included a pre-clearing with a CP serum (data not shown). In another series of experiments, labeled 0.5% NP-40 extracts from normal human keratinocytes were first preabsorbed with a PV serum, a normal human serum, or a CP serum with an immunoblotting pattern of 180 kD and then immunoprecipitated with the same CP serum that were used for preabsorption. Under these conditions, the CP serum still precipitates the 180-kD antigen after an irrelevant precipitation with a PV serum (data not shown), and, although faintly, after a previous precipitation with the autologous CP serum or a CP serum of identical immunoblotting pattern (Fig 4, *lanes 2* and *3*).

DISCUSSION

In recent years, considerable efforts have been made to establish the precise nature of the target-antigen(s) recognized by sera from patients with BP. Immunochemical studies have demonstrated that there were two major BP antigens of apparent molecular weight of 230 kD and 180 kD referred to as BP230 and BP180 antigens, respectively [15–21,30,31]. Two BP antigen cDNA clones have been recently identified corresponding to the BP230 and BP180

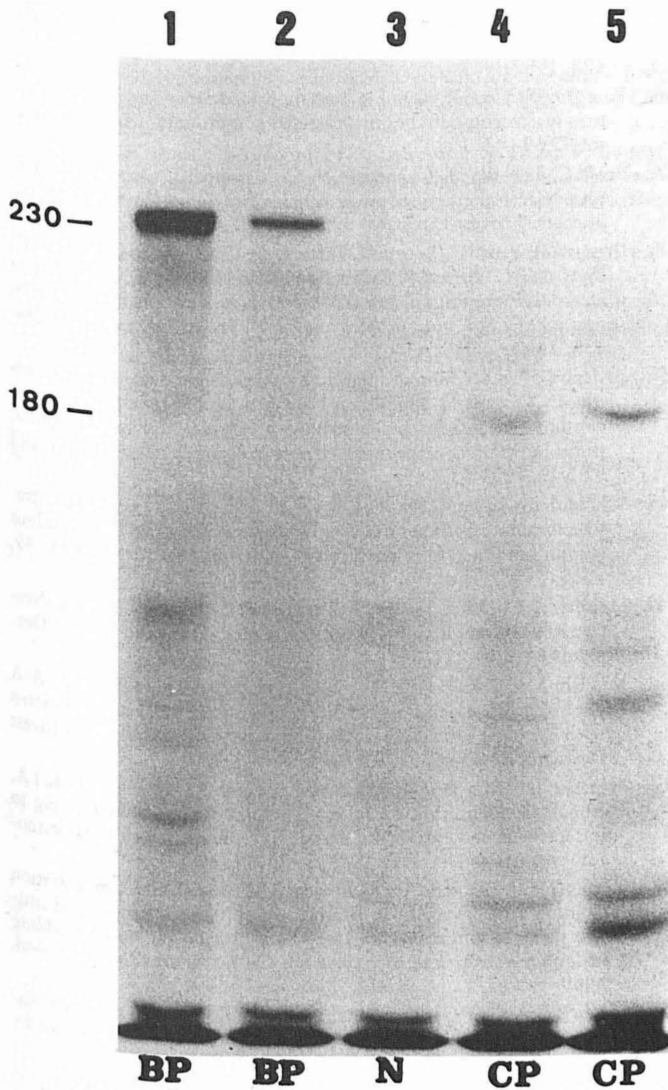

Figure 3. Fluorography of SDS-PAGE-separated, ^{35}S labeled Pam cells, immunoprecipitates with BP sera numbers 11 and 13 (*lanes 1* and *2*), CP sera numbers 5 and 9 (*lanes 4* and *5*), or normal human serum (*lane 3*). This figure illustrates the typical immunoprecipitation pattern of 230 kD with BP sera and 180 kD with CP sera.

epidermal antigens [22–25]. Northern blots have revealed that these two BP antigens are encoded by distinct RNA transcripts [24]. Sequence analysis has shown a significant homology between BP230 antigen and desmoplakin I and II, whereas BP180 antigen contains two collagen domains [25]. Both BP antigens are components of the hemidesmosomes, as demonstrated both by immunoelectron microscopic studies [9,12,13,22–24,30] and biochemical procedures [32].

In contrast, the nature of the target antigens involved in CP has been studied only by means of immunoblotting [8,15,16]. In the present study we have demonstrated that a 180-kD protein is the major target-antigen recognized by autoantibodies from patients with CP. By immunoprecipitation of extracts of cultured keratinocytes, all CP sera bound this 180-kD protein. Two of these CP sera also faintly bound a 230-kD protein of similar molecular weight as the major BP230 antigen. None of CP sera precipitates a 120-kD protein, previously reported as recognized by some CP sera using immunoblotting [16]. The CP 180-kD protein co-migrated with the BP180 antigen precipitated by some BP sera. As a rule, it has been shown in previous studies that BP sera only precipitated the major BP230 antigen [17,19], even when sera had an immunoblotting pattern of 180 kD [31]. With respect to these data, we were surprised to find that same pattern of 180 kD by immunoprecipitation for BP sera that reacted with the BP180 antigen by immunoblotting. However, in a recent report, the 180-kD BP antigen was also identified by immunoprecipitation of (^{14}C) keratinocyte extracts with BP sera [33]. Furthermore, our results concerning the immunoprecipitation of the 180-kD protein with some BP sera were confirmed by repeated assays using different extracts from normal human keratinocytes cultured in two different conditions or from a mouse epidermal cell line (Pam cells). Finally, heterogeneity of bullous pemphigoid antigen had been demonstrated even using immunoprecipitation of NP-40 extracts of cultured cells derived from a squamous cell carcinoma line [34].

We found differences between immunoblotting and immunoprecipitation patterns only in CP sera reacting by immunoblotting with the 230-kD antigen, which precipitated a 180-kD protein

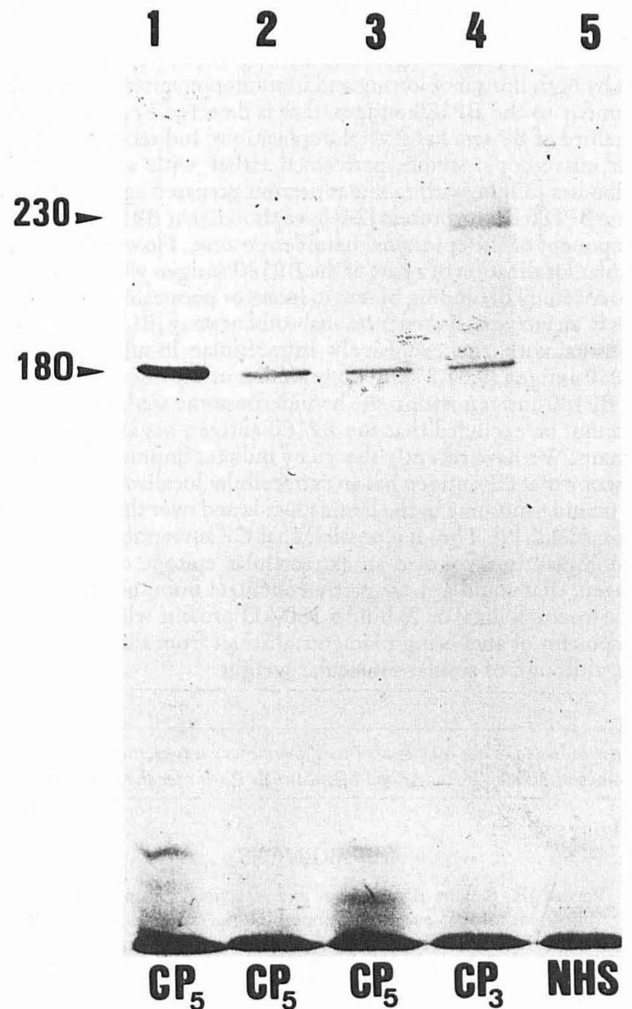

Figure 4. Immunoprecipitation of 0.5% NP-40 extracts from cultured keratinocytes with CP or normal human sera. Fluorography of SDS-PAGE-separated, ^{35}S-labeled immunoprecipitates with a CP serum number 5 (*lanes 1* to *3*), CP serum number 3 (*lane 4*) or normal human serum (*lane 5*). Labeled extracts were first preabsorbed with a normal human serum (*lanes 1, 4*, and *5*), CP serum number 5 (*lane 2*), and CP serum number 8 (*lane 3*). This figure shows that small but significant amounts of 180-kD CP antigen are left after a previous pre-clearing with CP sera with an immunoblotting pattern of 180 kD (*lanes 2* and *3*).

either alone or associated with the 230-kD protein. The discrepancies between these and previous results do not reflect major differences in techniques because we have used the same well-established immunoprecipitation procedure [17] used in other studies [19,30,31]. However, our results indicate that 180-kD and 230-kD proteins precipitated by CP sera have a molecular weight similar to that of the BP180 and BP230 antigens, respectively. Although it is established that the BP180 antigen is not a degradation product of the BP230 antigen [30,35,36], immunologic cross-reactivities between the BP230 and BP180 antigens have recently been demonstrated using affinity-purified antibodies against these antigens and immunoprecipitation technique [30]. The results of sequential immunoprecipitations of NP-40 protein extracts with successively BP and CP sera indicate immunologic cross-reactivities in addition to similar molecular weight. Unfortunately, we were not able to demonstrate the converse, i.e., that BP sera no longer precipitated the 230-kD or the 180-kD proteins when the technique included a pre-clearing with a CP serum. This is probably due to low amounts of CP antibodies (titer of anti-BMZ antibodies in CP sera of 1/10 by IIF, compared with 1/800 to 1/3200 for BP sera) that might have been insufficient to precipitate all BP antigen in the first step of these experiments. This was suggested by the result of a CP-CP sequential immunoprecipitation that showed that some CP antigen can be left after a first immunoprecipitation (Fig 4).

That the 180-kD antigen that is recognized by a majority of CP sera by both immunoblotting and immunoprecipitation techniques is similar to the BP180 antigen that is detected by approximately one third of BP sera has several implications. Indirect immunoelectron microscopic studies performed either with affinity-purified antibodies [30] or with rabbit antiserum prepared against the lysogenic BP180 fusion protein [24] have shown that BP180 antigen is a component of the epidermal hemidesmosome. However, an extracellular localization of a part of the BP180 antigen was suggested by a recent study of binding of sera to intact or permeabilized SCaBER cells in an enzyme-linked immunabsorbent assay [ELISA] [31]. This contrasts with the exclusively intracellular localization of the BP230 antigen [9,31,37]. Because studies of the fine localization of the BP180 antigen within the hemidesmosome are lacking to date, it cannot be excluded that the BP180 antigen has an extracellular domain. We have recently shown by indirect immunoelectron microscopy that CP antigen has an extracellular localization in front of the hemidesmosome in the lamina lucida and over the upper part of lamina densa [9]. Thus it is possible that CP autoantibodies either 1) predominantly recognize an extracellular epitope of the BP 180 antigen, that could at least partly conciliate immunochemical and ultrastructural data; or 2) bind a 180-kD protein which could be a component of anchoring filaments, distinct from the BP 180 antigen, although of similar molecular weight.

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