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How to cite

SKARIA, Mouna. La caractérisation de la réponse immune dans la pemphigoïde bulleuse. Doctoral Thesis, 2008. doi: 10.13097/archive-ouverte/unige:550

This publication URL: https://archive-ouverte.unige.ch/unige:550
Publication DOI: 10.13097/archive-ouverte/unige:550

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UNIVERSITE DE GENEVE
Département de neurosciences
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FACULTE DE MEDECINE
Section de médecine clinique
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Service de dermatologie

Thèse préparée sous la direction du Professeur Luca Borradori

La caractérisation de la réponse immune dans la pemphigoïde bulleuse

Thèse

présentée à la faculté de médecine de l'université de Genève

pour obtenir le grade de docteur en médecine

par

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de

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Thèse n **Méd. 10537**

Genève 2008

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Abréviations

BP230 : antigène de la pemphigoïde bulleuse 230

BP180 : antigène de la pemphigoïde 180

DH : dermite herpétiforme

EBA : épidermolyse bulleuse acquise

LP : lichen plan

PB : pemphigoïde bulleuse

IgA, E, G : immunoglobuline de type A, E, G

C3 : facteur du complément 3

IFD : immunofluorescence directe

IFI : immunofluorescence indirecte

IME : immunoélectronmicroscopie

kDa : kilodalton

PG : pemphigoïde gestationelle / gravidique (herpès gestationis)

PC : pemphigoïde cicatricielle

LEB : lupus érythémateux bulleux

LES : lupus érythémateux systémique

JDF : jonction dermo-épidermique

ECD : domaine extracellulaire de BP180

ICD : domaine intracellulaire de BP180

1) Introduction

Les maladies bulleuses auto-immunes sous-épidermiques constituent un groupe hétérogène de dermatoses caractérisées par la présence d'auto-anticorps dirigés contre des composants de la jonction dermo-épidermique. Actuellement, on distingue six formes de maladies bulleuses auto-immunes sur la base de caractéristiques cliniques, histologiques et immunopathologiques (**tableau I**). Leur classification repose sur l'antigène cible reconnu par les auto-anticorps. Leur incidence est plutôt faible avec environs 10 nouveaux cas sur 1 million par an.

Nos trois études se sont concentrées principalement sur la physiopathologie de la pemphigoïde bulleuse, la plus fréquente parmi ces maladies bulleuse auto-immunes.

2) Rappel historique

Les maladies bulleuses ont longtemps intéressé les médecins. Différentes appellations ont été utiliser pour désigner ces affections au cours du temps. Les grecques par exemple ont utilisé plusieurs termes, comme « *pemphix* », « *pomphos* », « *pompolyx* », « *phlyctaina* » et « *phlyzakion* »; les arabes « *nuffakha* » et les chinois « *thianpauh* ». Cela ne fait que 200 ans que des efforts pour mieux classer ces dermatoses ont été faits, notamment par Sauvages, Plenck et Willan par la suite (60, 65). C'est finalement en 1953, que Lever (65) a fait la distinction de la pemphigoïde bulleuse des autres maladies bulleuses sur la base de critères cliniques et surtout histologiques. En effet, la pemphigoïde bulleuse se différencie du pemphigus vulgaire par le développement d'une bulle sous-épidermique, sans le phénomène de l'acantholyse, reflétant la présence de kératinocytes détachés, flottant libres dans la cavité de la bulle. Puis, c'est en 1967 que Jordon et al. (52) ont décrit les images typiques d'immunofluorescence directe et indirecte de la pemphigoïde bulleuse. Ces examens d'immunofluorescence restent ce jour très importants pour le diagnostic de cette maladie.

3) Epidémiologie et clinique

La pemphigoïde bulleuse (PB) (synonyme : pemphigoïde) est une des plus fréquentes maladies bulleuses. Elle représente 70 % des dermatoses bulleuses auto-immunes sous–épidermiques. C'est une maladie qui touche les personnes âgées de plus de 65 ans, sans prédominance de sexe, ni prédisposition raciale, ethnique ou géographique. Cependant, des cas exceptionnels chez l'enfant ont été rapportés dans la littérature (7, 53, 60, 80, 104, 111).

Au début, les symptômes peuvent être trompeurs. Les patients se plaignent généralement de prurit intense à caractère chronique et insomnieux. A ce stade, les lésions sont souvent peu spécifiques : excoriations, placards eczématiformes ou urticariens. Plus tard, dans l'évolution de la maladie, une éruption bulleuse plus caractéristique peut s'observer (56, 60, 69). On retrouve typiquement des bulles tendues à contenu clair, de grande taille (0,5 cm), pouvant apparaître sur une peau saine ou un fond érythémateux, sans signe de Nikolski (induction d'un décollement bulleux du frottement de la peau apparemment saine). D'autres lésions peuvent être trouvées : lésions maculeuses, papules érythémateuses ayant parfois un aspect urticarien, lésions en cible, érosions post-bulleuses et croûtes. Les lésions se situent particulièrement sur les surfaces d'extensions des bras, la face antéro-interne des cuisses, les aisselles, l'aine et l'abdomen de façon symétrique **(Figure 1)**. Les lésions guérissent sans cicatrices contrairement à d'autres maladies bulleuses notamment le pemphigus cicatriciel ou l'épidermolyse bulleuse acquise. Les muqueuses sont très rarement atteintes (10-20% des cas). Si c'est le cas, elles atteignent particulièrement la bouche (13, 60, 64).

Biologiquement, il existe souvent une hyperéosinophilie élevée (> 500/mm^3), parfois associée a une élévation des IgE sériques.

La présentation clinique de la pemphigoïde peut parfois être inhabituelle et trompeuse **(Figures 2,3)** . Par exemple, la *pemphigoïde nodularis* est caractérisée par la présence de lésions hyperkératosiques nodulaires excoriées. L'aspect clinique et histologique fait penser à un prurigo nodulaire, mais les examens d'immunofluorescence directe et indirecte sont typiques pour une pemphigoïde **(Figure 4).** La *pemphigoïde végétante* est une autre variante se présentant avec des lésions en plaque purulo-végétantes dans les aisselles et l'haine. Elle est cliniquement similaire au pemphigus végétant. La *pemphigoïde polymorphe* et *dysidrosiforme* (avec des lésions d'eczémas), la pemphigoïde *érythrodermique* (avec érythème diffus) et finalement « la pemphigoïde papuleuse » sont des autres

présentations trompeuses. Enfin, la pemphigoïde peut être déclenchée ou induite par la prise de certains médicaments, surtout les diurétiques et certains neuroleptiques (3, 60).

4) Pronostic et traitement

La pemphigoïde bulleuse a une évolution chronique évoluant par poussées (56, 60). Son pronostic n'est néanmoins pas très bon. En effet, le taux de mortalité reste relativement élevé à 1 an, allant de 10 à 30 % chez les patients traités (19, 50, 56, 89). Le risque de décès est d'autant plus élevé que l'affection survient à un âge avancé, avec un état général diminué. Il est possible que la mortalité soit en partie également imputable aux effets secondaires des traitements (50, 88).

Certains auteurs ont aussi décrit que la détection d'anticorps anti-Ag PB180 circulants par immunotransfert est un facteur de mauvais pronostic (8).

Des associations avec d'autres maladies ont été parfois décrites dans la littérature : diabète sucré, psoriasis, polyarthrite rhumatoïde, lupus érythémateux systémique, vitiligo et pemphigus vulgaire. Il s'agit le plus vraisemblablement d'anomalie fortuite (56, 60, 74, 102). Des études prospectives bien conduits sont nécessaires afin de mieux élucider ce point.

Récemment, une association de la pemphigoïde bulleuse avec des maladies inflammatoires ou neurodégénératives du système nerveux central, à type de sclérose en plaques ou de sclérose myotrophique latérale, a été rapportée dans des observations isolées ou dans des séries rétrospectives (17, 74). La relevance de ces observations reste à préciser. Néanmoins, il est important de souligner qu'un des antigènes cibles de la pemphigoïde bulleuse, BP230, est une protéine dont il existe de multiples isoformes. Certaines semblent être exprimées de façon spécifique dans le système nerveux central et périphérique. Pour ce motif, il n'est pas exclu que certains malades développent des auto-anticorps dirigés contre BP230 ayant une réactivité croisée contre des isoformes exprimées dans d'autres tissus, y compris le système nerveux central. Enfin, de multiples cas de pemphigoïde bulleuse associés à des néoplasies, surtout carcinome, ont été décrits dans la littérature. Néanmoins, dans des études contrôlées, le risque d'avoir une néoplasie sous-jacente en cas de pemphigoïde bulleuse n'est pas très significatif. La mise en évidence d'un cancer chez des maladies reflète dans la plupart des cas l'âge avancé du sujet atteint (68, 109).

Le traitement d'un patient atteint de PB n'est pas bien codifié. Il est plus basé sur l'expérience clinique que sur des études contrôlées, qui sont peu nombreuses (**tableau I**). La corticothérapie générale constitue le traitement le plus utilisé dans les différents pays (60), mais elle comporte un risque non négligeable dans cette population de sujets âgés. La corticothérapie locale forte constitue une alternative intéressante : son efficacité a récemment été confirmée par la plus grande étude contrôlée

disponible (50). Cette dernière prouve que la corticothérapie locale est aussi efficace que la corticothérapie générale, avec une meilleure tolérance, à savoir, moins de complications systémiques et moins de décès. Cependant, la corticothérapie locale pose parfois des problèmes de faisabilité en milieu non médicalisé, avec un risque de mauvaise observance. Elle n'est pas dénuée d'effets secondaires locaux (p.e. atrophie cutanée sévère, complications infectieuses) et systémiques (p.e. insuffisance surrénalienne). Sa place dans le traitement de la PB généralisée, bien que très prometteuse et défendue par l'école française, doit encore être précisée.

Des traitements adjuvants, voire alternatifs, sont régulièrement discutés (60). Le recours d'emblée aux immunosuppresseurs dans les formes généralisées reste controversé, certains auteurs les réservant pour les formes corticorésistantes ou en cas de contre-indication à la corticothérapie générale. Il est en tout cas à adapter en fonction de sa faisabilité, des éventuelles contre-indications et des habitudes du prescripteur. Notre attitude en présence d'une atteinte généralisée consiste à utiliser d'emblée une corticothérapie générale (par exemple, prednisone à 0.5 mg/kg/j) en association avec un immunosuppresseur (par exemple, chlorambucil ou azathioprine). Néanmoins, cette approche n'est pas validée par des études contrôlées.

5) Diagnostic différentiel

La pemphigoïde bulleuse fait partie du groupe des « pemphigoides ». Ces maladies bulleuses ont en commun le développement d'auto-anticorps dirigés contre les mêmes protéines des hémidesmosomes au niveau de la JDE.

Le diagnostic de la pemphigoïde bulleuse peut être difficile (60, 69). La présentation clinique de la phase du début peut être trompeuse avec des lésions urticariennes ou eczématiformes, non-bulleuses. En présence de bulles, le diagnostic différentiel se pose avec d'autres maladies bulleuses, comme la pemphigoïde cicatricielle (CP), l'épidermolyse bulleuse acquise (EBA), la pemphigoïde gestationnelle (PG), la dermatite herpétiforme (DH), la dermatose bulleuse à IgA linéaire, et finalement le lupus érythémateux bulleux (LEB). Enfin, les éruptions bulleuses induites par des médicaments peuvent être également discutées.

Pour poser un diagnostic, il est essentiel d'effectuer des études immunohistopathologiques (immunofluorescence directe et indirecte) et un examen histologique **(tableau I)**.

La **pemphigoïde gestationnelle** est une maladie de la femme enceinte et du post-partum. L'éruption débute caractéristiquement au niveau de l'ombilic et s'étend au tronc, paumes et plantes, avec des lésions papulo-urticariennes, puis vésiculeuses et bulleuses (49, 78, 95).

La **pemphigoïde cicatricielle** touche caractéristiquement les muqueuses. Les muqueuses orales, oculaires, et plus rarement de la sphère ORL et génitale, sont le siège d'une inflammation résultant en décollements, érosions chroniques et développement progressif de séquelles cicatricielles, parfois invalidantes (cécité par exemple) (14, 15, 26, 28, 54, 85).

L'**épidermolyse bulleuse acquise** est une maladie bulleuse pouvant s'accompagner par des décollements et fragilité touchant surtout les faces d'extension des membres. Les lésions guérissent en laissant des cicatrices et des grains de milium. Parfois, des formes inflammatoires similaires à la pemphigoïde s'observent. Sur le plan immunologique, cette affection est associée à une réponse humorale contre le collagène VII (12, 32, 77).

La **dermatite herpétiforme** touche surtout le jeune adulte avec développement de lésions prurigineuses ou bulleuses-vésiculeuses, localisées typiquement sur les faces d'extension des membres et le tronc. Cette maladie survient typiquement en association avec une intolérance au gluten (maladie coeliaque). L'IF directe retrouve des dépôts granulaires d'IgA dans les papilles dermiques de façon caractéristique.

La **dermatose bulleuse à IgA linéaires** comprend un groupe de plusieurs maladies caractérisées par la présence de dépôts linéaires d'IgA au niveau de la JDE (18, 83, 111, 112, 115). Ces dépôts d'IgA reflètent d'une réactivité contre des protéines de la JDE parfois différentes : BP180 et BP230, collagène type VII, ou plus fréquemment une protéine de 98/120 kDa correspondant au domaine extracellulaire de BP180 (43, 83, 90, 114).

Le **lupus érythémateux bulleux** peut parfois s'accompagner par des lésions bulleuses et la présence d'anticorps contre le collagène type II, l'antigène cible de l'EBA (32).

Les éruptions bulleuses médicamenteuses peuvent relativement facilement être différenciées de la PB des études d'immunofluorescence directe et indirecte, qui sont négatives.

6) Histologie

L'image histologique va dépendre du type de lésions biopsiées (56, 60). La plus caractéristique est l'image d'une bulle sous-épidermique sans nécrose du toit ni acantholyse. Son contenu est fibrineux et cellulaire avec prédominance de polynucléaires éosinophiles **(Figure 4A)**. Il existe un infiltrat dermique mixte composé de polynucléaires éosinophiles, de lymphocytes et d'histiocytes. Dans le derme papillaire et autour des vaisseaux des mastocytes sont rarement retrouvés. Dans environ 20 % des cas, une spongiose à éosinophiles et des micro-abcès papillaires à polynucléaires éosinophiles sont décrits. Le diagnostic différentiel histologique se pose avec toutes les maladies bulleuses auto-immunes des groupes dermiques : EBA, PC, DH et PG.

A) Immunofluorescence directe (IFD): **(figure 4B)**

L'examen d'IFD est fondamental pour le diagnostic de pemphigoïde (33). La biopsie se fait en peau périlésionnelle, congelée dans de l'azote liquide, dans le milieu de Michel, ou simplement dans du sérum physiologique. L'IFD montre la présence de dépôts linéaires à IgG (90 %) et/ou du C3 (100 %) le long de la jonction dermo-épidermique (JDE). Ceci est parfois associé à des dépôts d'IgM ou d'IgA.
Des études récentes ont trouvé que la présence de dépôts linéaires en IgG ou C3 le long de la jonction épidermique chez un malade âgé plus de 70 ans a une sensitivité et spécificité élevées pour une pemphigoïde bulleuse si l'éruption cutanée s'accompagne par une absence d'atteinte des muqueuses, une absence de lésion bulleuse du visage et du cou, et s'il n'y a pas de lésion cicatricielle atrophique (51, 106).

B) Immunofluorescence indirecte (IFI) :

Cet examen est effectué dans le but de détecter la présence d'auto-anticorps anti-JDE dans le sérum du patient. Le sérum d'un patient est mis sur un substrat épithélial, soit de la peau humaine normale clivée par IM NaCl, soit de l'œsophage de singe ou de lapin. Chez environ 70 à 90 % des patients, on met en évidence des anticorps circulants de la classe IgG dirigés contre la JDE. Si la peau est clivée par une solution saline permettant une séparation de la peau au niveau de la membrane basale, les auto-anticorps marquent caractéristiquement le versant épidermique correspondant au toit de clivage, ou parfois le versant épidermique et dermique (30).

C) Immunoélectronmicroscopie (IME) : **(Figure5A)**

Elle s'effectue sur une biopsie cutanée de peau périlésionnelle. Elle montre la présence d'immunoréactants (IgG et C3) localisés dans la partie supérieure de la lamina lucida. Plus précisément, les dépôts sont associés aux hémidesmosomes sur le pôle basal des kératinocytes basaux (5).

D) Immunotransfert et immunoprécipitation :

Ces deux techniques permettent de mieux caractériser les différents antigènes reconnus par les anticorps circulants et impliqués dans la physiopathologie de cette maladie (4, 6, 22, 24, 34, 35, 36, 66). Les antigènes cibles sont deux protéines des hémidesmosomes : l'antigène de la pemphigoïde bulleuse 230 (BP230) et l'antigène de la pemphigoïde bulleuse 180 (BP180). La BP230 est une protéine cytoplasmique associée à la plaque hémidesmosale. Elle interagit avec les filaments intermédiaires (39, 55, 97, 98). La BP180 est par contre une protéine transmembranaire avec un domaine extracellulaire collagénique impliquée dans l'ancrage des kératinocytes basaux avec les protéines de la matrice extracellulaire (29, 34, 35, 46, 43, 44, 55, 90).

Enfin, il faut noter que les malades souffrant de pemphigoïde bulleuse ont souvent des réactivités dirigées contre des autres protéines des hémidesmosomes ou de la jonction dermo-épidermique, comme la plectine ou la laminine 5 (61, 86). Il est bien possible que l'apparition d'autres réactivités au cours de la maladie reflète d'un phénomène de *intermolecular epitope spreading* (107).

E) Enzyme-linked immunosorbent assay (ELISA) :

Il s'agit d'une méthode très sensible et spécifique (37, 40, 45, 47, 58, 59, 76, 79, 84, 85, 89, 94, 97, 98, 101, 113, 114) utilisant des protéines recombinantes de BP180 et BP230. Les résultats obtenus sont semi-quantitatifs, et permettent de suivre l'évolution de la maladie car le taux de réactivité contre BP180 (domaine NC16A) reflète la sévérité et l'extension de la maladie (9, 40, 103).

7) Constituants biochimiques de la jonction dermo-épidermique (figure 5A-B)

Pour comprendre la physiopathologie de la pemphigoïde bulleuse, il est essentiel de connaître l'organisation de la JDE. La peau est composée de l'épiderme, du derme de l'hypoderme et de ses annexes. La jonction dermo-épidermique est sur le plan ultrastructurel composée de trois compartiments, de l'épiderme vers le derme :

- de la membrane cytoplasmique basale des kératinocytes de la couche basale avec des complexes d'adhésions appelés, les hémidesmosomes. Ces derniers sont des structures servant d'une part de site d'insertion pour les filaments de kératines (tonofilaments) à la membrane cytoplasmique basale et, d'autre part, ils assurent une liaison avec la membrane basale sous-jacente (9, 39).
- de la membrane basale comportant deux zones distinctes : la *lamina lucida* (côté épiderme) et de la *lamina densa* (côté derme). La lamina lucida apparaît comme une zone optiquement vide, contrairement à la lamina densa qui est une zone électron-dense. La membrane basale est constituée de nombreuses protéines comme les laminines, la fibronectine, et le collagène type IV. Elle semble être reliée aux hémidesmosomes par des structures filamenteuses appelées filaments d'ancrage correspondant vraisemblablement au domaine extracellulaire de BP180 (9).
- du derme papillaire superficiel avec le réseau fibreux dermique. Les fibres d'ancrage de la lamina densa sont des structures filamenteuses qui forment des « loops » avec des de demi-cercles, assurant la cohésion de la JDE avec le derme papillaire sous-jacent.

La séparation sous-épidermique et la formation de bulles dans la pemphigoïde résultent de l'interaction des auto-anticorps avec des antigènes qui constituent des composantes des hémidesmosomes **(figure 5B)**. Ces structures permettent un attachement solide des kératinocytes de la membrane basale au derme sous-jacent. La fixation de ces auto-anticorps déclanche une réaction inflammatoire avec infiltration de leucocytes neutrophiles et éosinophiles, aboutissant à des dégâts tissulaires et séparation dermo-épidermique (15, 20, 23, 92, 93, 96, 110). Les cibles des auto-anticorps sont l'antigène 180 de la pemphigoïde bulleuse (BP180) et l'antigène 230 de la pemphigoïde bulleuse (BP230).

La BP180 est une protéine trans-membranaire, appelée aussi collagène XVII, composée d'un domaine extracellulaire collagénique (DEC) et d'un domaine intracellulaire (DIC). La BP180 joue un rôle important dans la stabilité de l'HD en interagissant directement avec différentes protéines de l'HD,

notamment 6 4 intégrine, la plectine et BP230 (29, 34, 35, 43, 44, 46, 55, 90). L'importance de BP180 dans le maintien de l'intégrité de la jonction dermo-épidermique est bien illustrée par l'observation qu'un défaut congénital de son expression dû à des mutations dans son gène s'accompagne par une fragilité cutanée et la formation de bulles, avec un phénotype d'épidermolyse bulleuse jonctionnelle (75).

La BP230 est une protéine entièrement cytoplasmique. Son domaine COOH-terminal est important pour son association aux filaments de kératines. Par contre, son domaine NH2-terminal se lie à 6 4 intégrine et à BP180 (39, 55, 97, 98).

8) Pathogénie

Au cours des dernières années, nombreuses observations ont permis de confirmer l'origine auto-immune de la pemphigoïde bulleuse et de préciser sa physiopathologie :

1) Les patients ont des auto-anticorps et des cellules T autoréactives dirigés contre BP180 et BP230 (10, 63, 67).

2) Les dégâts tissulaires surviennent après la fixation des auto-anticorps aux antigènes cibles.

3) Des modèles *in vitro* ainsi que des modèles *in vivo* chez la souris ont clairement établi le rôle pathogénique des auto-anticorps et leur implication directe dans le déclenchement de la réaction inflammatoire (15, 16, 31, 42, 70, 71, 72, 81, 110).

4) Dans la pemphigoïde gestationnelle (gravidique), le transfert d'autoanticorps transplacentaire de la mère à l'enfant peut causer l'apparition de bulles chez le nouveau-né (78).

5) La maladie est fortement associée à la présence de génotypes HLA de classe II distincts, et répond au traitement immunosuppresseur (2, 21, 82).

Les études de « *antigen mapping* » ont montré que les anticorps dirigés contre BP180 reconnaissent préférentiellement quatre sites antigéniques situés dans le domaine NC16A. Ce domaine se situe près de la région transmembranaire sur la portion extracellulaire de la protéine. Toutefois, certains anticorps sont également dirigés contre des sites localisés sur d'autres régions des parties intracellulaires et extracellulaires de BP180 (34, 35, 84, 111, 112). Par rapport à BP230, la majorité des auto-anticorps reconnaissent des sites antigéniques situés sur la partie COOH-terminal (85, 94, 98, 100).

Sur le plan physiopathologique, l'injection des anticorps de lapin reconnaissant la partie extracellulaire de BP180 murin produit chez les souris nouveau-nées une séparation dermo-épidermique avec décollements bulleux similaires à ceux observés dans la pemphigoïde bulleuse (70, 71, 72).

La formation des bulles sous-épidermiques dépend critiquement de la présence du système du complément, de neutrophiles ainsi que la sécrétion de différentes gélatinases et métalloprotéinases (70, 72, 92, 93). Actuellement, les auto-anticorps dirigés contre la partie extracellulaire de BP180 semblent jouer un rôle initiateur critique dans le développement de la maladie, tandis que les auto-anticorps contre la partie cytoplasmique de BP180 et BP230 pourraient contribuer aux dégâts tissulaires dans une phase secondaire (60, 107). Toutefois, aucune étude jusqu'à ce jour n'a confirmé cette hypothèse.

En sachant que les cellules T autoréactives sont importantes dans la production d'auto-anticorps et leur régulation, des études (10) ont recherché la présence de cellules T autoréactives contre BP180 et BP230 chez les patients avec BP. Ces études ont confirmé la présence de cellules CD4+ autoréactives contre BP180 ou BP230. La capacité de réagir et proliférer en présence de BP180 et BP230 semble être restreinte par certains allèles HLA de classe II comme HLA-DQB1*0301.

9) Tableaux et Figures

Tableau 1 : Maladies bulleuses sous-épidermiques auto-immunes; diagnostic différentiel

Maladie	*Directe*	*Indirecte*	*Immuno-électronmicroscopie*	*Immunotransfert*[3] *Immunoprécipitation*	*Association*	*Traitement* (11, 25, 27, 38, 48, 57, 60, 77, 99, 108)
Pemphigoïde bulleuse	IgG, C3 (IgA, IgM), JDE linéaire (90 %)	IgG (IgA)	Hémidesmosomes, filaments d'ancrage, lamina lucida	230KD, 180 KD	Médicaments	Prednisone, Azatioprine, Tetracycline, Nicotinamide
Pemphigoïde cicatricielle	IgG (IgA) JDF (20 %) linéaire	IgG (IgA)	Hémidesmosomes, filaments d'ancrage, fibre d'ancrage	180 KD, laminine-5[6], 250 KD(?)		Dapsone, Cyclophosphamide
Pemphigoïde gestationnelle ou gravidique	C3(IgG) JDF linéaire	IgG		180 KD, (230 KD)	Grossesse	

Dermatite herpétiforme	IgA papilles granulaires	IgA/IgG anti-plectine IgA/IgG anti-transglutaminase	Derme superficiel		HLA B8, DRW3, DQW2, atrophie villositaire	Dapsone, Régime sans gluten
Dermatose à IgA linéaires	IgA JDF linéaire	IgA (negative)	Lamina lucida ou/et lamina densa	97 KD ou 120 KD (180 KD, 230 KD, 290 KD)	Médicaments lymphomes (?)	Daspsone Sulfamides Prednisone
Epidermolyse bulleuse acquise	IgG JDE linéaire	Négative ou IgG	Sous lamina densa	290 KD	Crohn, RCH, autres	Prednisone

(1)ZMB : zone de la membrane basale.

(2)FGH :facteur herpes gestationis.

(3)Poids moléculaire de l'antigène en kilodaltons (KD).

(5)Individualisation controversée.

(6)Le plus fréquemment réactivité avec la chaîne a3 (200 KD et 165 KD).

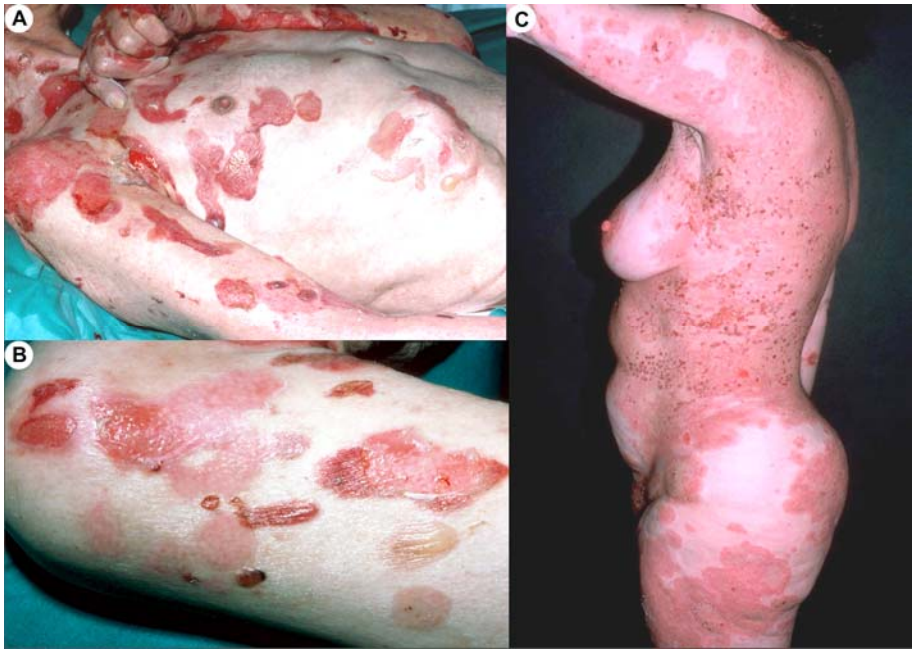

Figure 1

Pemphigoïde bulleuse dans sa présentation classique avec bulles et érosions généralisées.

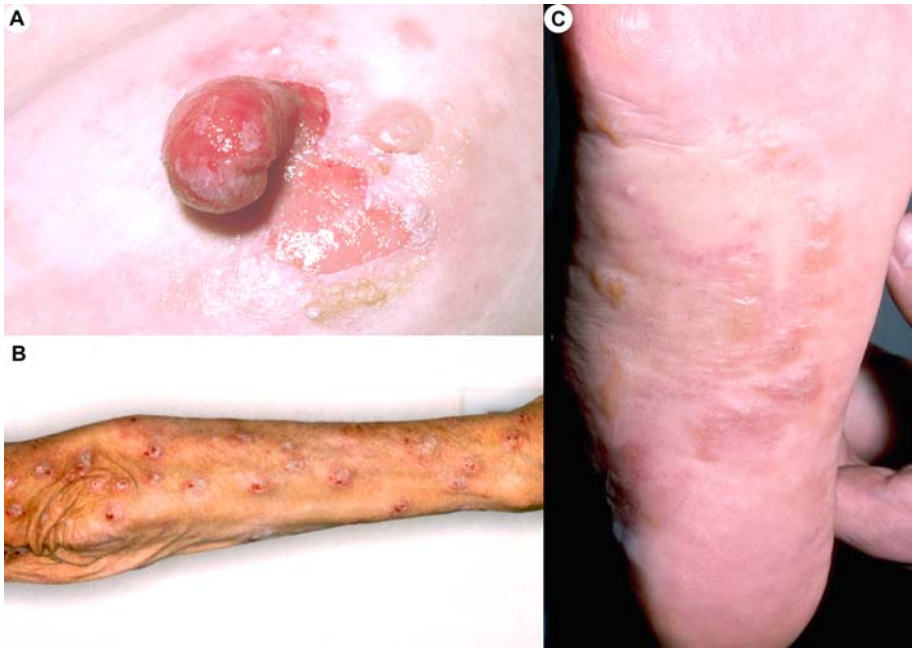

Figure 2

Forme inhabituelle et trompeuse de pemphigoïde bulleuse.

A. Bulles et lésions érosives localisées autour d'un stroma.

B. Pemphigoïde nodulaire avec lésions à type de prurigo excorié.

C. Pemphigoïde dishydrosique avec lésions eczématiformes bulleuses de la plante des pieds.

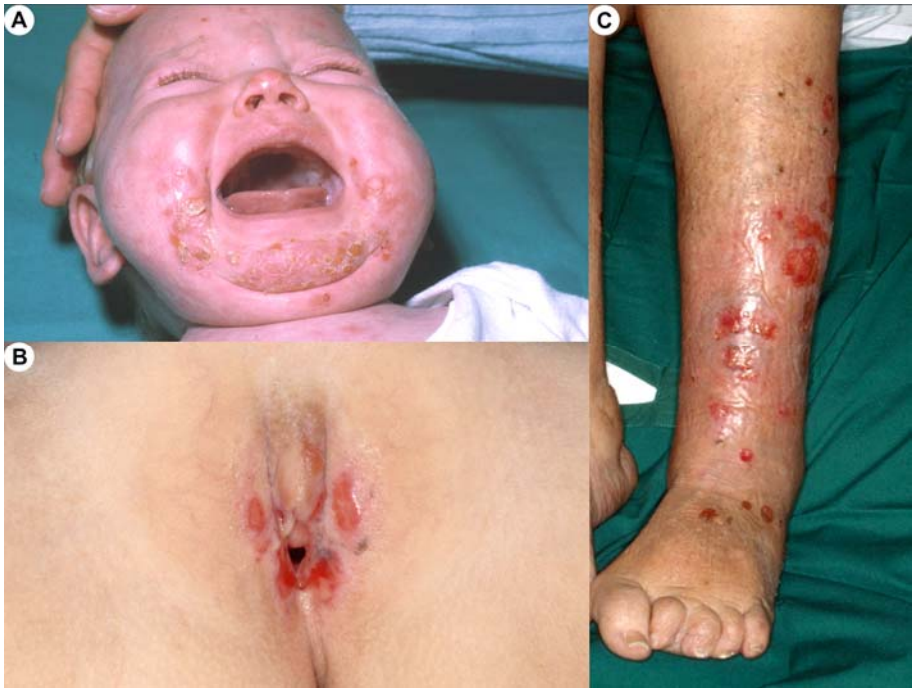

Figure 3

Forme inhabituelle de pemphigoïde bulleuse.

A. Pemphigoïde bulleuse de l'enfant avec atteinte du visage.

B. Pemphigoïde bulleuse de l'enfant avec lésions bulleuses et érosives isolées de la région vulvaire.

C. Pemphigoïde bulleuse localisée avec atteinte prétibiale chez une personne âgée.

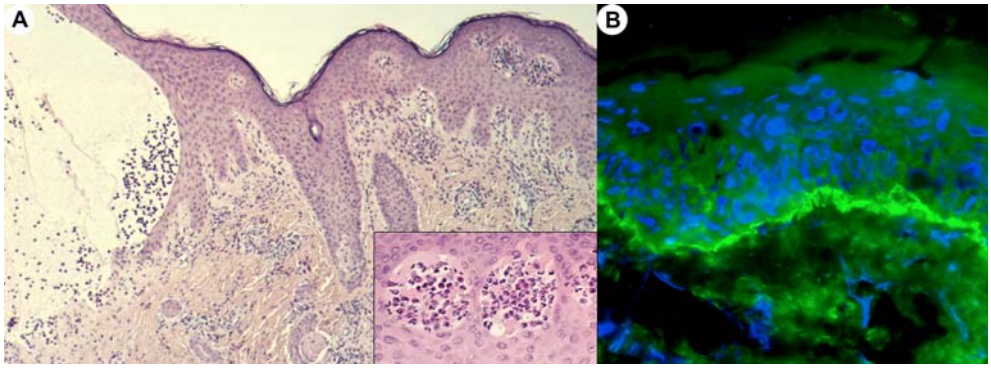

Figure 4

Examen histologique d'une lésion bulleuse dans la pemphigoïde bulleuse.

A. Décollement sous-épidermique avec infiltrat riche en neutrophiles et éosinophiles dans la cavité de la bulle et dans le derme superficiel. L'agrandissement met en évidence la bande d'infiltrats riches en polynucléaires neutrophiles et éosinophiles.

B. Examen d'immunofluorescence directe en peau périlésionnelle dans la pemphigoïde bulleuse. Dépôts linéaires d'IgG le long de la jonction dermo-épidermique.

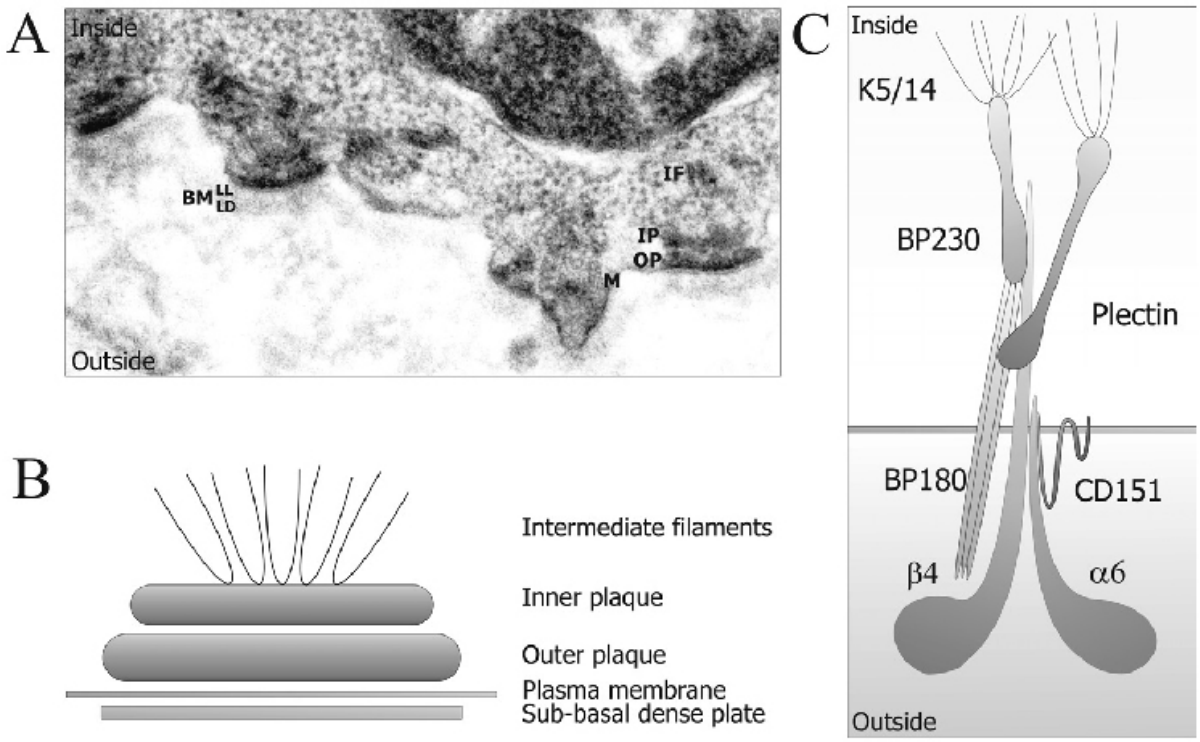

Figure 5

A. Aspect par microscopie électronique de la jonction dermo-épidermique avec mise en évidence de structures électrodenses sur la surface basale des kératinocytes correspondant aux hémidesmosomes. Ces derniers comprennent une plaque interne (IP) et une plaque externe (OP). La jonction dermo-épidermique (BM) comporte une *lamina lucida* (LL) et une *lumina densa* (LD).

B. Organisation structurelle des hémidesmosomes servant de site d'insertion pour les filaments intermédiaires et d'attachement pour les protéines de la membrane basale.

C. Organisation moléculaire des hémidesmosomes comprenant des protéines cytoplasmiques (BP230, plectine) et des protéines transmembranaires (BP180, intégrine 6 4 et la protéine CD151).

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11) Annexes

I) Caractérisation de la réactivité des anticorps anti-BP 230 dans la pemphigoïde bulleuse : les sites antigéniques immunodominants sont localisés dans la partie carboxy-terminale de BP230.

Introduction : La majorité des malades souffrant de pemphigoïde bulleuse ont des anticorps dirigés contre BP180 et BP230. Si les études antérieures avaient démontré que les anticorps dirigés contre BP180 réagissent de façon prédominante avec un site antigénique appelé domaine NC16A, localisé sur la portion extracellulaire de BP180, peu d'informations existent concernant les sites antigéniques reconnus par les anticorps dirigés contre BP230. Pour ce motif, nous avons étudié en utilisant des protéines recombinantes de BP230 couvrant la presque totalité de la molécule, la réactivité anti-BP230 d'un groupe de malades souffrant de pemphigoïde bulleuse.

Méthode : Nous avons analysée 25 sérums de patients ayant une pemphigoïde diagnostiquée sur des critères cliniques, des études histologiques, d'immunofluorescence directe et indirecte, et d'immunotransfert avec extraits kératinocytaires. Nous avons généré des protéines recombinantes par traduction *in vitro* avec des lysats de réticulocytes de lapin. Cinq protéines recombinantes, correspondant à différentes portions de BP230 ont été utilisées : le recombinant A (acide aminés 1 à 555 de BP230 (BP230 1-555) d'environ 63 kDa, le recombinant B (BP230 663-1293) de 74 kDa, le recombinant C(BP230 1593-1891) de 35 kDa, le recombinant D (BP230 1946-2649) de 79 kDa et le recombinant E (BP230 2203-2649) de 50 kDa. Les protéines ont par la suite été utilisées pour des études d'immunotransfert pour caractériser la réactivité des sérums à disposition.

Résultats : Parmi les 25 sérums réactifs avec BP230 par immunotransfert avec des extraits kératinocytaires, 8 sérums reconnaissent le recombinant A (32%), 8 (32%) le B, 1 (4%) le C, 21 (84%) le D et 16 (64%) le E.

Conclusion : Nos résultats confirment que le domaine COOH-terminal de BP230 est reconnu par la majorité des sérums réactifs avec BP230. En outre, notre étude démontre : 1) que d'autres régions antigéniques localisées sur la région amino-terminale (recombinant A) ou la région centrale (recombinant B) de la protéine sont reconnues ; 2) que la réponse anti-BP230 parmi les malades est très hétérogène. Il est vraisemblable que la présence de sites antigéniques multiples reflète d'un phénomène de « *intramolecular epitope spreading* ».Ces auto-anticorps de différentes spécificités

pourraient être, directement ou indirectement, impliqués dans le développement des dégâts tissulaires en interférant avec la fonction et/oul'assemblage des hémidesmosomes, et en participant à la réponse inflammatoire.

II) Les auto-anticorps IgG dirigés contre le domaine intracellulaire et le domaine extracellulaire de BP180 dans la pemphigoïde bulleuse appartiennent à la sous-classe IgG 1 et IgG 4

Introduction : Afin de mieux caractériser la réponse humorale dirigée contre BP180 au cours de la pemphigoïde bulleuse, nous avons étudié l'isotype IgG des anticorps dirigés contre le domaine extracellulaire et intracellulaire de BP180.

Méthode : Nous avons analysé la réactivité de 27 sérums de patients souffrant de pemphigoïde bulleuse par immunotransfert en utilisant des protéines recombinantes couvrant le domaine extracellulaire et intracellulaire de BP180. Pour déterminer l'appartenance des IgG aux différentes sous-classes, nous avons utilisé des anticorps monoclonaux, bien caractérisés, dirigés contre les IgG 1, les IgG 2, les IgG 3 et les IgG 4.

Résultats : Vingt-sept (100%) et 21 (77%) des 27 sérums ont montré une réactivité contre le domaine extracellulaire de BP180 avec des anticorps de sous-classe de type IgG1 et IgG4, respectivement. Quatorze (82%) et six (35%) dès 17 sérums de malade ayant réagit avec le domaine intracellulaire de BP180 avaient des anticorps de sous-classe de type IgG1 et IgG4, respectivement. La présence d'une réactivité de IgG2 et IgG3 contre BP180 était moins fréquente. Les patients avec une maladie de longue durée ont montré une tendance à une réponse surtout de type IgG1 mais aussi à de type IgG4.

Conclusions : Nos études confirment que les anticorps dirigés contre BP180 appartiennent de façon prédominante aux sous-classes IgG 1 et IgG 4. La mise en évidence presque constante d'anticorps IgG 1 dirigés contre le domaine extracellulaire et intracellulaire de BP180 a très vraisemblablement une importance physiopathologique. En effet, l'habilité de cette sous-classe d'activer le système du complément joue un rôle critique dans le déclenchement de la réaction inflammatoire et dans l'induction des dégâts tissulaires. Dans un modèle murin de pemphigoïde bulleuse, la présence d'un système de complément fonctionnel est en effet essentielle pour le développement de la maladie.

III. Caractérisation de la réponse contre BP180 dans une observation de lichen planus pemphigoides

Introduction : Le *lichen plan pemphigoides* constitue une forme particulière de pemphigoïde bulleuse survenant en association avec un lichen plan. Sur le plan clinique, les malades développent des lésions cutanées typiques pour un lichen, avec des papules polygonales, avec par la suite apparition de lésions vésiculo-bulleuses typiques pour une pemphigoïde. Sur Les examens immunopathologiques montrent la présence de dépôts d'IgG et/ou C3 le long de la jonction dermo-épidermique avec présence d'anticorps circulants dirigés contre la jonction dermo-épidermique. Les antigènes reconnus par ces anticorps circulants n'ont pas été jusqu'ici bien caractérisés. Afin d'étudier la réactivité d'un sérum de malade souffrant de *lichen plan pemphigoides*, nous avons pratiqué des études d'immunotransfert en utilisant des formes recombinantes de BP180, comprenant la partie extracellulaire et intracellulaire.

Résultats et conclusion : Par immunotransfert, nous avons démontré que le sérum du malade étudié contient des anticorps IgG dirigés contre BP180. Spécifiquement, les études d'*antigen « mapping »* indiquent que ces anticorps reconnaissent de façon prédominante le domaine N16A de BP180. Ce résultat suggère que les malades ayant un lichen planus pemphigoides ont une réponse immune très similaire, voire identique, aux malades ayant une forme inflammatoire classique de pemphigoïde bulleuse.

12) Résumé

La pemphigoïde bulleuse est une maladie chronique associée à une importante morbidité-mortalité. Cette affection représente la dermatose bulleuse auto-immune la plus fréquente en Europe et touche principalement les personnes de plus de 70 ans.

Elle se caractérise par la présence d'auto-anticorps dirigés contre deux molécules appelées BP180 et BP230. Ces deux antigènes sont des composants des hémidesmosomes, structures assurant l'adhésion entre l'épiderme et la membrane basale. La fixation de ces anticorps aux antigènes cibles induit une cascade d'événements inflammatoires avec dégâts tissulaires par la formation de bulles sous-épidermiques.

Pour ce travail de thèse, nous avons effectué différentes études visant la caractérisation de la réponse humorale dirigée contre BP180 et BP230 dans la pemphigoïde bulleuse et dans des affections apparentées comme le « lichen planus pemphigoides ».

En bref :

1) Nos résultats confirment que les anticorps dirigés contre BP230 reconnaissent de façon prédominante le domaine carboxyterminal de BP230. Néanmoins, plusieurs autres régions antigéniques localisées sur la région amino-terminale et centrale de la molécule sont reconnues par une proportion significative des sérums. La présence d'une réponse humorale dirigée contre multiples épitopes reflète vraisemblablement d'un phénomène de l'*intramolecular epitope spreading* .

2) Nous avons également effectué une analyse de la réponse humorale dirigée contre BP180, l'autre antigène cible de la pemphigoïde bulleuse. Spécifiquement, nous avons analysé l'isotype des anticorps dirigés contre le domaine extracellulaire et intracellulaire de BP180. Nos résultats indiquent que les anticorps IgG dirigés contre BP180 appartiennent de façon prédominante aux sous-classes IgG1 et IgG4, en ligne avec les études antérieures. La mise en évidence d'anticorps IgG1 a très probablement une importance physiopathologique directe car cette classe est capable d'activer le système du complément dont l'implication dans l'induction des dégâts tissulaires a été confirmée par des études dans un modèle murin de pemphigoïde bulleuse.

3) Enfin, nous avons eu l'occasion d'étudier la réactivité d'un sérum obtenu d'un malade ayant une forme particulière de pemphigoïde, appelée « lichen planus pemphigoides ». Nous avons démontré que le sérum du malade contient des anticorps dirigés contre BP180 reconnaissant le domaine NC16A de BP180. Cette région constitue également le site antigénique reconnu de façon prédominante par les malades ayant une pemphigoïde bulleuse classique. Le profil immunologique ne semble donc pas expliquer les différences cliniques observées entre le « lichen planus pemphigoides » et la pemphigoïde bulleuse.

En conclusion, bien que nos études aient contribué à une meilleure compréhension et caractérisation de la réponse humorale contre BP230 et BP180 dans le groupe des pemphigoïdes bulleuses, les mécanismes responsables pour la survenue de la maladie restent à ce jour seulement partiellement élucidés. Récemment, dans un modèle murin « humanisé », il a été démontré de façon indiscutable que les anticorps dirigés contre le domaine extracellulaire de BP180 sont directement impliqués dans le développement des lésions cutanées. Par contre, le rôle des anticorps dirigés contre BP230, une protéine cytoplasmique reste mal précisée. Un des défis futurs sera donc celui de développer des tests in vitro ou des modèles animaux permettant de mieux comprendre la contribution des anticorps contre BP230 dans la pemphigoïde bulleuse. Il est bien possible qu'une meilleure compréhension de la physiopathologie de cette affection s'accompagnant par une forte morbidité et mortalité puisse se traduire également par le développement de stratégies thérapeutiques plus ciblées sans les effets secondaires parfois néfastes des thérapeutiques utilisés aujourd'hui de routine (corticothérapie locale, corticothérapie systémique, immunosuppresseurs) pour le traitement de cette affection.

British Journal of Dermatology 2001; **144:** 760–768.

Autoantibodies to the extracellular and intracellular domain of bullous pemphigoid 180, the putative key autoantigen in bullous pemphigoid, belong predominantly to the IgG1 and IgG4 subclasses

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Accepted for publication 14 November 2000

Summary

Background Autoantibodies to the extracellular domain (ECD) of bullous pemphigoid (BP) antigen 180 (BP180) are thought to play a crucial part in the pathophysiology of BP.

Objectives As the various IgG subclasses have different biological properties, we have sought to assess the relative isotype distribution of IgG to BP180 and their reactivity against the ECD and intracellular domain (ICD) of BP180.

Methods The reactivity of 27 sera from patients with BP was assayed by immunoblotting against recombinant proteins covering the ECD and ICD of BP180.

Results Twenty-seven (100%) and 21 (77%) of 27 BP sera, respectively, contained IgG1 and IgG4 autoantibodies binding to the ECD of BP180. Fourteen (82%) and six (35%) of the 17 BP sera that were reactive with the ICD of BP180 had autoantibodies of the IgG1 and IgG4 subclass, respectively. The profile of the isotype restriction appeared to be similar when the response to the ECD vs. that to the ICD was compared. IgG2 and IgG3 reactivity with BP180 was found less frequently. Patients with BP of longer duration showed a tendency to have, in addition to IgG1, an IgG4 response.

Conclusions Consistent with prior evidence indicating that subepidermal blister formation in BP is dependent upon complement activation, the frequent finding of complement-fixing IgG1 autoantibodies to both the ECD and ICD of BP180 might have pathogenic relevance in BP. These findings provide new insights relevant for our understanding of the immune response to BP180, the putative key autoantigen in BP.

Key words: autoantibodies, BP180, bullous pemphigoid, immunoglobulin subclasses

Bullous pemphigoid (BP) is a subepidermal blistering disease associated with autoantibodies directed against the epidermal basement membrane (BM).[1,2] Patients' autoantibodies typically react with the BP antigen 230 (BP230, also termed BP antigen 1) and the BP antigen 180 (BP180, also termed BP antigen 2 or type XVII collagen),[2–4] two components of adhesion complexes called hemidesmosomes in stratified and some complex epithelia.[5] BP230, a cytoplasmic protein of the plakin family, is involved in linking the intermediate filament cytoskeleton to hemidesmosomes,[6] while BP180 is a transmembrane constituent with a large collagenous extracellular domain (ECD) serving as cell-surface receptor for extracellular matrix ligand(s).[7,8]

Recent studies have demonstrated that autoantibodies to BP180 recognize multiple epitopes on both the ECD and intracellular domain (ICD) of BP180. Nevertheless, the sera of most patients sera bind to the NC16A subdomain, an immunodominant region on the ECD close to the transmembrane domain.[9–12] In contrast, BP230-specific autoantibodies predominantly recognize sequences contained within the COOH-terminal region of the protein.[13–15]

Evidence has recently been provided suggesting that the presence of IgG antibodies against BP180 and, in particular, against its ECD, correlates with disease

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activity or outcome in BP patients.[16–18] In addition, passive transfer of IgG antibodies raised against the ECD of murine BP180 reproduces all key features of BP in neonatal mice.[19,20] In contrast, in patients with BP, the presence of circulating BP230-reactive autoantibodies does not appear to affect prognosis.[16] Furthermore, autoantibodies against BP230 cause an inflammatory reaction in rabbits only after additional injury to their epidermis.[21] Based on these observations, it is thought that autoantibodies against the ECD of BP180 are pathogenic, while antibodies against intracellular antigenic determinants on BP230 and on the ICD of BP180 represent a secondary event in the context of an epitope spreading phenomenon.

Studies from several laboratories have indicated that the autoantibody response in BP patients shows a predominant IgG4 and IgG1 subclass restriction.[22–29] However, most of these analyses have been limited by the use of immunofluorescence (IF) microscopy techniques, which did not allow any conclusions about the specific response to BP180 and BP230 to be reached. The determination of specific IgG subclasses is of importance because of their different functional effects and pathogenic potential. Hence, because of the putative key role of BP180 in disease initiation, to further our understanding of the pathophysiology of BP, we have sought in this study to assess the relative isotype distribution of the IgG response to BP180 and to investigate whether a particular subclass of autoantibodies recognizes antigenic determinants located on the ECD and ICD. To address these questions, the reactivity of BP patients' sera was assayed by immunoblotting (IB) against recombinant proteins covering the ECD and ICD of BP180 expressed in transfected COS-7 cells.

Materials and methods

Human sera

Serum samples were obtained from patients with BP ($n = 27$), pemphigus vulgaris ($n = 6$) and healthy volunteers ($n = 12$). The clinical diagnosis of BP was confirmed by histology, direct IF microscopy (deposits of IgG and/or C3 along the epidermal BM), and indirect IF microscopy (autoantibodies binding to the epidermal side of 1 mol L^{-1} NaCl-separated normal human skin).[30] The serum samples from BP patients were selected on the basis of their reactivity with BP180 by IB of keratinocyte extracts.[31] In addition, 10 of these BP sera also contained antibodies targeting BP230 (sera uniformly diluted at 1 : 20).

Antibodies

The following antibodies were used: mouse IgG1 monoclonal antibody (mAb) anti-FLAG™ M2 against the FLAG™ peptide (DYKDDDDK) (IBI, Eastman Kodak Company, New Haven, CT, U.S.A.); mAb MYC 1–9E10.2 against a defined c-myc epitope;[32] mouse IgG1 mAb 1A8c and mAb 233 directed against the ICD and ECD of BP180, respectively (donated by Dr K.Owaribe, Nagoya University, Japan); an antiserum raised against the COOH-terminus of BP230 (kindly provided by Dr J.R.Stanley, University of Pennsylvania, Philadelphia, PA, U.S.A.).[33] The mAbs against IgG subclasses included: clone NL16 for IgG1 (IUIS/WHO code HP6012; working dilution 1 : 200), clone GOM 1 for IgG2 (HP6008; dilution 1 : 200), clone ZG4 for IgG3 (HP6010; dilution 1 : 500) and clone RJ4 for IgG4 (HP6011; 1 : 500) (all from Oxoid AG, Basel, Switzerland). These mAbs were chosen on the basis of their specificities extensively evaluated in previous analyses.[34] In addition, these mAbs had been used in previous studies to examine the IgG subclass distribution in the BP group of disorders.[22,23,35] The sensitivity and specificity of these mAbs at their working dilution was pretested in IB experiments using equal amounts of IgG1–4 purified from myeloma proteins.[15,23]

cDNA constructs

Generation of eukaryotic expression vectors encoding the ECD (residues 490–1497) and the ICD (residues 1–466) of BP180 have been described.[12,36] Construction of a plasmid encoding the COOH-terminal region of BP230 (residues 1946–2649) has recently been reported.[15]

Transfection experiments

The African monkey kidney cell line COS-7 was transfected using the diethylaminoethyl-dextran method.[37] Gene expression was assayed after 48 h. Cells were lysed as described elsewhere.[12]

In vitro translation

The recombinant BP230 protein was produced by an *in vitro* transcription and translation system according to the manufacturer's recommendations (TNT® T7

Table 1. Clinical features of bullous pemphigoid (BP) patients and IgG reactivity of their sera against the extracellular domain (ECD) and intracellular domain (ICD) of BP180 at the time of the study

					BP180	
BP sera	Age (years)	Duration of the disease	Skin lesions	Treatment at the time of the study	ECD	ICD
BP1[a]	88	1 year	Generalized	PDN, Aza	+	–
BP2[a]	93	16 months	Scalp, limbs	None	+	–
BP3[a]	91	3 months	Generalized	PDN, Aza	+	++
BP4[a]	91	2 months	Back, trunk, limbs	CB, PDN	+	–
BP5[a]	77	10 years	Back, trunk, limbs	CB, PDN	+	–
BP6[a]	83	1 week	Limbs	None	+	+
BP7[a]	77	1 month	Generalized	None	+	+
BP8[a]	89	1 month	Trunk, limbs	PDN	+	–
BP9[a]	89	2 months	Limbs, trunk, axillary	PDN	+	–
BP10[a]	60	3 months	Generalized	PDN, Aza	+	–
BP11	74	2 months	Limbs, face	PDN, C	+	++
BP12	92	2 weeks	Trunk	PDN, Aza	+	++
BP13	97	6 years	Back	PDN, CB	+	+
BP14	86	6 years	Generalized	PDN, CB	+	+
BP15	76	5 months	Trunk, back, limbs	PDN, CB	+	+
BP16	86	3 months	Limbs, face	PDN, Aza	+	+
BP17	82	6 weeks	Trunk, limbs	PDN, Aza	+	(+)
BP18	86	1 month	Generalized	PDN, CB	+	+
BP19	82	2 years	Back, trunk	PDN, CB	+	+
BP20	87	5 weeks	Trunk	PDN, Aza	+	+
BP21	53	2 months	Trunk	PDN	+	(+)
BP22	70	2 years	Trunk, mouth	sulphapyridine	+	+
BP23	1	2 months	Generalized	PDN	+	+
BP24	76	3 years	Generalized	PDN, Aza, MM	+	–
BP25	66	2 months	Generalized	PDN, MM	+	–
BP26	75	1 month	Generalized	PDN, Aza	+	+
BP27	77	4 months	Generalized	None	++	–
Total					27/27 (100%)	17/27 (63%)

[a]Reactivity with BP230; PDN, prednisone; Aza, azathioprine; CB, chlorambucil; C, cyclophosphamide; MM, mycophenolate mofetil; ++, strong positive; +, positive; (+), faint positive; –, negative.

Coupled Reticulocyte Lysate System; Promega, Madison, WI, U.S.A.) as previously reported.[15]

Immunoblot analysis

Extracts of transfected COS-7 cells (50–100 μg) or 10 μL of the denatured translation reaction were loaded per well on a 5% or 7·5% sodium dodecyl sulphate–polyacrylamide gel, separated and electrophoretically transferred to nitrocellulose sheets.[38] The nitrocellulose paper was cut into strips and incubated with a 1 : 20 and 1 : 100 dilution of each tested serum in Tris-buffered saline (TBS) -0·5% Nonidet P40 for 2 h at room temperature. After washing in TBS-0·5% Nonidet P40, the strips were then incubated with peroxidase-conjugated goat anti-human IgG (H + L) antibody (Institut Pasteur, Marnes la Coquette, France), goat anti-mouse immunoglobulin (Amersham Life Sciences, Zürich, Switzerland) or goat anti-rabbit IgG Fab′ fragment (Cappel, Durham, NC, U.S.A.) for 2 h. For IgG subclass determination, the strips were incubated for 1·5 h in an intermediate step with the various IgG subclass-specific mAbs diluted (see above) in washing buffer. After washing, the strips were developed with 3,3′-diaminobenzidine-4 HCl (0·5 mg mL^{-1}) in 100 mmol L^{-1} Tris–HCl pH 7·4 containing 0·01% H_2O_2.

Statistical analysis

The disease duration between the different groups of patients was compared using the non-parametric Mann–Whitney *U*-test.

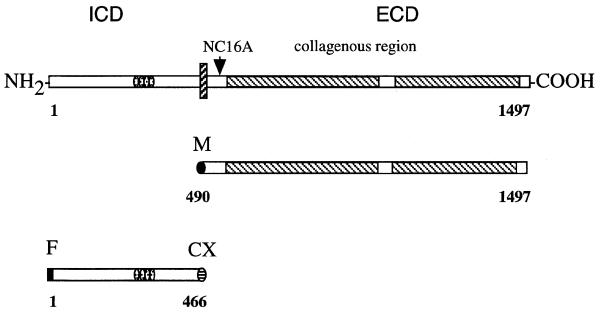

Figure 1. Schematic representation of bullous pemphigoid (BP)180 and of the recombinant proteins consisting of either the extracellular domain (ECD) or the intracellular domain (ICD) of BP180. The recombinant form of the ECD of BP180 (encompassing residues 490–1497) included a c-myc tag (M) at its NH_2-terminal end. The ICD of BP180 (residues 1–466), which is tagged at its NH_2-terminal end with the FLAG™ peptide (F), was expressed fused to the membrane localization sequence of K-Ras (CX) (GenBank accession numbers M54968 and M38506), as previously described.[12]

Results

IgG autoantibodies binding to the extracellular domain and intracellular domain of bullous pemphigoid 180

The 27 BP sera reactive with BP180 by IB of keratinocyte extracts were first assayed against recombinants encompassing either the ECD or the ICD of BP180 produced in transfected COS-7 cells (Table 1, Fig. 1). All 27 (100%) BP sera, including 22 previously tested sera,[12] contained IgG that reacted with an approximately 100-kDa protein corresponding to the ECD of BP180. This protein was also recognized by the mAb 233 specific for the ECD of BP180 (not shown) as well as the mAb MYC 1–9E10.2 directed against the c-myc tag present in the recombinant BP180 ECD protein (Fig. 1).

In addition, 17 (63%) BP sera bound to a 52-kDa

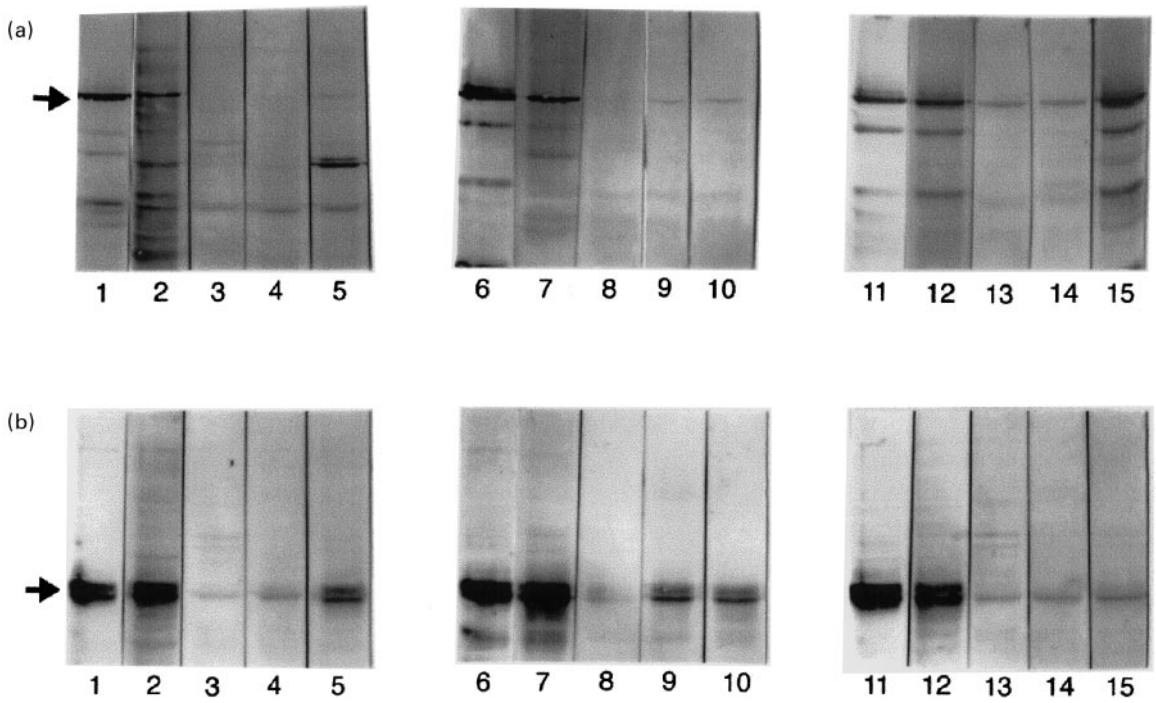

Figure 2. IgG autoantibodies binding to bullous pemphigoid (BP)180 belong predominantly to the IgG1 and IgG4 subclass. The subclass distribution of the IgG autoantibody response to BP180 was assessed by immunoblotting as described in Materials and methods. (a) Reactivity with the extracellular domain of BP180: monoclonal antibody (mAb) MYC 1–9E10.2 anti-c-myc epitope detecting a protein of approximately 101 kDa (lanes 1, 6 and 11; arrow), sera from patients BP20 (lanes 2–5), BP3 (lanes 7–10) and BP14 (lanes 12–15). (b) Reactivity with the intracellular domain of BP180: MAb anti-FLAG™ peptide detecting a protein of approximately 52 kDa (lanes 1, 6 and 11; arrow), sera from patients BP20 (lanes 2–5), BP3 (lanes 7–10) and BP11 (lanes 12–15). The sera were probed for IgG1 (lanes 2, 7 and 12), IgG2 (lanes 3, 8 and 13), IgG3 (lanes 4, 9 and 14) and IgG4 (lanes 5, 10 and 15). Some additional reactive bands were occasionally found with experimental sera and most probably represented either unspecific background or reactivity against proteins of unknown identity in COS-7 cell extracts. The intracellular domain of BP180 appeared as a doublet, most likely due to proteolytic degradation. Samples were separated by 7·5% sodium dodecyl sulphate–polyacrylamide gel electrophoresis under non-reducing conditions.

Table 2. Analysis of IgG subclass distribution of bullous pemphigoid (BP) sera reactive with the extracellular domain (ECD) and intracellular domain (ICD) of BP180

	ECD				ICD			
BP sera	IgG1	IgG2	IgG3	IgG4	IgG1	IgG2	IgG3	IgG4
BP1	++[a]	(+)	+	+				
BP2	+[a]	+	(+)	++[a]				
BP3	++	–	(+)	(+)	++[a]	(+)	+[a]	+[a]
BP4	+	–	–	–				
BP5	(+)	+	–	+[a]				
BP6	+	–	–	–	++	–	–	–
BP7	+[a]	+	–	+	(+)	–	–	–
BP8	++[a]	+	+	–				
BP9	(+)	–	(+)	(+)				
BP10	(+)	–	–	–				
BP11	+	–	–	(+)	++[a]	–	–	–
BP12	(+)[a]	(+)	–	–	++[a]	–	–	–
BP13	+	–	–	+	++[a]	–	–	++[a]
BP14	++[a]	+[a]	+[a]	++[a]	–	–	(+)	–
BP15	+	–	–	–	+	–	–	–
BP16	+[a]	–	–	+[a]	+	–	–	–
BP17	++[a]	–	(+)	+[a]	–	–	–	–
BP18	++[a]	+[a]	+[a]	+[a]	–	–	–	–
BP19	+[a]	(+)[a]	–	++[a]	++[a]	–	–	+[a]
BP20	+	–	–	(+)	++	–	–	+
BP21	++	–	–	+	(+)	–	–	–
BP22	+	–	–	++	+[a]	–	–	++[a]
BP23	++[a]	+[a]	(+)	(+)[a]	++[a]	–	–	+[a]
BP24	+	++	(+)	+				
BP25	+	++	–	+				
BP26	++[a]	+	+	(+)	+	–	–	–
BP27	(+)	–	(+)	(+)				
Total	27/27 (100%)	13/27 (48%)	12/27 (44%)	21/27 (77%)	14/17 (82%)	1/17 (6%)	2/17 (12%)	6/17 (35%)

Sera tested at dilution 1 : 20; [a]sera also reactive at dilution 1 : 100; ++, strong positive; +, positive; (+), faint positive; –, negative.

protein corresponding to the ICD of BP180, as assessed using either the mAb 1A8c specific for the ICD of BP180 (not shown) or the mAb anti-FLAG™ binding to the FLAG™ peptide tag present in this recombinant form of BP180. None of the control sera from normal volunteers ($n = 12$) and pemphigus patients ($n = 6$) showed reactivity.

IgG1 against the extracellular domain of bullous pemphigoid 180 predominates in the sera from bullous pemphigoid patients

Next, IB studies using mAbs for IgG subclasses were performed. All 27 (100%) BP sera contained IgG1 binding to the ECD of BP180, while autoantibodies of the IgG2, IgG3 and IgG4 subclasses were found in 13 (48%), 12 (44%) and 21 (77%) BP sera, respectively (Fig. 2, Table 2). In four sera, IgG1 was the only IgG subclass detectable, while in all cases in which IgG4 was found IgG1 was also present.

IgG1 is the major subclass targeting the intracellular domain of bullous pemphigoid 180

The isotypes of IgG autoantibodies binding to the ICD were then assessed. Fourteen (82%) of the 17 BP sera reactive with the ICD of BP180 contained IgG1 autoantibodies (Fig. 2, Table 2). In six (35%) BP sera, IgG4 autoantibodies binding to the ICD of BP180 were also found, always present together with IgG1. Finally, in a minority of patients, IgG2 (6%) and IgG3 (12%) were also detectable. The isotype profile of the response to the ICD appeared to parallel that to the ECD, as 14 of 14 (100%) patients showing IgG1 reactivity against the ICD also possessed IgG1 binding to the ECD of BP180, while six of six (100%) BP sera had IgG4 response to both the ECD and ICD of BP180.

IgG subclass profile and disease duration

No correlation could be established between the detection of an IgG autoantibody response to both the ECD and ICD of BP180 and severity (disseminated vs. localized involvement) or duration of the disease (Table 1).[12] However, when the presence of a given specific IgG subclass to BP180 was analysed, it appeared that the longer the duration of clinical manifestations then the more likely was the detection

Table 3. Detection of IgG1 and IgG4 autoantibodies to the extracellular domain (ECD) and intracellular domain (ICD) of BP180 according to disease duration

	ECD			ICD		
Isotype		Disease duration (months)			Disease duration (months)	
distribution	Sera	Mean	Median	Sera	Mean	Median
IgG1	6/27	1·9	1·5	8/14	1·8	1·5
IgG1 and IgG4	21/27	19·0	3·0	6/14	21·0	13·5
	$P = 0{\cdot}085$[a]			$P = 0{\cdot}043$[a]		

[a]Statistical comparison between disease duration of bullous pemphigoid (BP) patients with IgG1 reactivity and that of BP patients with both IgG1 and IgG4 reactivity. The non-parametric Mann–Whitney *U*-test was used.

of both IgG1 and IgG4 reactive with either the ECD or the ICD of BP180 (Table 3). In the six BP sera containing IgG1 but not IgG4 to the ECD of BP180 the mean and median duration of the disease was 1·9 and 1·5 months, respectively. In the 21 patients with both IgG1 and IgG4 response, the mean and median duration of the disease increased to 19 and 3 months, respectively, although the difference did not reach statistical significance ($P = 0{\cdot}085$). Furthermore, in patients with an isolated IgG1 response to the ICD the mean and median duration of the disease was 1·8 and 1·5 months, respectively, while the six patients with both an IgG4 and IgG1 response exhibited a mean and median duration of the disease of 21 and 13·5 months, respectively, with a difference that was statistically significant ($P = 0{\cdot}043$).

Isotype restriction of autoantibody response to bullous pemphigoid 230

Finally, the 10 BP sera that immunoblotted BP230 of keratinocyte extracts were also tested against a recombinant protein consisting of the distal 704-residue stretch of the COOH-terminus of BP230, that was previously found to contain immunodominant sequences.[15] Seven of these 10 (70%) BP sera recognized this recombinant (not shown), while none of the control sera from normal volunteers ($n = 12$) or pemphigus patients ($n = 6$) showed reactivity. Four (57%) and five (71%) of these seven BP sera had specific IgG1 and IgG4 response, respectively, while IgG2 and IgG3 were found in one and two sera, respectively.

Discussion

Our aim was to assess whether autoantibodies to the ECD of BP180 exhibit a particular subclass distribution compared with those binding to its ICD. Our results suggest that IgG1 and, to a lesser extent, IgG4 are the predominant subclasses in BP sera. All 27 (100%) and 21 of 27 (77%) BP180-reactive sera had autoantibodies of the IgG1 and IgG4 subclass to the ECD of BP180, respectively. Moreover, of the 17 BP sera reactive with its ICD, 14 (82%) and six (35%), respectively, contained IgG1 and IgG4 autoantibodies.

Previous semiquantitative IF microscopy studies have indicated that IgG anti-BM antibodies from patients with *bona fide* BP belong predominantly to the IgG4 and, less frequently, to the IgG1 subclass.[22,23,29] However, in line with our findings, Bernard *et al*. did not find an IgG4 predominance by IB analysis of epidermal extracts, as 90% and 80% of 10 BP180-reactive sera, respectively, contained autoantibodies of the IgG1 and IgG4 subclass.[23] Furthermore, IgG4 autoantibodies to BP230 were found more frequently than IgG1, that is, in 100% and 52% of 20 BP sera, respectively, suggesting a predominance of IgG4 to BP230, as observed in our study. In another study, autoantibodies were found to belong predominantly to the IgG4 subclass, but no distinction was made between the response to BP180 and BP230.[29] In the latter two studies, consistent with our results, autoantibodies of the IgG2 and IgG3 subclasses were detected less frequently. Finally, in a recent prospective study of Dopp *et al*.,[24] 66% and 50% of 18 BP sera, respectively, contained IgG4 and IgG1 autoantibodies immunoblotting the NC16A domain of BP180, suggesting a slight predominance of specific IgG4 immunoglobulins. It is likely that these differences might be due to various factors: (i) although we used well-characterized mAbs, the different mAbs to a specific IgG subclass exhibit variable sensitivity and specificity;[29,34] (ii) our assay detects autoantibodies targeting not only the NC16A domain, but also other antigenic regions of the ECD and ICD of BP180; and (iii) the disease stage of our retrospective BP population and the immunosuppressive treatments might have influenced the subclass profile. This latter possibility is supported by a preliminary report indicating that the isotype restriction of the autoantibody response to BP180 shows significant changes in the course of the disease.[39]

IgG subclasses have different biological properties. Specifically, IgG1 and IgG3, in contrast to IgG4 and IgG2, have a strong complement-fixing capacity by the classical pathway, as well as cytophilic properties with a high ability to bind to Fc receptors on the plasma membrane of leucocytes.[40] Because of its inability to fix complement, IgG4 has been considered rather as a non-inflammatory 'protective' antibody (reviewed[41]).

The frequent detection of IgG1 autoantibodies to BP180 in our study raises the question of whether this IgG subclass accounts for the pathogenic activity in BP. Support for this idea derives from the observations that: (i) the C3 complement component is detected in the epidermal BM of most BP patients; (ii) complement-fixing anti-BM antibodies seem to be important for subepidermal blistering in an *in vitro* model of BP;[30] (iii) in a murine model of BP, the pathogenic activity of anti-BP180 autoantibodies is dependent on complement-mediated neutrophil infiltration with

subsequent liberation of proteinases;[20,42] (iv) IgG1, together with IgG3, is also the predominant IgG subclass in gestational pemphigoid, a disease closely related to BP;[43] and (v) IgG1 autoantibodies have been implicated in the pathogenesis of other autoimmune disorders such as prediabetes,[44] chronic thyroiditis,[45] rheumatoid arthritis[46] and chronic active hepatitis.[47] In contrast, non-complement-fixing IgG4 autoantibodies predominate in the pemphigus group and anti-epiligrin cicatricial pemphigoid,[48,49] animal models of which indicate that tissue damage is not dependent on complement activation. In light of our own and previous published results, it is most likely that in BP anti-BP180 IgG4 antibodies are also implicated in disease initiation and perpetuation.[16,24]

As T-helper (Th) 1 cytokines such as interferon-γ are able to induce the secretion of IgG1 and IgG2, while Th2 cytokines such as interleukin (IL) -4, IL-5 and IL-13 have been shown to regulate the secretion of IgG4 and IgE,[50,51] the detection of BP180-specific IgG1, IgG4 and IgE in BP patients in the present and previous studies suggests that both autoreactive Th1 and Th2 cells[50] regulate the autoantibody response to BP180. We have recently found that BP180-specific autoreactive CD4+ cells produce both Th2 and Th1 cytokines.[51] Strikingly, autoreactive Th2 cells were found exclusively in BP patients, but not in healthy controls, who exhibited only Th1 responses to BP180. These findings suggest that Th2 cells are critical for initiating the production of pathogenic autoantibodies. It is possible that the apparently more frequent detection of specific IgG4 in patients with longer disease duration that was observed in our study reflects an isotype switch due to a shift in the Th1/Th2 balance in the chronic phase of BP. As the profile of the IgG subclasses to the ECD and ICD is similar, it is difficult to raise any speculation about the possibility that different T-lymphocyte cytokine subsets regulate the humoral response to putatively pathogenic and secondary, 'non-pathogenic', determinants on the ECD and ICD of BP180, respectively.

Our study suggests that autoantibodies to both the ECD and ICD of BP180 belong predominantly to the IgG1 and, probably to a lesser extent, to the IgG4 subclasses. Better definition of the pathophysiology of BP will require prospective studies of both cellular and humoral response to BP180 and BP230 in various disease stages. Furthermore, it will be important to employ sensitive techniques such as enzyme-linked immunosorbent assay as well as eukaryotic recombinant proteins or peptides predicted to encompass crucial determinants.

Acknowledgments

This work has been supported by a grant from the Swiss National Foundation for Scientific Research (32–51083.97 and 32–56727.99), the Fondation Touraine (Paris), TELETHON Action Suisse (Aubonne) and the Fondation de Reuter (Geneva). The authors are indebted to Drs P.Bernard (Reims, France), L.Bruckner-Tuderman (Münster, Germany), M.Hertl (Erlangen, Germany) and T.Hunziker (Berne, Switzerland), who provided some of the sera used in the study. The authors thank Drs M.Hertl and C.Hauser for helpful discussions.

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Rapid Communication

Dermatology

Dermatology 1999;199:253–255

Received: June 6, 1999
Accepted: August 9, 1999

IgG Autoantibodies from a Lichen planus pemphigoides Patient Recognize the NC16A Domain of the Bullous Pemphigoid Antigen 180

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Key Words
Lichen planus • Lichen planus pemphigoides • Bullous pemphigoid • Bullous pemphigoid antigen 180 (BP180) • Type XVII collagen

Abstract
Lichen planus pemphigoides (LPP) most likely encompasses a heterogeneous group of subepidermal autoimmune blistering disorders occurring in association with lichen planus. We describe the case of a 49-year-old patient with features characteristic of LPP. Direct immunofluorescence microscopy studies demonstrated linear deposits of C3 along the cutaneous basement membrane, while circulating IgG autoantibodies directed against the epidermal side of skin separated by 1 *M* NaCl were detected. The patient's serum contained IgG autoantibodies immunoblotting a recombinant form of bullous pemphigoid antigen 180 (BP180), but not the COOH-terminus of BP230. By using deletion mutants, it was found that IgG reactivity was restricted to the NC16A domain of BP180, the region harboring the major antigenic sites targeted by IgG autoantibodies from patients with the bullous pemphigoid group of disorders. Our findings provide support to the idea that a subset of patients with LPP have a distinct form of bullous pemphigoid associated with lichen planus.

Introduction

Lichen planus pemphigoides (LPP) is a rare disorder characterized by the presence of lichen planus lesions with vesicles and bullae arising on both involved and uninvolved skin [1–3]. Light microscopy studies of papular lesions demonstrate features typical of lichen planus, while biopsies obtained from bullous lesions reveal a subepidermal blister usually suggestive of bullous pemphigoid (BP) [1–4]. Direct immunofluorescence microscopy studies demonstrate linear deposits of C3 and/or IgG along the epidermal basement membrane zone [2–4]. Although LPP has been regarded by some authors as a distinct entity, it most likely denotes the occurrence of lichen planus in association with distinct subepidermal autoimmune bullous disorders, including most frequently BP [5–9]. This idea is supported by the observation that the targeted autoantigens frequently, but not always, correspond to 180-kD and 230- to 240-kD proteins showing the same electrophoretic motility of the BP antigen 180 (BP180) and of the BP antigen 230 (BP230), respectively [5–9]. Furthermore, the immunoreactants have usually been localized to the lamina lucida and the hemidesmosomal region, and, less frequently, to the lamina densa and the sublamina densa region of the epidermal basement membrane zone [6–12]. Nevertheless, LPP patients present with some peculiar common features, including a peak incidence of occurrence in the fifth decade, a predilection for an involvement of the distal extremities and a relatively benign course [reviewed in 6, 9].

In this report, to further understand the pathophysiology of LPP, we have sought to investigate the protein(s) targeted by IgG autoantibodies from a patient with LPP by immunoblotting analyses utilizing a series of recombinant forms of BP180 and BP230.

Case Report

A 47-year-old Indian patient was referred for evaluation of a severely itchy eruption of 3 months of duration. Her past history revealed arterial hypertension and Paget's disease of bones, for which the patient had been receiving enalapril and tiludronate for the last 12 and 7 months, respectively. On examination, she had typical lichen planus lesions located on her arms, legs, ankles, wrists and abdomen with involvement of the oral mucosa. Light microscopy studies of a lichenoid papular lesion demonstrated hyperkeratosis, hypergranulosis, acanthosis, with damage of the lowest cell layer of the epidermis and a band-like inflammatory infiltrate in the upper dermis. Direct and indirect immunofluorescence studies were negative. Full blood cell count, serum chemistry, urine analyses and protein electrophoresis were unremarkable. Antinuclear antibodies, anti-DNA antibodies and serology for hepatitis B, C and HIV were negative.

Enalapril treatment was discontinued and the patient was given two intramuscular injections of triamcinolone acetonide, 40 mg, at an interval of 2 weeks, with a slow improvement of the lesions and pruritus. Four weeks later, she developed 0.2- to 0.5-cm blisters on the

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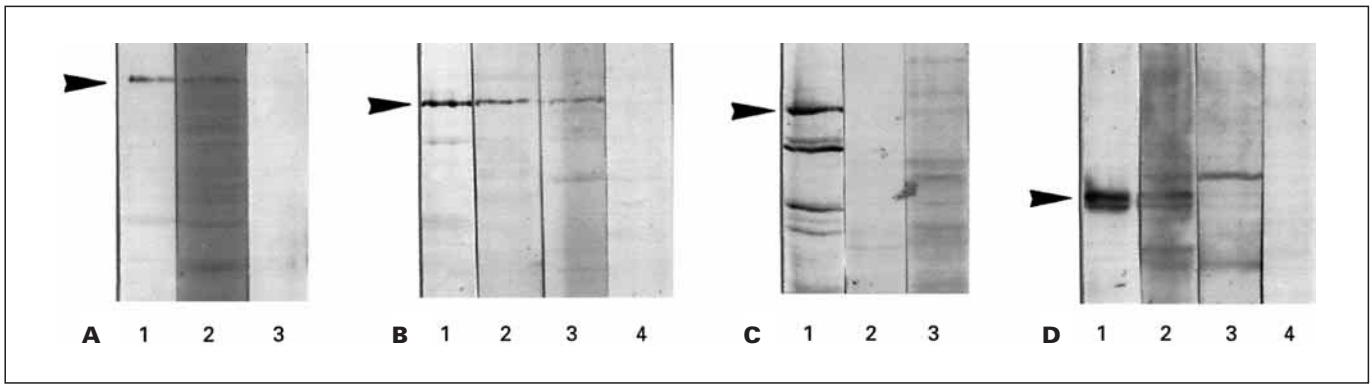

Fig. 1. Immunoblot of serum samples obtained from the LPP patient and control subjects utilizing BP180 recombinants expressed in transfected COS-7 cells. To identify the recombinants, the constructs were tagged at their 5′ end with either the *myc*-epitope or the FLAG peptide [17]. Samples were separated by 5% or 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis under nonreducing conditions. Recombinant proteins close to their predicted mass are recognized by MoAb 9E10 or MoAb anti-FLAG peptide (arrow). Some additional bands are occasionally found with both experimental and control sera and most likely represent proteolytic degradation of the recombinants or unspecific background. **A** Full-length BP180: lane 1 = MoAb anti-FLAG peptide; lane 2 = IgG reactivity of the patient's serum; lane 3 = reactivity of normal human serum. **B** Extracellular domain of BP180: lane 1 = MoAb 9E10 anti-*myc* epitope; lane 2 = IgG reactivity of a BP control serum; lane 3 = reactivity of the patient's serum; lane 4 = reactivity of a normal human serum. **C** Extracellular domain of BP180 devoided of the NC16A domain: lane 1 = MoAb 9E10 anti-*myc* epitope; lane 2 = IgG reactivity of the patient's serum; lane 3 = reactivity of normal human serum. **D** Intracellular domain of BP180: lane 1 = MoAb anti-FLAG peptide; lane 2 = IgG reactivity of a BP control serum; lane 3 = reactivity of the patient's serum; lane 4 = reactivity of a normal human serum.

right hand arising on involved and uninvolved areas. Light microscopy studies of a biopsy specimen of a bullous lesion showed a subepidermal blister with some lichenoid changes of the basal cell layer and a sparse inflammatory infiltrate in the upper dermis. Direct immunofluorescence microscopy studies of perilesional skin revealed strong linear deposits of C3 along the epidermal basement membrane zone. Triamcinolone acetonide was continued, but the patient was lost to follow-up.

Results

The patient's serum was tested for circulating autoantibodies. By indirect immunofluorescence microscopy, the patient's serum contained IgG that stained the epidermal side of normal human skin separated by 1 *M* NaCl (titer 1:50) [13]. Immunoblot analyses using keratinocyte and dermal extracts remained negative [14, 15]. In addition, no reactivity was detected against a recombinant protein encompassing the COOH-terminus of human BP230 (residues 1946–2649 of BP230) that was generated using a rabbit reticulocyte translation system (Promega, Madison, Wisc., USA). In contrast, the patient's serum immunoblotted a full-length recombinant form of BP180 expressed in transfected COS-7 cells as previously described (fig. 1) [15–17]. To further map the antigenic sites of BP180 recognized, a series of BP180 mutants were utilized [17]. The patient's serum was found to contain IgG that immunoblotted the entire extracellular domain of BP180 (residues 490–1497 of BP180), but neither its extracellular domain devoid of the NC16A region (residues 558–1497) nor its intracellular domain (residues 1–466; fig. 1).

Discussion

Our patient presented with clinical, histological and immunopathological features characteristic of LPP [1–6]. The patient exhibited a lichen planus eruption complicated by bullous lesions involving the extremities. In addition, she showed linear deposits of C3 along the epidermal basement membrane with circulating autoantibodies directed against the epidermal side of saline-separated skin. To define the targeted autoantigens, immunoblotting studies were performed. The results demonstrate that the patient had autoantibodies that specifically recognize BP180. Furthermore, by utilizing a series of BP180 recombinants, the reactivity was found to be restricted to the NC16A domain of BP180 as assessed under the denaturing conditions of the immunoblot technique. Strikingly, this region of BP180 has been shown to contain immunodominant antigenic sites targeted by IgG autoantibodies from patients with BP, gestational pemphigoid and cicatricial pemphigoid [18–22]. In addition, IgA autoantibodies from patients with linear IgA bullous dermatosis might also recognize this domain [23]. It is noteworthy that, in our patient, while no reactivity was found using keratinocyte extracts, the patient's serum was shown to contain IgG autoantibodies that immunoblotted BP180 utilizing eukaryotic expressed recombinants. This method appears to have a high sensitivity and specificity for the detection of anti-BP180 antibodies [15, 17].

Our results are in line with a recent study, in which IgG autoantibodies from 4 LPP patients reacted with the NC16A domain by both immunoblotting and ELISA utilizing prokaryotic expressed BP180 recombinants [24]. In this latter study, the 4 LPP sera were found to bind to amino acid stretches within the NC16A domain that differed from those typically targeted by BP sera [24]. Reactivity against the NC16A domain has recently been demonstrated in another patient with LPP [9]. Together, these findings demonstrate that a subset of LPP patients have an autoantibody response to the NC16A domain of BP180 that harbors the major antigenic sites recognized by sera from the BP group of disorders, suggesting that LPP might represent a distinct

form of BP occurring in association with lichen planus.

Although it has been conjectured that in LPP the autoantibody response to structural components of the dermo-epidermal junction results from an 'epitope-spreading phenomenon' secondary to the tissue damage by the inflammatory process of lichen planus [3, 25], the pathophysiological mechanisms leading to subepidermal blister formation remain unclear. Furthermore, it is unknown whether a distinct immunogenetic profile confers a susceptibility to the development of LPP. Finally, exogenous factors, such as drug exposure, might be critically implicated in disease initiation. It is not excluded that in our patient enalapril had a triggering effect, since angiotensin-converting enzyme inhibitors have been implicated in the induction of lichen-planus-like eruption, LPP and BP [26–30].

Acknowledgments

This work has been supported by a grant from the Swiss National Foundation for Scientific Research (32-51083.97, to L.B). The authors kindly thank Dr. John R. Stanley, University of Pennsylvania, Philadelphia, Pa., USA, for providing cDNAs encoding human BP230 that were used to generate expression vectors for BP230.

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IgG Autoantibodies from Bullous Pemphigoid Patients Recognize Multiple Antigenic Reactive Sites Located Predominantly Within the B and C Subdomains of the COOH-Terminus of BP230

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Bullous pemphigoid is a subepidermal bullous disorder characterized by an autoantibody response against the bullous pemphigoid antigen 230 (BP230) and the bullous pemphigoid antigen 180 (BP180), a cytoplasmic component and a transmembrane component, respectively, of hemidesmosomes. Although immunodominant sequences within the extracellular domain of BP180 have been identified, characterization of the antigenic sites on BP230 is still incomplete. To identify autoantibody-reactive sites on BP230 and to examine whether the targeted regions are contained within functionally important domains, recombinant fragments encompassing almost the entire BP230 were used to assess the reactivity of 25 bullous pemphigoid sera by immunoblotting. Our results demonstrate that (i) the region bearing the B and C subdomains of the COOH-terminus of BP230 contains immunodominant sequences recognized by the majority of bullous pemphigoid sera; (ii) additional autoantibody-reactive sites are present over extended regions of the NH_2-terminal half of BP230 without evidence for antigenic cross-reactivity between the NH_2- and COOH-termini of BP230; and, finally, (iii) autoantibodies reacting with the BP230 tail predominantly belong to the IgG_4 and IgG_1 subclasses, suggesting that both autoreactive TH_2 and autoreactive TH_1 cells regulate the autoantibody response to immunodominant sequences of BP230. As the COOH-terminus of BP230 mediates the attachment of keratin intermediate filaments to the hemidesmosomal plaque, whereas its NH_2-terminus contains sequences important for its interaction with other constituents of hemidesmosomes, autoantibodies to BP230 might precipitate subepidermal blister formation and perpetuate the disease not only by eliciting an inflammatory reaction but also by interfering with the function of BP230 and thus the stability of hemidesmosomes. ***Keywords: antigenic site/autoimmunity/BP180/BP230/bullous pemphigoid/epitope. J Invest Dermatol 114:998–1004, 2000***

Bullous pemphigoid (BP) is an autoimmune subepidermal blistering disease usually affecting the elderly (Lever, 1953). A hallmark of this disorder is the presence of autoantibodies targeting the bullous pemphigoid antigen 180 (BP180, also termed bullous pemphigoid antigen 2, or type XVII collagen) and the bullous pemphigoid antigen 230 (BP230, or bullous pemphigoid antigen 1) (Stanley *et al*, 1981, 1984; Labib *et al*, 1986; Mueller *et al*, 1989), two components of hemidesmosomes (HD). HD are multiprotein junctional complexes that promote adhesion of epithelial cells to the underlying basement membrane in stratified and other complex epithelia, such as skin and mucous membranes (Green and Jones, 1996; Borradori and Sonnenberg, 1999).

BP180 is a type II transmembrane molecule with a collagenous extracellular domain (ECD) serving as cell-surface receptor (Giudice *et al*, 1991; 1992; Hopkinson *et al*, 1992; Li *et al*, 1993). This idea is supported by the observation that mutations in the BP180 gene cause generalized atrophic benign epidermolysis bullosa, an inherited skin blistering disorder characterized by impaired dermo-epidermal cohesion (Jonkman *et al*, 1995; McGrath *et al*, 1995). Recent studies have identified sequences of BP180 important for its interaction with the $\alpha 6\beta 4$ integrin (Hopkinson *et al*, 1995; Borradori *et al*, 1997; Aho and Uitto, 1998; Schaapveld *et al*, 1998), the other transmembrane component of HD, and BP230 (Borradori *et al*, 1998).[1,2] BP230, a cytoplasmic component belonging to the plakin family of proteins (Stanley *et al*, 1988; Sawamura *et al*, 1991; Green *et al*, 1992; Ruhrberg and Watt,

Manuscript received September 20, 1999; revised November 23, 1999; accepted for publication November 25, 1999.

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Abbreviations: BP, bullous pemphigoid; BP180, bullous pemphigoid antigen 180; BP230, bullous pemphigoid antigen 230; ECD, extracellular domain; HD, hemidesmosomes.

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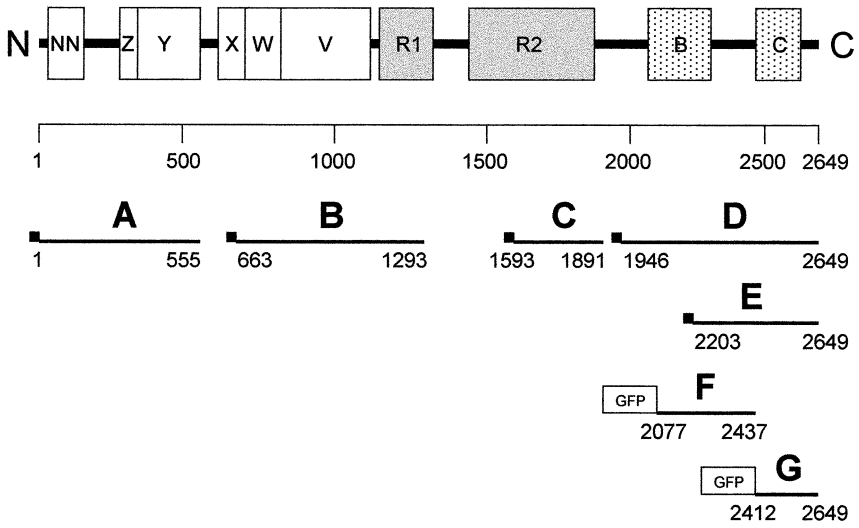

Figure 1. Schematic representation of wild-type and mutant forms of BP230. For wild-type BP230, the various subdomains represented in white for the NH_2-terminus, in gray for the coiled-coil domain, and in dotted boxes for the COOH-terminus are based on the predicted secondary structure (Green *et al*, 1992). The clones for recombinants A to E have been tagged at their 5′ end with c-myc, whereas recombinants F and G are fused to the COOH-terminus of the green fluorescent protein (GFP). The amino acid range of the segment of BP230 that is covered by the recombinant is indicated. The protein sequence of BP230 is numbered according to Sawamura *et al* (1991).

1997), is predicted to have a central coiled-coil region flanked by two globular end domains with several distinct subregions (Green *et al*, 1992; Ruhrberg and Watt, 1997) (**Fig 1**). The COOH-terminal domain of BP230 associates with keratin intermediate filaments (Yang *et al*, 1996), whereas its NH_2-terminus might interact, in addition to BP180, with the β4 subunit of the α6β4 integrin[1] (Koster J. and Favre B., unpublished results).

Immunoblot and immunoprecipitation studies have shown that almost all BP sera exhibit reactivity to BP230, and up to 60% to BP180 (Stanley *et al*, 1981; Labib *et al*, 1986; Bernard *et al*, 1989; Mueller *et al*, 1989). Although the majority of BP sera recognize immunodominant sequences located within the extracellular NC16A domain close to the transmembrane region, additional antigenic sites exist on both the ECD and the intracellular domain of BP180 (Giudice *et al*, 1993, 1994; Balding *et al*, 1996; Matsumura *et al*, 1996; Zillikens *et al*, 1997; Perriard *et al*, 1999). In contrast, the characterization of the autoantibody response to BP230 is poor. Initial studies have demonstrated significant reactivity against the COOH-terminal region of BP230. Nevertheless, these studies (i) have been limited by the use of either bacterial fusion proteins and synthetic peptides covering small portions of BP230 or by cDNAs encoding for murine BP230, and (ii) did not allow any conclusions to be drawn about the exact distribution of the major autoantibody-reactive sites on the entire autoantigen (Rico *et al*, 1990; 1996; Tanaka *et al*, 1991; Miller *et al*, 1993; Gaucherand *et al*, 1995; Ide *et al*, 1995).

Recently, it has been shown that rabbit antibodies raised against the murine BP180 ECD induce a blistering disorder mimicking BP (Liu *et al*, 1993, 1995) when passively transferred to neonatal mice. In contrast, autoantibodies against BP230 cause an inflammatory reaction in rabbits only after additional injury to their epidermis (Hall *et al*, 1993). These observations have led to the speculation that antibodies against the ECD of BP180 are pathogenetically critical, whereas the appearance of antibodies against intracellular antigenic determinants on BP230 (and on the intracellular domain of BP180) represents a secondary event. Even if this model is confirmed by experimental data, the presence of autoantibodies to BP230 may still be important for the perpetuation of the disease following membrane damage by either eliciting an inflammatory reaction and/or inhibiting the function of BP230.

To extend our understanding of the pathophysiology of BP, we examined more rigorously whether BP autoantibodies target functionally important regions of BP230 and determined their IgG subclass distribution. The reactivity of BP patients' sera was assessed by immunoblotting using a series of novel recombinants covering almost the entire BP230 that were expressed either by an *in vitro* translation system or by using extracts of transfected COS-7 cells.

MATERIALS AND METHODS

Human sera Serum samples were obtained from adult patients with BP (n = 25), pemphigus vulgaris (n = 6), and healthy volunteers (n = 12). The clinical diagnosis of BP was confirmed by histology, direct immunofluorescence microscopy (deposits of IgG and/or C3 in epidermal basement membrane), and indirect immunofluorescence microscopy (autoantibodies binding to the epidermal side of 1 M NaCl-separated normal human skin at titers of 1:20 or more) (Gammon *et al*, 1984). The serum samples from BP patients were selected for the study on the basis of their reactivity with BP230 by immunoblotting of keratinocyte extracts (Bernard *et al*, 1989). In addition, 10 of these BP sera also contained antibodies targeting BP180 (sera uniformly diluted at 1:20).

Antibodies The following antibodies were used: the monoclonal antibody (MoAb) MYC 1–9E10.2 against a defined c-myc epitope (Evan *et al*, 1985); a rabbit antiserum raised against a BP230 recombinant encompassing residues 1722–2114 of BP230 between the coiled-coil and the B subdomain (kindly provided by Dr. J. R. Stanley, University of Pennsylvania, Philadelphia) (Tanaka *et al*, 1990); the human MoAb 5E binding to the linker region between the B and C subdomains of the BP230 tail (generously given by Dr. T. Hashimoto, Keyo University School of Medicine, Tokyo) (Hashimoto *et al*, 1993); the anti-GFP polyclonal antibody (Clontech Laboratories, Palo Alto, CA). The MoAb against IgG subclasses included clone NL6 for IgG_1 (IUIS/WHO code HP6012; working dilution 1:200), clone GOM 1 for IgG_2 (HP6008; dilution 1:200), clone ZG4 for IgG_3 (HP6010; dilution 1:1000), and clone RJ4 for IgG_4 (HP6011; 1:1000) (Oxoid, Basel, Switzerland). These MoAb were chosen on the basis of their specificities, extensively evaluated in previous analyses (Jefferis *et al*, 1985). In addition, these MoAb had been used in previous studies to examine the IgG subclass distribution in the BP group of disorders (Bird *et al*, 1986; Kelly *et al*, 1989; Bernard *et al*, 1990). The sensitivity of the MoAb at their working dilutions was pretested in immunoblotting experiments using $IgG_{1–4}$ purified from myeloma proteins.

cDNA constructs The human BP230 nucleotide and protein sequences are numbered according to the published sequence of human BP230 (GenBank accession number M69225) and to Sawamura *et al* (1991), respectively. For cloning procedures, the cDNA constructs DN86 and DN157 (Stanley *et al*, 1988; Elgart and Stanley, 1993) as well as pcBPA-10 and pcBPA-4 (Sawamura *et al*, 1991), which were isolated in a λgt11 library screen, were used. To generate clone A, pcBPA-10 was utilized as a

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[2]Hopkinson SB, Jones JCR: BPAG2 (type XVII collagen) associates with the N-terminal domain of BPAG1 in hemidesmosomal plaques. *J Invest Dermatol* 112:527, 1999 (abstr.)

template in polymerase chain reaction (PCR) with recombinant *PfuTurbo* DNA polymerase (Stratagene, La Jolla, CA) using a 5′ primer that contained a *Not* I, *Sal* I, and a *Sma* I site (underlined), and the nucleotides corresponding to sequences 39–61 of BP230 including the endogenous initiation starting codon (GCCGGCGGCCGCAGGTCGACCCGGG-*ATGCACAGTAGTAGTTATAGTTA*), whereas the 3′ end primer contained a *Not* I site, a stop codon (bold), and nucleotides corresponding to sequences 1682–1703 of BP230 (CGATGCGGCCGC-**TTA***ATTCCTTGGCTTCAGTTGAATT*). The generated fragment was digested with *Not* I and cloned in frame into a *Not* I-opened modified pcDNA 3 vector (Invitrogen, San Diego, CA), which at the 5′ end of its multiple cloning site contained a sequence encoding the c-myc epitope (MEQKLISQQDL) (kindly provided by Dr. E. Sander, The Netherlands Cancer Institute, Amsterdam). Clone B was derived from DN157, a clone spanning nucleotides 2023–3960 of BP230. DN157 was used as a template in PCR using a 5′ primer that contained a *Not* I and the nucleotides corresponding to sequence 2024–2047 of BP230 (GCCGGCGGCCGC-*AATTCGAGCTAGCAATGTGGCTTC*), whereas the 3′ end primer contained a *Not* I site, a stop codon (bold), and nucleotides corresponding to sequences 3894–3917 of BP230 (CGATGCGGCCGC**TTA***CTGCTT TATCAGC TTCAAGAGTTC*). After *Not* I-digestion the PCR fragment was cloned in frame into the *Not* I-opened modified pcDNA3 vector. For generation of cDNA clones C and D, a first cDNA fragment was isolated by digestion with *Eco*R I from clone DN86 (positions 4813 and 6645 of BP230) (Stanley *et al*, 1988; Elgart and Stanley, 1993), and a second cDNA fragment was obtained by digestion of pcBPA-4 (Sawamura *et al*, 1991) using *Eco*RI (position 6645) and *Sac* II (position 8921). The two isolated cDNA fragments were then ligated in frame in the eukaryotic expression vector pGFP-C3 (Clontech Laboratories). For construction of clone C, the novel pGFP-C3 plasmid was cut with *Ecl* 136 II and *Eco*R V (cutting in the 5′ end of the multiple cloning site of pGFP-C3 and at 8060 of BP230, respectively). The *Ecl* 136 II-*Eco*R V fragment was then implanted in frame into the blunt-ended *Not* I site of the modified pcDNA3 plasmid. As clone DN86 contained a 2 bp deletion (position 5713–5714) resulting in a premature termination codon after four additional residues, the correct coding sequence encompassed nucleotides 4813–5711. For generation of clone D, the pGFP-C3 plasmid was digested with *Xho* I and *Apa* I (cutting at 5874 of BP230 and in the 3′ end region of the multiple cloning site of the vector, respectively). The *Xho* I-*Apa* I fragment was then cloned in frame into the *Xho* I and *Apa* I sites of the modified pcDNA 3 vector. For generation of clone E, construct pcBPA-4 was first digested with *Eco*R 1 (position 6645) and *Eco*R V (position 8060). The purified *Eco*R I and *Eco*R V fragment from this clone was then implanted in frame in the *Eco*R I and *Eco*R V sites of the modified pcDNA3 vector. Clone F was generated by ligating the BP230 fragment from *Bsa*B I (at 6265) to *Bsr*F I (at 7345), blunt-ended with *PfuTurbo* DNA polymerase (Stratagene), into pEGFP-C3 (Clontech Laboratories) cut with *Sma* I. This plasmid was then digested with *Bgl* II, the overhangs filled in with *PfuTurbo* DNA polymerase (Stratagene), and religated together to put the BP230 protein sequence in frame with the GFP. Clone G was prepared by inserting the BP230 fragment from *Mun* I (at 7270) to *Eco*RI (at 8930) into pEGFP-C3 digested with *Eco*R I. The correctness of the cloning sites and of the sequences corresponding to the used primers was verified by sequence analysis. cDNAs encoding the ECD (residues 490–1497) and the intracellular domain (residues 1–466) of BP180 have been described previously (Borradori *et al*, 1997; Perriard *et al*, 1999).

***In vitro* translation** BP230 mutants were produced by an *in vitro* transcription and translation system according to the manufacturer's advice (TNT T7 Coupled Reticulocyte Lysate System, Promega, Madison, WI). Each reaction tube contained rabbit reticulocyte lysate, TNT T7 RNA polymerase, RNasin ribonuclease inhibitor, a 20 μM amino acid mixture, and 0.5 μg of plasmid in a final volume of 50 μl. For radioactive control reaction, an amino acid mixture minus methionine with 40 μCi of [^{35}S]methionine was used. After incubation at 30°C for 90 min, the translation reaction was mixed with a sodium dodecyl sulfate (SDS) sample buffer and heated at 100°C for 3 min before use.

Transfection experiments The African monkey kidney cell line COS-7, which does not express endogenous BP230 and BP180 (Borradori *et al*, 1997), was transfected using the DEAE-dextran method as previously described (Cullen, 1987). Gene expression was assayed after 48 h. Cells were lyzed with 1% SDS in 25 mM Tris(hydroxymethyl)-aminomethane (Tris) HCl, pH 7.5, 4 mM ethylenediamine tetraacetic acid, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg leupeptin per ml, and 10 μg soybean trypsin inhibitor per ml. Protein concentration in the cell lysates was determined using the DC Protein assay (Biorad, Glattbrugg, Switzerland).

Western blot analysis Ten microliters of the denatured translation reaction or 50–100 μg of extracts of transfected COS-7 cells were subsequently loaded per well on a 5% or 7.5% SDS-polyacrylamide gel, separated, and electrophoretically transferred to nitrocellulose sheets (Sonnenberg *et al*, 1993). The nitrocellulose paper was cut into strips and incubated with a 1:20 dilution of each tested serum in Tris-buffered saline-0.5% Nonidet P40 for 2 h at room temperature. After washing in Tris-buffered saline-0.5% Nonidet P40, the strips were then incubated with peroxidase-conjugated goat antihuman IgG (H + L) antibody (Institut Pasteur, Marnes la Coquette, France), goat antimouse Ig (Amersham Life Sciences, Zürich, Switzerland), or goat antirabbit IgG Fab′ fragment (Cappel, Durham, NC). For IgG subclass determination, the strips were incubated for 1.5 h in an intermediate step with the various IgG-subclass-specific MoAb diluted (see above) in washing buffer. After washing, the strips were developed with 3,3′-diamino-benzidine-4 HCl (0.5 mg per ml) in 100 mM Tris-HCl, pH 7.4, containing 0.01% H_2O_2.

RESULTS AND DISCUSSION

Generation of recombinant fragments of BP230 for epitope mapping Previous studies have only provided a suggestion that the autoantibody reactivity to BP230 is restricted to its COOH-terminal domain, as large regions within both the NH_2-terminal and the central coiled-coil regions of BP230 were not systematically assayed. To more rigorously identify antigenic sites on BP230, we generated cDNA constructs for BP230 that were used for the production of recombinant fragments encompassing almost the entire 2649-amino acid autoantigen with an *in vitro* translation system (**Fig 1**). This approach was chosen as (i) the expression of BP230 recombinants using various cell lines has proved to be difficult, most probably due to an instability of the proteins (Yang *et al*, 1996; personal observations); and (ii) it allowed the harsh and time-consuming extraction procedure for purifying (large) bacterial BP230 recombinants to be avoided (Tanaka *et al*, 1990, 1991).

Circulating IgG from BP patients predominantly bind to the COOH-terminal region of BP230 The reactivity of 25 BP sera was assessed against the generated BP230 recombinant fragments by immunoblotting analyses. The results demonstrate that BP autoantibodies recognize multiple antigenic sites located not only on the COOH-terminus but also within the central coiled-coil and NH_2-terminal region of BP230 (**Table 1**). In extension to previous studies (Tanaka *et al*, 1991; Miller *et al*, 1993; Gaucherand *et al*, 1995; Ide *et al*, 1995), our findings demonstrate that the major autoantibody-reactive sites are indeed contained within the COOH-terminal region of BP230, at least as assessed under the denaturing conditions of the immunoblot technique. Twenty-one (84%) and 16 of 25 (64%) BP sera reacted with recombinant fragments D and E corresponding to distal 704- and 447-residue stretches, respectively, of the BP230 tail (**Fig 2**). None of the control serum samples from normal volunteers (n = 12) and pemphigus patients (n = 6) showed binding to these recombinant proteins. Similar reactivities were observed by Tanaka *et al*, who found that 84% and 61% of BP sera immunoblotted two recombinants encompassing 997-residue and 507-residue segments, respectively, of the tail of murine BP230 (Tanaka *et al*, 1991), as well as by Ide *et al* utilizing an enzyme-linked immunosorbent assay (ELISA) with these same recombinant proteins (Ide *et al*, 1995). Our results indicate that the antigenic sites are predominantly located downstream of residue 1891 of BP230, because the more proximal region close to the coiled-coil region covered by recombinant C (covering residues 1593–1891), to which only one of 25 (4%) BP sera bound, exhibits poor reactivity. Although this idea is supported by findings obtained with sera from Japanese BP patients, Rico *et al* found that 48% of BP sera from the United States (but only 5% of control sera) immunoblotted a bacterial recombinant containing residues 1623–1812 of BP230 (Rico *et al*, 1996). Differences in the studied BP populations and in their immunogenetic restriction may account for these discordant results. Finally, recombinants D and E were recognized by the MoAb 5E (Hashimoto *et al*, 1993), whereas a rabbit antiserum to BP230 (Tanaka *et al*, 1990) reacted with recombinants C and D (not shown).

Table I. Clinical features of bullous pemphigoid (BP) patients and reactivity of their sera in immunoblot analyses against various recombinants of BP230 expressed by an *in vitro* translation system[a]

BP sera	Age	Duration	Skin lesions	Treatment[b]	BP 230 recombinants				
					A	B	C	D	E
BP1	78	1 y	Back, limbs	PDN 10 mg	−[c]	−	−	+	−
BP2	68	4 mo	Limbs	PDN 7.5 mg, Aza 100 mg	+	−	−	+	+
BP3	94	3 wk	Limbs	None	−	+	−	+	+
BP4	88	1 mo	Generalized	PDN 100 mg	−	−	−	+	+
BP5[d]	88	1 y	Generalized	PDN 30 mg, Aza 50 mg	−	+	−	+	+
BP6[d]	93	1.5 y	Scalp, limbs	None	+	−	−	+	+
BP7[e]	91	3 mo	Generalized	PDN 40 mg, Aza 100 mg	+	+	−	+	+
BP8	72	3 y	Trunk, limbs	None	−	−	−	+	+
BP9	82	5 mo	Trunk, limbs	PDN 40 mg, CB 6 mg	−	−	−	+	+
BP10[d]	91	2 mo	Back, trunk, limbs	PDN 40 mg, CB 6 mg	++	++	−	−	−
BP11	50	2 y	Generalized	Thalidomide 100 mg	−	−	−	++	+
BP12[d]	77	10 y	Back, trunk, limbs	PDN 30 mg, CB 4 mg	−	+	−	+	−
BP13[e]	83	1 wk	Limbs	None	−	−	+	−	−
BP14	67	6 mo	Back, trunk, limbs	PDN 40 mg, CB 6 mg	+	−	−	+	+
BP15	82	1 wk	Back, limbs	None	++	+	−	++	+
BP16	75	6 mo	Face, limbs	None	−	−	−	+	+
BP17	95	1 wk	Limbs	PDN 20 mg, Aza 20 mg	−	−	−	+	+
BP18	75	6 mo	Generalized	Mn 200 mg, NA 120 mg	−	+	−	++	+
BP19[e]	77	1 mo	Generalized	None	−	−	−	+	−
BP20[d]	89	1 mo	Trunk, limbs	PDN 30 mg	−	−	−	+	+
BP21[d]	89	2 mo	Trunk, axillary, limbs	PDN 35 mg	−	−	−	+	−
BP22[d]	60	3 mo	Generalized	PDN 90 mg, Aza 100 mg	−	−	−	−	−
BP23	85	4 mo	Back, trunk, mouth	PDN, Aza	−	−	−	+	+
BP24	75	2 mo	Generalized	Mn 200 mg, MM 2 g	+	−	−	+	−
BP25	76	1 y	Generalized	PN 60 mg, Dapsone 100 mg	+	+	−	−	−
Positive sera					8/25 (32%)	8/25 (32)	1/25 (4%)	21/25 (84%)	16/25 (64%)

[a]As described in *Materials and Methods*. The regions covered by the recombinant fragments are depicted in Fig 1.
[b]PDN, prednisone; Aza, azathioprine; CB, chlorambucil; Mn, minocycline; NA, nicotinamide MM, mycophenolate mofetil.
[c]Intensity of labeling; ++, strong positive; +, positive; −, negative.
[d]Serum sample also reactive against the extracellular domain of BP180 as specified in the *Materials and Methods*.
[e]Serum sample also reactive against both the extracellular and the intracellular domain of BP180.

BP sera also react with multiple antigenic reactive sites on the NH_2-terminal half of BP230 Our study shows that a substantial number of BP230-reactive sera display IgG reactivity to the head of BP230 (**Table I**). Eight (32%) of 25 of the BP sera had IgG that recognized recombinant A containing a 555-residue NH_2-terminal stretch of BP230, whereas the same percentage of BP sera bound to recombinant B, a more distal fragment close to the coiled-coil domain (**Fig 2**). In line with the latter findings, a bacterial fusion protein encompassing residues 1003–1193 adjacent to the NH_2-terminal end of the coiled-coil region was bound by 38% of the BP sera (Rico *et al*, 1996). To exclude the possibility that autoantibodies directed against the COOH-terminus of BP230 cross-reacted with its NH_2-terminus, IgG from a representative patient with autoantibodies binding both the NH_2- and COOH-terminus of BP230 was affinity-purified against recombinant D and tested against recombinant A. Patient IgG affinity-purified against the COOH-terminus of BP230 did not immunoblot the NH_2-terminus of BP230 (not shown), indicating that there was no antigenic cross-reactivity between the NH_2- and COOH-termini of BP230. This idea was further supported by the observation that two of 25 (8%) BP sera had an antibody response restricted to the NH_2-terminal half of BP230 (BP10 and BP25). Based on these findings, it can be anticipated that screening of BP sera utilizing recombinant fragments encompassing only the BP230 tail would miss some autoreactivities. Finally, the observation that one of the tested sera (BP22) did not bind to BP230 recombinants A to E suggests that this patient's autoantibodies reacted with sequences not present in the expressed recombinant fragments, or, alternatively, reflects loss of reactivity due to sample storage. Preliminary findings indicate that immunoblot analysis using BP230 recombinants expressed by an *in vitro* translation system has a sensitivity at least comparable to that of conventional immunoblotting using keratinocyte extracts.

Autoantibody-reactive sites are clustered within the B and C subdomains of BP230 To better characterize the location of the immunodominant sequences within the tail of BP230, we expressed recombinant fragments encompassing either the B subdomain with the linker region (recombinant F) or the C subdomain alone (recombinant G) in transfected COS-7 cells. For this purpose, the recombinants were fused to a green fluorescent protein to increase their stability (Smith and Fuchs, 1998). Our results showed that 18 of 21 (85.7%) BP sera reacting with recombinant D also immunoblotted recombinant F, a segment of 361 amino acids, whereas 16 (76%) sera bound to the 238-residue recombinant G (**Table II**, **Fig 3**). None of the control normal human sera (n = 8) exhibited binding to these recombinant fragments. Interestingly, the segment spanning the B, the linker, and the C subdomains contains several amino acid stretches (residues 2285–2288, 2295–2297, 2344–2347, 2394–2397, 2467–2469, 2493–2498, 2595–2598, and 2607–2611) predicted to have a high antigenic index (Jameson and Wolf, 1988). In line with this prediction, a synthetic peptide encompassing residues 2287–2302 was found to be recognized by 35% of 37 BP sera in an ELISA (Rico *et al*, 1990). Moreover, the linker region of BP230 and of the other plakin members envoplakin, periplakin, and desmoplakin, which show high homology with each other, is frequently targeted by autoantibodies from patients with paraneoplastic pemphigus (Mahoney *et al*, 1998), an autoimmune mucocutaneous disease with a peculiar autoantibody response to plakins. Together with

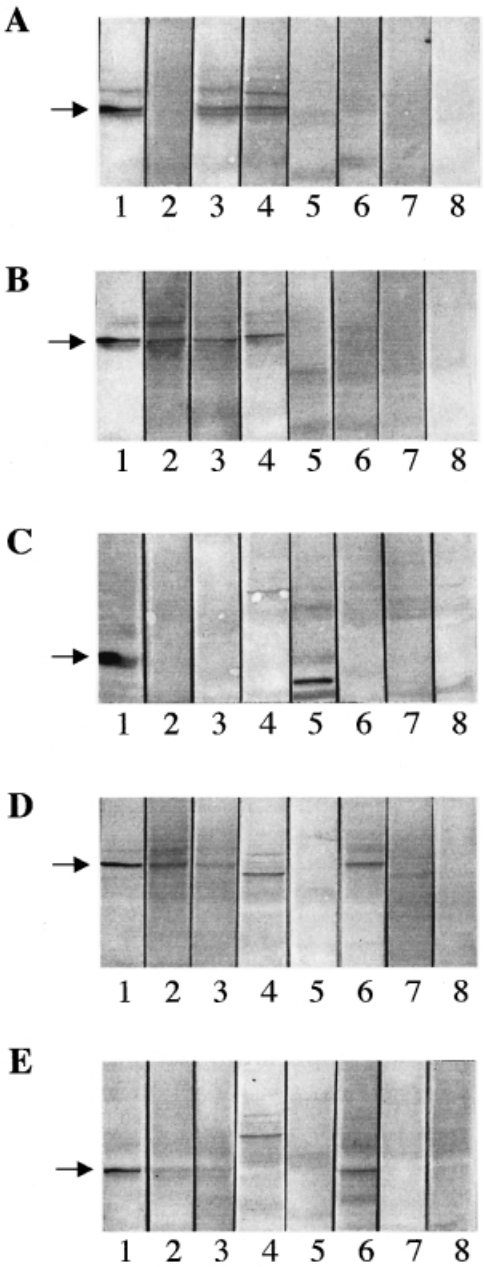

Figure 2. BP sera react with multiple antigenic epitopes throughout BP230. Reactivity of 25 BP sera was assessed by immunoblotting as described in *Materials and Methods* against BP230 recombinants (listed in **Fig 1**) that were expressed by an *in vitro* translation system. Reactivity against recombinants A (*A*), B (*B*), C (*C*), D (*D*), and E (*E*). *Lanes 1–8* correspond to MoAb 9E10 anti-c-myc-epitope, and sera from BP patients BP3, BP7, BP10, BP13, BP17, and two normal volunteers, respectively. The apparent molecular weights of the recombinants (*arrow*), each epitope tagged with c-myc at their 5′ end, were slightly smaller than the sizes predicted on the basis of the corresponding sequences, i.e., 63 kDa, 75 kDa, 35 kDa, 79 kDa, and 50 kDa for recombinant A, B, C, D, and E, consistent with the abnormal electrophoretic migration of BP230 and other plakins (Wiche *et al*, 1993). Each of the 5 BP sera exhibits a different pattern of immunoreactivity and is representative of the reactivity patterns observed with other BP sera. Note that some additional reactive bands were occasionally found with both experimental and control sera and most probably represented either unspecific background or reactivity against proteins in the lysate translation system. Samples were separated by 7.5% SDS-PAGE under nonreducing conditions.

these findings, our results provide strong evidence that the region between the B and C subdomains of BP230 harbors several clustered antigenic sites.

Table II. Immunoblot reactivity of BP sera binding to recombinant D against distinct regions encompassing the B domain and the linker (recombinant F) and the C domain of BP230 (recombinant G)[a]

	BP 230 recombinants	
	F	G
BP sera	18/21 (85.7%)	16/21 (76%)
Control sera	0/8 (0%)	0/8 (0%)

[a]Expressed in transfected COS-7 cells as specified in *Materials and Methods*.

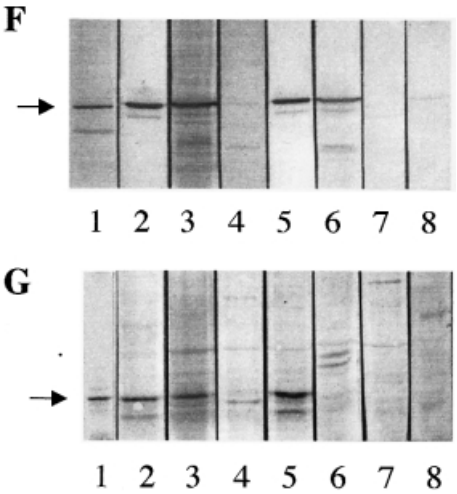

Figure 3. Immunodominant sequences are located within the B domain, the linker, and the C domain of BP230. Reactivity of the 21 BP sera binding to the COOH-terminal domain (recombinant D) were assayed by immunoblotting as described in *Materials and Methods* against recombinants F and G (**Fig 1**) that were expressed by transfected COS-7 cells. Reactivity against recombinants F (*F*) and G (*G*). *Lanes 1–8* correspond to rabbit anti-GFP, and sera from BP patients BP3, BP7, BP12, BP17, BP21, and two normal volunteers, respectively. Proteins close to their predicted mass of 61 kDa and 45 kDa, respectively, are recognized by the rabbit antiserum against GFP. The 5 BP sera are representative of the reactivity patterns observed with other BP sera. Note that some additional reactive bands were occasionally found with both experimental and control sera and most probably represented either unspecific background or reactivity against proteins in COS-7 extracts. Samples were separated by 7.5% SDS-PAGE under nonreducing conditions.

IgG autoantibodies binding to the BP230 tail predominantly belong to the IgG_4 and IgG_1 subclasses Previous studies have reported a predominance of the IgG_4 and IgG_1 subclasses in circulating BP autoantibodies (Bird *et al*, 1986; Yamada *et al*, 1989; Bernard *et al*, 1990; Soh *et al*, 1991). The specific subclass pattern of autoantibodies to either BP230 or BP180, however, was not investigated except in one study. Therefore, we qualitatively assessed the IgG subclass distribution of the autoantibody response in all but one BP serum sample reactive with the immunodominant BP230 tail (recombinant D). Seventeen (85%) and 16 (80%) of the 20 BP sera available for testing exhibited autoantibodies of the IgG_4 and IgG_1 subclass, respectively, binding to recombinant D (**Fig 4**). In 14 patients, both IgG_4 and IgG_1 were present. Only one (5%) BP serum contained IgG_2 autoantibodies, whereas five of 20 sera (25%) had autoantibodies of the IgG_3 subclass. Our findings are in apparent contrast with a previous study, in which a predominant IgG_4 restriction for BP230 was found (Bernard *et al*, 1990). As the MoAb utilized were the same, such difference is most probably due to the fact that the latter study tested BP sera at higher dilutions (1:100), providing support to the idea that IgG_4 is present in higher concentrations than IgG_1. Nevertheless, the presence of IgG_1 and

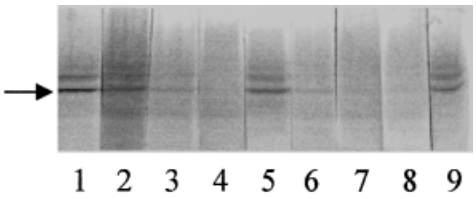

Figure 4. IgG autoantibodies binding to the BP230 tail belong predominantly to the IgG_4 and IgG_1 subclasses. The subclass distribution of the IgG autoantibody response to recombinant D was assessed by immunoblotting as described in *Materials and Methods* for 20 BP sera. Reactivity with MoAb 9E10, *lane 1*; with BP7 serum, *lanes 2–6*; and with BP18 serum, *lanes 5–8*. The sera were probed for IgG_1 (*lanes 2* and *5*), for IgG_2 (*lanes 3* and *7*), for IgG_3 (*lanes 4* and *8*), and for IgG_4 (*lanes 5* and *9*). Samples were separated by 7.5% SDS-PAGE under nonreducing conditions.

IgG_4 suggests that both autoreactive Th_1 and autoreactive Th_2 cells (Romagnani, 1992) are involved in the regulation of the autoantibody response to the immunodominant region of BP230. In fact, Th_2 cytokines such as IL-4 and IL-13 have been shown to regulate the secretion of IgG_4, whereas the Th_1 cytokine interferon-γ induces the secretion of IgG_1 (reviewed in Romagnani, 1992; Büdinger *et al*, 1998). Although no data are yet available for BP230, BP180-specific autoreactive T cell lines were found to produce both Th_2 and Th_1 cytokines (Büdinger *et al*, 1998) in keeping with the observation that IgG_4 and IgG_1 are also the major subclasses in autoreactivity to BP180 (Bernard *et al*, 1990).

"Epitope spreading," BP230 antigenic sites, and disease activity In the course of an autoimmune disease, B and T cell responses are not restricted to a unique "immunodominant" epitope, but recognize additional "secondary" epitopes within the same protein or distinct molecules that might play a key role for the progression and perpetuation of the disease (Vanderlugt and Miller, 1996; Chan *et al*, 1998). Hence, the identification of several antigenic sites throughout BP230 (and BP180) in the present and previous studies most probably reflects this "epitope spreading" phenomenon. It remains unclear, however, which epitopes of BP180 and/or BP230 are crucial for disease initiation. In this retrospective analysis, no obvious correlation could be established between disease duration and activity and the epitope reactivity of anti-BP230 autoantibodies (**Table I**). Prospective studies utilizing more sensitive techniques such as ELISA, in which the antigens are assayed under native conditions allowing detection of autoantibodies against conformational epitopes, together with investigations of T cell responses against BP230 and BP180 will hopefully provide insights crucial for the identification of pathogenetically relevant epitopes. The idea that conformation-dependent epitopes on BP230 exist is indeed supported by the observation that immunoprecipitation studies, in which the antigen-antibody reaction occurs before the denaturation of SDS polyacrylamide gel electrophoresis (PAGE), were found to be more sensitive than immunoblotting for detecting immunoreactivity with BP230 (Mueller *et al*, 1989).

The targeted domains of BP230 are functionally important It is tempting to correlate our epitope mapping results with recent cell biologic studies. A 768-residue stretch of the BP230 tail has been shown to contain sequences required for the interaction of BP230 with the intermediate filament cytoskeleton (Yang *et al*, 1996). In line with these findings, BP230-null mutant mice exhibit an impaired anchorage of keratin intermediate filaments to HD in basal keratinocytes with discrete signs of blistering, most probably because of reduced mechanical strength (Guo *et al*, 1995). In contrast, the NH_2-terminal domain of BP230 appears to be important for the localization of the molecule to HD, by containing binding site(s) critical for its association with the cytoplasmic domain of BP180 and the $\beta 4$ subunit of the $\alpha 6\beta 4$ integrin (**Fig 1**). Based on these findings, it is conceivable that upon tissue injury – or even by penetrating intact cells (Alarcón-Segovia *et al*, 1996) – autoantibodies to BP230 get into the cell, bind to the target antigen, and contribute to subepidermal blister formation and disease perpetuation not only by eliciting an inflammatory response but also by interfering with the function and molecular interactions of BP230, and, by this means, with HD stability. Electron microscopy studies showing disappearance of the cytoplasmic plaque of HD and anchoring filaments with degenerative alterations within the cytoplasm of basal cells in noninflammatory bullae of BP do not exclude this possibility (Jakubowicz *et al*, 1970).

In conclusion, our study represents a comprehensive epitope mapping analysis of BP230 utilizing recombinants encompassing almost the entire autoantigen. Multiple reactive antigenic sites exist over the entire molecule. Nevertheless, the region spanning from the B to the C subdomain within the BP230 tail contains immunodominant sequences recognized by the majority of BP sera. The identification of autoantibodies predominantly belonging to the IgG_4 and IgG_1 subclasses suggests that both autoreactive TH_2 and autoreactive TH_1 cells are involved in the regulation of the autoantibody response against the immunodominant BP230 tail. These results provide additional insights relevant for our understanding of the pathophysiology of BP.

This work has been supported by a grant from the Swiss National Foundation for Scientific Research (32–51083.97 to L.B.) and by the German Research Council (grants 1604/5–1 and 1604/5–2 to M.H., and SFB 293/B3 to L.B-T.). L.B. is the recipient of grants of the Fondation Touraine (Paris), TELETHON Action Suisse (Aubonne), and the Fondation de Reuter (Genève). The authors are particularly indebted to Dr. J.R. Stanley (University of Pennsylvania, Phildelphia) and Dr. J. Uitto (Jefferson Medical College, Philadelphia), who generously provided cDNAs for human BP230.

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