UNIVERSITATEA DE MEDICINĂ ŞI FARMACIE
“IULIU HAŢIEGANU”CLUJ-NAPOCA

REZUMAT TEZA DE DOCTORAT

Implicarea patogenetica a autoanticorpilor in afectiunile buloase autoimune, in special pemfigoidul bulos

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Cuprins

INTRODUCERE

Tesutul epitelial este format din mai multe straturi, realizand o adeziune puternica intre celule, cu substanta intercelulara redusa. Acest teSut formeaza o serie de structuri care acopera in intregime suprafata corpului, dar totdata captuseste si cavitatea organelor interne. Ca structura de organ, pielea pastreaza matricea de bistratatlitate, cele doua straturi fiind dealtfel doua tesuturi distincte: epidermul si dermul, separate printr-o membrana bazala. Fiziologia tesutului epitelial este foarte complexa, principalele funcții fiind de: bariera de protectie, reglare a temperaturii, protecție mecanica si funcție imunologica sau senzoriala. Arhitectura epidermala, necesara pentru funcția de bariera a pielii, este perturbata in diverse afecțiuni cutanate, printre care si afectiunile buloase autoimune.

Integritatea pielii este asigurata prin diverse tipuri de adeziuni, atat la nivelul keratinocitelor cat si intre keratinocite si mebrana bazala. Proteinele transmembranare si matriceale care asigura aderența intre celule si matrix au fost denumite molecule de adeziune, fiind grupate in mai multe complexe de legatura: joncțiunile stranse, desmozomi, jonctiunile “gap”, adeziuni focale si hemidesmozomi.

Cercetarile in aceasta directie au demonstrat ca prin intermediul acestor legaturi celula-celula sau celula-matrice extracelulara, se transmit informații din exteriorul celulei spre interiorul celulei. Absența contactului cu mediul si a informațiilor din mediu unei celule poate constitui in multe cazuri o cauza pentru intrarea celulei in procesul de apoptoza.

Epidermul este separat de derm printr-o membrana bazala, in compoziția careia intra proteine ale matricei extracelulare, cum sunt integrina α6β4, laminina 5, colagenul IV si VII si fibronectina. La nivelul țesutului cutanat, membrana bazala are ca si corespondent microscopic jonctiunea dermo-epidermala (DEJ). Adeziunea keratinocitelor la nivelul membranei bazale este determinata prin intermediul adeziunilor focale si a hemidesmozomilor.

Hemidesmozomii practic asigura adeziunea epiteliului uni- sau pluristratificat de membrana bazala, structural fiind formați din placa citoplasmatica, proteine transmembranare, precum si o serie de proteine asociate membranei bazale. Placa citoplasmatica are rol de a lega proteinele care apartin citoscheletului de proteinele transmembranare si mai departe prin acestea, asigura legarea de membrana bazala. Conține mai multe proteine precum: antigenul pemfigoidului bulos, plectina si o serie de proteine mai putin definite precum HD1, IFAP300 si P200.

Antigenul pemfigoidului bulos (BP230) este o proteina care, ca si plectina aparține familiei plakinelor, fiind principala ținta a autoanticorpilor in pemfigoidul bulos. Capatul COOH se asociaza cu filamentele de keratina la nivelul placii hemidesmozomale, iar capatul NH2 se asociaza cu proteine de adeziune transmembranare cum ar fi: β4 integrina sau colagenul de tip XVII (BP180).

STADIUL ACTUAL AL CUNOASTERII

Afecțiunile buloase autoimune sunt un grup de afectiuni rare, potențial fatale, caracterizate clinic prin prezența de leziuni buloase, fiind asociate cu un raspuns imun impotriva proteinelor structurale ale pielii care mentin adeziunea intercelulara in epiderm si adeziunea keratinocitelor bazale de membrana bazala si dermul subiacent.

In funcție de trasaturile histopatologice si imunopatologice, afectiunile buloase autoimune sunt clasificate in doua categorii: buloze intraepiteliale si respectiv, subepidermale. Prima categorie, denumita si "pemfigus" este caracterizata de apariția de vezicule/bule intraepiteliale, declansate de pierderea adeziunii intre celulele epiteliale, imunologic definita de prezenta autoanticorpilor impotriva jonctiunilor celulare. A doua categorie, denumita "pemfigoid" rezulta in urma detasarii keratinocitelor bazale de membrana bazala, caracterizata imunologic prin apariția autoanticorpilor impotriva proteinelor de adeziune de la nivelul jonctiunii dermo-epidermale.

Pemfigoidul bulos (BP) este o boala cutanata buloasa, subepidermala, autoimuna, cu implicarea mai rara si a mucoaselor. Imunologic este caracterizat de prezenta in serul pacientilor a autoanticorpilor circulanți specifici pentru antigenele hemidesmozomale. Aspectul clinic al acestei afectiuni implica formarea de bule sau flictene la nivelul joncțiunii dermo-epidermale. Diagnosticul este confirmat prin evidențierea depozitelor de IgG si complement C3 la nivelul membranei bazale prin tehnici de imunofluorescenta. Mecanismul patogenetic consta in fixarea autoanticorpilor la nivelul membranei bazale cu formarea de complexe imune la acest nivel. Ulterior, aceste complexe imune activeaza mediatorii inflamatori si complementul, cu efecte chemotactice pentru celulele inflamatorii. Astfel, ajunse la acest nivel, celulele imune vor elibera enzime proteolitice (proteaza), capabile sa degradeze proteine hemidesmozomale precum si afectarea integrității keratinocitelor bazale. In final, procesul inflamator susținut va determina formarea de bule si vezicule.

Desi Colagenul de tip XVII (BP180/BPAg2) a fost identificat ca antigenul major implicat in patogeneza pemfigoidului bulos, studiile experimentale au evidențiat o posibila contribuție a proteinei hemidesmozomale BP230, sau mai puțin implicate proteinele α6 integrina si lamina-5.

CONTRIBUTIE PERSONALA

Ipoteza de lucru

Este bine cunoscut rolul patogenetic al anticorpilor anti BP180 in generarea fenotipului clinic al pemfigoidului bulos, prin legarea de aceste antigene, cu formarea ulterioara de complexe imune. Ca rezultat al prezenței acestor complexe imune la nivelul joncțiunii dermo-epidermale, se genereaza activarea complementului in cascada cu producerea unor imunoreactanți capabili sa recruteze si sa activeze granulocitele la acest nivel.

Desi studiile efectuate asupra proteinei BP230 o califica ca si o proteina cu importanța patogenetica minora, rolul autoanticorpilor sintetizați impotriva acestui antigen este putin studiat si cu rezultate uneori contradictorii.

Utilizand acceasi strategie ca si Liu (Liu et al, 1993) pentru investigarea unui posibil potential patogenetic al anticorpilor anti BP230, Kiss et al, au folosit un model experimental de transfer pasiv la soareci neonatali, rezultatele demonstrand potențialul patogenic al acestor autoanticorpi de a induce leziuni tegumentare. Cu toate acestea, autoanticorpii folosiți pentru modelul *in vivo* au fost sintetizati prin injectarea la iepuri a unui compus de proteine recombinante, conținand atat fragmente de BP180 cat si de BP230. Dupa 24 h, ulterior fricționarii usoare a epidermului, s-a constat faptul ca unul din cei 7 soareci neonatali injectați cu 5 mg anticorpi purificați, a dezvoltat leziuni persistente de tip bulos la acest nivel. Astfel, ignorand un posibil triger dat de acțiunea fizica asupra tegumentului, un efect indirect al anticorpilor anti BP230 mediat indirect de posibili anticorpi anti BP180 nu poate fi exclus, chiar daca serul a fost preabsorbit fața de proteina BP180.

Folosind o alta abordare, Hall et al, in urma imunizarii unor iepuri cu un fragment sintetic de 18 aa a antigenului uman BP230, a aratat ca acestia erau rezistenți la inducerea fenotipului clinic de BP. Rezultatele s-au schimbat doar dupa aplicarea de radiații, in acest fel anticorpii legati la nivelul antigenului tegumentar au determinat activarea complementului si a altor tipuri de celule imune, culminanad cu aparitia de leziuni necrozante la acest nivel.

Pentru a aborda aceste neclarificari, in studiul prezent am recurs la investigarea potențialului patogenic al anticorpilor anti BP230 in pemfigoidul bulos. Atfel, dupa expresia ununi fragment recombinant al proteinei murine BP230, anticorpii generați fața de acest antigen, au fost evaluați in privința potențialului patogenic de a induce leziuni tegumentare “spontane” in urma transferului pasiv la soareci adulti sau neonatali.

Material si metode

Pentru studiul actual am produs o forma recombinanta suprapusa a regiunii terminale (COOH) a proteinei murine de 230 kDa implicata in patologia autoimuna din cadrul pemfigoidului bulos. Ulterior, aceasta proteina recombinanta am exprimat-o in bacterii chimiocompetente (*E. Coli Top 10*).

In vederea expresiei acestui antigen (BPAg1/ BP230), cDNA-ul care codifica fragmentul corespunzator acestei proteine (denumit C2) a fost produs prin reacția in lant a polimerazei (PCR) folosind ca matrița o biblioteca de cDNA produs prin revers transcripția mARN-ului izolat din keratinocite de cultura de origine murina (PAM212 cell). Secvențele specifice pentru endonucleazele *SalI* si *BamHI* au fost introduse prin primeri sintetici atat la nivelul insertului (mBP230-C2) cat si la nivelul vectorului (pGEX-SP1).

Vectorul pGEX (conținand secvența "GST" si gena "rezistenta la Ampicilina") impreuna cu fragmentele de cDNA au fost amplificate prin metoda PCR si ulterior supuse digestiei cu enzime de restricție. In urma ligarii, au rezultat vectori recombinanti/plasmide (pGEX-mBP230-C2), fiind inserați la nivelul celulelor bacteriene *E Coli Top 10*. Dupa transformare, bacteriile au fost cultivate pe mediu Luria-Bertani cu carbenicilina, iar la final testate prin PCR pentru prezenta insertului de DNA.

Proteinele de fuziune produse in E Coli au fost purificate prin cromatografie de afinitate folosind o matrice de glutation pentru proteina fuzionata cu GST. Ulterior expresiei proteinei recombinante in cantitate suficienta, studiul initial s-a focusat pe obtinerea de anticorpi fata de proteina BP230 si observarea patogenitatii acestor anticorpi . Anticorpii policlonali obtinuti astfel prin injectarea unor iepuri de laborator cu proteina exprimata, au fost purificați prin cromatografie (coloane de G sefaroza).
Analiza la nivel molecular a specificitații autoanticorpilor a fost efectuata folosind metode: Western blotting, ELISA si Imunofluorescenta.

Experimentele ulterioare cuprinse in studiul al doilea au urmarit evaluarea interacțiunii autoanticorpilor cu complementul si leucocitele umane la nivelul jonctiunii dermo-epidermale. In acest sens, am folosit modele in vivo si in vitro.

Modelul *in vivo* s-a realizat prin transferul pasiv de autoanticorpi anti BP230, la soreci adulți si neonatali, folosindu-se totodata controale negative si pozitive. Lotul de soareci neoantali a fost injectat cu 10mg IgG purificat in decurs de 24 ore, timp de 3 zile, iar soarecii din lotul de adulți au fost injectati cu 15mg/24 ore IgG, pe parcursul a 10 zile.

Modelul *in vitro*, de evaluare a activarii leucocitelor, a constat in secțiuni la criostat de piele umana/murina, incubate cu anticorpii anti BP230 de iepure, urmate de spalare si reincubare cu leucocite si complement de la persoane sanatoase.

Rezultate

Primul set de experimente s-a bazat pe expresia formei recombinante a unui fragment antigenic a proteinei BP230, si o caracterizare imunologica ulterioara a anticorpilor generați la iepuri, imunizați cu proteina recombinanta (GST-mBP230-C2).
Prin analiza imunoblot, am observat o inalta specificitate in recunoasterea formei recombinante a proteinei BP230-C2, atat de catre serul pacientilor cu BP, cat si de anticorpii policlonali generați in laborator.

In continuare, pentru a evalua capacitatea acestor anticorpi in a recunoaste forma nativa a proteinei BP230, am incubat țesut de soarece neonatal cu ser imun de iepuri, iar ulterior cu un anticorp secundar anti IgG. Rezultatele imunofluorescenței au aratat ca anticorpii de tip IgG din serul imun de iepure se leaga la nivelul membranei bazale intr-un patern caracteristic pemfigoidului bulos, in contrast cu anticorpii IgG din serurile pre-imune. Deasemenea, in urma testarii pentru localizarea specifica, antigenul recunoscut de anticorpii imuni de iepure s-a dovedit a fi la nivelul porțiunii epidermale a epiteliului detasat artificial (incubare in solutie NaCl), dealtfel in concordanta cu localizarea binecunoscuta intracelular si respectiv deasupra membranei bazale.
Totodata, prin tehnica de imunoflorescenta am arata ca acesti anticorpi prezinta capacitatea de a fixa complementul, atunci cand am folosit atat ser de soarece, cat si uman ca sursa de complement.

In urmatorul set de experimente am investigat potențialul anticorpilor IgG de iepure anti GST-mBP230-C2 de a induce fenotipul clinic al pemfigoidul bulos. In acest fel, pentru modelul in vivo am folosit anticorpi de iepure anti BP230 si respectiv un control pozitiv reprezentat de anticorpi anti BP180, precum si anticorpi din ser pre-imun ca si control negativ. Transferul de anticorpi s-a realizat la soareci BALB/c la fiecare 12 ore pe o perioada de 3-10 zile. La finalul perioadei, s-a constata ca nici unul din lotul de soareci neonatali sau adulti au dezvoltat leziuni tegumentare de tip vezicule/bule sugestive BP.
Totusi, analiza histopatologica a confirmat prezenta detasarii epidermale in lotul de soareci neonatali imunizati cu anticorpi anti BP180.

Pentru a investiga mai departe aceasta lipsa de patogenicitate a anticorpilor de a induce leziuni clinice, am analizat prin imunofluorescenta, prezența anticorpilor circulanți din serul soarecilor imunizați, precum si prezența depozitelor de IgG si complement la nivelul membranei bazale. Rezultatele au aratat ca anticorpii IgG anti BP230 si anti BP180 recunosc antigenul in vivo, in schimb doar anticorpii anti BP180 au reusit sa activeze complementul.

Aceasta lipsa de patogenicitate a anticorpilor anti BP230 a fost evaluata si prin capacitatea acestora de a activa granulocitele, cu generarea ulterioara de enzime proteolitice si inducerea detasarii dermo-epidermale. Asa cum am precizat la testele de imunofluorescenta, anticorpii anti BP230 si anti BP180 prezinta capacitatea de recunoastere si legare a antigenelor tegumentare, cu formarea de complexe imune la nivelul jonctiunii dermo-epidermale.

Incubarea sectiunilor de piele conținanad complexele imune fixate la nevelul membranei bazale, cu granulocite umane, s-a generat reducerea NBT cu formarea de precipitate de formazan doar de anticorpii anti BP180 si de la pacienti pemfigoid bulos, in contrast cu anticorpii anti BP230 si cei preimuni. Mai departe, pe acelasi model de activare a granulocitelor, am incubat secțiunile de piele ce conțineau complexe imune cu granulocite umane, cu o incubare prelungita. In final, s-a observat faptul ca, doar anticoprii anti BP180 si cei de la pacienți BP au indus activarea granulocitelor la nivelul membranei bazele, culminand cu formarea detasarii dermo-epidermale.

Discutii

Anticorpii policlonali de iepure s-au legat la nivelul jonctiunii DEJ, inducand ulterior activarea complementului prin formarea de complexe imune.
In urma transferului pasiv la soareci neonatali/adulti, acesti anticorpi nu au reusit sa reproduca trasaturile clinice ale pemfigoidului bulos. Mai departe, aceasta lipsa de patogenitate a fost deasemenea confirmata in modelul *ex vivo* de BP folosind asa numita tehnica a “criosectiunilor” pe piele umana.

Fara a exclude in totalitate un posibil rol al anticorpilor anti BP230 in patogeneza pemfigoidului bulos, in acest studiu am adus evidențe solide asupra lipsei de potențial patogenic al acestor autoanticorpi in modele *in vivo* si *ex vivo*.

Aceasta incapacitate de a induce leziuni tegumentare este data de un posibil potențial limitat in legarea antigenului ținta *in vivo*. Pentru ca BP230 este o proteina intracelulara localizata la nivelul placii hemidesmosomale, autoanticorpii nu se pot lega promt la acest nivel si ca atare au nevoie de un triger prealabil pentru axpunerea antigenului. In felul acesta, in prima etapa pare sa fie necesara prezența autoanticorpilor fața de BP180, care prin legare de acest antigen determina activarea mediatorilor proinflamatori cu lezarea ulterioara a integrității membranei keratinocitare, astfel proteina BP230 fiind expusa sistemului imun. Deoarece, este o proteina complet intracelulara, BP230 poate avea o antigenicitate mai inalta decat antigenul BP180.

Alta posibila explicație pentru lipsa de patogenicitate a acestor autoanticorpi poate fi data de lipsa specificitatii in activarea complementului si a granulocitelor.

O alta explicatie, asa cum sugerea Iwata intr-unul din studii (Iwat, 2009), anticorpii fața de BP230 pot induce apariția bolii printr-un mecanism mai subtil , non-inflamator, respectiv epuizarea antigenului la nivelul jonctiunii dermo-epidermale, ca rezultat al formarii de complexe imune.

Nu in cele din urma, prin expunerea antigenului BP230, autoanticorpii pot interfera capacitate functionala a acestei proteine de a asigura adeziunea ferma la nivelul jonctiunii dermo-epidermale.

Concluzii

In concluzie, rezultatele obținute arata faptul ca anticorpii generați impotriva unui fragment al antigenului BP230 au capacitatea sa induca printr-un raspuns inflamator, distructii tisulare “spontane” in modelul experimental de BP. Cu toate acestea, nu putem exclude intru totul potențialul acestor anticorpi de a induce fenotipul clinic in alte modele experimentale sau la pacienți. Mai multe experimente menite sa clarifice potențialul patogenic al autoanticorpilor specifici BP230, ar trebuie sa-si dovedeasca utilitatea in intelegerea mecanismului imunopatogenetic al acestei afectiuni, precum si crearea unui fundament solid in dezvoltarea de noi strategii terapeutice pentru afectiunile buloase inflamatorii mediate de auto-anticorpi.

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SUMMARY OF THE DOCTORAL THESIS

Pathogenic relevance of autoantibodies in autoimmune bullous diseases, particularly bullous pemphigoid

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INTRODUCTION

The epithelial tissue is built from many layers, realizing a strong adhesion between the cells, with the reduced intercellular substance. This tissue forms a series of structures, which covers the bodysurface completely, but also covers the cavity of the internal organs. As an organstructure, the skin keeps the specific matrix of a double-layer structure, the two layers beeing built from two different tissues: the derma and epiderma, separated through a basal membrane. The phisiology of the epithelial tissue is very complex, having the following main functions: to function like a protection barrier, to regulate the temperature, mechanical protection, immunologic and sensorial function. The epidermal architecture, necessary for the skins protective function, is disturbed in different diseases of the skin, including the autoimmune bullous diseases.

The integrity of the skin is ensured through different types of adhesions, at the keratinocyte level, but also between the keratinocyte and the basal membrane. The transmembrane and matrix proteins, which ensure the adherence between the cells and the matrix, were named adhesion molecules, beeing gruped in many connection complexes: the tight junctions, desmosomes, the "gap" junctions, focal adhesions and hemidesmosomes.

Various studies in this direction proved, that through this cell-cell connections or cell-extracell matrix , informations from outside the cell are transmitted towards the inner part of the cell. The absence of any contact with the medium and informations from outside the cell can be in many cases a cause for the cell to enter in the apoptosis process.

The epiderma and the derma are separated through a basal membrane, which contains in its composition proteins from the extracellular matrix such as α6β4, laminin 5, collagen type IV, VII and the fibronectine. At the level of the skin tissue the basal membrane has its microscopic correlation in the dermo-epidermal junction. (DEJ). The adhesion of the keratinocytes from the basal membrane is determinded by the focal adhesions and the hemidesmosomes.

The hemidesmosomes practically ensure the adhesion of the single- or manylayered epithelial tissue to the basal membrane, structurally formed from a cytoplasmatic plaque, transmembrane proteins, but also a series of proteins, associated with the basal membrane. The cytoplasmatic plaque plays a role in connecting the proteins, which belong to the cytoskeleton, with the transmembrane proteins and further through these, ensures the connection with the basal membrane. It contains many proteins like: the bullous pemphigoid antigen, the plectin protein and a series of less defined proteins like HD1, IFAP300 and P200.

The bullous pempgigoid antigen(BP230) is a protein which, like the plectin, belongs to the plaktin family, beeing the main target of the autoantibodies in the bullous pemphigoid. The COOH extremity connects itself with the keratin fillaments from the hemidesmosomal plaque, and the NH2 extremity connects itself with the transmembrane adhesion proteins like: β4 integrin or the type XVIIcollagen (BP180).

THE STATE OF CURRENT KNOWLEDGE

The autoimmune bullous diseases are a group of rare, potential lethal affections, clinically characterized through the presence of bullous lesions, beeing associated with an immune response against the structural proteins of the skin, which maintain the intercellular adhesion in the epiderma and the adhesion of the basal keratinocytes and the basal membrane and the subadjacent dermal tissue.

Depending on the histopathological and immunopathological features, the autoimmune bullous disorders are classified in two cathegories: intraepithelial and subepidermal bullouses. The first group, also known as "pemphigus" is characterized through the appearance of intraepithelial vesicles, caused by the loss of adhesion between the epithelial cells, which is immunologically defined through the presence of autoantibodies against the cellular junction. The second group is known as "pemphigoid" and results after the detachment of the basal keratinocytes from the basal membrane, immunologically characterized through the appearance of the autoantibodies against the adhesion proteins at the dermo-epidermal junction.

The bullous pemphigoid (BP) is a supepidermal autoimmune cutaneous bullous disease, with less frequently implication of mucous membrane. It is immunologically characterized of the circulant autoantibodies, which are specific for the hemidesmosomal antigenes. The clinical aspect of this affection involves the formation of bullae or flictenes at the level of dermo-epidermal junction.

The diagnosis is confirmed through the evidence of IgG deposits and complement C3 at the basal membrane zone, by immunofluorescent analysis. The pathogenic mechanism is realized through the fixation of the autoantibodies on the basal membrane with the formation of immune complexes at this level. Afterwards, these immune complexes activate the inflammatory mediators and the complement with chemotataxis effect for the immune cells. After they achieved this level in the presented manner, the immune cells will release protheolytic enzymes (protheosis), with capacity of degrading the hemidesmosomal proteins, but also being able to affect the integrity of the basal keratinocytes. At the end, the sustained inflammatory process will determine the formation of vesicles.

Although, type XVII (BP180/BPAg2) collagen was identified as the major antigen involved in the pathogenese of the bullous pemphigoid, the experimental studies have evidenced a posible contribution of the hemidesmosomal BP230 protein, or the less involved the α6 integrin and lamine 5.

PERSONAL CONTRIBUTION

Working hypothesis / AIM

It is wel known the pathogenic role of antibodies aginst BP180 in the generation of clinical phenotype of the bullous pemphigoid, through binding to these antigens, with the following formation of immune complexes. As a result of the presence of these immune complexes at the dermo-epidermal junction, the complement cascade is generated with the production of immunoreactants, that are able to recrute and activate the granulocytes at this level.

Although the previous studies regarding the BP230 protein qualify it as a protein with a minor pathogenetic importance, the role of the synthetized antibodies against this antigen is less studied and with contradictory results.

Using the same strategy as Liu (Liu et al, 1993) for the investigation of a pathogenic potential of the anti BP230 antibodies, Kiss et al, used an experimental model of passive transfer to neonatal mice. In this regards, the results have proved the pathogenic potential of this autoantibodies to induce tegumental lesions. Nevertheless, the antibodies used for the *in vivo* model were generated through injection of rabbits with a recombined protein compound, including both fragments of BP180 but also fragments of BP230. 24 hours after injections, following soft frictioning of the epidermal tissue, it was concluded, that 1 of 7 neonatal mice, which were injected with 5mg purified antibodies, developed persistent bullous lesions at this level. Thereby, ignoring a possible triger given by the physical action on the tegument, an indirect effect of the antibodies anti BP230, indirectly mediated by possible antibodies anti BP180 cannot be excluded, even if the serum was preabsorbed regard the protein BP180.

Using another approach, Hall et al, after the immunization of some rabbits with a synthetic fragment contained 18 aa of the human antigen BP230, showed that mice were resistent to induction of BP clinical phenotype. The results changed only after the apllication of radiation, thus the antibodies bound to the epidermal antigen coused the complement activation other types of immune cells, culminating with the appearance of necrotizing lesions at the this level.

To address these unclarified aspects, in the present study we aim to investigate the pathogenic potential of autoantibodies specific to BP230 in bullous pemphigoid disease. Thereby, after expression of a recombinant fragment of murine BP230 protein, polyclonal antibodies generated against this antigen were assess for the capacity to induce “spontanenous” epidermal lesions after the passive transfer experminents in adult and neonatal mice.

Materials and methods

For the present study, I produced an overlapping recombinant form of the terminal (COOH) region of the murine 230 kDa proteine involved in the autoimmune pathology of bullous pemphigoid. Afterwards, I expressed this recombined protein in chimiocompetent bacteria *(E. Coli Top 10)*.

In order to express this antigen (BPAg1/ BP230), the coding cDNA for the corresponding fragment of this protein (also called C2) was produced through the polymerase chain reaction, using as as a library, a matrix cDNA produced by revers transcription of mARN isolated from the murine keratinocytes of culture (PAM 212 cell). The specific sequences for the SalI and BamHI endonuclease were introduced through synthetic primers, both at insert level (mBP230-C2) and at vector level (pGEX-SP1) respectively.

The pGEX vector (containing the "GST" sequence and the gene "resistent to Ampiciline) together with the fragments of cDNA were amplified through the PCR method and afterwards bent to digestion with restriction ensymes. After the ligation, recombinant vectors (pGEX-mBP230-C2) resulted, beeing inserted into the cellular bacteria *E Coli Top 10*. After the transformation, the bacteria were cultivated in Luria Bertani media with carbeniciline, and at the end they were tested through PCR for the presence of insert DNA.

The fusion proteins produced in E Coli have been purified through chromatography affinity, using a glutathion matrix for the fusion GST-protein. After the expression of the recombined protein in enough quantity, the initial study was focused on obtaining antibodies against the BP230 protein and observing the pathogenic potential of these antibodies. The polyclonal antibodies, obtained by injecting laboratory rabbits with the expressed protein, were purified through chromatography (sepharose G columns). The molecular analysis of the antibodies specificity was realized using the following methods: Western blotting, ELISA and Immunofluorescence.

The subsequently experiments contained in the second study, assess the antibodies interaction with the complement and the human leucocytes at the dermo-epidermal junction. For this purpose we used *in vivo* and *in vitro* models.

The *in vivo* model was realized through passive transfer of autoantibodies anti BP230 on adult and neonatal mice, using negative and positive controls at the same time. The neonatal mice were injected with 10 mg purified IgG within 24 hours during 3 days, and the adult mice were injected with 15 mg/24 hours IgG, during 10 days.

The *in vitro* model for the leucocyte evaluation, consisted in cryostat sections of human skin, incubated with antibodies anti BP230 from rabbits, followed by washing and re-incubating with leucocytes and complement from healthy people.

Results

The first set of experiments was based on the expression of a recombinant form of BP230 protein, followed by immunological characterization of specific plyclonal antibodies generated in rabbits by immunisation with a recombined protein (GST-mBP230-C2). Through the immunoblot analysis, we observed a high specificity in recognizing the recombinant form of the protein BP230-C2, both from the serum of BP patients, but also from the polyclonal antibodies generated in the laboratory.

Further, to evaluate the capacity of these antibodies to recognize the native form of the BP230 protein, we incubated neonatal tissue mice with immune rabbit serum and afterwards with a secondary anti IgG antibody. The results from the immunofluorescence showed that the IgG antibodies from the immune rabbit serum bind at the basal membrane in a characteristic pattern for the bullous pemphigoid, in contrast with the IgG antibodies from the pre-immune serum. Likewise, after testing the specific localization, the antigen recognized by the immune rabbit antibodies, proved to be at the epidermal level of the artificially dettached epithelial tissue (incubation in NaCl solution), otherwise in concordance with the wellknown intracellular localization, and above the basal membrane, respectively. At the same time, through the immunofluorescence analysis, we showed that these antibodies present the capacity to fix the complement, when we used both mouse and human serum as a source of complement system.

In the next set of experiments, we investigated the potential of the IgG rabbit antibodies anti GST-mBP230-C2 to induce the clinical phenotype of the bullous pemphigoid. In this manner for the *in vivo* model we used rabbit antibodies anti BP230 and a positive control represented by antibodies anti BP180, as well as antibodies purify from pre-immune sera, as a negative control. The transfer of the antibodies was realized on BALB/c mice every 12 hours during 3-10 days. At the end of experiments, we observed that none of the neonatal and adult mice develope tegumental vesicles-like lesions sugestive for the BP.

Although, the hystopathological analysis confirmed the presence of the epidermal dettachment at the neonatal mice immunized with the anti BP180 antibodies.

For further investigation on this lack of pathogenicity of the antibodies to induce clinical lesions, we analyzed the presence of the circulating antibodies from the serum of the immunized mice by immunofluorescence analysis, but also the presence of IgG and complement deposits at the basal membrane level. The results showed that the IgG anti BP230 antibodies and the BP180 antibodies recognize the *in vivo* antigen, instead only the anti BP180 antibodies managed to activate the complement.

This lack of pathogenicity of the anti BP230 antibodies was also evaluated through their capacity to activate the granulocytes, with a further production of proteolytic enzymes and induction of dermo-epidermal dettachment. As it was mentioned in the immunofluorescence tests, the anti BP230 and anti BP180 antibodies present the capacity of recognition and binding to the epidermal antigens, with followed formation of immune complexes on the dermo-epidermal junction. The incubation of skin contained the fixed immune complexes at the basal membrane, with human granulocytes generated a reduction of NBT with formation of formazan precipitate only by anti BP180 antibodies and from the pacients with bullous pemphigoid, in contrast with the anti BP230 and pre-immune antibodies. Further on the same pattern of granulocyte activation, we incubated the skin sections, which contained immune complexes with human granulocytes, with a longer incubation. At the end it was observed, that only the anti BP180 antibodies and antibodies from patients with BP

induced the activation of granulocytes on the basal membrane, culminating with the dermo-epidermal dettachment.

Discussions

The polyclonal antibodies from rabbits connected themselves on the DEJ junction level, inducing afterwards the complement activation through formation of immune complexes.

After the passive transfer to neonatal/ adult mice , these antibodies didn't manage to reproduce the clinical features of the bullous pemphigoid. Further, this lack of pathogenicity was also confirmed in the *ex vivo* model of experminetal BP, using the so called "criosections" technique on human skin.

Without a total exclusion of a possible role of these anti BP230 antibodies in the pathogenicity of bullous pemphigoid, in this study we brought clear evidence on the lack of pathogenic potential of these autoantibodies using *in vivo* and *ex vivo* models of experiments.

This lack of capacity to induce tegumental lesions is given by a possible limited potential to connect the target antigen *in vivo*. Because the BP230 is an intracellular protein located on the hemidesmosomal plaque, the autoantibodies cannot immediately connect at this level and in conclusion they need a preliminary trigger for the exposure of the antigen. In this manner, in the first stage, the presence of the antibodies against anti BP180 seems to be necessary. These antibodies determine through connection the activation of the proinflammatory mediators, with the forward lesion on the integrity of the keratinocytal membrane, so the BP230 proteine beeing exposed to the immune system. Being a completely intracellular proteine, BP230 can have a higher antigenicity in comparrison with the BP180 antigen.

Another possible explanation for the lack of pathogenicity of these antibodies may be given through the lack of specificity in the activation of the complement and granulocytes.

Another explanation, as Iwata suggested in one of his studies (Iwat, 2009), the antibodies against BP230 can induce the appearance of the disease through a more subtile non-inflammatory mechanism, by depleting of antigen at the dermo-epidermal junction, as a result of immune complexes formation.

Nevertheless, through exposure of the BP230 antigen, the autoantibodies can interfere with the functional capacity of this protein to ensure the ferm adhesion at the dermo-epidermal junction level.

Conclusions

In conclusion, our data show that antibodies generated to an antigenic fragment of murine BP230 are not able to induce "spontaneous" tissue distruction in an inflammatory manner in our experimental BP model. However, we cannot completely exclude the potential capacity of such antibodies to induce disease in other experimental settings or in patients. Further experiments aimed at clarifying the pathogenetic potential of BP230 specific antibodies should prove helpful for understanding disease pathogenesis and creating a solid base for developing novel therapeutic strategies for autoantibody-mediated inflammatory blistering diseases.