Departament de Medicina / Universitat Autònoma de Barcelona

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Títol: Desenvolupament d'un nou model murí humanitzat de pemfigoide ampul·lós obtingut a partir de cèl·lules mare humanes fol·liculars

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Treball de Recerca

Convocatòria: setembre de 2011

CERTIFICAT DEL DIRECTOR I CO-DIRECTOR DEL TREBALL DE RECERCA

El Dr. Ramon M. Pujol Vallverdú, Professor del Departament de Medicina de la Universitat Autònoma de Barcelona i Cap de Servei del Departament de Dermatologia del Parc de Salut Mar, així com el Dr. Josep E. Herrero Gonzalez, com a co-director,

FAN CONSTAR,

que el treball titulat: *"Desenvolupament d'un nou model murí humanitzat de pemfigoide ampul·lós obtingut a partir de cèl·lules mare humanes fol·liculars"* ha estat realitzat sota la nostra direcció per la llicenciada **Mª Estela Martínez Escala**, trobant-se en condicions de poder ser presentat com a treball d'investigació de 12 crèdits, dins del Programa de Doctorat en Medicina Interna/Diagnòstic per la Imatge (curs 2010-2011), a la convocatòria de **setembre**.

Barcelona, 1 de setembre de 2011.

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Key words: animal model, bullous pemphigoid, humanized skin, NC16A, skin graft, type XVII collagen.

Paraules clau: col·làgena XVII, empelt cutani, model animal, NC16A, pell humanitzada pemfigoid ampul·lar.

Resum: El pemfigoid ampul·lar és una malaltia cutània autoimmune. La majoria dels pacients presenten autoanticossos contra proteïnes de la membrana basal de la pell, concretament en contra de la col·làgena XVII, específicament envers el epítop immunodominant, l'NC16A. La patogenicitat dels anticossos ha estat demostrada mitjançant experiments *in vitro* i *in vivo*. L'escassa homologia existent entre l'NC16A i el seu homòleg murí (NC14A), ha dificultat l'el·laboració de models animals d'aquesta malaltia. En aquest treball demostrem que el sèrum de pacients amb pemfigoid ampul·lar produeix separació dermo-epidèrmica en pell de ratolí humanitzada obtinguda a partir de cèl·lules mare humanes del provinents fol·licle pil·lós.

Summary: Bullous pemphigoid is an autoimmune blistering disease. Most patients present autoantibodies against basement membrane proteins, specifically against type XVII collagen, which contains the immunodominant epitope NC16A. The pathogenesis of these antibodies has been demonstrated by in vitro and in vivo experiments. The divergence detected between NC16A and its murine homologue (NC14A) hinders the development of different animal models. In this article we demonstrate that patients' sera affected of bullous pemphigoid induce dermal-epidermal separation over humanized murine skin produced by human epidermal stem cells from hair follicle.

DEVELOPMENT OF A NEW HUMANIZED MURINE ANIMAL MODEL FOR BULLOUS PEMPHIGOID OBTAINED FROM HUMAN EPIDERMAL STEM CELLS

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Key words: animal model, bullous pemphigoid, humanized skin, NC16A, skin graft, type XVII collagen.

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ABSTRACT

Introduction. Bullous pemphigoid is an autoimmune disease. Patients have autoantibodies against proteins of the basement membrane zone. Autoantibodies against type XVII collagen are the most frequently detected, and their pathogenicity has been demonstrated by *in vitro* and *in vivo* experiments. Herein we present a novel animal model of bullous pemphigoid based on producing skin derived from human epidermal stem cells on mice.

Patients and methods. Sera from seven patients with a diagnosis of bullous pemphigoid were collected. Humanized murine skin was obtained through co-incubation of human epidermal stem cells and murine fibroblasts on the back of Swiss nude mice. Indirect immunofluorescence studies and dermal-epidermal separation with these sera were performed on human skin, humanized murine skin and native murine skin.

Results. Serum IgG from 6/7 patients bound to the dermal-epidermal junction by IIF on both human and humanized murine skin. In 5/7, IgG deposition was observed on native murine skin, although with a lesser extent. All of the 7 sera were capable to induce dermal–epidermal separation on human and humanized murine skin. The presence of multiple hair follicles on native murine skin hindered the evaluation of this test; however, subepidermal separation was observed in 2/4 patients' sera.

Discussion. The non-collagenous 16A (NC16A) domain of type XVII collagen contains most of the immunodominant epitopes in human bullous pemphigoid. The murine homologue of NC16A, called NC14A, is completely different from its human counterpart, so, initial attempts to induce blisters in mice by the injection of human bullous pemphigoid antibodies failed. Our model circumvents this difficulty, since the resulting tissue expresses epithelial and basement membrane zone proteins of human origin.

Conclusion. The present work presents a novel humanized animal model for bullous pemphigoid, of interest to further dissect the pathogenetic mechanisms of disease, and study the efficacy and side effects of potential therapies for bullous pemphigoid and other pathologically-related disorders.

INTRODUCTION

Bullous pemphigoid (BP) is the most frequent autoimmune blistering disease, typical of the elderly and characterized by circulating and tissue-bound autoantibodies against the dermal-epidermal junction. Autoantibodies in BP patients are mainly directed to a couple of hemidesmosomal proteins: BP230 (so-called BPAG1), an intracellular protein constituent of the hemidesmosomal plaque[1], and the transmembrane protein BP180 (also termed BPAG2 or type XVII collagen)[2].

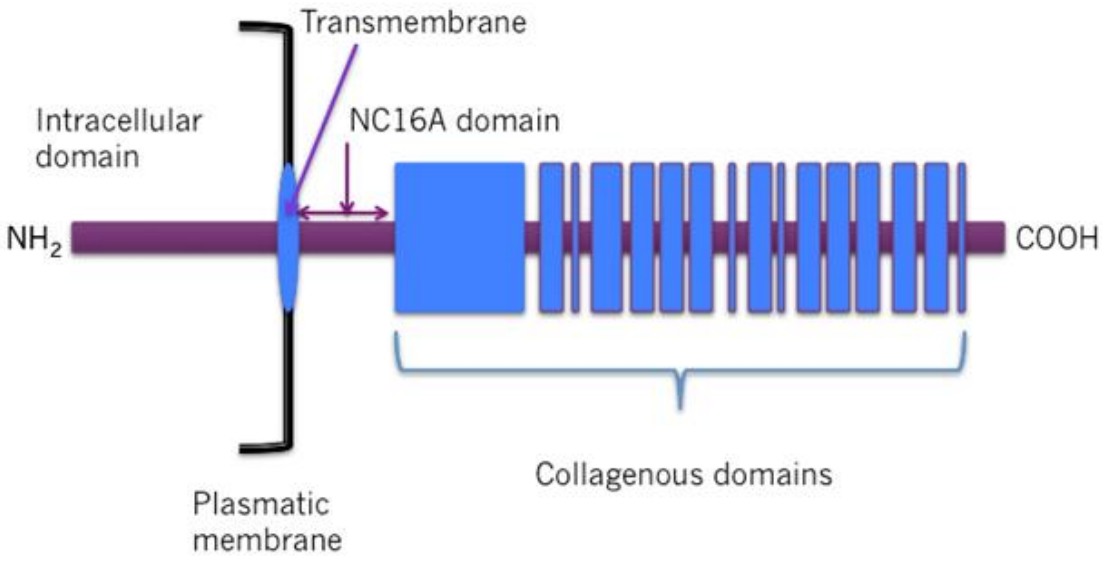
Patients with BP present with tense blisters on skin and occasionally on mucous membranes. A skin biopsy demonstrates a subepidermal blister with an eosinophil- and neutrophil-rich infiltrate in the superficial dermis. Deposition of C3 and/or IgG is detected by direct immunofluorescence (DIF) on healthy perilesional skin. Circulating antibodies against the basement membrane zone (BMZ) can be detected by indirect immunofluorescence (IIF) in 90% of the patients. Specific autoantibodies against BP230 can also be detected by immunoblot (IB) with epidermal or cultured keratinocytes' extracts.

Type XVII collagen (BP180) is a homotrimeric type II-orientation transmembrane protein, with a molecular weight of 180-kd. Its carboxy-terminal portion consists of 15 collagen domains of variable length that are separated from one another by short stretches of non-collagenous sequences (Figure 1)[3,4]. Most BP sera recognize the membrane-proximal non-collagen linker domain (so-called non-collagenous 16A domain, NC16A)[5], which is considered to contain the immunodominant epitopes, as in pemphigoid gestationis[6]. Besides, the pathogenic role of anti-NC16A autoantibodies has been demonstrated in several *in vitro* and *in vivo* experiments[7,8].

In vitro experiments

Gammon et al[9] originally developed an *in vitro* technique to study the potential of patients' sera to induce subepidermal blisters on human skin cryosections.

Briefly, these sections were incubated with patients' sera and leukocytes and a source of complement from healthy donors. The experiments were successful, however, at that moment the antigenic specificity of the pathogenic antibodies could not be demonstrated. Twenty years later, Sitaru *et al*[8] demonstrated that IgG antibodies against the NC16A domain were responsible for blister formation by a similar *in vitro* approach.



hBP180 NC16A --- R S I L P Y G D S M D R I E ---

mBP180 NC14A --- - - V L Y H D V Q M D K S N ---

Figure 1. At the top: schematic diagram showing the structural representation of the BP180 protein based on sequence analysis of the human cDNA[10]. At the bottom: comparison of the amino acid sequence alignment of the human and murine forms of BP180 in the region containing the major epitopes recognized by BP autoantibodies. Identical residues are marked in yellow, and conservative substitutions are highlighted in green[7].

In vivo experiments

Animal models of autoimmune disease are classified upon the origin of antibodies responsible for tissue damage (Table 1).

Table 1. *Classification of animals models of autoimmune blistering diseases.*

Spontaneous	Pathogenic autoantibodies spontaneously produced. BP has been described in horses and Yucatan minipigs.
Passive transfer (*Table 2*)	Antibodies are obtained from patients' sera or from other animals previously immunized (e.g. rabbits, sheeps, chickens, etc.) and injected into different recipient animals (e.g. mice).
Active disease (*Table 3*)	The animal itself produces antibodies by transfer of autoreactive lymphocytes/splenocytes, or by protein immunization procedures (e.g. injection of a recombinant or synthetic form of the autoantigen with or without other adjuvants of the immune response)

Specific BP animal models that have already been published are described in Tables 2 and 3.

Passive transfer animal models

The pathogenic relevance of antibodies in autoimmunity is directly demonstrated when antibodies from patients are injected into animals and they develop clinical lesions consistent with the human disease phenotype, together with compatible histological and DIF features. This technique did work in pemphigus vulgaris, pemphigus foliaceus and epidermolysis bullosa acquisita [11-13], but was unsuccessful in BP[14,15]. Interestingly, BP patients' IgG passively transferred into neonatal mice[14] or monkeys[15] exhibited little or no binding to the

cutaneous BMZ of the injected animals and produced no skin lesions. This phenomenon suggests that pathogenic human BP autoantibodies might not crossreact with its autoantigen counterpart in the experimental animal. It has already been shown that NC16A, the human BP180 immunodominant domain containing the major epitopes for BP, exhibits an unusually high degree of sequence divergence with its murine homologue (NC14A)[7] (Figure 1), despite the high overall homology of human and murine BP180 (81.6%)[7]. For this reason, successful BP animal models are necessarily more elaborated, ranging from the use of human skin grafts to human NC16A transgenic models.

Table 2. *Passive transfer animal models of BP.*

Authors	Antibodies origin	Recipient animal	Features
Anhalt GJ et al (1981)[16]	IgG isolated from BP patients	Intracorneally into New Zealand white rabbits	After 24h: SE blisters. Positive DIF
Liu et al (1993)[7]	IgG isolated from immunized rabbits	Neonatal mice	After 24h: SE blisters. Positive DIF
Zillikens D et al (2001)[17]	IgG isolated from patients and immunized rabbits	SCID adult mice with human skin graft	Blisters not induced. Negative DIF
Yamamoto K et al (2002)[18]	IgG anti-hamster type XVII collagen from immunized rabbits	Neonatal hamsters	Microscopic subepidermal blisters. Positive DIF
Nishie et al (2007)[19]	Sera and IgG purified from BP patients	Humanized NC16A knock-out mice	After 48h: Erythema, blisters. Positive DIF
Liu et al (2008)[20]	IgG isolated from BP patients sera	Humanized NC16A knock-out mice	SE blisters, with inflammatory infiltrate. Positive DIF
Nishie et al (2009)[21]	Transplacental transfer of mother antibodies	Neonatal humanized NC16A knock-out mice	SE blisters. Positive DIF

SE: subepidermal, DIF: direct immunofluorescence, SCID: severe combined immunodeficiency.

Active animal models

Active animal models are well suited to investigate the pathogenetic mechanisms of autoimmune diseases (initial loss of immune tolerance, factors that influence the perpetuation of the autoantibody response, effector mechanisms of tissue injury) and to study the efficacy and safety of long-term therapeutic interventions. Nevertheless, despite these models duplicate the clinical phenotype, the histological, ultrastructural and immunological aspects of human disease, we should be aware that these are fully murine systems since autoantibody production occurs within the same organism[22]. In short, the role of human autoantibodies cannot be investigated.

Table 3. *Active animal models of BP.*

	Authors	Immunization technique	Receipt animal	Features
Transgenic models	Olasz et al (2007)[23]	Autoantibodies	Wild type mice with skin grafts from human Col17$^{+/+}$ transgenic mice	Histological changes of BP. Positive DIF
Transfer of autoreactive lymphocytes	Ujiie et al (2010)[24]	Spleen lymphocytes from Wild-type C57BL/6 with transplanted skin from mCol17 deficient mice	Rag2$^{-/-}$ mice	Skin lesions resembling BP
Forced immunization models	Hall RP et al (1993)[25]	Injection of a protein encoding a BP230 epitope, plus ultraviolet-B irradiation	Rabbits	Inflammatory reaction on the irradiated skin. Deposition of IgG and C3

In this work, we describe a new animal model consisting of growing skin from epidermal human stem cells on Swiss nude mice. Herein we present the results of *in vitro* experiments that validate its potential use as a model for BP and other autoantibody-induced related skin diseases. We demonstrate the *in vitro* ability of human BP sera to bind to the DEJ and induce dermal-epidermal separation.

PATIENTS AND METHODS

Patients' sera. Serum samples from 2 healthy donors and 7 BP patients prior to the initiation of therapy were collected. All BP patients were characterized by: a) skin blisters, b) subepidermal blisters on the skin biopsy, c) deposition of C3 +/- IgG at the DEJ by DIF study of healthy perilesional skin. An enzyme-linked immunoabsorbent assay (ELISA) was performed for each sample to determine anti-BP180 antibody levels (Table 4).

Table 4. *BP180 ELISA results from 7 BP patients (positive if >9 U/ml).*

Patients	Age	Gender	BP180 ELISA
BP1	83	M	159.03
BP2	77	F	13.00
BP3	82	F	27.90
BP4	80	M	2.00
BP5	87	F	45.90
BP6	86	M	124.90
BP7	82	M	9.00

Preparation of skin cryosections. Human stem-cell-derived skin grown on Swiss nude mice, native skin from C57BL/6 mice, and neonatal human foreskin obtained from routine circumcision, were washed in cold phosphate-buffered saline (PBS), cut in pieces of 5 x 15 mm, embedded in optimum cutting temperature (OCT) compound and stored at -80ºC. Four cryosections of 6 µm were placed in the centre of each Superfrost Plus microscope slide (Menzel-

Gläser, Braunschweig, Germany)[9]. IIF studies were also performed on 1 M NaCl-split human skin following a previous protocol[26].

Humanized skin on Swiss nude mice. Humanized skin was developed on the back of Swiss nude mice (athymic mice) by in situ coincubation of epidermal cells from the bulge of human hair follicles and cutaneous or mucous fibroblasts from C57BL/6 mice. Briefly, a mixture of both cell types was transplanted and incubated for one week within a silicon chamber sticked over the mice back after performing an incisional wound. After 8 to 10 weeks, skin biopsies were obtained and processed as explained above. Humanized skin produced with mucous fibroblasts was used, since it lacked hair follicles.

Indirect immunofluorescence microscopy studies (Figure 2). Sera dilutions 1:10 in PBS with 1% bovine serum albumin were incubated for 60 minutes in a humidified dark chamber at 37ºC. After three washes of 7 minutes with PBS, slides were incubated with a FITC-labelled goat anti-human IgG (Sigma-Aldrich, St Louis, MO) for 30 minutes at 37ºC. After extensive washing with PBS, slides were topped with glass covers (Menzel-Gläser, Braunschweig, Germany) after the addition of fluorescent mounting medium (DAKO Cytomation, Glostrup, Denmark). Slides were observed with a fluorescence microscope (Olympus BX51, Olympus, San Diego, CA).

IIF results were evaluated as follows (subjective quantification):

-	An intermittent or continuous linear IgG deposition was not detected along the DEJ
+	Intermittently positive linear IgG deposition along the DEJ
++	Slight continuous IgG deposition along the DEJ
+++	Moderate continuous IgG deposition along the DEJ
++++	Strong continuous IgG deposition along the DEJ

(Subjective quantification by two observers)

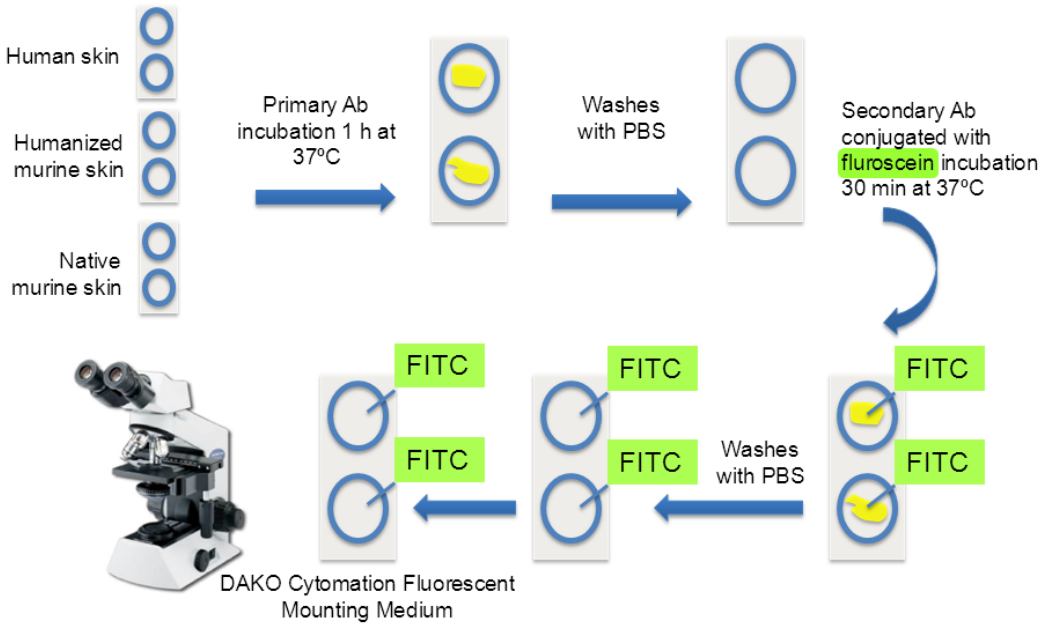


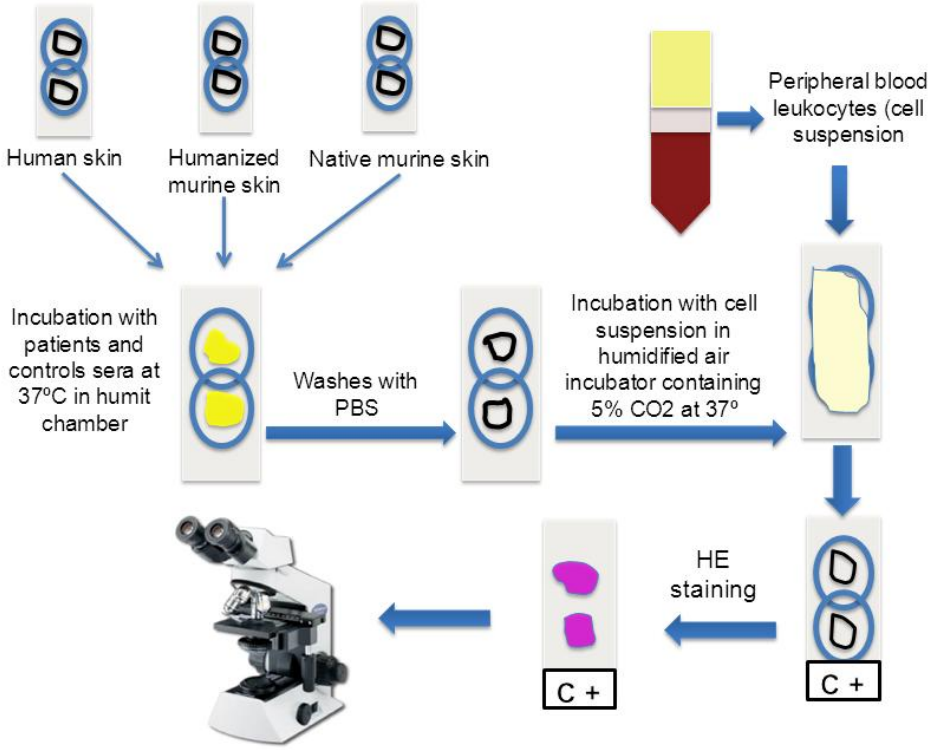
Figure 2. Indirect immunofluorescence protocol (Ab: antibody, FITC: Fluorescein isothiocyanate)

Peripheral blood leukocytes. Peripheral blood leukocytes from healthy donors were isolated by sedimentation gradient containing a 3% 500 mM dextran solution (Nycomed, Oslo, Norway). Cells were harvested, washed twice in RPMI 1640 (Life Technologies, Karlsruhe, Germany) and resuspended in the same medium. Approximately, a concentration of $1x10^7$ cells/ml in the culture medium is needed to perform the dermal-epidermal separation assay. The cell suspension was kept on ice and cell viability was tested using trypan blue staining; only preparations with viability higher than 95% were used.

Induction of dermal-epidermal splits by BP patients' sera and leukocytes on cryosections of human skin, humanized murine skin and native murine skin (Figure 3)[8,9]. Briefly, cryosections were rehydrated with PBS for 10 minutes to remove the embedding medium. Slides were incubated with patients' and control sera diluted 1:1 in NeutrAB (Medion Diagnostics, Miami, FL) in a humidified chamber at 37ºC for 120 – 180 minutes. After washing the sections with PBS twice, slides were covered with a second slide leaving a 0.03 mm space in between. Approximately 500 µl of the cell suspension were introduced and incubated for 90 to 180 minutes at 37ºC in a humidified air incubator containing 5% CO_2. Chambers were finally disassembled, sections were

washed in PBS for 10 minutes, air-dried for 10 minutes, fixed in formalin and stained with hematoxylin and eosin.

Figure 3. Induction of dermal-epidermal separation on human skin, humanized murine skin and native murine skin sections.



Sections were evaluated on a light microscope as follows:

-	No split is observed
I	0 – 25% of dermal-epidermal separation
II	25 – 50% of dermal-epidermal separation
III	50 – 75% of dermal-epidermal separation
IV	75 – 100% of dermal-epidermal separation

Percentages expressed above consider the length of dermal-epidermal separation with regard to the total DEJ length.

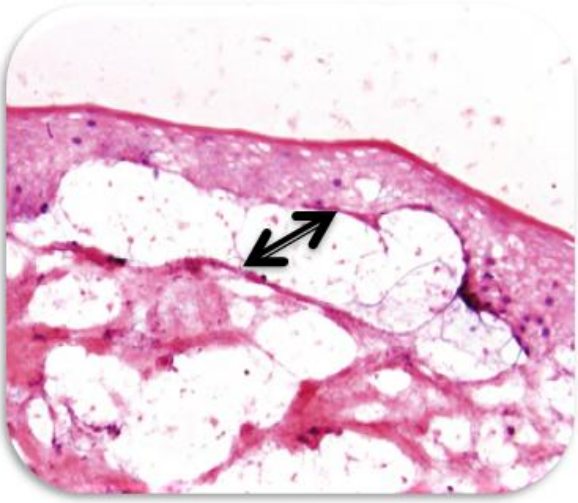


Figure 4. Hematoxylin and eosin staining, 400x. A close-up view of a focus of dermal-epidermal separation (arrow).

RESULTS

Results are summarized in Table 5.

Serum antibodies from BP patients bind to both humanized and native murine skin

IIF study of 6/7 BP sera on human salt-split skin showed a strong fluorescence on the roof of the blister. Results were similar for the same six patients on humanized murine skin. One of the patients' sera (BP2) demonstrated a combined staining of both the roof and the floor of the split. Five of seven patients' sera were also positive on native murine skin, though to a lesser extent compared with human skin and humanized murine skin. These results failed to correlate with the BP180 ELISA levels.

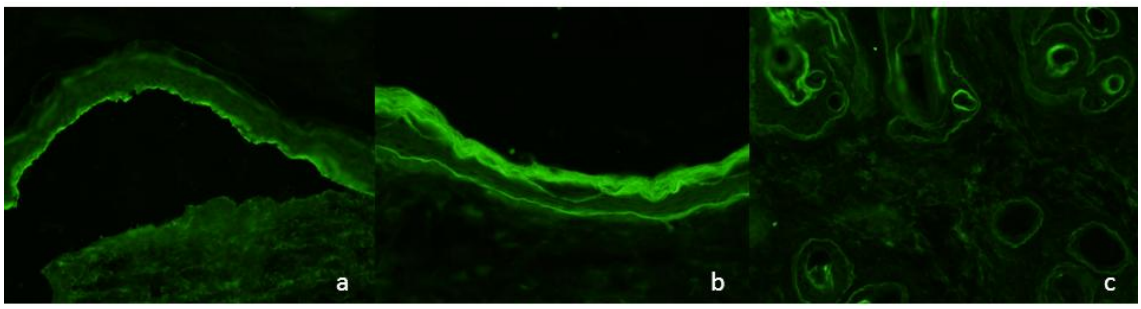


Figure 5. IIF of BP1 serum on the 3 different skin substrates.

a) 200x. A moderate deposit of IgG on the roof of the blister is observed on SSS of human foreskin. b) 400x. Moderate continuous deposit of IgG in the BMZ on humanized murine skin. c) 200x. Intermittent deposition of IgG in the BMZ on native murine skin

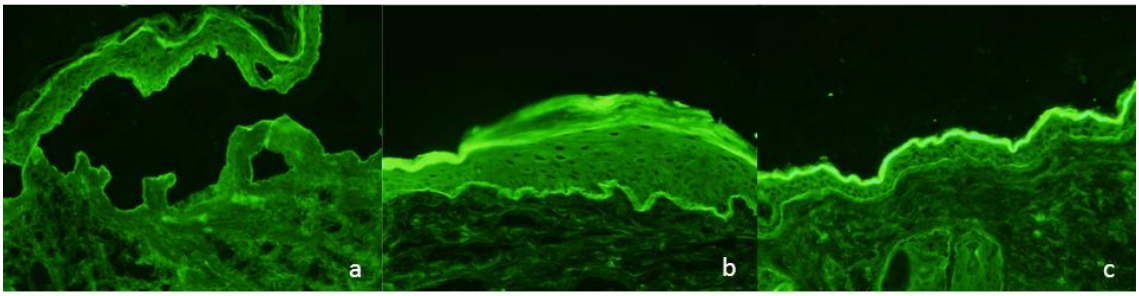


Figure 6. IIF of BP2 serum on the 3 different skin substrates.

a)200x. Moderate continuous deposit of IgG in a mix pattern (roof an floor) is observed SSS human foreskin. b)400x. Slight continuous deposit of IgG in BMZ on native murine skin. c)200x. Intermittent deposition of IgG in BMZ on native murine skin

Sera from BP patients induce subepidermal splits in cryosections of humanized and non-humanized murine skin

All BP patients' sera incubated with leukocytes induced dermal-epidermal detachment in humanized murine skin, to a similar extent to that found on human skin. The presence of hair follicles in native murine skin hindered a correct assessment of the dermal-epidermal separation, although we were able to observe detachment in 2 out of 4 sections that were adequate for evaluation. The degree of dermal-epidermal separation did not significantly correlate with the levels of anti-BP180 IgG as detected by ELISA.

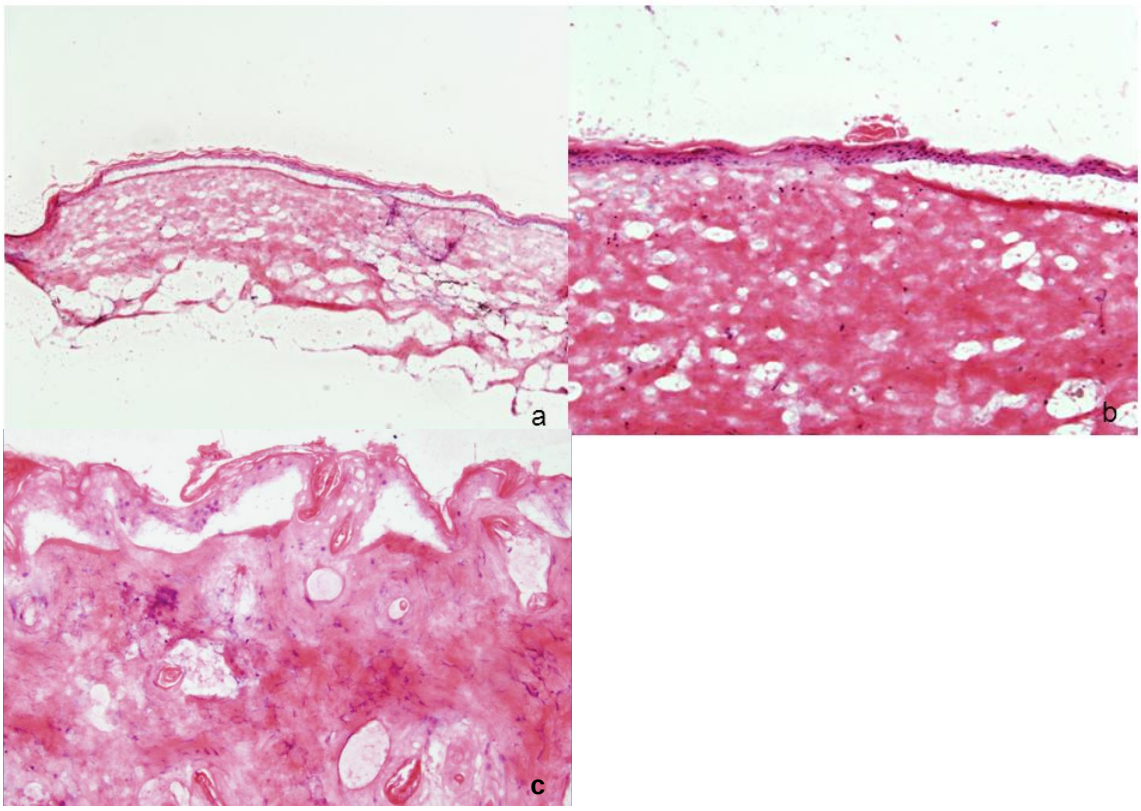


Figure 7.

a) HE 100x. A whole-length dermal-epidermal separation is induced by BP6 serum on humanized skin. b) HE 200x. A close-up view of blister formation by BP7 serum. c) HE 200x. Dermal-epidermal separation induced by BP6 serum on native murine skin.

Table 5. *Summary of IIF and dermal-epidermal separation study results.*

Patients	BP180 ELISA values (U/ml)	Skin substrate	IIF	Subepidermal separation
BP1	159.03	HS	+++	IV
		HMS	++	IV
		NMS	-	-
BP2	13.00	HS	++ (Mixed pattern)	IV
		HMS	++	III
		NMS	+	NA
BP3	27.90	HS	-	IV
		HMS	-	III
		NMS	-	NA
BP4	2.00	HS	+++	IV
		HMS	++	II
		NMS	++	NA
BP5	45.90	HS	++	II
		HMS	++	II
		NMS	-	-
BP6	124.90	HS	++++	IV
		HMS	+++	III
		NMS	++	II
BP7	9.00	HS	+++	III
		HMS	++	III
		NMS	+	I

HS: human skin, HMS: humanized murine skin, NMS: native murine skin, NA: not available

DISCUSSION

We demonstrate by IIF study that IgG binds to the BMZ of humanized skin, similar to what happens in human skin. Interestingly, IgG linear deposition is also observed when using native murine skin, although to a much lesser degree. This IgG staining of the BMZ on native murine skin might be explained by two mechanisms: 1) BP IgG contains anti-BMZ antibodies other than those against type XVII collagen (e.g. anti-BP230), and 2) there might be some degree of cross-reactivity between certain human antibodies and murine BMZ proteins, based on inter-species protein homology. In other words, BP sera are immunologically characterized by non-restricted autoantibody response, since they contain several antibodies against other epitopes different from NC16A, as well as antibodies against other BMZ molecules different from type XVII collagen, which are also present in native murine skin. Nonetheless, the role of these latter autoantibodies in the pathogenesis of BP has not yet been demonstrated[27].

Specifically regarding the antigen-antibody reactivity of anti-type XVII collagen antibodies among species, available studies are controversial. For instance, in the study by Liu et al[28], rabbit anti-human (hBP180) antibodies reacted with a recombinant form of human BP180 (mBP180) or mouse skin, by IB and IF studies, respectively. Conversely, rabbit anti-mBP180 antibodies reacted with recombinant mBP180 and mouse skin, but did not bind to hBP180 or human skin. In contrast, a recent study has found reactivity of human anti-NC16A antibodies with both the human NC16A domain and its murine homologue, NC14A (Sesarman et al., unpublished data). Our results are in line with last findings. Further studies are needed to clarify this aspect.

With regard to the question whether is an association between the intensity of the BMZ staining (by IIF) and type XVII collagen ELISA levels, we failed to detect a significant correlation. This might also be explained by the presence in BP sera of antibodies against epitopes and proteins other than NC16A and type XVII collagen, respectively[27].

In our last set of experiments, dermal-epidermal separation was similarly induced by BP sera on both humanized murine skin and human skin. Importantly, a certain degree of dermal-epidermal detachment was observed in two out of four cryosections of native murine skin (other slides could not be evaluated due to the presence of hair follicles that hindered the artificial split induction).

Mechanisms of antibody-induced blister formation in BP are dependent upon the activation of complement, degranulation of dermal mast cells and generation of neutrophil-rich infiltrates, which release proteinases and reactive oxygen species (ROS)[7,29,30,31]. Experiments transferring pathogenic BP antibodies into mice failed to induce a blistering phenotype when performed in mice deficient in complement, Fcγ-receptors (receptors of the constant portion of γ-immunoglobulins)[32], neonatal mice with immature innate immune system, a BP-like eruption is not observed (Table 6). It is already known that T and B lymphocytes are implicated in loss of tolerance and the initiation of autoimmune response. Nevertheless, T and B lymphocytes are not required for effector mechanisms leading to blister formation, which is demonstrated by experiments showing that the injection of BP IgG induces blistering in mice deficient in T cells (T-cell deficient nude mice) and mice deficient in both T and B cells (C57BL/6J Rag1 ^{tm1Mom})[33].

Table 6. *Main requirements for blister induction.*

Requirements antibody-blisters formation
1. Crossreactivity between antibody-antigen • Human antibody against transgenic human NC16A mouse • Rabbit IgG against murine NC14A (homologue of human NC16A)
2. Innate immune system preserved • Complement activation • Fcγ-receptors • Neutrophils • Mast cells • Release of proteinases • Release of oxygen metabolites

Different animal models of BP are available, each one with its own specificities. These specific features make one model adequate or not to study certain aspects of disease immunopathology (Table 7). For instance, passive transfer models are appropriate to dissect the pathogenetic mechanisms of the effector systems leading to tissue damage. In contrast, active immunization models are best preferred to study the phenomena responsible for the loss of immune tolerance to autoantigens, as well as for therapeutic assays addressed to modulate the chronic autoimmune response[22].

Table 7. *Research purpose for each animal model.*

Passive transfer in neonatal and adult mice	Tissue damage induced by antibodies (insights of inflammatory events)
Passive transfer into mice transplanted with human skin or humanized skin	Tissue damage induced by antibodies (insights of inflammatory events) Characterization of clinical, histological and immunological features of human BP
Autorreactive lymphocytes transfer, active model	Study cells of immune system that are mediating autoimmune response, development of cell-based therapy
Transgenic models, active models	Allows to study all aspects of autoimmune blistering disease and long-term effect of therapeutic interventions

The use of human skin grafts on mice represents an attempt to make animal models of inflammatory skin diseases closer to what really happens in human diseased skin.

To our knowledge, only one study using this approach has already been described for BP, unfortunately, with negative results. This study, performed by Zillikens and colleagues[17], consisted of engrafting human skin from healthy donors onto SCID immunodeficient mice. Interestingly, anti-BP180 IgG

autoantibodies from BP patients' sera and from a rabbit immunized with a recombinant form of human BP180, strongly bound to the DEJ of the graft, weakly fixed murine complement and induced a mild neutrophil-rich infiltrate on the upper dermis, but failed to induce clinically evident subepidermal blisters or dermal-epidermal separation on histopathological study. Thus, this model cannot really be considered a model of BP.

Two other experimental studies using skin grafts of humanized murine skin have been reported.

The first model using grafts of murine skin expressing human type XVII collagen, was described by Olasz et al[23]. Transgenic skin grafts onto wild-type mice induced an IgG response that labeled the basement membrane zone of human skin and transgenic humanized murine skin, but not skin from wild-type mice, by indirect IF study. This IgG was deposited at the DEJ of the recipient mice, which subsequently developed C3 deposition at the same level, recruited neutrophils to the dermal-epidermal junction and the papillary dermis, and finally, frank subepidermal blister formation. In this model, in contrast to ours: (a) human skin or human-derived skin is not used, and (b), this is an active experimental model.

The second model, by Ujiie and colleagues[24], consisted of engrafting murine skin expressing human type XVII collagen (from transgenic COL17$^{m-/-,h+}$ mice) on wild-type mice to induce an autoimmune response against human type XVII collagen. Splenocytes from the immunized wild-type mice were injected into Rag-2$^{-/-}$ / COL17$^{m-/-,h+}$ mice (expressing human type XVII collagen), which led to a continuous production of anti-human type XVII collagen IgG antibodies and a blistering phenotype clinically and immunopathologically typical of BP (except for the lack of an eosinophil infiltrate). Therefore, this model represents an active model of BP, in which: (1) no human skin graft is used, but a murine skin graft from COL17-humanized mice, and (2) this murine graft is not used in the recipient mice showing the blistering phenotype, but as the method to immunize wild-type animals with the human-sequence autoantigen, to serve them as

simple providers of reactive splenocytes to be transferred into COL17-humanized knock-out mice. These are indeed some of the differences with our model.

Interestingly, in both the last models[23,24], $CD4^+$ cells were necessary for the development of the blistering phenotype, in contrast to CD8+ cells, which were not relevant.

After comparing with the above-mentioned studies, we can conclude that our model, although in a preliminary stage, is unique novel model since it is the only passive transfer model in which the recipient animals carry a skin graft obtained from human epidermal stem cells (Table 8 summarizes the main features of the animal model presented). In this regard, our grafts can be considered a chimeric skin formed by the interaction and differentiation of human stem cells and murine fibroblasts.

Our grafts potentially express a variety of human epidermal proteins, as it was demonstrated with involucrin, a specific human protein that differentiate human from murine keratinocytes. We have not directly demonstrated that all BMZ proteins are of human origin, however, we assume this fact based on previous studies[34-36], which shows, first, that keratinocytes produce almost all proteins present in the BMZ (including type XVII collagen), and second, that fibroblasts just modulate this synthesis. It is worthwhile to remark this last feature, in contrast to other models using COL17-humanized transgenic mice, in which the only human protein expressed is type XVII collagen. Based on this fact, this model would allow us to study the pathogenetic role of other autoantibodies which bind to other autoantigens other than type XVII collagen. This possibility would be of great interest, since BP and other related diseases are commonly associated to a heterogeneous response to more than one autoantigen, such as BP230, integrins, etc.

Finally, the next experiments that we must perform to better characterize our model are: (1) *in vitro* assay of the induction of subepidermal separation on cryosections of chimeric skin grafts with affinity-purified anti-NC16A IgG, and (2)

in vivo injection of total IgG and affinity-purified anti-NC16A IgG antibodies from BP patients' sera.

One limitation of our experimental work presented here is that these data only form in vitro experiments, where human leukocytes are used to induce dermal-epidermal separation, in contrast to the in vivo situation where components of the innate immunity (granulocytes, complement and coagulation system) are provided by mice.

Table 8. *Summary of features of our probably new animal model.*

Passive transfer animal model
Adult mice
Athymic nude mice
Innate immune system preserved
Humanized grafted skin (epidermal and BMZ human proteins)

CONCLUSIONS

The pathogenic relevance of antibodies in autoimmune diseases is demonstrated when patients' antibodies are injected into animals and they develop signs of tissue injury that are clinically and immunopathologically suggestive of the human disease.

With regard to experimental BP, initial attempts to induce a blistering phenotype by the passive transfer of patients' IgG were unsuccessful, fact that is partially explained by the lack of cross-reactivity of human autoantibodies with the murine antigen counterpart. For this reason, experimental BP models are more elaborated and use different approaches, ranging from human full skin grafts to human NC16A transgenic mice.

In contrast to previous studies, in our model epidermal proteins are originated from human stem cells –thus, human proteins are expressed- while obviating the need of genetic manipulation. Future passive transfer experiments must be performed to consider the present model as a valid experimental BP model.

Finally, we hope that this study may help dissecting the effector mechanisms of tissue damage in BP and identifying potential therapeutic targets.

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