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Identification of Ocular Cicatricial Pemphigoid Antibody Binding Site(s) in Human β4 Integrin

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Purpose. To identify specific site(s) on human β4 molecule to which sera from ocular cicatricial pemphigoid (OCP) patients bind and to determine its role in the process of blister formation.

Methods. Clone the fragments representing the extracellular and intracellular domain of β4 molecule from normal human conjunctival mRNA into an expression vector; map the region to which sera from OCP patients bind by Western blot analysis. Determine the role of the immunodominant region in pathogenesis by demonstrating the ability of the rabbit antibody to the immunodominant region to produce separation of basement membrane zone (BMZ) from the basal epithelial layer when incubated with normal human conjunctiva in an in vitro organ culture model.

Results. Majority of the OCP sera tested bound to the C-terminal end of the intracellular domain (IC3.0) of the human β4 integrin. Further subcloning of IC3.0 demonstrated that a smaller fragment extending from 1489 aa to 1572 aa (IC3.4) was responsible for this binding. This region may have multiple antibody binding sites. Antibody to human IC3.0 and IC3.4 produced in rabbit, resulted in BMZ separation, histologically identical with that observed when normal human conjunctiva was cultured with OCP sera in an human conjunctival organ culture model.

Conclusions. These observations identify IC3.4 as the antibody binding site for sera of OCP patients and suggest a possible role for it in blister formation. Indirectly it highlights certain important aspects of the structural and functional dynamics of the biology of the hemidesmosomes and basement membranes. (*Invest Ophthalmol Vis Sci.* 2001;42:379–385)

Mucous membrane pemphigoid (MMP) is a multisystemic autoimmune inflammatory disease that affects mucous membrane derived from stratified squamous epithelium including the conjunctiva and occasionally the skin.[1,2] In some patients, the involvement of the conjunctiva is more prominent than other mucous membranes. Such a subset of MMP patients are referred to as having ocular cicatricial pemphigoid (OCP). Progressive subepithelial fibrosis follows the chronic conjunctivitis, resulting in severe dryness of the eye, ocular keratinization, and blindness secondary to corneal scarring. Sera of patients with subepithelial blistering diseases have demonstrable levels of circulating antibodies that bind to different antigens within the basement membrane zone (BMZ) of skin and mucosa.[3]

Our recent observations suggest that human β4 integrin may play an important role in the pathogenesis of OCP.[4,5] Integrins comprise of a large family of heterodimeric receptors that mediate cell adhesion to extracellular matrices and other cells.[6] In addition, they are involved in extracellular signal transduction and hemidesmosomal assembly.[7,8,9] Integrins probably play an important role in adherence of basal epithelial cells to underlying basement membranes.[10]

There are several human autoimmune diseases in which the target autoantigens are identified as being intracellular in location.[11,12,13] The exact mechanism by which the autoantibodies enter the cell and bind to these target antigens is not clearly known or understood. The purpose of the present study is to identify specific site(s) on human β4 molecule to which sera from OCP patients bind and to determine its role in the process of blister formation. In the present study, we demonstrate that OCP sera binds to intracellular regions (IC3.0 and IC3.4) of the human β4 molecule. Rabbit antibodies to these fragments produces BMZ separation in an in vitro human conjunctiva organ culture model.

Materials and Methods

Sera

The method section confirms adherence to the Declaration of Helsinki. Sera used in this study were obtained from 20 patients with active mucous membrane pemphigoid before beginning of systemic treatment. These patients had the pemphigoid disease process involving multiple mucosa but not the skin. Ocular involvement was the most prominent symptom, resulting in blindness in many of these patients. The clinical diagnosis of OCP was established by routine histology and confirmed by direct immunofluorescence of the conjunctiva. The presence of IgG and or complement was detected in the conjunctival BMZ. Sera of these OCP patients binds to the epidermal side of the salt split skin. Control sera were obtained from 20 healthy individuals, 5 patients each with confirmed bullous pemphigoid (BP) and pemphigus vulgaris (PV). Blood samples were collected after informed consent, and the study was approved by the institutional review board.

RNA Preparation and RT-PCR

Total RNA was extracted from normal human conjunctiva (NHC), using Trizol followed by ethanol precipitation.[14] Approximately 3 μg of total RNA was used to make cDNA using Reverse Transcriptase Superscript II (Gibco, BRL, Gaithersburg, MD) and gene-specific primer (5′-GGGGCAGGGTGCGGTCAAGTGTTTGGAAGAAC-3′) was designed based on the C-terminal sequence of the β4 molecule.[4] The resulting cDNA was used as the template for all the RT-PCR reactions. RT-PCR conditions were as follows: 94°C melting temperature; 60°C annealing temperature for 1 minute; 72°C extension temperature for 1 minute; and this entire procedure was repeated for 25 cycles. The reaction was performed with a hot start. Products obtained after 25 cycles of amplification in a DNA thermal cycler (Perkin–Elmer Corp., Norwalk, CT) were extracted using magic PCR purification resin.

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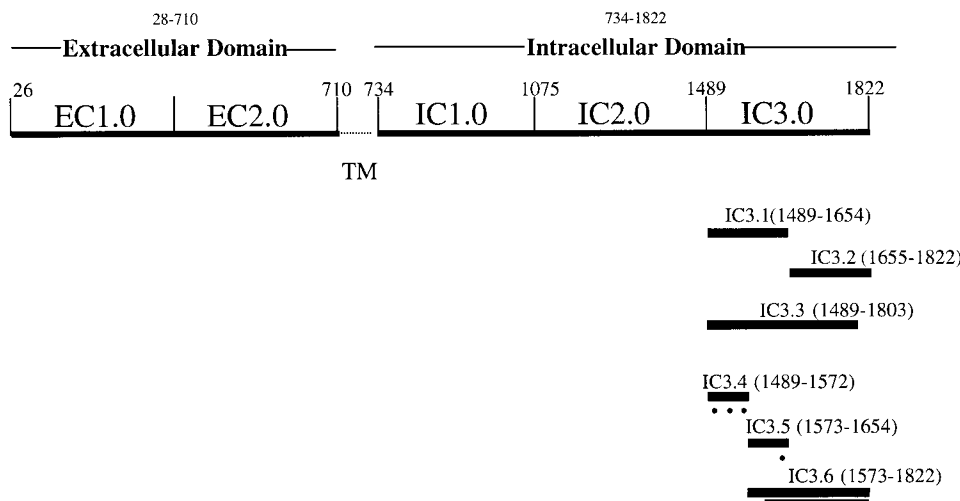

FIGURE 1. Schematic representation of the extracellular and intracellular domains of the β4 molecule from normal human conjunctival RNA and the constructs used in identifying the antibody binding site(s). EC, extracellular domain (EC1.0 and EC2.0); IC, intracellular domain (IC1.0, IC2.0, and IC3.0); TM, transmembrane region. All numbers are in terms of amino acid. *Underlined* portion of IC3.6 represents the clone initially selected from the keratinocyte library.[4] (●), the antigenic sites as predicted by Jameson and Wolf.[22]

Cloning and Characterization of the Different Fragments Representing the Extracellular and Intracellular Domain of the β4 Molecule from Normal Human Conjunctival RNA

The resulting cDNA from the conjunctival RNA was used for PCR amplification. The different fragments representing the extracellular (EC) and intracellular (IC) domain of human β4 integrin molecule were amplified and sequenced by the di-deoxy chain termination method at the Molecular Biology Core Facility of the Dana Farber Cancer Institute, Boston, MA. For accuracy purposes, both the strands were sequenced. The sequence of the fragments were compared to the sequence of the human β4 integrin molecule, Accession No. 53587.[15]

The fragments representing the extracellular domain are designated as EC1.0 (28 aa–366 aa), and EC2.0 (367 aa–710 aa); and those representing the intracellular domain are designated as IC1.0 (734 aa–1074 aa), IC2.0 (1075 aa–1488 aa), and IC3.0 (1489 aa–1822 aa). The fragments used in this study are schematically represented in Figure 1. The PCR products were resolved on 1.2% agarose gel, purified using Promega PCR prep resin, digested with the respective restriction enzyme (*Eco*RI and *Xho*I), and ligated into pET28a (Novagen Inc., Madison WI). The restriction sites *Eco*RI and *Xho*I were created in the 5′ and 3′ primers, respectively, to facilitate the subcloning procedure. The ligated products were transformed into *Escherichia coli* DH5α, and the positive clones were identified by restriction endonucleases analysis.[14] The selected recombinants were expressed in *E. coli* BL21λDE3p*lysS*. IC3.0 (1489 aa–1822 aa) was further subcloned as IC3.1 (1489 aa–1654 aa), and IC3.2 (1655 aa–1822 aa). Based on a recent report, which determined that the very end of the C-terminal portion of the intracellular tail of β4 was an important site for interaction between β4 and plectin.[10] Hence we produced a construct designated IC3.3 (1489 aa–1803 aa), which eliminates the last 19 aa of β4 to determine its capacity to bind to OCP sera. For the purpose of epitope-mapping, IC3.1 was subcloned into two fragments as IC3.4 (1489 aa–1572 aa) and IC3.5 (1573 aa– 1654 aa). Finally, another construct, IC3.6, extending from 1573 aa–1822 aa (representing a combination of IC3.2 and IC3.5) was made. This construct was made to verify the binding of the OCP antibody to a cDNA clone selected while screening of a keratinocyte cDNA library in λgt11 as reported in our earlier study.[4]

Expression and Purification of the Fusion Proteins Representing the Different Domains of β4

The different fragments of the intracellular and extracellular domains were expressed in BL21λDE3p*lysS*. The pET-28a (Novagen Inc.) allows expression, by means of the bacteriophage T7 Φ10 promoter, of a His-Tag sequence fused to the N-terminus of the different fragments.[16] Cells of the resultant transformants were grown to an OD_{610} of ~0.4 at 37°C in LB containing kanamycin (40 μg/ml), induced by the addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After a 3-hour incubation under induction conditions, the cells were harvested by centrifugation at 3000*g* for 20 minutes at 4°C. The cell pellets were resuspended and solubilized in bug-buster buffer as per manufacturer's instructions (Novagen, Inc.).

Identification and Characterization of Fusion Proteins by Western Blot Analysis

Characterization of the fusion proteins was done by standard Western blot analysis.[4] The primary antibodies consisted of the panel of patient's sera, control sera, and antibodies to extracellular and intracellular domain of human β4 molecule raised in rabbit (provided by F. Giancotti, Memorial Sloan Kettering Cancer Center, New York, NY). The binding was detected using ECL reagents (Amersham-Pharmacia Biotech, Inc., Piscataway, NJ).

Immunization and Production of Rabbit IgG

New Zealand rabbits were immunized SC as described earlier[17] with 100 μg of purified EC1.0, EC2.0, IC1.0, IC2.0, IC3.0, and IC3.4 fragments of β4 molecule. Pre- and postimmunization sera were collected from the respective rabbits.

Indirect Immunofluorescence

Indirect immunofluorescence was performed using normal human conjunctiva as substrate as described earlier[5] to test the presence of anti-BMZ antibodies. Test reagents included OCP sera ($n = 20$), and rabbit antibodies to EC1.0, EC2.0, IC1.0, IC2.0, IC3.0, and IC3.4. Sera from normal human individuals and pre-immune rabbit sera served as negative control, whereas sera from patients with PV ($n = 5$) served as a positive control in this assay.

In Vitro Culture of Normal Human Conjunctiva

Normal human conjunctiva was obtained during cataract surgery. Then, 4 to 5 mm^2 size pieces of normal human conjunctiva were incubated in a 24-well tissue culture plate in complete RPMI-1640 medium supplemented with different test reagents. Next, 30% v/v of sera from patients with OCP ($n = 5$), PV ($n = 5$), normal human individuals ($n = 5$), pre-immune rabbit sera, and rabbit antibodies to EC1.0, EC2.0, IC1.0, IC2.0, IC3.0, and IC3.4 were added to the wells and incubated with 5% CO_2 for 24, 36, and 48 hours. The optimal concentrations and timings were obtained from our earlier studies.[5] After incubation, tissue samples were examined by routine histology. The experiment was repeated three times.

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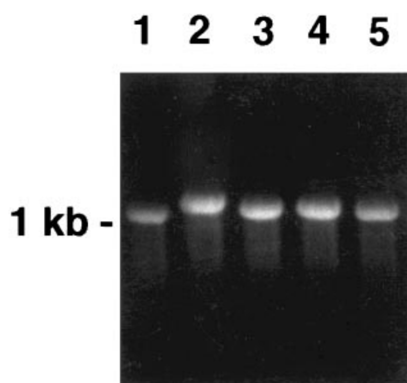

FIGURE 2. RT-PCR products representing the extracellular and intracellular domain of the β4 molecule from normal human conjunctival RNA. Human conjunctival RNA was used to synthesize cDNA using gene-specific primer. The resultant cDNA was used as template to amplify the fragment of DNA representing the different domains of the gene. The PCR products were resolved in a 1.2% agarose gel. *Lanes 1, 2, 3, 4,* and *5* represent EC1.0 (1.0 kb), EC2.0 (1.05 kb), IC1.0 (1.0 kb), IC2.0 (1.1 kb), and IC3.0 (1.0 kb), respectively.

RESULTS

Sequence Analysis of the cDNA from Human Conjunctival RNA

The nucleotide sequence of the fragments representing the extracellular and intracellular domain of β4 molecule obtained from human conjunctival cDNA was identical with the published sequence of the human β4 molecule (Accession No. 53587). We have demonstrated in our previous study that immuno-affinity–purified OCP autoantibody was able to recognize a putative clone from the keratinocyte cDNA library in λgt11. The sequence of the putative clone was identical with the C-terminal end of the β4 molecule.[15] Cloning of the different fragments representing the extracellular and intracellular domain of β4 molecule from cDNA of normal human conjunctiva was done primarily to determine whether there were any additional binding sites on the β4 integrin molecule for OCP autoantibody, besides the one described earlier.[4]

Molecular Characterization of the Fragments of Human β4 Molecule

The various fragments representing the extracellular and intracellular domains are schematically represented in Figure 1 and were PCR amplified and resolved in 1.2% agarose gel. All the amplified fragments were approximately 1 kb in length. These were: EC1.0, 1.0 kb; EC2.0, 1.06 kb; IC1.0, 1.0 kb; IC2.0, 1.2 kb; and IC3.0, 1.0 kb. These PCR products representing the extracellular and intracellular domain of the putative OCP gene are shown in Figure 2.

Expression of Extracellular and Intracellular Fragments of β4 Molecule

The various fragments representing the extracellular and intracellular domains of the β4 molecule obtained from human conjunctiva were expressed in *E. coli* expression vector. The respective fusion proteins were resolved on a 10% SDS-PAGE gel and stained with Coomassie Blue. The protein encoded by these fragments were of the following sizes: EC1, ~ 39 kDa; EC2, ~ 40 kDa; IC1, ~38 kDA; IC2, ~ 42 kDa; IC3, ~ 37 kDa (data not shown).

Binding of OCP Sera to the Fragments of β4 Molecule

Binding pattern of OCP sera to various fragments is presented in Figure 3. All the OCP sera ($n = 20$) demonstrated binding to IC3.0. However, five OCP sera samples bound to both IC2.0 and IC3.0, and two OCP sera reacted to all three fragments IC1.0, IC2.0, and IC3.0 (data not shown). The reactivity to IC1.0 and IC2.0 was significantly less than IC3.0. OCP sera did not demonstrate binding to the fragments (EC1.0 and EC2.0) representing the extracellular domain of β4 molecule. Polyclonal antibody to the extracellular domain of β4 demonstrated binding to EC1.0 and EC2.0. Antibody to intracellular domain of β4 demonstrated binding only to IC3.0. This demonstrates that the binding pattern of antibody to intracellular domain of β4 is identical with the binding pattern of OCP sera. The polyclonal antibody to the intracellular domain of β4 used in this study was raised against the terminal 33 aa of β4.[18,19] The anti-intracellular antibody did not bind to either EC1.0 or EC2.0. Normal human sera and sera from patients with bullous pemphigoid (BP) and pemphigus vulgaris (PV) did not exhibit any detectable level of binding to any of the cloned fragments of β4. This demonstrates the specificity of the binding of the OCP sera to the intracellular domain of β4 molecule.

Binding of OCP Sera to Subfragments of Intracellular Domain (IC3.0) of the Human β4 Molecule

The IC3.0 was subcloned as IC3.1 and IC3.2. OCP sera ($n = 20$) demonstrated binding to IC3.1 and no binding to IC3.2 (Fig. 4). In contrast, antibody to the intracellular domain of β4 molecule demonstrated binding to IC3.2 and no binding to IC3.1. IC3.1 was subcloned into IC3.4 and IC3.5. All the OCP sera ($n = 20$) exhibited binding to IC3.4 and weak binding to IC3.5. We had reported previously that immunoaffinity purified OCP autoantibody bound to a cDNA clone, isolated from keratinocyte library, encoded the intracellular portion of β4.[4] To confirm that observation, we created a construct IC3.6 (consisting of IC3.2 and IC3.5), which represents the cDNA clone used in our earlier study.[4] OCP sera ($n = 20$) bound to IC3.6.

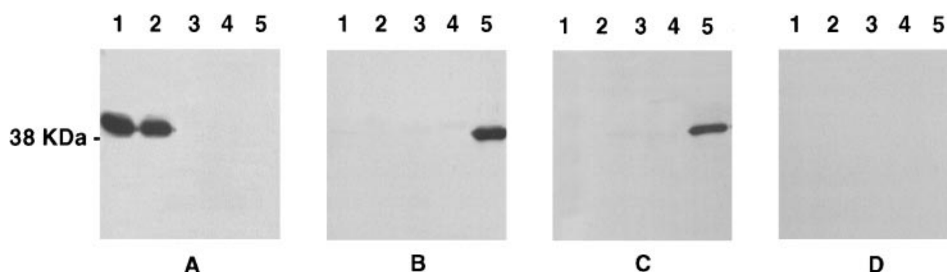

FIGURE 3. Immunoblot analysis of the various fragments representing the different domains of the human β4 molecule. *Lanes 1, 2, 3, 4,* and *5* represent EC1.0 EC2.0, IC1.0, IC2.0, and IC3.0, respectively. Cell lysates containing equal amount of the recombinant proteins were resolved on a 10% SDS-PAGE gel, electroblotted, and probed with antibody to the extracellular domain of β4 molecule in (**A**), antibody to intracellular domain of β4 molecule in (**B**), OCP sera in (**C**), and normal human sera in (**D**). Note that the anti-extracellular antibody binds only to EC1.0 and EC2.0 (**A**, *lane 1* and *lane 2*); anti-intracellular domain antibody and OCP sera binds only to IC3.0, *lane 5* in (**B**) and (**C**), respectively.

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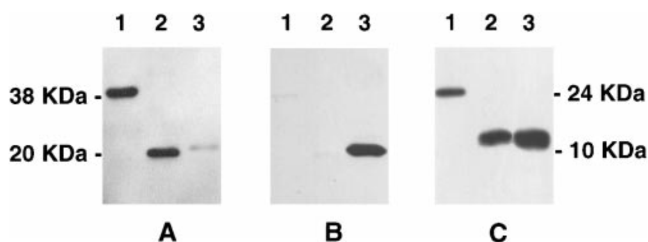

FIGURE 4. Subcloning of IC3.0 of β4 to identify the antibody binding sites of OCP sera using immunoblot analysis. IC3.0 of the intracellular domain was further subcloned as IC3.1, IC3.2, and IC3.3. IC3.1 was further subcloned as IC3.4, IC3.5, and IC3.6. *Lanes 1, 2,* and *3* in (**A**) and (**B**) represent IC3.3, IC3.1, and IC3.2, respectively. *Lanes 1, 2,* and *3* in (**C**) represent IC3.6, IC3.5, and IC3.4 respectively. The cell lysates containing equal amounts of the recombinant protein from IC3.1, IC3.2, and IC3.3 were resolved on a 10% polyacrylamide gel; and IC3.4, IC3.5, and IC3.6 were resolved on a 15% polyacrylamide gel. (**A**) and (**C**) were reacted with OCP sera, and (**B**) was reacted with antibody to intracellular domain of the β4 molecule. Note strong binding of OCP sera to IC3.3 and IC3.1 (**A**, *lane 1* and *lane 2,* respectively) and binding of anti-intracellular antibody to IC 3.2 (**B**, *lane 3*). OCP sera also exhibit strong binding to IC3.4 (**C**, *lane 3*) and less binding to IC3.5 (**C**, *lane 2*) and IC3.6 (**C**, *lane 1*).

To determine the importance of the C-terminal end of the intracellular portion of β4, a construct IC3.3 was made. It consists of the entire IC3.0 without the terminal 19 aa. OCP sera bound to IC3.3 but the anti-intracellular antibody did not bind to it. This would suggest that the terminal 19 aa of the intracellular tail of β4 are important in the binding of the anti-intracellular antibody to it. This confirms the earlier studies.[18,19]

Indirect Immunofluorescence

Normal human conjunctiva sections incubated with OCP sera and rabbit antibodies to IC3.0 and IC3.4 demonstrated a smooth homogenous linear BMZ staining (Fig. 5). PV sera exhibited binding to intercellular cement or epithelial cell surface of the normal human conjunctiva. No binding was observed in the sections of normal human conjunctiva incubated with normal human serum and pre-immune rabbit serum. Rabbit antibodies to EC1.0, EC2.0, IC1.0 and IC2.0 bound to BMZ on normal human conjunctiva.

In Vitro Organ Culture Study

Subepithelial separation at the BMZ was observed in normal human conjunctiva after 24 hours of organ culture with antibodies to IC3.0, IC3.4, and OCP sera (Fig. 6). Then, 75% of the explants showed BMZ separation when cultured with rabbit antibodies to IC3.0 and IC3.4, compared to 55% when cultured with OCP sera. The pattern of BMZ separation was similar to that reported in a conjunctiva organ culture model for BMZ separation.[5] Normal human conjunctiva cultured with rabbit antibodies to IC1.0, IC2.0, EC1.0, EC2.0, pre-immune rabbit serum, and normal human sera did not produce any BMZ separation. Sections of normal human conjunctiva cultured with PV sera showed acantholysis of epithelial cells (Fig. 6).

Discussion

In this study, we cloned fragments representing the extracellular and intracellular domains of β4 molecule from normal human conjunctival cDNA into an *E. coli* expression vector. Sequence analysis of these fragments exhibited 100% homology to the published sequence of β4.[15] In an earlier study, we reported that a clone from human keratinocyte cDNA library

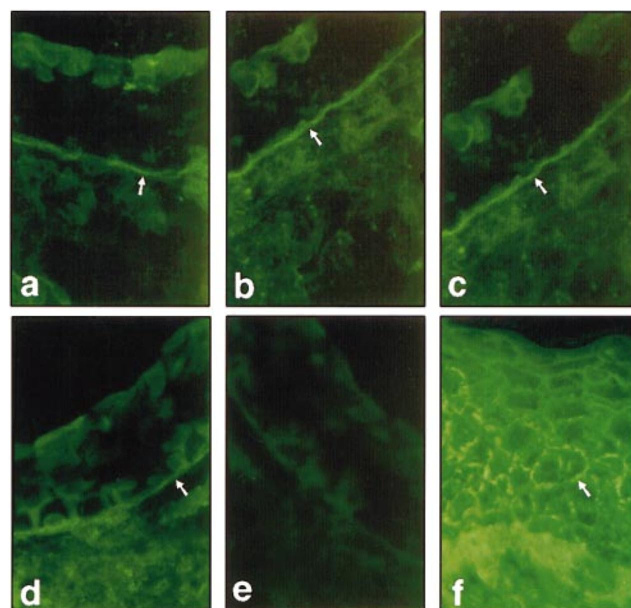

FIGURE 5. Indirect immunofluorescence assay. Normal human conjunctiva sections were incubated with different test reagents: (**a**) OCP sera; (**b**, **c**, and **d**) rabbit antibody to IC3.0, IC3.4, and EC1.0, respectively; (**e**) pre immune rabbit sera; (**f**) PV sera. A smooth homogenous linear BMZ staining is observed in (**a**), (**b**), (**c**), and (**d**). Magnification, ×20. There is no BMZ staining in (**e**). Note the intercellular staining of conjunctival epithelial cells when incubated with PV sera in (**f**). Magnification, ×40.

identified by immunoaffinity purified OCP autoantibodies had complete homology with a portion of intracellular domain of human β4 integrin.[4] Hence clones of smaller fragments of

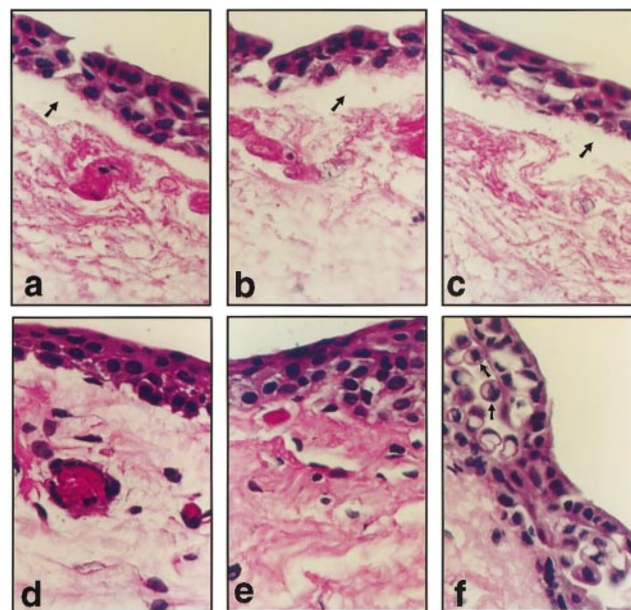

FIGURE 6. In vitro conjunctiva organ culture model. Here, 4 to 5 mm^2 pieces of normal human conjunctiva were incubated with different test reagents: (**a**) OCP sera; (**b**, **c**, and **d**) rabbit antibody to IC3.0, IC3.4, and EC1.0, respectively; (**e**) pre-immune rabbit sera; (**f**) PV sera. Note that both IC3.0 and IC3.4 (**b** and **c**) produce BMZ separation, which is histologically identical with that produced by OCP sera (**a**). The rabbit antibody to EC1.0 and pre-immune rabbit sera did not produce any BMZ separation (**d** and **e**, respectively). PV sera demonstrates acantholysis of conjunctival epithelial cells (**f**). Magnification, ×20.

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human β4 were produced to determine the epitope(s) to which OCP sera bind.

In the present study, we demonstrated that the intracellular domain of human β4 molecule contains peptides to which OCP sera bind. All the sera tested bound to IC3.0, 25% bound to both IC2.0 and IC3.0 and only 10% bound to the entire intracellular fragment IC1.0, IC2.0 and IC3.0, suggesting that the important epitope(s) lie within IC3.0, with the presence of minor or secondary epitope(s). However, our in vitro organ culture studies indicated that antibodies to these minor epitopes are probably not as important in BMZ separation.

OCP sera demonstrate strong binding to IC3.1. Further subcloning of this region, demonstrated that a smaller peptide IC3.4 may account for most of this binding. A 19 aa-stretch, extending from 1801 aa to 1822 aa or IC3.3, may be important for the binding of the anti-intracellular antibody, indicating that multiple epitopes may be possibly involved. In our indirect immunofluorescence assay we demonstrated that rabbit antibodies to all these fragments specially IC3.0 and IC3.4 binds to the conjunctival BMZ in a pattern similar to OCP sera, confirming our binding in the immunoblot assay.

In our in vitro conjunctival organ culture model, rabbit antibody to IC3.0 and IC3.4 were capable of producing BMZ separation, identical with that produced by OCP sera. The demonstration of the production of acantholysis of epithelial cells by PV sera is our positive control. The production of in vitro acantholysis in skin organ culture eventually lead to the demonstration of the ability of PV sera to produce clinical disease in vivo in neonatal mice.[20,21]

Model of the algorithm for antigenic determinants proposed by Jameson and Wolfe[22] suggests that regions containing IC3.4 and IC3.5 have a high probability of being antigenic. IC3.4 contains three stretches of highly antigenic region (residues 1497–1510, 1518–1531, and 1546–1558), whereas IC3.5 contains only one stretch of highly antigenic region extending from 1605 to 1616. Furthermore, the regions extending from 1497–1510, 1546–1558 (IC3.4), and 1605–1616 (IC3.5) are hydrophilic as predicted by the hydrophilicity plot.[23]

The binding to IC3.4 is stronger than IC3.5 or IC3.6. Hence it may indicate that there is epitope hierarchy and possible phenomenon of epitope spreading. There is significant evidence for the presence of multiple epitopes for binding of autoantibodies in human autoimmune diseases such as SLE,[24] myasthenia gravis,[25] and in animal models for autoimmune diseases such as nonobese diabetic (NOD)[26,27] and murine experimental autoimmune encephalomyelitis.[28]

A group of patients with MMP-like disease, now called anti-epiligrin MMP involving multiple mucous membranes, have anti-epiligrin antibodies that bind to multiple epitopes within laminin 5.[29,30] The sera of these patients bind to the dermal side of salt split skin. In immuno-electron microscopy (IEM) this sera demonstrates the deposition of immuno-reactants on the lower lamina lucida and lamina densa.[3,31,32] The sera of patients tested in the present study do not contain autoantibodies to laminin 5 (unpublished data) and bind to the epidermal side of salt split skin. Such sera bind to the hemidesmosome and the cytoplasm of the basal keratinocyte between hemidesmosomes and the inner plasma membrane of keratinocyte or their junction.[3,33,34] Therefore, the subset of MMP patients in this report are distinct and different from anti-epiligrin MMP patients.

MMP is a heterogeneous disease with a wide spectrum of phenotypic presentation.[2] We realize that there may be several subsets of MMP and that each subset may correlate with one or more autoantibody systems. Hence it is likely that sera of some patients with MMP may bind to the molecules other than β4. Investigators have demonstrated the binding of MMP sera to BP180 (BPAg2).[35] The importance of such binding and its role in the pathogenesis of MMP is not clear. Neonatal Balb/C mice injected with antibodies to BP180 do not demonstrate mucosal disease.[17] BP sera containing antibodies to BP180 do not produce any subepithelial BMZ separation in mucous membrane in organ culture models.[5] When BP patients containing antibody to BP180 are examined, the majority of them produce subepidermal blisters only on the skin.[36] These observations would suggest that BP and MMP are clinically very distinct,[37] and different diseases though the antigens recognized or targeted by their autoantibodies may lie in the BMZ.

The large cytoplasmic domain of β4 displays a high level of structural and functional complexity.[38,39] The observation that OCP sera bind to a 165 aa-stretch (IC3.1) in the cytoplasmic portion of the C-terminus of the β4 molecule may provide insight into the molecular pathogenesis of BMZ separation. Adjacent on either side of the region of β4 (IC3.4) where the OCP sera bind, are the sites where BP180, BP230, and β4 interact with each other.[10,40] On one side, BP230 and BP180 bind to each other; and on the other side, BP230 and β4 bind to BP180.[41,42,43] Therefore, it is possible that binding of OCP sera to IC3.4 results in instability of the hemidesmosomes, and its interaction with the basement membrane proteins and may ultimately influence the process of BMZ separation.

In this and earlier studies,[44] we have demonstrated that the OCP sera binds preferentially to the intracellular region of β4. There was no detectable binding to the extracellular domain. We recognize that such binding is possible and that we are unable to detect it due to the limitations of the methodology and technology used. In these studies, the fusion proteins were expressed in *E. coli,* and protein expressed in mammalian systems could provide different results.

These observations would indicate that BMZ autoantibodies can penetrate cell membrane of basal epithelial cells and reach their intracellular binding site (IC3.4). The ability of autoantibodies to penetrate living cells has been well established.[45] Investigators have demonstrated that anti-RNP and anti-DNA antibodies,[11, 46] anti-La/SSB, anti-Ro/SSA, and anti-Scl70[12,13] can penetrate cells, bind to target antigens, and modify cell functions. The mechanism by which the autoantibody enters the cell may be different for each autoantibody, and the resultant effects on the functions of the cell may be different for each cell and each disease process.

The significance of the observations made in this study are threefold. First, they identify human β4 integrin as a possible autoantigen. The congenital absence of β4 has been associated with development of blister in epidermolysis bullosa and confirmed in β4 knockout mice.[47,48] However, this study would implicate a direct role for human β4 in an autoimmune disease with a clinical distribution limited to mucous membranes and occasionally the skin. Second, it provides some meaningful insights into the important sites of interactions, connections and interdependency of cytoplasmic structures that maintain hemidesmosomal stability and integrity. Third, it identifies a specific molecular event during the pathogenesis of OCP and concomitant BMZ separation. Several events must be present between the systemic production of the anti-BMZ antibody and its binding to BMZ antigens in mucosal tissue eventually producing subepithelial blisters. Once these multiple events are identified and characterized, it will be possible to devise strategies and create models that would facilitate development of disease and site specific therapeutic intervention.

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