Ocular cicatricial pemphigoid (OCP) is an uncommon, chronic, subepithelial, scarring systemic autoimmune disease that mainly affects the conjunctiva and other mucous membranes derived from stratified squamous epithelium and (occasionally) the skin. The pathologic process of chronic conjunctivitis and the accompanying progressive subepithelial fibrosis, results in trichiasis, distichiasis, conjunctival chronic conjunctivitis and the accompanying progressive subepithelial fibrosis, keratinization, xerosis, and, eventually, blindness secondary to epithelial fibrosis, results in trichiasis, distichiasis, conjunctival chronic conjunctivitis, cicatricial conjunctivitis; and normal human serum (NHS) were used.

METHODS. Indirect immunofluorescence assay and in vitro organ culture method using NHC were used. Sera and IgG fractions from 10 patients with OCP; immunoaffinity-purified OCP autoantibody; antibodies to human β4, β1, α6, and α5 integrins; and sera from patients with pemphigus vulgaris, bullous pemphigoid (BP), and chronic atopic and chronic ocular rosacea cicatrizant conjunctivitis; and normal human serum (NHS) were used.

RESULTS. Nine of 10 OCP sera or IgG fractions, immunoaffinity-purified OCP autoantibody, antibodies to human β4 and α6 integrins, and sera from patients with BP showed homogenous, smooth linear binding along the basement membrane zone (BMZ) of the NHC. NHS, antibodies to other integrins, and sera from patients with chronic cicatrizant conjunctivitis from other causes showed no such binding. When NHC was first absorbed with OCP sera and then reacted with anti-β4 antibodies or vice versa, the intensity of the BMZ binding was dramatically reduced or completely eliminated, indicating that there were autoantibodies in OCP sera specific for the β4 integrin. BMZ separation developed 48 to 72 hours after addition of total OCP sera, IgG fractions from OCP sera, immunoaffinity-purified autoantibodies from sera of patients with OCP, or anti-β4 antibodies to the NHS cultures, but not after addition of normal control sera, sera from patients with chronic cicatrizant conjunctivitis from causes other than OCP, or sera from patients with OCP in clinical remission.

CONCLUSION. Circulating anti-β4 integrin antibody may have an important role in the pathogenesis of OCP. (Invest Ophthalmol Vis Sci. 1999;40:2283–2290)
antibody to a 205-kDa protein in OCP sera recognizes the cytoplasmic domain of human β4 integrin. These observations collectively suggest that sera of patients with OCP specifically contain antibodies to human β4 integrin. A subset of patients with OCP with clinical features similar to CP has been characterized by the presence of autoantibodies against epiligrin, which is now identified as the α3 subunit of laminin 5,6,10 a ligand for α6β4 integrin. Binding of oral pemphigoid autoantibodies to the α6 integrin subunit has been reported.12

The purpose of this study was to investigate whether the anti-BMZ autoantibodies in sera of patients with OCP specifically bind to the human β4 integrin present in human conjunctiva. We describe an in vitro model that may facilitate understanding of some of the events that produce vesicular lesions in conjunctival epithelium in OCP.

MATERIALS AND METHODS

Serum Samples

The method section confirms adherence to the Declaration of Helsinki. Sera used in this study were obtained from 10 patients with OCP in the acute and active phase of the disease before the institution of therapy. All patients were evaluated in the Immunology Service at the Massachusetts Eye and Ear Infirmary between July 1 and December 31, 1997. The diagnosis of OCP was confirmed in each patient by direct and indirect immunofluorescence (IIF) analysis of bulbar conjunctiva biopsy specimens, as previously described.4 Four patients had involvement of other mucous membranes or the skin. Oral mucosa was involved in three patients. In one patient there was oral and skin involvement. All the sera studied bound to a 205-kDa protein (β4 integrin) on an immunoblot assay using human conjunctiva as substrate.5,13 Additionally, when tested on salt-split skin, the anti-BMZ autoantibody at 1:20 dilution bound to the epidermal side of the split.14

Sera were obtained from 10 normal healthy people, from 10 patients with established BP (cutaneous only, without oral involvement) who had anti-BMZ antibody of 1:1280 or greater titer, when tested by IIF with monkey esophagus as substrate;15 from 3 patients with pemphigus vulgaris (PV); from 2 patients with chronic active cicatrizizing conjunctivitis secondary to rosacea or atopy; and from 3 patients with OCP in remission, monoclonal (10 g/ml) and polyclonal antibodies to human β4 integrin (1:100 dilution), and monoclonal antibodies to α6, α5, and β1 integrins (10 μg/ml). Phosphate buffered saline (PBS) and normal human serum (NHS) and sera from patients with chronic active cicatrizizing conjunctivitis secondary to rosacea or atopy served as negative controls. Positive controls for this assay included sera from patients with PV and BP. Four-micrometer sections of the conjunctiva were first incubated at room temperature with normal goat serum 1:20 dilution for 25 minutes and then washed three times with PBS. Primary antibodies were diluted in PBS-bovine serum albumin 1% in 1:10 to 1:320 dilutions and incubated at room temperature for 1 hour. The sections were then washed three times in PBS, incubated at room temperature with the appropriate fluorescein isothiocyanate (FITC)–conjugated secondary antibody for 30 minutes, and viewed under a fluorescence microscope. Incubation with the primary antibody was omitted in PBS control experiments.

Absorption and Blocking Studies

Blocking studies were performed as follows: Multiple sections of NHC were preabsorbed for 60 minutes repeatedly with the following six reagents: sera of patients with OCP that contained high titer (1:1000 by immunoblot) of anti-BMZ antibodies, the IgG fraction from OCP sera, sera from patients with BP containing antibodies to BPAG1 and BPAG2, monoclonal antibodies to human α6 integrin, monoclonal antibody to human β1 integrin, and normal human serum. These sections were then washed four times with PBS and incubated with monoclonal or polyclonal antibodies to the human β4 integrin. The sections were then stained with the appropriate FITC-conjugated secondary antibodies and viewed under a fluorescence microscope to assess binding at the BMZ. In the reverse experiment, multiple sections of NHC were repeatedly preabsorbed with monoclonal and polyclonal antibodies to human β4 integrin, washed four times with PBS, and reacted with the six reagents listed earlier in the paragraph. The sections were washed and then stained with the appropriate FITC-conjugated secondary antibodies and viewed under the fluorescence microscope for BMZ staining.

In Vitro Culture of Normal Human Conjunctiva

The in vitro model used to evaluate the effects of specific reagents in the pathogenesis of OCP was based on earlier
models that used skin in organ culture to study the effects of PV autoantibody on intercellular adhesion.18,19 Pieces of NHC (2–3 mm²) obtained during cataract surgery were floated in 2 ml complete RPMI-1640 medium in a 12-well tissue culture plate immediately after surgical excision. Three different concentrations of the test reagent were studied. Total serum (10%, 20%, and 30% by volume); purified IgG fractions; 100, 200 and 300 μg of immunoaffinity-purified autoantibodies from sera of patients with OCP; and 10, 20, and 50 μg of anti-β4 and anti-α6 integrin antibodies were added to the wells and incubated at 37°C with 5% CO₂ for 12 to 72 hours. These optimal volumes and concentrations were determined by pilot experiments using the three concentrations to titrate the ability of test reagents to produce BMZ separation. In these pilot experiments, the cultures were terminated at 12, 24, 48, 72, and 96 hours. The conjunctival sections were processed for routine histology using hematoxylin and eosin stain and were examined for subepidermal blister formation. There was some degree of variability in the BMZ separation, but the best results were seen in cultures that were incubated for at least 48 hours. NHS and normal human IgG and sera of three patients with PV and three patients with BP with high titers of antibodies to BPAg1 and BPAg2, two patients with chronic active cicatrizing conjunctivitis secondary to rosacea or atopy were used as controls. Sera from three patients with disease in prolonged clinical remission who had received no therapy for 3 years were also evaluated. Sera of the patients with OCP in clinical remission did not show the presence of anti-BMZ binding using immunoblot assays.13 After incubation, the tissue samples were examined by routine histology, using hematoxylin and eosin staining procedures. The experiment was repeated five times using the same panel of reagents.

Blocking of Blister Formation in an In Vitro Model

OCP sera, immunoaffinity-purified OCP antibody, antibody to human β4 integrin, and sera from patients with PV were absorbed with lysates from β4 integrin–expressing cell lines (UM-SC-22), as previously described6,7. Thereafter, they were tested in the immunoblot assay for the presence of antibody to human β4 integrin.8 These antibodies were then used in the in vitro organ culture system for BMZ separation, as described earlier.

Sera from patients with active PV and with high titers of PV autoantibody were absorbed with UM-SC-22 cell lines. Absorbed and nonabsorbed PV sera were used in the in vitro NHC culture model.

RESULTS

Indirect Immunofluorescence

NHC sections incubated with sera from patients with OCP showed homogenous, continuous linear BMZ staining indistinguishable from that observed during direct immunofluorescence analysis of conjunctiva from 9 of 10 patients with OCP who had active disease (Fig. 1). Strikingly similar observations were made when NHS was incubated with purified IgG fractions of sera from patients with active OCP, with immunoaffinity-purified OCP antibodies, with monoclonal and polyclonal antibodies to human β4 integrin, and with monoclonal antibodies to the α6 integrin. The monoclonal antibody to β4 integrin exhibited more intense staining than did the polyclonal antibody to β4 integrin and OCP sera. Sera of patients with active PV showed binding to intercellular spaces between the epithelial cells of the NHC (data not shown). Sera of patients with active BP that contained antibodies to BPAg1 and BPAg2, determined by immunoblot analysis, showed smooth linear binding to BMZ of NHC sections (data not shown). The marked reduction in background was caused by prior absorption of the NHC sections with goat serum for the IIF assay and NHS for the blocking experiments. No binding was observed in sections of NHC incubated with normal human serum and PBS (Fig. 1) and normal human IgG (Fig. 2D). No staining was observed with monoclonal antibodies to the α5 or β1 integrins or sera of patients with chronic active cicatrizing conjunctivitis other than OCP.

Blocking Experiments with Indirect Immunofluorescence

When the NHS sections were first repeatedly preabsorbed with OCP sera and then incubated with antibodies to β4 integrin, the intensity of the binding of the anti-β4 antibody to the BMZ was reduced, indicating that OCP sera contained antibodies binding to the same epitope as anti-β4 integrin antibodies. When the NHC sections were first repeatedly preabsorbed with monoclonal or polyclonal antibodies to human β4 integrin and then incubated with sera or IgG from patients with OCP, BMZ binding was eliminated (Fig. 2F). When the NHC sections were preabsorbed with NHS or antibody to β1 integrin and then reacted with OCP sera or antibody to human β4 integrin, the BMZ binding was unaffected (Fig. 2). This suggests that the continuous linear BMZ staining produced by sera from patients with active OCP competed with monoclonal and polyclonal antibodies to β4 integrin. The absorption of NHS with either OCP sera or with antibodies to the β4 integrin reduced but did not eliminate the binding of antibodies to the α6 integrin in the BMZ. Absorption of NHS sections with high-titer BP sera reduced but did not eliminate the binding of antibodies to α6 and β4 integrins. Binding of the PV sera to the intercellular cement substance of the NHC was unaffected by earlier absorption of NHS sections with both OCP sera and antibodies to β4 integrin (data not shown).

In Vitro Culture of Normal Human Conjunctiva

No morphologic changes were observed before 24 hours of culture. NHC cultured for 48 to 72 hours showed subepithelial separation from the BMZ in the presence of OCP sera. IgG fractions from sera of patients with OCP, immunoaffinity-purified antibody from OCP sera, but not in the presence of NHS (Fig. 3), sera from patients with chronic active cicatrizing conjunctivitis secondary to rosacea or atopy, or sera from patients with OCP in clinical remission. The effect of treatment with OCP sera, immunoaffinity-purified OCP antibody, and antibody to β4 integrin on induction of BMZ separation is summarized in Table 1. The data presented are the mean results of five experiments, using the same panel of reagents. In treatment with 10% volume of OCP sera, only 5% to 10% of explants showed BMZ separation after 48 to 72 hours of incubation. The maximum explants showing BMZ separation (60%–65%) were observed in treatment with a 30% volume of OCP sera during 48 to 72 hours of incubation. In treatment with 100, 200, and 300 μg of immunoaffinity-purified OCP antibody,
no BMZ separation was observed during a 12- to 24-hour incubation period. However, BMZ separation was observed in 45% to 50% of explants when treated with 300 μg immunoaffinity-purified OCP antibody and incubated for 48 to 72 hours. In treatment with 10, 20, and 50 μg of the monoclonal antibody to human β4 integrin, no BMZ separation was observed in the explants incubated for 12 to 24 hours. Using 20 μg, only 15% to 20% of explants showed BMZ separation during 48 to 72 hours’ incubation. Forty percent to 50% of explants showed BMZ separation when treated with 50 μg of antibody to β4 integrin and incubated for 48 to 72 hours. Prominent tissue necrosis was observed in explants incubated for 96 hours. BMZ separation was observed in sections of organ culture of NHC incubated with antibodies to human β4 integrin (Fig. 4).

The morphologic and histologic characteristics of the BMZ separation in the organ culture sections were indistinguishable from those observed in clinical OCP, except for the absence of infiltration of inflammatory cells in the submucosa. Such pathology was not observed in sections of NHC cultured with antibodies to α5, α6, and β1 human integrins or antibodies to BPAg1 or BPAg2. Sections of NHC cultures with serum from patients with acute PV showed typical acantholysis (Fig. 4). This positive control indicates the validity of the experimental design and technique. No BMZ separation was observed in the presence of NHS, normal human IgG, and sera of patients with OCP in prolonged clinical remission.

**Blocking of Blister Formation in an In Vitro Model**

OCP sera, immunoaffinity-purified OCP antibodies, and antibody to human β4 integrin absorbed with lysates of β4 integrin–expressing cell lines (UM-SC-22) did not produce blister formation in the in vitro model. These antibodies did not bind to the 205-kDa protein in an immunoblot assay using normal human conjunctiva and skin as substrate.

Sera from patients with PV demonstrated binding to 130-kDa protein in the same substrates. When sera from patients with PV were similarly absorbed with lysate of β4 integrin–expressing cell lines and used in the in vitro model, it resulted in production of acantholysis of conjunctival epithelial cells, identical with that produced by unabsorbed sera. No BMZ separation was seen in NHC cultures treated with absorbed or unabsorbed PV sera.
opportunity to study the mechanism of separation of epithelial cells from underlying subepithelial structures and in so doing to provide indirectly information on factors that contribute to the integrity of conjunctival BMZ.

Indirect immunofluorescence and blocking experiments using IIF assays clearly indicated that OCP sera, IgG fractions from OCP sera, and immunoaffinity-purified OCP autoantibodies from OCP sera bound to the target antigen identically. The IIF assay also showed that conjunctival BMZ contained α6, BPag1, and BPag2, along with other integrins and adhesion molecules, and that OCP sera did not cross-react with them. This experimental strategy was similar to that previously published in studying the process of acantholysis in PV. In those studies, sera from patients with PV bound to the intercellular cement substance (desmoglein III) of the skin in a pattern identical with that seen in vivo in patients with PV. The validity of our model is further enforced by the observation that when NHC was incubated with serum from patients with PV, the pemphigus autoantibody bound in a manner identical with that seen on the normal human epidermis or in vivo in patients with PV. Furthermore, BP sera bound to conjunctival BMZ in a smooth linear manner identical with that of the skin. Normal human sera and sera of patients with other cicatrizing ocular diseases did not bind to the conjunctiva in a similar pattern. Observations in this report may suggest that the subset of patients with OCP presented herein may be a distinct subset, because they had a high frequency of extraocular involvement and universal presence of immunoreactants on conjunctival BMZ. Therefore, it appears that the possibility of such patients having a higher concentration of circulating autoantibodies in their sera to different specificities was more likely to be detected in our assay system than from other patients with OCP.

Interestingly, several investigators have demonstrated that sera of patients with OCP contain antibodies to BPag1 and BPag2. However, our studies clearly indicated that the antibodies to BPag1 and BPag2 did not block the binding of the antibodies to conjunctival BMZ by OCP sera. The binding sites for BP antibodies and CP antibodies were on the different epitopes of BPag2.20

Studies indicate that β4 and α6 form a heterodimeric molecule associated with hemidesmosomes.21 Yet, our blocking experiments demonstrated that, within the limitations of the IIF assay, OCP sera and anti-β4 antibodies did not block the binding of anti-α6 integrin antibody to conjunctival BMZ. This observation further supports the hypothesis that production of anti-β4 antibodies in patients with OCP is a specific, early, disease-related event and is not a consequence of damage or immune injury. Additional support for this notion of a pathogenic role of anti-β4 integrin antibodies in OCP comes from the observation that such antibodies were not present in the sera of patients with disease in prolonged clinical remission who were receiving no systemic therapy.

In our in vitro conjunctival organ culture model, antibodies to β4 integrin were capable of causing BMZ separation that was histologically similar to that produced by incubation of NHC with OCP sera, IgG from patients with OCP, and immunoaffinity-purified OCP antibodies. These microvesicles created in vitro had histology fairly identical with or similar to that observed in the conjunctiva of patients with OCP early in the course of the disease. Interestingly, antibody to α6 integrin did not produce any microvesicles, indicating that antibody to β4 integrin was specific in its action. Further evidence for the
specificity of antibody to human β4 integrin–producing blister came from the blocking experiments. OCP sera, immunoaffinity-purified OCP antibodies, and antibody to human β4 integrin absorbed with lysate of β4 integrin–expressing cell lines did not produce lesions seen in incubation with unabsorbed antibodies. These experiments, therefore, within the limitation of the organ culture system, provide additional potent evidence that anti-β4 integrin antibody may play an important role in the production of vesicles or bullae in the conjunctiva of patients with OCP.

The concept that this in vitro model is a reasonable mechanism to study events that may mimic the in vivo phenomenon in tissue dysadhesion is valuable. Evidence for such a hypothesis comes from the experiments in which typical acantholysis, which is a histologic hallmark of PV, was observed in conjunctiva incubated with PV sera. The results observed in the conjunctiva were identical with previously published observations when normal human skin was incubated with PV sera. Absorption of PV sera with β4-expressing cell lines did not affect the ability of PV sera to produce acantholysis. The earlier in vitro model culture for the study of PV eventually provided valuable insights in understanding the molecular pathogenesis of PV.

In our conjunctival organ culture, we did not observe any vesicle formation when NHC was incubated with BP sera. Using normal human skin, Gammon et al. have advanced our understanding of the pathogenesis of BP, showing that BP sera alone is incapable of causing skin BMZ separation but requires the addition of complement and polymorphonuclear leukocytes for lesion production. The data on the relationship between the duration of incubation and concentration of antibodies indicate that these events require time for the biologic processes to evolve. The process appeared to be dose dependent, indicating that an appropriate concentration of antibody is required to bind to available antigen sites to accomplish the separation of the epithelium from the submucosa. Similarly, the process of blister formation was time dependent, because no separation was seen in 12 to 24 hours. Our organ culture model permits the evaluation of the specific roles of polymorphonuclear leukocytes, complement, cytokines, and other bi-

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<th>Incubation Time (h)</th>
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<th>Immunoaffinity-Purified OCP Antibody*</th>
<th>Antibody to Human β4 Integrin*</th>
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<tr>
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<td>10%†</td>
<td>20% 30% 100 μg 200 μg 300 μg</td>
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<td>72</td>
<td>5 45 60</td>
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Data are represented as percentage of sections showing presence of vesicles.

* Twenty histologic sections were prepared from each explant and examined for presence of vesicles.
† Concentration (vol/vol).
‡ Concentration per ml.
ologic agents in the process of BMZ separation mediated by an autoantibody.

The pathogenesis of OCP is a complicated process that is probably heterogeneous and may involve multiple immunologic events and biologic agents. Our in vitro organ culture model is simply one that allowed us to study the binding of circulating antibodies to the conjunctiva and the phenomenon associated with the separation of epithelial cells from the underlying submucosa and tissue matrix. There are no animal models for OCP. At least two interesting possible explanations emerge from our observations on BMZ separation: first, that autoantibody in patients’ sera or exogenously added antibody dissolves β4 integrin and that vesicles are a result of physical dissolution of the binding of β4 integrin molecule to its ligand; and second, that binding of antibody to β4 integrin on the conjunctival epithelial cell surface may trigger an intracellular signal that ultimately results in the movement of the epithelial cells away from the basement membrane, a consequence of multiple intracellular events. Precedence for such observations has been reported recently. Antibodies to α6β4 integrin heterodimer can influence the movement of tumor cells. Earlier observations in the study of PV demonstrated that the breaking of cell adhesion in the epidermis (acantholysis) is not caused by dissolution of desmoglein III, but rather by activation of plasminogen to plasmin. This activation occurs as a direct consequence of unregulated serine protease production in the suprabasal epidermal cells. The signal for the upregulation of serine protease production is generated by the binding of PV antibodies to desmoglein III on epidermal cell surfaces. The in vitro model presented in this study allows investigators to examine closely the consequences of the binding of anti-β4 integrin antibodies to β4 integrin on conjunctival epithelial cell surfaces and the processes that occur secondary to such binding.

**Acknowledgment**

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**References**