Antibasement Membrane Antibody-Mediated Experimental Conjunctivitis

Melvin I. Root,* Stephanie P. Alstdt,* A. Betts Carpenter, † Nirmala SundarRaj,* and Richard A. Thofr*

In ocular cicatricial pemphigoid, the binding of circulating antibodies to conjunctiva is believed to initiate an antibody-mediated cytotoxic response that results in inflammation and tissue damage. To develop a model of antibody-mediated conjunctival inflammation, we examined the effect on conjunctiva of local or systemic administration of a murine monoclonal antibody against basement membrane of stratified squamous epithelium. Neonatal rabbits were given either a single subconjunctival or intraperitoneal injection of the antibody. Eyes were graded clinically for inflammation and conjunctival biopsies were performed. After subconjunctival injection, clinical and histologic inflammation as well as murine antibody and rabbit complement binding to conjunctival basement membrane were detected. With systemic administration there was post-injection clinical inflammation, and conjunctival basement membrane-bound murine antibody was detected. There was no difference observed in conjunctival mitotic rate or goblet cell frequency between treatment groups and controls, following either route of administration. We have created, therefore, a model for antibody-mediated conjunctivitis in rabbits by local or systemic administration of a monoclonal antibody against a component of stratified squamous epithelial basement membrane. Invest Ophthalmol Vis Sci 31:168-175, 1990

Cicatrical pemphigoid is a scarring disease of the conjunctiva and other mucous membranes with occasional involvement of the skin.1-10 This potentially blinding disease is characterized by a chronic conjunctivitis, ocular surface epitheliopathy, conjunctival epithelial hypermitosis and subepithelial fibrosis.2-11 Localized acute conjunctivitis with hyperemia, edema and polymorphonuclear leukocyte infiltration often precedes focal symblepharon formation.4,7,8 Immunoglobulin bound in a linear pattern to the basement membrane of conjunctiva has been demonstrated in patients with ocular cicatrical pemphigoid.4,5,8-10 Circulating antibody to basement membrane of stratified squamous epithelium has been detected in patients with ocular cicatrical pemphigoid4,8,10 and titers may correlate with clinical activity.12 The tissue injury in cicatrical pemphigoid is believed to be initiated by the binding of these circulating antibodies to the basement membrane of conjunctiva.4,5,8 This antibody-mediated cytotoxic (type II hypersensitivity) mechanism may result from fixation of complement, and infiltration and degranulation of effector cells, leading to clinical inflammation.4,5,8,13-17

 Conjunctivitis is part of the symptom complex of other closely related diseases that are believed to result from the same mechanism of antibody-mediated cytotoxicity, including bullous pemphigoid,2-4,8,10,18 pemphigus vulgaris,8,19-24 epidermolysis bullosa acquisita8,25-28 and dermatitis herpetiformis.8,29 The epithelial antigen involved in each of these diseases is believed to be different.1,2,10,23-28,30 In vivo cutaneous animal models of antibody-mediated cytotoxic diseases have been developed.18-22 Each uses concentrated human sera from patients with bullous pemphigoid18,22 or pemphigus vulgaris.19-21 The concentrated autoantibodies were administered locally18,22 or systemically.19-21

The development of an ocular experimental animal model to study the pathogenesis and mechanism of tissue injury could result in better, more specific treatments for ocular antibody-mediated cytotoxic diseases, including ocular cicatrical pemphigoid. To develop this model, we examined the effect of a monoclonal antibody to basement membrane of stratified squamous epithelium31,32 on conjunctiva of neonatal rabbits, when administered locally or systemically. The time courses of clinical inflammation, immunopathology, histologic inflammation, epithe-
llial mitotic rate and goblet cell frequency of the conjunctiva were evaluated.

Materials and Methods

Experimental Animals

Neonatal Dutch belted rabbits of either sex were obtained within 24 hr of birth. The animals weighed between 30–50 g. Animals were maintained and handled in accordance with the ARVO Resolution on the Use of Animals in Research.

Preparation and Characterization of Monoclonal Antibodies: Monoclonal Antibody 63 (MAb 63) and Monoclonal Antibody 33 (MAb 33)

MAb 63 is a murine monoclonal IgG 1 which binds to lamina densa of rabbit stratified squamous epithelium basement membrane. MAb 63 has no activity against laminin, type IV collagen, fibronectin or any component of vascular endothelium. MAb 33 (MAb 33 was previously referred to as MAb 1HP3G33) was used as an immunoglobulin control, and is a murine monoclonal IgG 1 with no activity against any rabbit antigen. Hybrids secreting MAb 63 and MAb 33 were grown in ascites fluid in BALB/c mice. Ascites fluid was collected and monoclonal antibodies were purified by ammonium sulfate fractionation, DEAE ion exchange chromatography, S-200 Sephacryl column chromatography and sterilized by filtration (Research Biogenics, Inc., Bastrop, TX). Antibody solutions were tested for purity by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

The immunoglobulin concentrations were determined by the Coomassie blue reaction, measuring the absorption at 650 nm using a spectrophotometer. Murine IgG (Cappel, Malvern, PA) was used as a standard. Monoclonal antibodies were diluted with sterile PBS (pH 7.2) to the test solution concentration of 0.15 mg of IgG/ml. Antibase membrane antibody titers of MAb 63 and MAb 33 were determined immunohistochemically, by indirect immunofluorescence using normal Dutch rabbit conjunctiva as the tissue substrate. MAb 63 demonstrated staining with an end point titer of 1:128,000. There was no immunofluorescent staining observed with MAB 33.

Injection Technique and Dosages

Local administration: Seventy-three neonatal Dutch belted rabbits, less than 24 hr old, were randomly divided into four groups. Group 1 received MAb 63, Group 2 received MAb 33, Group 3 received PBS and Group 4 was untreated. Both eyes of each rabbit received the same treatment and each eye in the treatment group received a total of 0.1 ml of the test solution. Using a lateral canthal percutaneous approach, 0.05 ml of the test solution was injected into the upper and lower bulbar conjunctiva by a 30-gauge needle. For many rabbits less than 8 days old, blunt dissection was required to open the eyelids to allow the necessary visualization to place the entire bolus subconjunctivally.

Systemic administration: Forty-nine neonatal Dutch belted rabbits less than 24 hr old were randomly divided into three groups. Group 1 received MAb 63, Group 2 received MAb 33 and Group 3 was untreated. Each rabbit in a treatment group received 3 mg of IgG/kg of either MAb 63 or MAb 33 by intraperitoneal injection.

Clinical Evaluation

At 1, 2, 3, 4 and 6 hr and 1, 3, 5, 7 and 9 days post-injection, eyes were examined in a masked fashion for signs of clinical inflammation. Three eyes were evaluated at each time point for each group. Hyperemia, edema, thickness and rigidity of the upper and lower conjunctiva at the time of biopsy were assessed on a 0–4 scale, with the sum representing the grade of clinical inflammation.

Tissue Evaluation

At 1, 2, 3, 4 and 6 hr and 1, 3, 5, 7 and 9 days post-injection the rabbits were anesthetized with ketamine 50 mg/kg, xylazine 10 mg/kg, which was supplemented with topical proparacaine 0.5%. Three eyes from different animals were evaluated at each time point for each group. A 4 × 6 mm piece of inferior bulbar conjunctiva was excised for histology and autoradiography. A similar piece of superior bulbar conjunctiva was excised for direct immunofluorescence.

Direct Immunofluorescence

Unfixed conjunctiva was embedded in O.C.T. Compound (Miles Scientific, Naperville, IL), 8 µm thick sections were cut with a cryostat, transferred to gelatin-coated glass slides and incubated with fluorescein isothiocyanate conjugated (FITC) goat anti-mouse IgG antibody (Cappel) or FITC goat anti-rabbit C3 antibody (Cappel) for 45 min at room temperature. Slides were observed in a masked fashion under a fluorescent microscope with epi-illumination, and assessed on a 0, 1, 2, 3 scale for absent, discontinuous, weak and strong staining, respectively, with the sum representing the grade of immunofluorescence.
Histology

Conjunctival biopsies were sectioned and stained with hematoxylin and eosin, and examined by light microscopy for number and type of inflammatory cells. Inflammatory cells were counted in a masked fashion in four random high power fields (HPF). High power represents the x400 magnification and a field of 0.113 mm². The average number of cells per HPF represents the grade of histologic inflammation.

Mitotic Rate and Goblet Cell Frequency

Conjunctival biopsies were incubated in 1 ml of Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO Laboratories, Grand Island, NY), with 40.45 μCi of tritiated thymidine, with a specific activity of 80.9 Ci/mmol (New England Nuclear, Boston, MA), for 2.5 hr in 95% air, 5% CO₂ at 37°C followed by 30 min of incubation in isotope-free DMEM. The specimens were then fixed in 10% formalin, embedded in paraffin and 7 μm unstained sections were prepared by standard histologic technique. Slides were dipped in scintillation cocktail for aqueous samples (Formula-963, New England Nuclear), then into nuclear track emulsion (NTB-2, Kodak, Rochester, NY). Slides were stored at -20°C for 24 hr, developed (D19 Kodak developer, Kodak), and then stained with periodic acid Schiff (PAS) using hematoxylin as the counterstain. The mitotic rate was determined by counting the number of tritiated thymidine labeled cells per 100 basal epithelial cells. The goblet cell frequency was the number of PAS-positive goblet cells per 100 basal epithelial cells.

Statistical Analysis

Data were evaluated for statistical significance using one-way analysis of variance with groups as the factor, for each route of administration and each time point.

Non-Ocular Tissue

Two non-ocular stratified squamous epithelia (dorsal thoracic skin and oral mucosa) and one non-ocular, nonstratified squamous epithelium (peritoneum) were examined histologically and with direct immunofluorescence 1 and 3 days after intraperitoneal injection as described for conjunctiva. Skin and oral mucosa were examined at 1, 2, 3, 4 and 6 hr and 1, 3, 5, 7 and 9 days post-injection for signs of clinical inflammation.

Results

Local Administration

The results of the local administration are summarized in Figures 2, 4 and 5.

Clinical inflammation: Hyperemia and edema of the conjunctiva were noted after all local injections. The signs of clinical inflammation tended to decrease with time. There was significantly more clinical inflammation 3 hr to 9 days post-injection of MAb 63 when compared with controls (P < 0.05) (Figs. 1, 2).

Direct immunofluorescence: After injection of MAB 63, a linear pattern of murine immunoglobulin (IgG) was detected at the conjunctival basement membrane within 1 hr and persisted up to 9 days post-injection (Figs. 3, 4) (P < 0.001). At 1 and 2 hr murine immunoglobulin was also detected in the subepithelial tissue. Rabbit complement (C3) was detected in a linear pattern at the conjunctival basement membrane at 2 hr in one of three rabbits and at 3 hr in one of three rabbits. No murine immunoglobulin or rabbit complement was detected after injection of MAb 33, PBS or in untreated eyes.

Histologic inflammation: The predominant infiltrating cell type was the polymorphonuclear leuko-
cyte and rarely a lymphocyte was seen. The polymorphonuclear leukocytes were observed in the epithelium and subepithelial tissue. There were significantly more polymorphonuclear leukocytes/HPF, 3 hr to 7 days post-subconjunctival injection of MAb 63 when compared with controls ($P < 0.05$) (Fig. 5).

Mitotic rate and goblet cells: There was no significant difference in mitotic rate or goblet cell frequency among treated and untreated groups. In all groups goblet cell frequency was found to increase progressively with age.

Systemic Administration

The results of systemic administration are summarized in Figures 7, 9 and 10.

Clinical inflammation: There was significantly more clinical inflammation between 6 hr and 9 days after intraperitoneal injection of MAb 63 than there was in controls ($P < 0.05$) (Figs. 6, 7). Control groups demonstrated very little inflammation. However, the inflammation seen after intraperitoneal injection of MAb 63 was slower to appear, less intense and shorter in duration than after subconjunctival injection of MAb 63.

---

**Fig. 3.** Linear binding of murine immunoglobulin at the conjunctival basement membrane 3 days after a single percutaneous subconjunctival injection of MAb 63 (direct immunofluorescence, original magnification $\times 200$).

**Fig. 4.** Total mean grade of immunofluorescence, representing binding of murine immunoglobulin (IgG) at the conjunctival basement membrane, after a single percutaneous subconjunctival injection of the test solution (for each group $n = 3$, at each time point).

**Fig. 5.** Mean number of polymorphonuclear leukocytes per high power field ($\times 400$) of rabbit conjunctiva, representing histologic inflammation, after a single percutaneous subconjunctival injection of the test solution (for each group $n = 3$, at each time point).
Fig. 6. Hyperemia and edema of the conjunctiva of a neonatal rabbit 3 days after a single intraperitoneal injection of MAb 63 (3 mg of IgG/kg).

Direct immunofluorescence: Murine immunoglobulin was detected after intraperitoneal injection of MAb 63 (Figs. 8, 9). It was detected as a linear pattern of fluorescence at the conjunctival basement membrane within 3 days and persisted up to 9 days after injection. No immunoglobulin was detected after injection of MAb 33 or in untreated controls ($P < 0.001$). No rabbit complement (C3) was detected in any of the groups after intraperitoneal injection.

Histologic inflammation: The predominant cell type was polymorphonuclear leukocytes and rarely a lymphocyte was seen. There was no significant difference in histologic inflammation between groups, at any time, in contrast to the histologic findings after local injections (Fig. 10).

Mitotic rate and goblet cell frequency: There was no significant difference in mitotic rate or goblet cell frequency for any group after intraperitoneal injection.

Non-ocular tissue: At day 1 there was linear binding of murine immunoglobulin and rabbit complement (C3) at the basement membrane of the hair follicles of the skin. No murine immunoglobulin or rabbit complement (C3) were detected at the basement membrane of the epidermis on day 1 or at any site in the skin on day 3. Murine immunoglobulin but no rabbit complement was bound in a linear pattern to the basement membrane of oral mucosa on day 3 but not on day 1. No murine immunoglobulin or rabbit complement (C3) binding was detected on the peritoneum on day 1 or 3. No inflammatory cells were present in epithelium or subepithelium of the non-ocular tissues examined. No cutaneous or oral mucosal bullae, or sign of cutaneous or oral mucosal inflammation was observed clinically at any time 1, 2, 3, 4 and 6 hr and 1, 3, 5, 7 and 9 days post-injection.

Figure 7. Effect of systemic administration of a single intraperitoneal injection of test solutions, on total mean grade of clinical inflammation (for each group $n = 3$, at each time point).

Discussion

The development of cutaneous animal models of antibody-mediated cytotoxic diseases has greatly increased the understanding of pathogenesis, mechanism of tissue injury and therapy for these diseases. Ogawa and coworkers administered sera from patients with bullous pemphigoid by intradermal injection into guinea pigs. Local inflammation, dermal–epidermal separation, antibody binding and complement fixation were detected. In 1981, Anhalt, Labib, Sugar, Diaz and coworkers injected sera from patients with bullous pemphigoid into the corneal stroma of rabbits. Antibody and complement was detected at the corneal basement membrane. Also, an inflammatory infiltrate of neutrophils and subepithelial blister formation was observed. In 1982, Anhalt, Labib, Diaz and others developed an excellent model for pemphigus vulgaris. Sera from patients with pemphigus vulgaris was administered by intraperitoneal injection into neonatal mice. The mice developed clinical, histologic and immunopathologic features identical to cutaneous pemphigus vulgaris in humans.

By using neonatal rabbits and an antibody with activity against a rabbit antigen, we have designed a model that mimics the immunopathology of an autoimmune disease. We postulate that the changes we have noted occur though the following chain of events: after injection of murine immunoglobulin into neonatal rabbits, tolerance is established wherein the rabbit's immune system will not recognize or respond to the murine immunoglobulin as foreign; alternatively, if tolerance is incomplete the rabbit's immune system has insufficient time during the period studied to respond to the protein presented. This murine immunoglobulin binds to its target rabbit conjunctival basement membrane, resulting in activation of this antibody.
polymorphonuclear leukocytes respond to the bound, activated murine immunoglobulin as if it were a bound, activated rabbit immunoglobulin.\textsuperscript{13-15,18-22,24} Subsequent fixation of complement leads to chemotaxis and cellular damage.\textsuperscript{15,16} Polymorphonuclear leukocytes interact strongly with the activated Fc portion of the bound immunoglobulin, resulting in degranulation.\textsuperscript{13-15,24} Degranulation releases enzymes, radicals, etc., which leads to tissue damage, further chemotaxis and amplification of the inflammatory cascade.\textsuperscript{13,15} This leads to clinical inflammation and a conjunctivitis produced by this antibody-mediated mechanism.\textsuperscript{15}

The low weight of the neonatal rabbits has made it possible to achieve relatively high concentrations of monoclonal antibodies.\textsuperscript{19,24} The cutaneous models of antibody-mediated cytotoxic diseases have been developed by using autoantibodies purified from patients' sera.\textsuperscript{18-22} The monoclonal antibodies used in our model have an advantage in that they are available in virtually endless supply and their characteristics remain constant. Use of the same antibody allows a degree of standardization and reproducibility in repeated trials and future experiments not possible with autoantibodies derived from human sera.

Compared with pooled IgG controls used in other models, our MAb 33 immunoglobulin control may
be considered superior; since every molecule of MAb 33 is identical, it presents fewer epitopes for induction of tolerance than pooled IgG controls.

Local injections were made via a percutaneous subconjunctival route. This allowed a bolus of MAb 63 to be placed subconjunctivally without loss of solution or direct manipulation of conjunctival epithelium or its basement membrane, which might affect the grade of histologic inflammation or mitotic rate.

The relative delay of appearance of antibody bound to conjunctival basement membrane after systemic administration is a reflection of the time required for absorption from the intraperitoneal space, transport by the systemic circulation and time required for the antibody to accumulate to detectable levels. The comparatively weaker clinical inflammation and lack of significant histological inflammation may be the result of a lower concentration of MAb 63 in the conjunctiva because of dilution after systemic administration as well as absorption of MAb 63 by basement membranes of other stratified squamous epithelium.

The relatively weak immunofluorescence of MAb 63 to the basement membrane of non-ocular stratified squamous epithelium and absence of histologic or clinical signs of inflammation may be the result of the relative affinity of MAb 63 for ocular tissue (which is the tissue it was raised against) or the compositional and structural differences between conjunctiva, skin and oral mucosa.

MAb 63 is a murine IgG 1 that has relatively poor complement fixing activity. However, complement binding was demonstrated at the conjunctival basement membrane at 2 and 3 hr. This may reflect the peak concentration of bound antibody and thus, maximal complement binding. C3 may be present at other time points but at concentrations below the sensitivity of our direct immunofluorescent technique. Clq and C4 may have been present, but we did not test for these complement components. The fixation of complement, however, is not required in an antibody-mediated cytotoxic response, or for the resultant inflammation or tissue damage.13-15,17

Cicatricial pemphigoid is characterized by an increased conjunctival mitotic rate4,5,8,11 and a markedly decreased goblet cell frequency, which is most often zero.3,8,11 In our model, no differences were detected in mitotic rate or goblet cell frequency between treated and control groups. This is likely a reflection of a single injection and short follow-up. The changes in cellular differentiation and mitotic activity and decrease in goblet cell frequency in cicatricial pemphigoid may be the result of ongoing inflammation, rather than the pulse of inflammation produced in this model. Nevertheless, during episodes of acute activity in ocular cicatricial pemphigoid there is redness, edema, binding of immunoglobulin and complement at the conjunctival basement membrane, and an inflammatory infiltrate consisting mainly of polymorphonuclear leukocytes.4,7,8 These clinical, immunopathologic and histologic features are very similar to those of our model. The model we developed also resembles other antibody-mediated cytotoxic diseases with conjunctival inflammation, including bullous pemphigoid,2,4,9,10,18 pemphigus vulgaris,8,19-24 epidermolysis bullosa acquisita,8,25-28 and dermatitis herpetiformis.8,29

By modulating specific parameters of the inflammatory cascade in our model, such as leukocyte migration and complement depletion, it will be possible to investigate the factors responsible for tissue damage in acute disease.13-17,21,22 The study of these processes may lead to the development of more specific or local therapies for diseases whose ocular complications have been treated with systemic steroids or cytotoxic immunosuppression.3,4,6,8,10,21,22 Long-term experiments may provide models for the more important and destructive changes of chronic ocular cicatricial pemphigoid.

Key words: experimental conjunctivitis, basement membrane, stratified squamous epithelium, antibasement membrane antibody, antibody-mediated, cicatricial pemphigoid

Acknowledgment

We thank Chaloppdi V. Sundar-Raj for developing monoclonal antibodies MAb63 and MAb33 (MAb 1HP3G).

References


