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Chronic exposure to the anti-M3 muscarinic acetylcholine receptor autoantibody in pemphigus vulgaris contributes to disease pathophysiology

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Pemphigus vulgaris **(PV) is a potentially lethal autoimmune mucocutaneous blistering disease characterized by binding of IgG autoantibodies (AuAbs) to keratinocytes (KCs). In addition to AuAbs against adhesion molecules desmogleins 1 and 3, PV patients also produce an AuAb against the M3 muscarinic acetylcholine (ACh) receptor (M3AR) that plays an important role in regulation of vital functions of KCs upon binding endogenous ACh. This anti-M3AR AuAb is pathogenic because its adsorption eliminates the acantholytic activity of PV IgG; however, the molecular mechanism of its action is unclear. In the present study, we sought to elucidate the mode of immunopharmacologic action of the anti-M3AR AuAb in PV. Short-term exposures of cultured KCs to PV IgG or the muscarinic agonist muscarine both induced changes in the expression of keratins 5 and 10, consistent with the inhibition of proliferation and upregulated differentiation and in keeping with the biological function of M3AR. In contrast, long-term incubations induced a keratin expression pattern consistent with upregulated proliferation and decreased differentiation, in keeping with the hyperproliferative state of KCs in PV. This change could result from desensitization of the M3AR, representing the net antagonist-like effect of the AuAb. Therefore, chronic exposure of KCs to the anti-M3AR AuAb interrupts the physiological regulation of KCs by endogenous ACh, contributing to the onset of acantholysis. Since cholinergic agents have already demonstrated antiacantholytic activity in a mouse model of PV and in PV patients, our results have translational significance and can guide future development of therapies for PV patients employing cholinergic drugs.**

Pemphigus vulgaris (PV) is a potentially lethal autoimmune mucocutaneous blistering disease characterized by IgG autoantibodies (AuAbs) binding to keratinocytes (KCs) and inducing devastating blisters affecting oral and/or esophageal surfaces and, sometime, also the skin. Although the incidence of PV is only 1 to 16 per million population per year (1, 2), this disease represents a significant burden to health care professionals and the health care system (3). Prior to the introduction of therapy with oral corticosteroids in the 1950s, pemphigus had a dismal natural course with a 50% mortality rate at 2 years and 100% mortality rate by 5 years after onset of the disease. While corticosteroid treatment is life-saving, the high dose and prolonged courses required for disease control are associated with significant adverse effects, including death (4, 5). Mortality remains at a relatively high rate, ranging from 5 to 13%, due to differences in patient care in different parts of the world (6–8). Pemphigus vulgaris patients develop intraepidermal cell-cell detachment (acantholysis) above the basal cell layer, blisters, and nonhealing erosions. The initial event of acantholysis is basal cell separation from each other and immediate suprabasal KCs. Under an AuAb attack, basal cells shrink, causing intercellular separation, but remain attached to the epidermal basement membrane, forming a unique pattern known as "tombstoning" (9).

In typical PV, AuAbs recognize desmosomal protein desmoglein 3 (Dsg3) and sometime also desmoglein 1 (Dsg1). However, on average, 10% of acute PV patients with anti-KC AuAbs detectable by direct and/or indirect immunofluorescence are negative for Dsg1/3 AuAbs by ELISA (reviewed in (10)). There are no known clinical and pathological differences between PV patients with *versus* without anti-Dsg AuAbs. Anti-KC AuAbs in patients with atypical, that is, anti-Dsg1/3 AuAb-negative PV (non-Dsg PV in short), are pathogenic because their IgGs induce skin blistering in neonatal mice due to suprabasal acantholysis (11). Keratinocytes in the lowermost epidermal layers are the primary target for AuAbs in both typical and atypical PV. In typical PV, basal KCs are believed to be selectively targeted because they express a bulk of Dsg3, whereas in atypical PV, the predominant pathogenic target on basal KCs remains unknown. Our studies suggest that the M3 muscarinic class of acetylcholine (ACh) receptors (M3AR) is the most likely candidate.

The M3AR is preferentially coupled to activation of pertussis toxin–insensitive G proteins of the Gαq/11 family, which activates phospholipase C (PLC) and produces inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DG). These second messengers elicit activation of PKC and trigger the release of

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Ca^{2+} from intracellular stores ($[Ca^{2+}]_i$). M3 muscarinic acetylcholine receptor is predominantly expressed in the lowermost epidermal layer wherein it regulates vital function of KCs, including proliferation, migration, and adhesion (reviewed in (12, 13)). It has been demonstrated that M3AR controls cytoskeleton assembly and formation of cell–cell attachments (14–16) and that $M3AR^{-/-}$ KCs feature abnormal expression of the intercellular adhesion molecules (17).

Among the known species of anti-KC AuAbs detectable in PV patients (reviewed in (18)), the presence of AuAbs against KC muscarinic ACh receptors (mAChRs) has been known for 3 decades (19). The pathogenic significance of anti-M3AR AuAb in PV is suggested by the following lines of evidence: (1) proteomic studies demonstrated anti-M3AR AuAb in a large number of PV sera (20, 21); (2) its titer correlates with disease stage of PV (11, 22); (3) while absorption of PV IgGs on recombinant M3AR prevents skin blistering in the passive AuAb transfer mouse model of PV, the acantholytic activity of preabsorbed PV IgGs can be restored by adding back the eluted AuAb (11); and (4) acantholysis and cutaneous Nikolskiy sign (23) in mouse skin (*i.e.*, erosions induced by gentle rubbing with a pencil eraser) can be induced by anti-M3AR AuAb in combination with two other AuAbs affinity purified from non-Dsg PV sera, antidesmocollin 3 AuAb, and anti-secretory pathway Ca^{2+}/Mn^{2+}-ATPase isoform 1 protein AuAb, with which anti-M3AR AuAb synergizes (11, 24). In PV, anti-M3AR AuAb may also synergize with AuAbs against other adhesion and signaling molecules, such as other subtypes of mAChRs and various subunits of the nicotinic class of ACh receptors (20, 25–29) as well as antimitochondrial AuAbs (30–32). However, the exact mechanism of pathogenic action of anti-M3AR AuAb in PV remains poorly understood and requires further investigation.

The role for autoimmunity against KC M3AR in non-Dsg PV was evaluated using 1-day old $M3AR^{-/-}$ mice (24). Compared to WT mice, the intact M3AR KO mice demonstrated an increase of intercellular spaces due to mild shrinkage of epidermal cells. Similar subtle morphologic changes were observed in WT pups injected with the affinity-purified anti-M3AR AuAb. Injection of PV IgGs caused spontaneous skin blistering and superficial erosions in both WT and $M3AR^{-/-}$ mice. However, while injection of PV IgGs to a WT mouse predictably caused suprabasal acantholysis, the entire basal cell layer was missing at the bottom of blister cavity in $M3AR^{-/-}$ mice. These results suggested that in non-Dsg PV, anti-M3AR AuAb determines the suprabasal location of the intraepidermal split. As we have previously demonstrated (17, 33), constant physiologic stimulation of KC M3AR with endogenous ACh maintains attachment of basal cells to the basal membrane, in part, due to upregulation of sedentary (hemidesmosomal) integrins anchoring KCs to the basal membrane. Lack of the M3AR-mediated physiologic control of cell-substrate attachment by endogeneous ACh explains disappearance of the basal cells in blisters induced by PV IgG in $M3AR^{-/-}$ mice. Since basal cells remained attached to the basal membrane in WT mice treated with affinity-purified anti-M3AR AuAb or with whole PV IgG fraction that contained anti-M3AR AuAb as well as other species of anti-KC AuAbs, it is likely that anti-M3AR AuAb exhibited an agonist-like immunopharmacological effect, by analogy with the thyrotropin receptor AuAb that stimulates the thyroid function in Graves' hyperthyroidism.

Our research goal is to understand how anti-M3AR AuAb affects adhesion of basal KCs. Based on the overwhelming evidence that ACh to M3AR signaling pathway is an essential physiologic regulator of cell adhesion in the epidermis (reviewed in (12, 13)), elucidation of immunopharmacologic aspects of autoimmunity against KC M3AR in PV should open a door for evaluation of cholinergic drugs for steroid-sparing therapy of PV patients and patients with some other diseases of skin adhesion featuring acantholysis. In the present study, we sought to elucidate the exact mode of immunopharmacologic action of pathogenic anti-M3AR AuAb on basal KCs. Cumulative results of our immunohistochemical, immunoblotting, and radioligand-binding experiments indicate that anti-M3AR AuAb produced in non-Dsg PV patients exhibits an agonist-like effect that leads to receptor desensitization (a.k.a. tachyphylaxis), which, in turn, abolishes the physiologic regulation of KCs with endogeneous ACh thus contributing to acantholysis. Therefore, the net pathobiologic effect of anti-M3AR AuAb is an antagonist-like effect. These findings provide new insights into the immunopharmacologic mechanisms of initiation of acantholysis in PV, and also lay a groundwork for future translational studies evaluating the antiacantholytic potential of cholinergic drugs.

Results

Chronic treatment of mice with anti-M3AR AuAb decreases the level of receptor in murine epidermis

Since the effect of chronic exposure to PV IgGs on the expression of KC M3AR could not be evaluated *in vivo* due to death of neonatal mice, we treated mice with affinity-purified anti-M3AR AuAb. The neonatal C57BL/6 mice were injected intradermally with anti-M3AR AuAb every 12 h over 3 days, after which the M3AR was visualized in the epidermis by indirect immunofluorescence using commercial rabbit anti-M3AR antibody. Qualitative analysis of the intensity of fluorescence demonstrated dramatic decrease from the baseline in experimental mice treated with anti-M3AR AuAb (Fig. 1). We sacrificed mice every 12 h after injections but did not see any appreciable differences until after 72 h. The control mice that received injection of equal concentration of normal IgG (NIgG) or plain saline did not develop any visible changes of the staining pattern (data not shown). We also stained murine epidermis for M1, M4, and M5 mAChR subtypes expressed in KCs (34) and did not observe any changes from controls (data not shown). These results suggested that chronic stimulation of KCs with anti-M3AR AuAb leads to disappearance of the targeted receptor from the cell surface.

Anti-M3AR AuAb and PV IgG decrease concentration of M3AR protein but not mRNA in murine KCs

To elucidate whether decreased expression of M3AR in murine epidermis occurred at the mRNA or the protein levels,

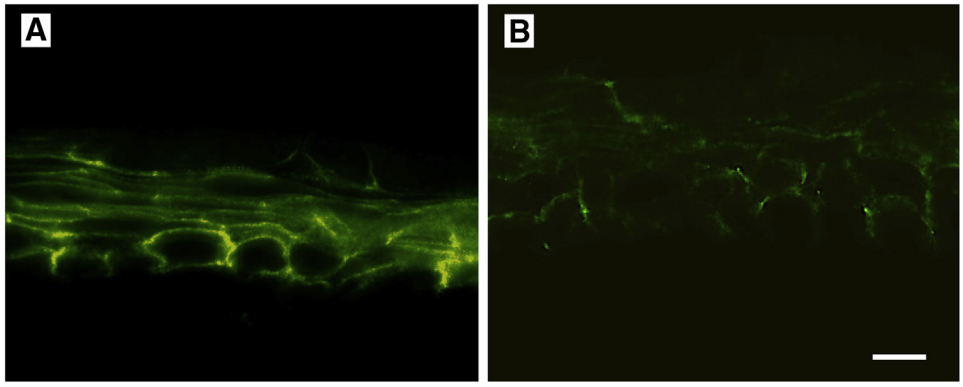

Figure 1. Analysis of the effect of anti-M3AR AuAb on the expression of keratinocyte M3AR by indirect immunofluorescence. The representative images of M3AR in the epidermis of intact (*A*) and experimental neonatal C57BL/6 mice repetitively injected with anti-M3AR AuAb (*B*). The experimental 1-day old mice were injected intradermally with anti-M3AR AuAb every 12 h during 72 h, after which the M3AR was visualized in the epidermis by rabbit anti-M3AR primary and FITC-labeled secondary antibodies as detailed in Experimental procedures. Note the disappearance of M3AR immunoreactivity in experimental mice. The control mice that received injection of equal concentration of NIgG or plain saline did not develop any visible changes of staining (data not shown). The scale bar represents 10 μm. AuAb, autoantibody; M3AR, M3 muscarinic acetylcholine receptor; NIgG, normal IgG.

or both, we performed a series of real-time quantitative PCR (qPCR) and In-cell Western experiments with cultured murine epidermal KCs. The cells were cultivated in the culture medium for 24, 48, or 72 h in the presence of PV IgG isolated from the pooled sera of PV patients who did not develop anti-M3AR AuAb, those who did as well as affinity-purified anti-M3AR AuAb or the pan-muscarinic agonist muscarine, after which the levels or receptor mRNA and protein were quantitated and compared with intact controls (Fig. 2). The results demonstrated statistically significant ($p < 0.05$) decrease in the receptor expression at the protein but not mRNA levels when the cells were exposed to affinity-purified anti-M3AR

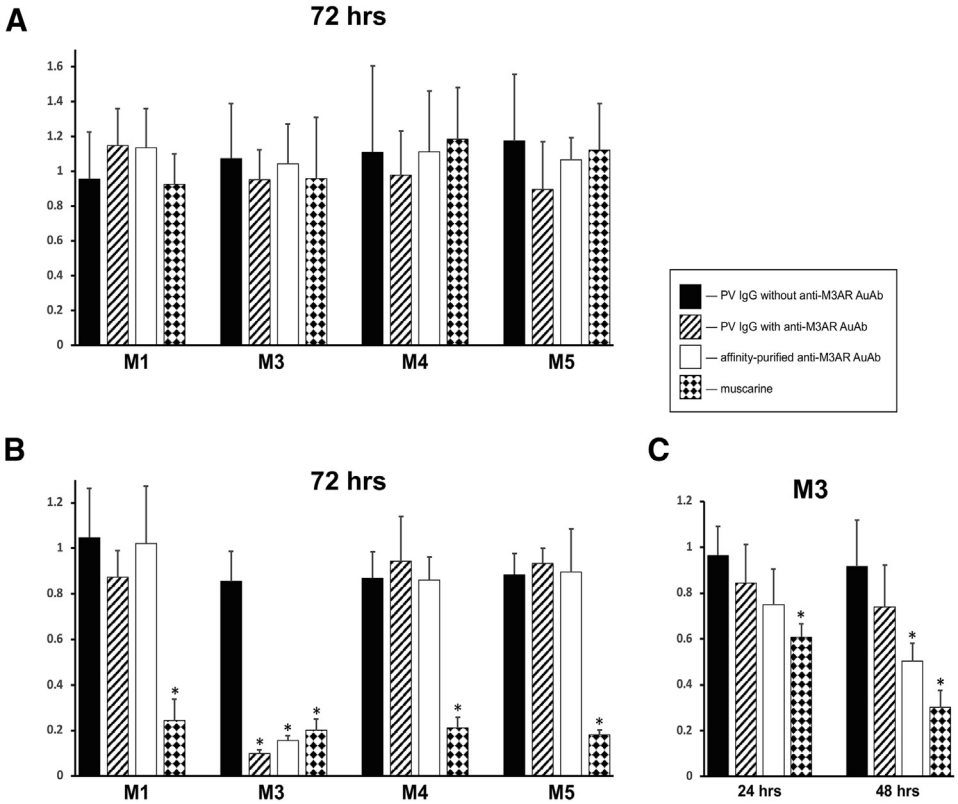

Figure 2. Alterations in the M3AR gene expression in cultured murine KCs. Quantitative PCR (*A*) and ICW (*B* and *C*) analysis of the expression of M1, M3, M4, and M5 mAChR subtypes after incubations of confluent cultures of murine epidermal KCs in 96-well plates with PV IgG without anti-M3AR AuAb, PV IgG with anti-M3AR AuAb, affinity-purified anti-M3AR AuAb, or 1 μM muscarine for the time periods indicated on the graph. The relative amounts of mRNA transcript and protein levels of M3AR were measured as described in the Experimental procedures section. To standardize the analysis, the gene expression ratios in the intact KCs (control cells) were taken as 1. Triplicate experiments were performed with KCs from each of the 3 cell donors ($n = 3$). *Asterisks* indicate significant ($p < 0.05$) differences from intact control KCs. AuAb, autoantibody; ICW, In-cell Western; KCs, keratinocytes; M3AR, M3 muscarinic acetylcholine receptor; PV, Pemphigus vulgaris.

AuAb, PV IgG containing anti-M3AuAb, and the muscarinic agonist muscarine, but not PV IgG without anti-M3AR AuAb. In a time-course study of M3AR protein, we found that the degree of receptor loss correlated with duration of incubations, so that longer exposures resulted in more profound changes. None of the exposure conditions led to any significant alterations of the mRNAs or proteins of other mAChR subtypes expressed in KCs (Fig. 2). These results confirmed the specificity of the effect of PV AuAbs on the KC M3AR subtype.

Pemphigus vulgaris IgG depletes the cellular content of M3AR in cultured human KCs

The effect of PV IgG on the expression of M3AR in human KCs was analyzed by immunoblotting. The monolayers of Het-1A cells were incubated for 72 h in the growth media supplemented with equal amounts of PV IgG containing anti-M3AR AuAb, or NIgG, or no additions, and the M3AR protein bands were visualized by immunoblotting with commercial anti-M3AR antibody (Fig. 3). As expected, the M3AR protein band appeared at approximately 65 kDa (34). We observed a dramatic reduction in the relative amount of M3AR in KCs exposed to PV IgG. These results suggested that the pathophysiology of PV includes an AuAb-induced reduction of M3AR signaling in epidermal KCs.

Dichotomous effect of PV IgG on the M3AR signal transduction in human KCs

Next, we evaluated the immunopharmacologic action of PV IgG on KC M3AR. We preincubated the monolayers of Het-1A cells with PV IgG or NIgG for 1 h or 24 h, washed, and measured effects of the pan-muscarinic agonist muscarine and the M3-preferring antagonist 4-DAMP on the downstream effectors of M3AR signaling PLC, DG, and IP3 (Fig. 4). The maximum changes from receptor activation were determined in the intact cells stimulated with muscarine and taken as 100%. The specificity of the effects to ligation of M3AR was confirmed by the ability of 4-DAMP to abolish the effects of muscarine. Exposure to PV IgG for 1 h caused significant ($p < 0.05$) elevation of all second messengers. In marked contrast, the longer exposure to PV IgG (24 h), but not NIgG, resulted in the significant ($p < 0.05$) decrease of the cell responsiveness to stimulation with muscarine and PV IgG (Fig. 4). These results suggested that the anti-M3AR AuAb produced by PV patients exhibits an agonist-like pharmacologic effect and leads to receptor desensitization.

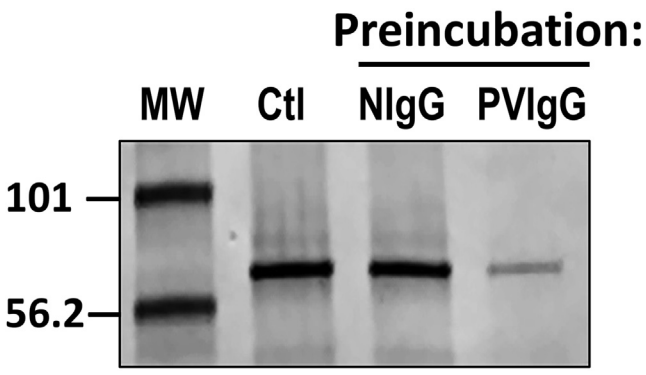

Figure 3. Analysis of the effect of PV IgG on the expression of keratinocyte M3AR by immunoblotting. The representative images of the protein bands visualized by rabbit anti-M3AR AuAb in the monolayers of Het-1A cells incubated for 72 h in the growth media, containing equal amounts of PV IgG, or NIgG, or no additions (Ctl), washed, and harvested, and the amount of M3AR was probed in western blots as detailed in Experimental procedures. Note an appreciable reduction of the relative amount of the M3AR protein band that expectedly appeared at approximately 65 kDa (34). AuAb, autoantibody; M3AR, M3 muscarinic acetylcholine receptor; NIgG, normal IgG; PV, Pemphigus vulgaris.

Pemphigus vulgaris IgG decreases the number M3AR ligand-binding sites on human KCs

To identify the role for desensitization of KC M3AR in PV pathophysiology, we analyzed the effects of PV IgG *versus* NIgG on binding of a muscarinic radioligand to human KCs. The monolayers of Het-1A cells were preincubated at 37 °C or 4 °C for 1 h in the growth medium containing equal amounts of PV IgG or NIgG and used in the radioligand-binding assay with the specific muscarinic ligand [^{3}H]QNB (35). At 37 °C, the cycling of ligated receptors is rapid, whereas at 4 °C, receptor translocation is prevented (36). Preincubation of Het-1A cells with PV IgG at 37 °C, but not 4 °C, decreased specific binding of [^{3}H]QNB by about 80% ($p < 0.05$) (Fig. 5). Preincubation with NIgG did not alter the radioligand-binding parameters. These results suggested that desensitization of KC M3AR in PV due to its ligation by anti-M3AR AuAb is followed by receptor internalization and subsequent degradation.

Dichotomous effect of PV IgG on the muscarinic regulation of KC proliferation and differentiation

Since the physiologic regulation of basal cell differentiation through M3AR is well-established (reviewed in (37)), we analyzed the effects of short-term (*i.e.*, 24 h) and long-term (*i.e.*, 72 h) exposures to PV IgG on the expression of mRNAs of the KC differentiation markers cytokeratins K5 and K10. In these series of experiments, instead of nondifferentiating immortalized Het-1A cells, we used normal human epidermal KCs grown to ~80% confluence in KC growth medium containing 1.6 mM Ca^{2+}. A short-term exposure to PV IgG, but not NIgG, caused significant ($p < 0.05$) decrease of K5 and an increase of K10 mRNAs (Fig. 6). The effect of long-term preincubation was opposite, manifested by significant ($p < 0.05$) increase of K5 and decrease of K10 mRNAs. Because the effect of muscarine was similar to that of PV IgG (Fig. 6), it can be concluded that chronic stimulation of basal KCs with anti-M3AR AuAb alters maturation of KCs.

Discussion

In this study, we demonstrate for the first time that chronic exposure to anti-M3AR AuAb depletes M3AR in mouse epidermis and cultured human KCs, and that this AuAb produces an agonist-like effect on the M3AR-mediated signaling. Taken together, these findings indicate that desensitization of KC M3AR in PV results from its ligation by an AuAb with its

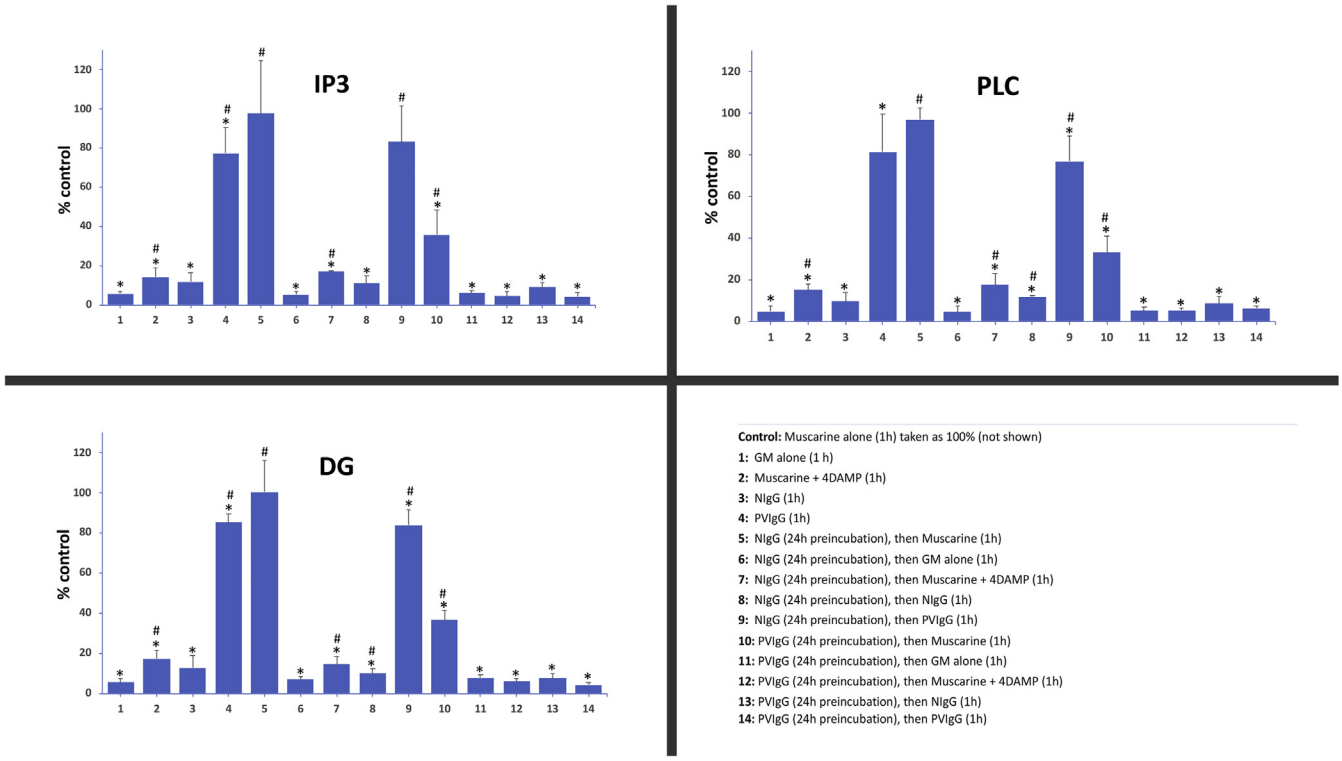

Figure 4. Analysis of the effect of PV IgG on the signaling by keratinocyte M3AR. The monolayers of Het-1A cells were preincubated in the growth medium containing test agents as shown on the graph, washed, and used in the PLC, DG, and IP3 assays as detailed in Experimental procedures. *Asterisk* = $p < 0.05$ compared to control cells stimulated with 1 μM muscarine to activate M3AR (taken as 100%); *pound sign* = $p < 0.05$ compared to intact cells representing the baseline activity of M3AR. DG, diacylglycerol; IP3, inositol 1,4,5-triphosphate; M3AR, M3 muscarinic acetylcholine receptor; NIgG, normal IgG; PLC, phospholipase C; PV, Pemphigus vulgaris.

subsequent internalization and intracellular degradation. The dichotomous effect of PV IgGs on the cholinergic regulation of KC differentiation by ACh through M3AR observed in the present study suggests that a long-term exposure of basal KCs to anti-M3AR AuAb that interrupts physiologic regulation of KCs by non-neuronal ACh disturbs normal unfolding of the KC differentiation program in the epidermis of PV patients and thus contributes to acantholysis. These results help explain how acantholysis can occur in the absence of anti-Dsg1/3 AuAbs and shed new light on more broad mechanisms of KC damage in PV.

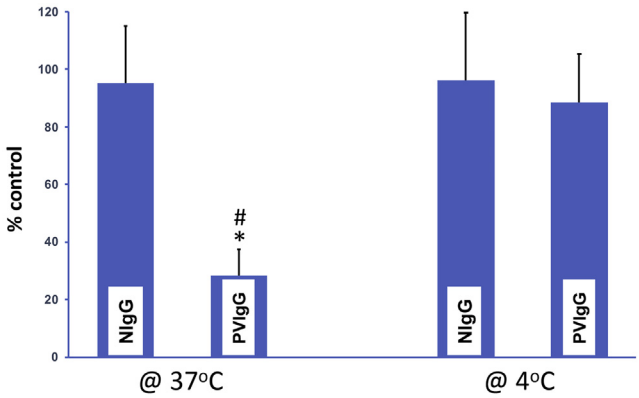

Figure 5. Analysis of the effect of PV IgG on the ligand-binding properties of keratinocyte M3AR. The monolayers of Het-1A cells were preincubated for 1 h at 37 °C or 4 °C in the growth medium containing equal amounts of PV IgG *versus* NIgG and used in the radioligand-binding assays with [^{3}H]QNB as detailed in Experimental procedures. *Asterisk* = $p < 0.05$ compared to specific [^{3}H]QNB binding to intact control cells taken as 100%; *pound sign* = $p < 0.05$ compared to cells preincubated with NIgG at the corresponding temperature. [^{3}H]QNB, [^{3}H]L-Quinuclidinyl [phenyl-4-3H] benzilate; M3AR, M3 muscarinic acetylcholine receptor; NIgG, normal IgG; PV, Pemphigus vulgaris.

The results obtained in the present study elaborate on the pathogenic role of non-Dsg AuAbs in PV. A recently published critical appraisal of different known triggering pathways in PV pathophysiology (38) emphasized that the pathogenesis of this serious disease is complex and requires an in-depth understanding of the various predisposing and provoking factors and effector mechanisms to improve the efficacy and safety of current treatments. Numerous studies conducted worldwide over the last 3 decades on anti-Dsg AuAbs convincingly demonstrated that these AuAbs can serve a reliable diagnostic marker of PV. Unfortunately, thus far this knowledge did not help to improve treatment of PV patients. The growing body of evidence about non-Dsg immunophenotype phenotypes of PV has prompted alternative explanations of the disease mechanisms and a search for new potential therapeutic targets (18). The non-Dsg PV represents a unique model for elucidation of non-Dsg self-antigens in the physiologic regulation of KC cell–cell adhesion and blister development. By now, more than 50 non-Dsg target antigens have been identified (reviewed in (39)). Since composition of the pool of the most common non-Dsg AuAbs appears to be similar among PV patients with or

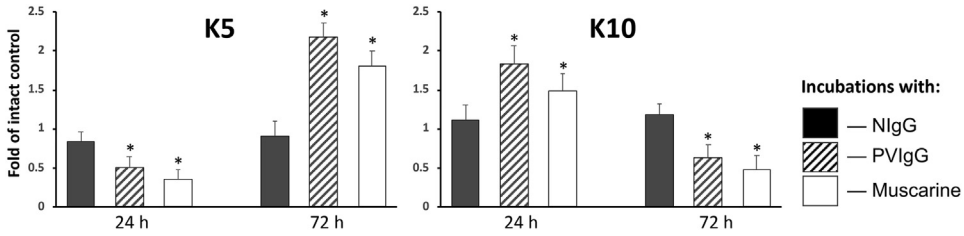

Figure 6. Analysis of the effect of PV IgG on keratinocyte maturation. Normal human epidermal KCs grown in culture to ~80% confluence were incubated for 24 or 72 h in KGM-containing 1.6 mM Ca^{2+} without any additions (control, taken as 1) or in the presence of PV IgG, NIgG, or 1 μM muscarine. The qPCR analysis was used to detect alterations in the expression of keratin 5 and keratin 10 genes at the mRNA level. The results were normalized relative to the rates of expression of corresponding genes in the control samples, taken as 1. *Asterisks* indicate changes that significantly ($p < 0.05$) differ from the control. KCs, keratinocytes; KGM, keratinocyte growth medium; NIgG, normal IgG; PV, Pemphigus vulgaris; qPCR, quantitative PCR.

without anti-Dsg AuAbs, that is, both in typical and atypical PV, elucidation of the mechanisms of pathogenic actions of non-Dsg AuAbs in non-Dsg PV sheds new lights on the immunopathology of conventional variants of PV. Among the hypothetical mechanisms currently considered to explain blister formation in PV, the autoimmune attack on KC M3AR fits with the following four hypotheses of acantholysis onset: basal cell shrinkage hypothesis, signal transduction hypothesis, apoptolysis hypothesis, and multiple hit hypothesis. The anti-M3AR autoimmunity appears to be in the center of congregation of different triggering pathways leading to suprabasal acantholysis in PV. Hence, further studies of the immunopharmacologic action of anti-M3AR AuAb should lead to better understanding of the complex pathophysiology of this serious disease and identification of new specific treatment option utilizing pharmacologic ligands of M3AR.

It has been well documented that the outside-in signaling elicited due to binding of PV IgGs to KCs proceeds *via* different pathways and involve several types of downstream effector molecules (reviewed in (40)). Several research groups have convincingly demonstrated that binding to PV IgGs to KCs activates PLC, elevates DG and IP3 levels, upregulates PKC activity, and increases the concentration of $[Ca^{2+}]_I$, and that these signaling events play an important role in pemphigus pathophysiology by altering the cell–cell adhesion of KCs (41–44). Although various KC cell membrane proteins targeted by PV IgGs can trigger signaling cascades involving one or more of the above messengers (39), ligation of M3AR by an AuAb can explain and reconcile previously reported findings.

Since PV IgGs cause acantholysis without complement and inflammatory cells (reviewed in (45)), the type of autoimmune damage of M3AR in PV is that of receptor/ligand interactions known as "Inactivation/Activation of Biologically Active Molecules" (46), according to which an AuAb may activate, inactivate, protect, or have no effect on the biological function of targeted receptor. A mimetic, or agonist-like, effect may result from its interaction with an active site of M3AR in imitation of the natural ligand, that is, ACh, leading to conformational changes that are similar to those occurring due to binding of the agonist ACh. A blocking, or antagonist-like, effect inactivating ACh signaling may result from each of the following three types of M3AR/AuAb interactions: (i) steric hindrance at the ligand-binding site, so that ACh binds either not at all or with diminished affinity; (ii) accelerated internalization and degradation of the antibody/receptor complex, decreasing the number of functional M3ARs on the cell surface (a.k.a. tachyphylaxis); and (iii) structural alteration affecting the ligand-binding ability of M3AR. Since a short-term exposure of KCs to either PV IgGs or the muscarinic agonist muscarine in both cases resulted in elevation of the M3AR second messengers PLC, DG, and IP3, the mode of binding of anti-M3AR AuAb to its target receptor was an agonist-like. The ability of the M3AR antagonist 4-DAMP to abolish effects of muscarine confirmed that the observed signaling events indeed occurred downstream of M3AR activation. On the other hand, since a long-term preincubation with PV IgGs significantly decreased the ability of KCs to respond to M3AR stimulation, the pathophysiological outcome of the AuAb action on M3AR was an antagonist-like. The disappearance of the AuAb-bound M3ARs from the cell membrane of mouse and human KCs indicated that receptor desensitization abolished the physiologic regulation of KCs by endogenous ACh through M3AR.

Our observation that the long-term effect of PV IgGs on the differentiation markers K5 and K10 demonstrated that KC proliferation and differentiation were disturbed. The fact that the long-term effect of muscarine was very similar to that of PV IgGs sheds lights on the mechanisms of dysregulation of KC differentiation in PV. The changes induced by both PV IgG and muscarine observed by us were in keeping with a marked increase of the proliferation marker K14 and a decrease of K10 in the epidermis of patients with fully developed PV symptoms (47). Further, the differentiation-dependent mechanisms induced by PV IgG within KCs have been shown to contribute to epidermal blistering in an *in vitro* model of PV (48). Since the effects of PV IgGs and muscarine coincided, one may conclude that a hyperproliferative state of KCs in the skin of PV patients results from desensitization of M3AR that constitutes the net antagonist-like effect resulting from the originally agonist-like ligation of the targeted receptors by the AuAbs. In our study, the agonist-like action of anti-M3AR AuAb that preceded receptor depletion was manifested by a decrease of K5 and an increase of K14 after short-term incubations with PV IgG or muscarine. Noteworthily, inhibition of cell proliferation is within the biologic functions of M3AR (49).

The development of a better treatment regimen for PV patients is urgently needed. While the "multi-drug" protocol

provides for about 87% of drug-free complete remission for five and more years (50), the remaining problems are the following: (1) the need to use high doses of systemic steroids at the beginning of the treatment to facilitate the healing of existing erosions; (2) high costs and inconvenience of long lasting infusions of intravenous immunoglobulin for elimination of AuAbs; (3) high toxicity of rituximab and antimetabolite and alkylating immunosuppressors required to prevent the rebound effect from intravenous immunoglobulin; and (4) long duration of therapy, about 2 years, required for the critical mass of AuAb producing B clones to naturally die off based on their life span (51). The results of the present study help explain the mechanisms of therapeutic action of cholinergic agents in PV (reviewed in (52)). For instance, we have previously reported that the cholinergic agonist and acetylcholinesterase inhibitor carbachol alleviated symptoms in mice with passively transferred PV and that the acetylcholinesterase inhibitor pyridostigmine bromide (Mestinon) produced the therapeutic effect in a PV patient (53). In both circumstances, elevation of the tissue levels of endogeneous ACh apparently opposed the pathogenic action of anti-M3AR AuAb. Thus, our results have translational significance as they can guide future studies toward the development of better therapies for PV patients employing cholinergic drugs.

Experimental procedures

Pemphigus sera, cells, and reagents

We used three pooled serum specimens from patients with non-Dsg PV. This research had been approved by Institutional Review Board of University of California Irvine (2009-7049). Pemphigus vulgaris IgG and NIgG were isolated by FPLC protein G affinity chromatography (54). The anti-M3AR AuAb was obtained through immunoaffinity purification of pooled PV IgGs as detailed by us elsewhere (11). The antigen specificity of affinity-purified AuAbs was verified by visualizing a single protein band with expected molecular mass in immunoblots of homogenized monolayers of human KCs purchased from ThermoFisher Scientific and grown in the serum-free keratinocyte growth medium containing 5 ng/ml epidermal growth factor and 50 mg/ml bovine pituitary extract (Gibco-BRL) in accordance with the protocol provided by the vendor. The Het-1A cell line—an established clonal population of SV40-immortalized human esophageal squamous epithelial cells (*i.e.*, KCs) (55)—was purchased from American Type Culture Collection (catalog no. CRL-2692) and propagated in the Clonetics brand bronchial cell medium without retinoic acid (Cambrex Bio Sciences), as detailed by us elsewhere (56). Murine KCs were isolated from the epidermis of 2 to 4 days old C57Bl/6 mice and grown at 37 °C and 5% CO_2 in 25 cm^2 Falcon culture flasks using the cell culture techniques optimized for mouse KCs, as detailed by us elsewhere (57, 58). The muscarinic agonist muscarine and antagonist 4-DAMP were purchased from Sigma-Aldrich Corp. The ELISA kits for the measurements of PLC, DAG, and IP3 were purchased from MyBioSource, Inc. All ELISA assays were performed following protocols provided by the manufacturers in triplicate (n = 3). Rabbit polyclonal anti-human/mouse M3AR IgG antibody was purchased from GeneTex, Inc. and used in the indirect immunofluorescence experiments with mouse skin and immunoblotting experiments with homogenized Het-1A cells as detailed by us elsewhere (34).

Radioligand-binding assay

The M3AR binding studies with Het-1A cells exposed to PV IgG *versus* NIgG were performed using [^{3}H]L-Quinuclidinyl [phenyl-4-3H] benzilate (specific activity 30 Ci/mmol; Lot 200,831) purchased from American Radiolabeled Chemicals in accordance to a standard protocol detailed by us elsewhere (59). After incubation, cell monolayers were washed 5 times and solubilized in a solution containing 0.2 N NaOH, 1% SDS, 5 mM EDTA, 0.2 mM DTT, and 0.5% Triton X-100, and the total protein concentration was identified in each sample using bovine serum albumin as standard. Total binding and nonspecific binding were determined in triplicates. Nonspecific binding was determined in the presence of 100 μM atropine (Sigma-Aldrich Corp.). Saturation isotherms were analyzed according to a model of one-site binding using the nonlinear regression analysis program Prism (GraphPad Software).

Real-time quantitative PCR

The qPCR assays of the gene expression for human cytokeratins K5 and K10 and murine mAChR subtypes M1, M3, M4 and M5 expressed KCs (34) were performed as detailed by us in the past (60). Briefly, total RNA was isolated using the RNeasy Mini Kit (Qiagen), treated with DNase (RNase-free DNase Set; Qiagen) and concentrated by RNeasy MinElute cleanup kit (Qiagen). Two micrograms of total RNA was reverse transcribed into 20 μl cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR was performed in triplicate at the Applied Biosystems 7500 instrument using the TaqMan Gene Expression Assays and TaqMan Universal Master Mix reagent (Applied Biosystems) in accordance with the manufacturer's protocols. To correct for minor variations in mRNA extraction and load amount, the gene expression values were normalized using the housekeeping gene GAPDH. The data from triplicate samples were analyzed with a sequence detector software (Applied Biosystems) and expressed as mean ± SD of test mRNA relative to that of control.

In-cell Western

The In-cell Western, a high throughput quantitative assay of cellular proteins, was performed *in situ*, as described by us in detail elsewhere (61), using the reagents and the Odyssey Infrared Imaging System from LI-COR Biosciences. The freshly isolated mouse KCs were seeded into 96-well U plates at a concentration of 2 × 10^5 cells/well, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton solution. The cells were incubated with the Odyssey blocking buffer for 1.5 h, treated overnight at 4 °C with the primary rabbit antibodies to individual mAChRs, washed, and stained for 1 h at room temperature with a secondary LI-COR IRDye 800CW

goat anti-rabbit antibody, diluted 1:800. A combination of Sapphire700 (1:1000, LI-COR Biosciences) and DRAQ (1:10,000, Cell Signaling) was used to normalize for cell number/well. The antibodies to the M1, M3, M4, and M5 mAChR subtypes-expressed KCs (34) were from R&D Antibodies. The antibodies were diluted 1:100 to 1:200 in the Odyssey blocking buffer according to manufacturer's recommendations.

Statistical analysis

The data were analyzed using ANOVA against an α level of 0.05 and presented as mean ± SD. The graphs were created using GraphPad Prism 5.

Data availability

The data sets used for the present study are available from the corresponding author upon reasonable request.

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Abbreviations—The abbreviations used are: ACh, acetylcholine; AuAb, autoantibody; DG, diacylglycerol; IP3, inositol 1,4,5-triphosphate; KC, keratinocyte; M3AR, M3 muscarinic acetylcholine receptor; mAChR, muscarinic ACh receptor; NIgG, normal IgG; PLC, phospholipase C; PV, Pemphigus vulgaris; qPCR, quantitative PCR.

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