Background: Pemphigus vulgaris (PV) is an autoimmune–mediated disease of skin and mucosa leading to progressive blistering and nonhealing erosions. Patients develop autoantibodies to adhesion molecules mediating intercellular adhesion and to keratinocyte cholinergic receptors regulating cell adhesion.

Observations: To determine whether a cholinergic agonist can abolish PV IgG–induced acantholysis, litter mates of neonatal athymic nude mice were injected with PV IgG together with carbachol (0.04 µg/g body weight). None of these mice developed skin lesions. Through in vitro experiments, we measured the expression of adhesion molecules in monolayers of normal human keratinocytes incubated overnight in the presence of 0.25mM carbachol using semiquantitative Western blot and immunofluorescence. Carbachol caused an elevation of the relative amount of E-cadherin in keratinocytes (P < .05) without changing that of plakoglobin (P > .05). The phosphorylation level of E-cadherin and plakoglobin was increased by PV IgG, whereas this effect of PV IgG was attenuated in the presence of 0.5mM carbachol. Pyridostigmine bromide, an acetylcholinesterase inhibitor, produced effects similar to those of carbachol, which helps explain its clinical efficacy in a patient with active PV that was resistant to treatment with systemic glucocorticosteroids. Treatment with pyridostigmine bromide (360 mg/d) in a patient with PV allowed to keep his disease under control at a lower dose of prednisone than that used before starting pyridostigmine bromide treatment.

Conclusion: Elucidation of the cholinergic control of keratinocyte adhesion merits further consideration because of a potential for the development of novel antiacantholytic therapies using cholinergic drugs.

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METHODS

PASSIVE TRANSFER OF PV

Neonatal athymic nude mice were used to test direct antiacantholytic effects of cholinergic agonists. At the third day of life, athymic nude mice weigh approximately 1.5 g and can develop gross skin blisters on passive transfer of PV antibodies. This study had been approved by the University of California Davis Review Committee on the Use of Animals in Research, Sacramento, Calif. We injected 52 mice intraperitoneally through a 30-gauge needle with PV (ex-
A recently reported case of PV that had improved by cigarette smoking and a study showing successful use of nicotinamide as a steroid-sparing agent in pemphigus suggested that pharmacological regulation of keratinocyte acetylcholine (ACh) axis may be a novel anticantholytic therapy for pemphigus because (1) cigarette smoke contains the cholinomimetic agent nicotine and (2) nicotinamide exhibits cholinomimetic effects owing to the stimulation of ACh release and inhibition of acetylcholinesterase (AChE). To determine whether a pharmacologic stimulation of keratinocyte cholinergic receptors can be used as a steroid-sparing regimen in the treatment of pemphigus, we administered pyridostigmine bromide (Mestinon; ICS Pharmaceuticals, Costa Mesa, Calif; 60-mg tablets) to a patient with active PV at the dose of 360 mg/d. The use of Mestinon in a patient with PV had been approved by the University of California Davis Human Subjects Review Committee. This patient, an 82-year-old white man, had been treated for almost 8 years with a mid-dose of prednisone, ranging from 15 to 30 mg/d, and occasional intralesional corticosteroid injections. He had a calcific erosion on his nose, which would never completely heal and would become active (ie, turn red and/or get painful, enlarge in size, and produce exudate). No other lesions were seen. The lesion began to improve starting from the third week of treatment with pyridostigmine bromide. After 2 months of treatment with pyridostigmine bromide, the patient's
condition dramatically improved (Figure 1). The dose of pyridostigmine bromide was then decreased to 300 mg/d. The patient was treated with pyridostigmine bromide for an additional 3 months. Occasional redness and/or itching, burning, or tingling sensations of the skin lesion could be alleviated by increasing the pyridostigmine bromide dose from 300 to 360 mg/d without changing the dose of prednisone. While receiving pyridostigmine bromide treatment, the daily dose of prednisone was tapered to 10 mg. Further decrease of prednisone dose was associated with a flare of his skin lesion. No other therapy, except for prednisone tapering, was used. The abdominal skin was then examined by light microscopy and direct immunofluorescence, revealing intraepidermal splitting due to extensive acantholysis and intercellular epidermal staining consistent with binding of PV IgGs to murine keratinocytes, respectively. None of the 4 negative control mice that were injected with pooled normal human IgG at the daily dose of 10 mg/g of body weight for 2 days developed any gross or microscopic signs of pemphigus or showed any deposition of human IgGs in their skin during 40 hours of observation. To standardize assessment of the extent of acantholysis, we computed the areas of intraepidermal splitting in the images of skin harvested from the euthanized mice at the umbilical level 20 hours after the injection. The results showed that in mice treated with PV IgG alone, the acantholysis extended to 73.4%±11% of the epidermis (Table). To determine whether a cholinergic agonist can abolish PV IgG–induced acantholysis, litter mates of the athymic nude mice were injected with the same dose of PV IgG together with carbachol (0.04 µg/g body weight). In addition to being a mixed, nicotinic, and muscarinic agonist, carbachol is also a reversible AChE inhibitor.20 We chose carbachol because in the past this cholinomimetic agent has been shown to antagonize PV IgG–induced acantholysis in keratinocyte monolayers.7 In contrast to mice in the positive control group, none of the mice injected with PV IgG together with carbachol developed any visible skin lesions 40 hours after the first injection (Figure 2B). At this point, our most vigorous efforts to induce Nikolskiy sign failed in the skin of 4 of 7 mice in this sub-

Figure 1. Clinical results in a patient with pemphigus vulgaris treated with pyridostigmine bromide (Mestinon; ICS Pharmaceuticals, Costa Mesa, Calif). A, Before pyridostigmine bromide treatment; B, 2 months later. Prior to starting treatment with pyridostigmine bromide, this patient took prednisone at the dose of 20 mg/d. Pyridostigmine bromide was administered at the daily dose of 360 g. While the patient was receiving pyridostigmine bromide treatment, the dose of prednisone was decreased to 10 mg/d.

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group. Microscopic examination of the skin from euthanized mice, however, was performed 20 hours after the injection because at this time visible skin lesions were also absent in the positive control mice. Compared with an extensive intraepidermal splitting seen in the positive control litter mates (Figure 2C), skin samples from carbachol-treated mice showed only limited areas of epidermal acantholysis (Figure 2D). Morphometric analysis revealed that administration of carbachol resulted in a significant ($P<.001$) decrease of the extent of epidermal splitting from approximately 73% to 37% (Table).

Since epidermal keratinocytes synthesize and release ACh,21,22 which serves as an endogenous agonist of both the nicotinic and muscarinic classes of cholinergic receptors expressed in keratinocytes,23,24 we sought to determine whether increasing the level of free ACh in the epidermis can ameliorate the signs of experimental pemphigus. Toward this goal, we injected a group of mice with PV IgG

<table>
<thead>
<tr>
<th>Treatment (Control/Experimental)</th>
<th>No. of Mice</th>
<th>Morphometric Assay of the Extent of Acantholysis, %</th>
<th>Semiquantitative Assay of Epidermal IgG Binding (Relative Values)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PV IgG/PV IgG + cholinomimetic carbachol</td>
<td>4/7</td>
<td>73.4 ± 11/36.8 ± 2.4</td>
<td>67.0 ± 9.3/106.87 ± 7.9</td>
</tr>
<tr>
<td>PV IgG/PV IgG + pyridostigmine bromide</td>
<td>4/7</td>
<td>78.2 ± 1/31.3 ± 7.2</td>
<td>116.8 ± 7.6/188.9 ± 7.3</td>
</tr>
</tbody>
</table>

*Data are mean ± SD value in control/experimental mice. The extent of acantholysis and the intensity of epidermal staining for human IgG were estimated 20 hours after a single injection of pemphigus vulgaris (PV) IgG with or without test drug. $P<.001$ for all results.

Figure 2. Carbachol inhibits pemphigus vulgaris (PV) IgG–induced skin blistering of 3-day-old athymic nude mice. A, Positive control: a representative 3-day-old homozygous athymic nude mouse 40 hours after injection of 7 mg/g of body weight per day of PV IgG showing gross skin blistering. B, Experiment: a representative mouse of the same progeny after injection of 7 mg/g body weight per day of PV IgG together with 0.04 µg/g of body weight per day of carbachol. Note lack of gross skin blistering. C, Positive control: extensive acantholysis in murine epidermis 20 hours after injection of PV IgG alone (hematoxylin-eosin, scale bar=100 µm). D, Experiment: limited acantholysis in murine epidermis 20 hours after injection of PV IgG together with 0.04 µg/g body weight per day of carbachol (hematoxylin-eosin, scale bar=100 µm). E and F, Direct immunofluorescence staining of the epidermis in positive control (E) and experimental (F) mice 20 hours after injection of 7 mg/g body weight per day of PV IgG together with 0.04 µg/g body weight per day of carbachol, using fluorescein isothiocyanate–labeled goat antihuman IgG antibody (scale bar=50 µm). Note that the pemphiguslike epidermal staining is present in both cases.
together with the AChE inhibitor pyridostigmine bromide25 (0.1 µg/g body weight). The protective effect of pyridostigmine bromide on PV IgG–induced acantholysis was found to be similar to that of carbachol. The gross skin lesions did not appear, Nikolskiy sign was negative, and the extent of acantholysis in the skin of mice treated with pyridostigmine bromide decreased significantly (P<.001) compared with the positive control litter mates (Table).

Since patients with PV develop antibodies to keratinocyte ACh receptors, along with autoantibodies to desmosomal cadherins (reviewed by Grando6), one of the hypothetical mechanisms that could explain the antiacantholytic effect of carbachol and pyridostigmine bromide (which, similar to carbachol, can act directly on ACh receptors in addition to reversibly inhibiting AChE)26 was the direct competition of these drugs with PV IgG for binding to keratinocytes. To test this hypothesis, we measured the intensity of fluorescent staining of the epidermis of mice injected with PV IgG alone (positive control) or together with carbachol or pyridostigmine bromide (experiment), using fluorescein isothiocyanate–labeled goat antihuman IgG antibody (Figure 2E and F). Quantitative analysis of the intensity of specific staining showed an increased amount of PV IgG in the epidermis of cholinergic agonist–treated mice compared with that determined in positive control mice (Table), indicating that steric hindrance could not account for the antiacantholytic effect of carbachol and pyridostigmine bromide. An unexpected increase of the intensity of fluorescent staining of the epidermis in experimental mice could be explained through a hypothesis that cholinergic agonists up-regulated expression of keratinocyte adhesion molecules targeted by PV IgG.

UP-REGULATION OF E-CADHERIN EXPRESSION

To investigate molecular mechanisms of antiacantholytic action of cholinergic agonists, we measured the expression of adhesion molecules in monolayers of normal human keratinocytes incubated overnight with 0.25mM carbachol or pyridostigmine bromide. Since acantholysis in pemphigus starts at non-desmosomal areas of the keratinocyte cell membrane,27-30 we studied E-cadherin, a protein involved in assembly of the adherence junctions, and plakoglobin (ie, γ-catenin), which contributes to both the adherence and the desmosomal junction complexes and reportedly plays an important role in mediating the pathophysiological effects of PV IgG.31 Formation of the adherence junctions is a prerequisite for the formation of desmosomes.22 Although plakoglobin may not be the binding site of pemphigus autoantibodies, it is present in the autoantigenic complex precipitated by PV IgG32 and its expression is altered in PV.34 The semiquantitative indirect immunofluorescence assay demonstrated that treatment with cholinergic agonists significantly (P<.05) increased the relative amount of E-cadherin in keratinocytes (Figure 3 and Figure 4). The relative amount of plakoglobin did not increase. These indirect immunofluorescence findings were confirmed by the results of Western blot, which showed that the protein level of E-cadherin was increased in cultures treated with cholinergic agonists compared with nontreated control monolayers (Figure 5). Both carbachol and pyridostigmine bromide caused elevation of the relative amount of E-cadherin (P<.05) without changing that of plakoglobin (P>.05).

INHIBITION OF PV IgG–INDUCED PHOSPHORYLATION

Since phosphorylation has been recently recognized as a major factor in regulation of intercellular adhesion,33 particularly in disassembly of keratinocyte intercellular

Figure 3. Fluorescence images of E-cadherin (scale bar=50 µm). Results of semiquantitative indirect immunofluorescence analysis of the relative amounts of E-cadherin in cultured human foreskin keratinocytes incubated overnight without (control) (A) or with 0.25mM carbachol (B) or pyridostigmine bromide (C). After incubation, keratinocyte monolayers were washed and immunostained. The images were analyzed using software for semiquantitative image analysis.

Figure 4. Cholinergic effects on the expression of adhesion molecules by keratinocytes. The results are expressed as the relative amounts of fluorescence intensity expressed by experimental vs nontreated cells (control). Error bars indicate SD.
lytic activity of cholinomimetic drugs observed in vitro7 of 0.5mM carbachol or pyridostigmine bromide, this effect phosphorylation of keratinocyte adhesion molecules.37-41 ecules and, therefore, is customarily used to study phosphorylation of E-cadherin and plakoglobin.36-38ward this end, we measured the effects of carbachol and pyridostigmine bromide on PV IgG–induced phosphorylation of E-cadherin and plakoglobin using the DJM-1 cutaneous squamous cell carcinoma cell line that features high levels of phosphorylation of adhesion molecules and, therefore, is customarily used to study phosphorylation of keratinocyte adhesion molecules.37-41 Incubation of keratinocyte monolayers for 1 hour with 1 mg/mL of pooled PV IgG resulted in 1.8- and 8.6-fold increase of the phosphorylation level of E-cadherin and plakoglobin, respectively (Figure 6). In the presence of 0.5mM carbachol or pyridostigmine bromide, this effect of PV IgG was attenuated, indicating that antiancho hypertonic activity of cholinomimetic drugs observed in vitro7,41 and in vivo (Figure 2) may stem from pharmacological regulation of both the expression of E-cadherin and their ability to attenuate PV IgG–induced phosphorylation of E-cadherin and plakoglobin.

This study demonstrates that activation of the keratinocyte ACh axis can ameliorate pemphigus acantholysis and up-regulate the expression of adhesion molecules and protect them from PV IgG–induced phosphorylation. The knowledge on the pathophysiology of acantholysis converges with that on the physiology of keratinocyte adhesion. We hypothesized that a nonsteroidal treatment of pemphigus can be achieved by pharmacologically interceding at the site of intracellular biochemical events that mediate the acantholytic effects of pemphigus autoantibodies, and studied the immunopharmacology of pemphigus IgG action on keratinocytes. In the past, we reported that PV IgG–induced phosphorylation of keratinocyte adhesion molecules could be abolished in the presence of the corticosteroid methylprednisolone.42 Increased phosphorylation of desmoglein 3 in pemphigus may lead to the formation of desmoglein 3–depleted desmosomes and altered adhesion.36,37 In addition to the phosphorylation of desmoglein,43 desmocollin,44 and desmoplakin,45 assembly and disassembly of desmosomal junctions also involves phosphorylation of the keratin- and vimentin-intermediate filaments (reviewed by Eriksson et al46). For instance, while phosphorylation of classic cadherins on tyrosine disables the adherence-type junctions, leading to cell-cell detachment,47,48 experimentally inhibiting tyrosine-specific phosphatases results in a major changes in cell morphology, as manifested by a rapid rounding up of the cells, followed by reorganization of the cell monolayer.48

A list of known targets for pemphigus antibodies includes both adhesion molecules (eg, desmogleins 1, 2, and 3, desmocollins, and plakoglobin) and the receptor molecules (FcεRⅠα, α3 and α9 nicotinic ACh receptor subunit, pemphaxin, and other annexins)(reviewed by Grando). Anti-ACh receptor antibodies are found in pa-
PV-like lesions can be induced in neonatal mice in the intercellular space between keratinocytes in the epidermis, corroborated by results showing enlargement of the inflammatory infiltrate. Because of the lack of a strong correlation between the clinical phenotype of PV and the presence of anti-desmoglein 1 and 3 antibodies and since PV-like lesions can be induced in neonatal mice in the absence of these antibodies, the immunopathogenesis of PV can be explained through the "multiple hit" hypothesis. We propose that acantholysis in PV results from synergistic and cumulative effects of autoantibodies targeting keratinocyte cell membrane antigens of different kinds, including (1) molecules that regulate cell shape and adhesion (eg, ACh receptors) and (2) molecules that mediate cell-to-cell adhesion (eg, desmosomal cadherins). Severity of the disease and exact clinical picture depend on the ratio of different kinds of autoantibodies in each particular patient. Antibodies to ACh receptors can weaken desmosomal junctions by inducing phosphorylation of adhesion molecules, cause desmosome shedding owing to the apoptosis-related cleavage of desmosomal cadherins, and prevent desmosomal reassembly owing to the activation of the cytolytic cascade. In turn, the binding of pemphigus IgG to desmosomal cadherins may prevent formation of new desmosomes because it blocks the extracellular domains of desmogleins mediating homophilic adhesion.30,31

We have previously reported that PV IgG–induced acantholysis can be treated in culture with cholinergic agonists.32 While glucocorticosteroids or protease inhibitors can only block, but not reverse, acantholysis,1,3,53 cholinomimetics are the only drugs capable of reversing PV IgG–induced acantholysis. The cholinergic effects on cell adhesion observed in cell monolayers23 have been corroborated by results showing enlargement of the intercellular space between keratinocytes in the epidermis treated with the nicotinic antagonist tubocurarine54 or the muscarinic antagonist atropine.55 The fact that patients with PV produce autoantibodies to ACh receptors expressed by keratinocytes, along with the fact that these ACh receptors regulate intercellular adhesion of these cells (the function that is altered in pemphigus), prompted us to use cholinomimetic drugs to prevent skin blistering in mice with experimentally induced pemphigus.

Passive transfer of PV IgG to neonatal Balb/c mice caused gross and microscopic changes consistent with experimental pemphigus in vivo.56 In pilot studies,57 we found that 3- to 5-day-old Balb/c mice may respond to anticaantholytic treatments with corticosteroids or cholinomimetics, but untreated positive controls do not always produce PV lesions because rapidly developing hair follicles may reinforce their epidermal integrity. Therefore, we sought to develop a more reliable animal model and tested athymic nude mice. The 1- to 2-day-old mice died despite any anticaantholytic treatments, whereas older mice responded to treatments and survived subsequent injections of PV IgG with a test drug. Therefore, we selected 3-day-old athymic nude mice as a model for testing anticaantholytic drugs. The cholinergic drugs used in the in vivo experiments were first tested in intact mice to assure that the doses at which they are used in adult humans are not lethal for neonatal mice. Once a safe dose of each drug was established, it was used for experimental treatment of pemphigus. We found that the cholinergic agonists carbachol and pyridostigmine bromide partially inhibit PV IgG–induced acantholysis by passive transfer of autoantibodies without altering the binding of IgG to the keratinocyte cell membrane.

The cholinergic drugs exerted their anticaantholytic action through yet poorly understood intracellular signaling pathways. Both drugs can reversibly inhibit AChE and ligate ACh receptors. While carbachol is a well-known mixed muscarinic and nicotinic agonist,20 pyridostigmine bromide interacts with the ACh-ionic channel complex, blocking it in open conformation.48 Thus, an important role of phosphorylation of adhesion proteins for normal reorganization of cadherin-cytoskeletal interactions, along with the fact that ligation of ACh receptor types expressed in keratinocytes (eg, a9 ACh–gated ion channels) has been reported to induce phosphorylation of the cell membrane-associated proteins,59 suggests that cholinomimetics ameliorate acantholysis in keratinocytes exposed to PV IgG by inhibiting phosphorylation-mediated alterations in the assembly and disassembly of intercellular attachment units. Additionally and/or alternatively, activation of the keratinocyte ACh axis with cholinomimetic drugs could up-regulate expression of adhesion molecules targeted by pemphigus antibodies, as suggested by increased binding of PV IgG in the epidermis of mice treated with cholinergic agonists. Indeed, we have recently found that ACh receptors expressed by keratinocytes couple a signaling pathway leading to activation of the adhesion molecules that mediate intercellular attachment of these cells.13,60 The expression of desmogleins 1 and 3 was increased in keratinocytes treated with carbachol or pyridostigmine bromide.13 Therefore, we speculate that pemphigus acantholysis in the skin of patients with PV could be treated by pharmacologically stimulating keratinocyte ACh axis. Elucidation of the cholinergic control of keratinocyte adhesion has a potential for the development of treatment regimens using safer drugs to control blistering in a variety of other skin diseases. First results of the clinical trial of pyridostigmine bromide in patients with pemphigus have been recently published.61

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