The Plant Lectin Wheat Germ Agglutinin Inhibits the Binding of Pemphigus Foliaceus Autoantibodies to Desmoglein 1 in a Majority of Patients and Prevents Pathomechanisms of Pemphigus Foliaceus In Vitro and In Vivo

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The Plant Lectin Wheat Germ Agglutinin Inhibits the Binding of Pemphigus Foliaceus Autoantibodies to Desmoglein 1 in a Majority of Patients and Prevents Pathomechanisms of Pemphigus Foliaceus In Vitro and In Vivo

Susana Ortiz-Urda,1*‡ Adelheid Elbe-Bürger, † Josef Smolle,‡ Yvonne Marquart,* Yakov Chudnovsky,‡ Todd W. Ridky, ‡ Pamela Bernstein, ‡ Klaus Wolff,* and Klemens Rappersberger*†

Pemphigus foliaceus (PF) is a life-threatening autoimmune blistering skin disease caused by pathogenic IgG autoantibodies against desmoglein 1 (dg1), a desmosomal cadherin-type adhesion glycoprotein. Using lectins and glycosidases, we have shown that dg1 displays an N-glycosylation pattern of the complex triantennary type. We have found that lectins and glycosidases interfere with N-bound sugar residues on the amino-terminal ectodomain of dg1 and completely abolish, in vitro, the antigenicity of dg1 in most of the patients’ sera. Moreover, in an ex vivo model using punch biopsies from normal human skin, we demonstrate that preconditioning of the epidermis in wheat germ agglutinin (WGA) prevents PF autoantibody binding, acantholysis, and subcorneal blistering. In addition, we show that topical treatment with WGA inhibits PF autoantibody binding to keratinocytes in both newborn BALB/c mice and in organotypic human epidermis grafted onto the back of SCID mice. The epidermis of these pre-treated animals displays a regular morphology, whereas control animals develop the immunopathologic phenotype of PF. These findings suggest that WGA may interfere with autoantibody binding to dg1, preventing experimental PF without affecting the adhesive function of dg1. Our observations may provide a new approach to the therapy of PF. The Journal of Immunology, 2003, 171: 6244–6250.

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1 Address correspondence and reprint requests to Dr. Susana Ortiz-Urda, Program in Epithelial Biology, Stanford University School of Medicine, Center for Clinical Sciences and Research Building, Room 2145, Stanford, CA 94305. E-mail address: sortiz@cmmg.stanford.edu
2 Abbreviations used in this paper: PF, pemphigus foliaceus; dg1, desmoglein 1; WGA, wheat germ agglutinin; KGM, keratinocyte growth medium; IF, immunofluorescence.

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carbohydrate moieties with WGA could abolish the binding of PF autoantibodies to dg1, an effect that was also observed after deglycosylation of dg1 in vitro. Using the BALB/c mouse (19, 26) and SCID/human-xenograft models (27), we then provide evidence that topically applied WGA penetrates through the epidermis, binds to the surface of keratinocytes, and inhibits PF autoantibody binding, preventing acantholysis and blister formation.

Materials and Methods

Synthesis of reagents

**Tissues.** Normal human skin from patients that underwent breast reduction was provided by plastic surgeons. Keratomed strips of skin and 4-mm punch biopsies were used for biochemical studies or immediately incubated in keratinocyte growth medium (KGM) (Clonetics, San Diego, CA) and further processed for in vitro, ex vivo, and in vivo studies.

**Sera.** Sera were obtained from 12 PF patients. Selection criteria were as follows: clinical symptoms characteristic of PF, presence of acantholysis within the granular cell layer upon histological examination, positive direct and indirect immunofluorescence (IF), and exclusive reaction with dg1 by immunoblotting, immunoprecipitation, and ELISA. ELISA index of positive PF patients’ sera was between 140 and 200 (28). For control, we used sera from healthy human volunteers.

**Antibodies.** Mouse mAb to dg1 (DG 3.10 and Dsg1 P 23) were purchased from Progen (Heidelberg, Germany). Rat Abs specific for human 6 integrin (Mac-1/72) were purchased from Chemicon International (Temecula, CA). As second-step reagents, we used affinity-purified FITC-labeled goat anti-human-IgG, FITC-labeled rabbit anti-mouse-IgG, tetramethylrhodamine-isothiocyanate-labeled goat anti-human IgG, and FITC-labeled rabbit anti-mouse IgG as detector Abs (Promega, Madison, WI).

**Lectins.** Different lectins were used as FITC- or digoxigenin-labeled or unlabeled reagents: Con A type 4 (Sigma-Aldrich, St. Louis, MO), WGA, soybean agglutinin, peanut agglutinin, Sophora japonica agglutinin, Banisteria simplicifolia 2, Ulex europaeus agglutinin 1, Maackia amurensis, Datura stramonium, and Aleuria aurantia (all from Vector Laboratories, Burlingame, CA). For the detection of tissue-bound unlabeled lectins we used FITC-conjugated rabbit IgG to WGA, and Con A, U. europaeus agglutinin 1, and soybean agglutinin (Dakopatts, Copenhagen, Denmark). Inhibiting sugars used were as follows: 200 mM α-methyl mannoside/200 mM α-methyl glucoside mixture for Con A; and 500 mM N-acetylglucosamine, chitin hydrolysat, or 100 mM acetic acid for WGA.

**Glycosidases.** To hydrolyze glycoconjugates, we used the following panel of glycosidases: N-glycosidase F, endoglycosidase-F2/F1, peptide-N-acetyl-β-D-glucosaminyl) asparagine amidae F of Flavobacterium meningosepticum, recombinant from Escherichia coli; O-glycosidase from Diplococcus pneumoniae; endoglycosidase-H from Streptomyces pilatus; α-mannosidase from jack beans (Canavalia ensiformis); β-galactosidase from bovine testes (all from Boehringer Mannheim, Mannheim, Germany).

**Light- and IF microscopy, immunoblotting, and immunoprecipitation of epidermal protein extracts**

These studies were performed according to routine methods (29).

**Deglycosylation of protein extracts and structural characterization of sugar residues**

Protein extracts were deglycosylated according to routine methods (30). The carbohydrate structures of blotted glycoproteins were analyzed with digoxigenin-labeled lectins and an alkaline-phosphatase-labeled anti-digoxigenin Ab.

**Lectin binding studies**

For the detection of sugar residues, cryosections of normal human skin were incubated for 20 min at 20°C with FITC-conjugated and unconjugated lectins, diluted to a concentration of 0.1 mg/mL in 0.1 M PBS (pH 6.8). Sections labeled with unconjugated lectins were subsequently incubated with PF sera as well as with the two different anti-dg1 mAbs.

**Deglycosylation of tissue sections**

Five-micrometer cryosections were incubated with 0.4 U of glycosidase in PBS for 2 h at 20°C. After thoroughly rinsing in PBS, sections were incubated with either PF sera or anti-dg1 mAb, and assayed for single and double IF microscopy.

**Ex vivo model and animal model**

For ex vivo studies, 4-mm punch biopsies from normal human skin were incubated with either PF sera or with normal human serum in KGM for 3 days in a tissue incubator and then snap frozen in liquid nitrogen.

**Animal model**

To investigate whether lectins topically applied to the skin may also inhibit PF autoantibody binding in vivo, we took advantage of the pemphigus mouse model (19, 26). In the first step, we partially removed the cornified cell layer of 96 BALB/c mice with 3% urea in Vaseline and oochulof (Tegaderm, Borken, Germany) for 6 h. Subsequently, 36 newborn BALB/c mice were treated with WGA and 12 with Con A at concentrations ranging from 0.5 to 2.5 mg/ml in propylene glycol under occlusive plastic dressing. As a control, 27 animals were treated with vehicle alone, and 21 animals were treated with E. coli 1. Twelve hours later, the animals were injected i.p. with PF sera of 11 different PF patients. We injected a total of 63 animals with PF sera and 33 animals with normal human sera through a 30-gauge needle in doses of 12 mg of protein per gram of body weight per day. The injections were given twice daily on 3 consecutive days. Animals were examined daily and photodocumented and sacrificed 12 h after the last injection. Skin samples were taken and further processed for immunomorphological studies. At the time of the biopsy, sera were obtained and assayed by indirect IF microscopy for titers of circulating human IgG.

**Cell cultures and organotypic skin**

Keratinocytes and fibroblasts from sterile skin samples were cultured separately on regular collagen culture dishes and further cultured in the presence of 1.5 mmol of Ca++ Complete growth of a mature, multilayered, and differentiated epithelium was achieved within 24–96 h. Then, the tissue was harvested and further processed (31).

**SCID/human-xenograft model**

Six-week-old SCID mice were obtained from a colony maintained in our laboratory. Human skin equivalents were grafted on the backs of 12 mice, secured with sutures, and dressed with gauze. Three weeks later, bandages were removed. First, we partially removed the cornified cell layer with 3% urea in Vaseline and oochulof for 6 h. Subsequently, 9 mice were treated overnight with WGA, and 3 were treated with U. europaeus 1 under similar conditions. Mice were then injected with PF sera dissolved in 300 μl of PBS. Injections were repeated every 24 h. Mice were examined every 12 h and killed 48 h after the first injection. Skin samples were taken and further processed for immunomorphological studies. At the time of the biopsy, sera were obtained and assayed by indirect IF microscopy for titers of circulating human IgG.

**Results**

Dg1 displays a N-glycosylation pattern of the triantennary complex type

In the first set of experiments, we analyzed the carbohydrate structure of dg1. Digestion of dg1 with N-glycosidase F, which hydrolyzes all types of N-glycan chains, reduced the molecular mass by 10–15 kDa (Fig. 1a, lanes 4 and 5). In contrast, treatment of dg1 with endoglycosidase F1/F2, which is selective for N-glycosylation of biantennary complex- and high manno-type sugars, or with endoglycosidase H, which is selective for N-glycosylation of high manno-type sugars, did not affect the molecular mass of the protein (Fig. 1b, lanes 3 and 4). Deglycosylation of O-linked sugar moieties resulted in a slight reduction of the molecular mass of dg1 (Fig. 1b, lane 5). The degree of deglycosylation was monitored with digoxigenin-labeled lectins (Fig. 1a, lane 9).

To specify the carbohydrate moieties of dg1, we then precipitated protein lysates with the mAb Dsg1 P 23 and subsequently
In certain patients with PF, the autoantigenicity of dg1 is determined by carbohydrate moieties, in in vitro and ex vivo organ cultures

In immunoprecipitation experiments, we confirmed that PF autoantibodies react with a protein that migrates with dg1 at 160 kDa, as detected by labeling with mAb DG 3.10 directed to intracellular epitopes of dg1 and PF sera (Fig. 1, lanes 2 and 3). After deglycosylation with N-glycosidase F, dg1 migrated to a molecular mass of 140–145 kDa as shown by labeling with both mAb, Dsg1 P 23, and DG 3.10 (Fig. 1, lanes 4 and 5). However, labeling of the deglycosylated protein with PF sera was reduced (Fig. 1, lane 6) or negative (lanes 7 and 8). Next, we investigated the sera of 12 PF patients and showed that 8 sera (including the two patients shown in Figs. 1a, lanes 7 and 8, and 2b, lanes 3 and 4) did not react with deglycosylated dg1, whereas four sera labeled deglycosylated protein (Fig. 2b, lanes 10–13). One of the sera reacted only weakly with the deglycosylated protein (Fig. 1a, lane 6).

These findings were consistent with deglycosylation experiments using N-glycosidase F on cryostat sections of normal human skin. Whereas binding of mAb Dsg1 P23 was not affected, deglycosylation completely abolished the consecutive binding of those PF sera that did not react with deglycosylated dg1 in immunoprecipitation experiments (Fig. 3, a and b). Identical results were achieved when skin sections were preincubated with WGA (2.5 mg/ml sodium chloride). Immunobinding of mAb Dsg1 P23 was not affected; however, the binding of the seven PF sera was completely abolished (Fig. 3, c and d). In contrast, the binding properties of the four PF sera that were reactive with deglycosylated dg1 in immunoprecipitation, was not affected and showed typical PF staining (data not shown). In control experiments, preincubation of lectins with their specific sugars abolished their ability to block PF sera binding on cryostat sections. Identical results were achieved when cryostat sections were incubated with lectins without affinity to dg1, such as soybean agglutinin (Fig. 3, e and f).

Preincubation of PF autoantibodies with WGA did not affect subsequent binding of PF autoantibodies to the surface of keratinocytes of cryostat sections from normal human skin (data not shown). In the next set of experiments, we used the PF ex vivo organ culture model in which 4-mm punch biopsies of normal human skin were incubated with PF sera. H&E-stained sections revealed subcorneal split with acantholytic keratinocytes within the blister fluid. IF studies revealed PF autoantibodies bound to the cell membranes of keratinocytes (Fig. 4, a and b). Strikingly different results were obtained when these experiments were performed after overnight preincubation of organ cultures in WGA. We found that WGA strongly labeled the surface of the keratinocytes in a pemphigus-like pattern, but we did not observe binding of PF autoantibodies from sera that were unreactive with deglycosylated dg1 (Fig. 4, c and d). In H&E-stained sections, acantholysis and intraepidermal split formation were absent (Fig. 4f). However, staining with the four PF sera that did react with deglycosylated dg1 in vitro (see above) as well as with mAb Dsg1 P 23 was unaffected by WGA treatment of epidermis (Fig. 4e). Incubation in normal human sera did not induce any immunopathological changes.

WGA, topically applied, prevents pathobiologic effects of certain PF sera in vivo, in a mouse model (Table I)

Twelve hours after the last injection of PF sera, all animals that were topically treated either with vehicle alone or U. europeaus 1, a lectin without affinity to dg1, developed a PF-like disease, with generalized erythema and blisters (Fig. 5a, right mouse). Examination of cryostat sections of the skin of these animals showed

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**FIGURE 1.** Immunoprecipitation of epidermal protein lysates with mAb to dg1 before and after deglycosylation with N-, F1/F2-, H-, and O-glycosidases. a, In consecutive immunoblotting experiments, dg1 in its native form appears at a molecular mass of 160 kDa, as indicated by labeling with mAb Dsg1 P 23 and with PF serum (lanes 2 and 3, arrow). After deglycosylation, dg1 appears at a reduced molecular mass of 140–145 kDa, as detected by labeling with mAb DG 3.10 and Dsg1 P 23 (lanes 4 and 5). The deglycosylated protein is recognized only weakly or not at all by different PF sera (lanes 6–8) or WGA (lane 9). Molecular mass markers are shown in lane 1. b, The molecular mass of dg1 was not affected by digestion with endoglycosidase F1/F2 or endoglycosidase H (lanes 3 and 4). After O-deglycosylation, there is a slight reduction in the molecular mass of dg1 (lane 5). Lane 2, Labeling of dg1 with mAb Dsg1 P 23 is indicated.

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**FIGURE 2.** Immunoprecipitation of epidermal protein lysates with dg1 and subsequent labeling with digoxigenin-labeled lectins. a, dg1 (arrow) displays reactivity with M. amurensis (lane 3), WGA (lane 4), Con A (lane 5), and peanut agglutinin (lane 6). dg1 does not react with Ulex 1 (lane 7). Labeling with mAb Dsg1 P 23 is indicated (lane 2). The molecular mass standards are shown (lane 1). a and b. After deglycosylation of dg1 with N-glycosidase F, seven sera (a, lanes 7 and 8, and b, lanes 3 and 4) did not react with deglycosylated dg1, whereas four sera labeled deglycosylated protein (b, lanes 10–13).
PF-like autoantibody binding to the surface of keratinocytes and subcorneal blisters with numerous acantholytic keratinocytes (Fig. 5, b and c). Also, two of six animals treated with Con A displayed a PF-like clinical and immunomorphological phenotype. One animal died early after the first injection and was excluded from the study. However, clinical and immunomorphological examination of the remaining three animals did not show any pathological features reminiscent of PF. Moreover, 19 of 21 WGA-treated mice that were injected with sera that did not react with deglycosylated dg1 displayed no signs of a PF-like disorder. The morphology of the epidermis appeared completely normal in H&E staining (Fig. 5, d). IF microscopy revealed WGA staining throughout all layers of the epidermis, whereas no in vivo binding of human IgG was detected (Fig. 5, e and f). Two animals in this group died; investigation of their skin did not display any conclusive findings. In contrast, all four animals injected with glycosylation-independent sera developed PF-like clinicopathological features indistinguishable from those shown in Fig. 5, a–c.

The skin of mice injected with normal human sera did not display any pathological changes regardless of whether they were treated with either WGA, Con A, or other lectin (for details, see Table I). Sera obtained from the mice, as assayed by indirect IF microscopy, contained circulating human IgG (data not shown).

**WGA prevents pathologic effects of certain PF sera in a SCID/human-xenograft disease model**

Human epidermal equivalents were grafted onto the backs of SCID mice. One portion of the animals was topically treated with WGA and the other group with *U. europeaus* 1. Twelve to 18 h after the last injection, all animals injected with glycosylation-independent PF sera, as well as all animals that were topically treated with *U. europeaus* 1, developed PF-like clinicopathological features (Fig. 6, a and b). In contrast, seven of nine WGA-treated mice that were injected with sera unreactive with deglycosylated dg1 were unaffected (Fig. 6, c and d). WGA labeled the upper layers of the epidermis (Fig. 6, e). The human origin of the grafts was verified with human-specific mAb to α6 integrin (Fig. 6, f).

**Discussion**

PF is a chronic disabling bullous autoimmune disease mediated by Abs that impair adhesion of keratinocytes. Therefore, treatment of
PF is performed with various aggressive immunosuppressive regimens, with their associated deleterious side effects. In this study, we asked whether the autoantigen of PF, dg1, might be addressed as a possible therapeutic target.

Most PF autoantibody binding sequences are localized between dg1 residues 50 and 219. As shown by Sekiguchi et al. (13), removal of autoantibodies against the 161 N-terminal residues of dg1 by immunoadsorption eliminates the ability of PF sera to induce cutaneous blisters in neonatal mice. This portion of dg1 includes two potential N-glycosylation sites on positions 61 and 131, two calcium-binding motifs, represented by residues 95–104 and 129–138, and the RAL sequence (aa 79–81). These peculiar biochemical features, and the fact that PF autoantibody binding sites are conformational epitopes, led us to speculate whether targeting of N-linked carbohydrate moieties in the antigenic epitopes of dg1 may affect PF autoantibody binding, in a manner similar to that shown with pemphigus vulgaris (32).

Using epidermal protein lysates from normal human skin, we first demonstrated that dg1 displays a triantennary complex type glycosylation pattern that predominantly consists of galactose, mannose, N-acetylglucosamine, and, on the outermost position, several molecules of sialic acid. We then examined by immunoprecipitation and immunoblotting the binding properties of PF autoantibodies to native and deglycosylated dg1, in vitro, and showed that N-deglycosylation of protein lysates reduced or inhibited the consecutive binding of 75%. However, the binding of four other serum probes to dg1 was clearly shown to be independent of the presence of carbohydrate moieties.

These observations were further confirmed in several in vitro, ex vivo, and in vivo experiments. We showed that either deglycosylation of the epidermis or labeling of epidermal carbohydrates with WGA abolished the subsequent autoantibody binding of 7 of 12 PF sera with associated prevention of PF pathology. In contrast, the pathobiologic effects mediated by 4 glycosylation-independent PF

### Table 1. In vivo testing of lectins in a mouse model

<table>
<thead>
<tr>
<th></th>
<th>Con A-Treated Mice</th>
<th>WGA-Treated Mice</th>
<th>Vehicle-Treated Mice</th>
<th>Ulex europeaus-Treated Mice</th>
<th>Total No. of Animals</th>
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<tbody>
<tr>
<td>PF sera nonreactive with deglycosylated dg1 (7)</td>
<td>6 sera tested</td>
<td>7 sera tested</td>
<td>7 sera tested</td>
<td>7 sera tested</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>6 animals</td>
<td>21 animals</td>
<td>14 animals</td>
<td>14 animals</td>
<td></td>
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<tr>
<td></td>
<td>3 animals remained healthy.</td>
<td>19 animals remained healthy.</td>
<td>All developed disease.</td>
<td>All developed disease.</td>
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<tr>
<td></td>
<td>2 animals developed disease.</td>
<td>2 died.</td>
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<td></td>
<td>1 died within 24 h.</td>
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<tr>
<td>PF sera reactive with deglycosylated dg1 (4)</td>
<td>4 sera tested</td>
<td>4 sera tested</td>
<td>0</td>
<td>4 animals</td>
<td>8</td>
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<td></td>
<td>4 animals</td>
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<td>4 animals</td>
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<td></td>
<td>0</td>
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<td>All developed disease.</td>
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<td>11 animals remained healthy.</td>
<td>9 animals remained healthy.</td>
<td>All developed disease.</td>
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<td></td>
<td>7 animals remained healthy.</td>
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<td>Normal human sera</td>
<td>6 animals remained healthy.</td>
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<tr>
<td>Total no. of animals</td>
<td>12</td>
<td>36</td>
<td>27</td>
<td>21</td>
<td>96</td>
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sera remained unaffected by the topical application of WGA. Identical PF pathology was seen in experiments with BALB/c mice treated with *U. europeaus* agglutinin 1, a lectin without affinity to dg1, where all animals developed a PF-like pathology. Taken together, these findings strongly indicate that WGA has a protective function against the pathobiologic effects of certain PF autoantibodies, in vitro and in vivo.

This phenomenon can be explained by the peculiar localization of *N*-linked sugar residues flanking the RAL motif in close proximity to the Ca$^{2+}$ binding sites. Bound lectins may spatially block the consecutive targeting of antigenic epitopes by PF autoantibodies. Similar effects have been demonstrated in leukemic mast cells, where WGA binding to the Fcε receptor RI inhibits the subsequent binding of a specific monoclonal reagent (33). Alternatively, there also exists the possibility that lectin binding alters the conformation of dg1 with reduction in its antigenicity. This hypothesis is supported by experiments showing that the PF autoantigen is conformation dependent (11, 12). However, Amagai et al. (12) have demonstrated that Ca$^{2+}$ exclusively, but not carbohydrates, may play the decisive role in the conformation of dg1 as PF autoantigen. This obvious contradiction to our observations might be explained by the heterogeneity of certain PF sera in their binding to selected epitopes, as shown in previous investigations (11) and demonstrated in this study. Moreover, a recent study has shown a disease-dependent intramolecular epitope spreading phenomenon in the course of endemic PF, a South American variant of the disease (34). Our findings suggest that, in addition to Ca$^{2+}$-dependent epitopes of dg1, certain dg1 epitopes are determined by carbohydrate moieties. To address this issue, it would be of interest to clarify to which dg1 epitopes the various Ca$^{2+}$- and carbohydrate-dependent or -independent PF autoantibodies bind, and whether there is any association with disease severity.

In addition, there exists the possibility that WGA binding to dg1 indirectly exerts its effects by interference with Ca$^{2+}$ homeostasis (24, 25). However, this hypothesis seems rather unlikely, because we added Ca$^{2+}$ in our in vitro experiments. Moreover, we never observed morphological changes in keratinocytes during in vivo experiments with WGA over a period of 3 days. If topically applied WGA exerts influences on Ca$^{2+}$ homeostasis in the epidermis, one would expect certain changes in keratinocyte differentiation and morphology, at least at the light-microscopic level (35). In addition, because it is known that both heterophilic and to a lesser degree homophilic desmoglein protein binding is Ca$^{2+}$ sensitive, disturbance of Ca$^{2+}$ concentrations in the murine and human epidermis by topically applied WGA would influence the adhesive properties of dg1 (10, 36).

Taken together, our observations in a human autoimmune skin disease suggest that it is possible to prevent autoantibody binding to a defined autoantigen by the topical application of WGA and thus interrupt pathobiologic mechanisms.

**Acknowledgments**

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