Abstracts of The National Pemphigus Foundation and The American Autoimmune Related Disease Association International Meeting: Pemphigus As a Model of Organ-Specific Humoral Autoimmune Diseases

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ORGANIZING COMMITTEE

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PURPOSE STATEMENT

Recent progress in the understanding of human autoimmunity provides a solid scientific foundation on which to base rational therapies for autoimmune diseases. Pemphigus has emerged as a classic example of an autoantibody mediated disease, and provides an excellent model to study the mechanisms of such diseases and to develop new options to treat them.

This meeting brought together scientists working on basic aspects of human autoimmunity with those working on pemphigus. Its goal was to review the current understanding of the causes and treatments of pemphigus, and identify the most important areas for future research on both pemphigus and other autoantibody mediated diseases.

The meeting focused on finding answers to the following questions:

- What is the etiology of autoimmune diseases in general, and pemphigus in particular?
- What are the factors affecting the autoimmune response characteristic of pemphigus?
- What are the most current treatments for pemphigus?
- What are the prospects for new treatments for pemphigus?

001

Cleavage by Granzyme B is Strongly Predictive of Autoantigen Status: Implications for Initiation and Progatation of Autoimmunity

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Systemic autoimmune diseases are a genetically complex, heterogeneous group of disorders in which the immune system targets a diverse but highly specific group of intracellular autoantigens. The molecules targeted are not unified by common structure, function or distribution in control cells but become clustered and concentrated in surface blebs when cells undergo apoptosis. We have demonstrated that the majority of autoantigens targeted across the spectrum of human systemic autoimmune diseases are efficiently cleaved by granzyme B *in vitro* and during cytotoxic lymphocyte granule-induced death, generating unique fragments not observed during other forms of apoptosis. These molecules are not cleaved by caspase-8, although this protease has a very similar specificity to granzyme B. The granzyme B cleavage sites in autoantigens contain amino acids in the P_2 and P_3 positions that are preferred by granzyme B but are not tolerated by caspase-8. In contrast to autoantigens, nonautoantigens are either not cleaved by granzyme B or are cleaved to generate fragments identical to those formed in other types of apoptosis. This striking ability of granzyme B to generate unique fragments is therefore an exclusive property of autoantigens and unifies the majority of molecules targeted in this spectrum of diseases. Several autoantigens targeted in tissue-specific autoimmune diseases (e.g. tyrosinase) are also specifically cleaved by granzyme B. These results focus attention on the role of the cytotoxic lymphocyte granule-induced death pathway in the initiation and propagation of systemic autoimmunity.

003

What is Pemphigus?

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Historical perspective The term pemphigus was most likely in use in the ancient world, but the first recorded instance was by hippocrates (460–370 BC) who described pemphigoid fever as ''pemphigodes pyertoi''. Galen (AD 131–201) named a pustular disease of the mouth as ''febris pemphigodes''. In 1637, zacutus again uses the term ''febris pemphigodes'' to describe patients with blisters of short duration. Desauvages (1760) described patients with high fever and blisters of short duration as having ''pemphigus maior''. None of the above conditions is considered to be true pemphigus, as their disease was of short duration and all patients recovered.

The first recorded cases that probably represent true pemphigus were by McBride (1777) and Wichmann (1791). Two of McBride's cases died of ''bloody ichor'' and ''putrid ulcers''. Wichmann applied the term ''pemphigus'' to his patients and accurately describes flaccid bullae and painful oral ulcerations.

Pemphigus foliaceus was first recognized by Cazenave in 1844, as a special, superficial, rapidly spreading form of pemphigus. Neumann, in 1886, describes a form of the disease with ''warty granulations'' as pemphigus vegetans. Senear and Usher, in 1926, describe pemphigus erythematosus, combining features of both pemphigus and lupus erythematosus. Auspitz (1881) first described disruption of epidermal cells in patients with pemphigus, but not as a specific finding. Civatte, in 1943, clearly delineated this histopathologic hallmark and labeled it acantholysis. He describes acantholysis (loss of cohesion) and intraepidermal bulla formation in pemphigus vulgaris, pemphigus vegetans, and pemphigus foliaceus. These important pathologic findings clearly separated pemphigus from other blistering skin diseases. In 1953, Lever defined the disease entity bullous pemphigoid both clinically and histopathologically, clearly distinguishing it from pemphigus. This ''pemphigus-like'' disease affected primarily elderly patients and was characterized by subepidermal bulla formation.

The next breakthrough occurred in 1964 with the reporting of autoantibodies in the sera of pemphigus patients, reactive with an ''intercellular substance'' of skin and mucosa, by Beutner and Jordon using indirect immunofluorescence. They later showed these same autoantibodies fixed in pathologic sections using direct immunofluorescence methods. In 1967, they also demonstrated autoantibodies in sera and skin specimens from patients with bullous pemphigoid but reactive with the basement membrane zone. These latter findings clearly separate bullous pemphigoid from pemphigus, and establish it as a distinct bullous skin disease.

005

Paraneoplastic Pemphigus – Autoimmunity and Cancer

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Paraneoplastic pemphigus (PNP) is a recently described form of pemphigus that is defined by the following: (a) the presence of mucosal ulcerations and blisters and a polymorphous skin eruption in the context of an occult or known neoplasm (b) histologic findings of vacuolar interface change, keratinocyte necrosis, and intraepidermal cell-cell detachment (suprabasilar acantholysis) (c) deposition of IgG and C3 on epidermal cell surfaces and variably also along the basement membrane zone (d) serum autoantibodies that bind to the cell surface of stratified squamous epithelia and also to simple, columnar, and transitional epithelia (e) serum autoantibodies that recognize a characteristic antigen complex of 250, 230, 210, 190, and 170 kDa peptides. These are now known to represent desmoplakins I and II (250, 210 kDa, the bullous pemphigoid antigen 1(230 kDa), envoplakin (210 kDa) and periplakin (190 kDa). Recent studies demonstrated that these patients also produce antibodies against desmoglein 3 (Dsg3) and Dsg1, and that anti-Dsg3 IgG plays a primary pathogenic role in inducing loss of cell adhesion of keratinocytes to cause blister formation in paraneoplastic pemphigus. The 170 kDa antigen is a transmembrane cell surface protein, which is yet to be identified. Serologic definition of the disease is dependent upon demonstration of antibodies against the plakin proteins, by immunofluorescent or immunochemical techniques. Refractory stomatitis is the most characteristic clinical feature of the disease, and in the past, many cases of erythema multiforme, Stevens Johnson syndrome or toxic epidermal necrolysis associated with hematologic neoplasms were probably unrecognized PNP.

Associated neoplasms are non-Hodgkin's lymphoma, chronic lymphocytic leukemia (CLL), Castleman's disease, thymomas, and poorly differentiated spindle cell sarcomas. In patients less than 20 years of age, essentially all cases are associated with Castleman's disease. Non – Hodgkin's Lymphomas and chronic lymphocytic leukemias are ''frequently'' complicated by autoimmune phenomena, such as autoimmune cytopenias, and we suspect that PNP is another example of induction of autoimmunity by these specific neoplasms. PNP also occurs in domestic animals (horses and dogs), with similar associated tumors and similar outcomes.

In patients with a benign neoplasm that can be completely resected by surgery, recovery is anticipated, but over a period of 12–24 months. The mortality of PNP associated with malignant neoplasms still approaches 95% by two years post diagnosis. Treatment of the underlying neoplasm by chemotherapy does not improve the autoimmune disease, once it is initiated by the tumour. Some cases of CLL are responsive to combined treatment with prednisone, cyclosporine and cyclophosphamide, but cases of NHL do not respond reliably to any therapy. Many fatalities are now recognized to be secondary to pulmonary involvement. There is evidence that PNP affects the epithelium of large and small airways, with sloughing of the respiratory epithelium, resulting in fatal bronchiolitis obliterans.

Future challenges include better definition of the mechanisms by which these tumors induce autoimmunity, and more effective therapies for those patients with associated lymphomas.

002

Genetic Predisposition to Autoimmune Diseases

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Most autoimmune diseases are linked to specific alleles of Class II histocompatibility complex proteins. The association suggests that the linked allele is directly involved in the genesis of the disease, although this has been proven in only a few cases. In the case of pemphigus vulgaris (PV), the linkage is to a subtype of HLA-DR4, DRA/DRB1*0402. This subtype is distinguished from other subtypes of DR4 by the presence of negatively charged residues in the P4 pocket, which mandates a positively charged residue at P4 of the peptide. The immunodominant epitope of desmoglein 3, amino acid residues 190–204, was predicted, verified experimentally, and recently substantiated by examination of additional patient material. The linkage to DRB1*0402 is found in Ashkenazi Jews as well as in Iranian patients, but in non-Jewish patients of European descent and in India and Pakistan, PV appears to be primarily linked to DQB1*0503. In addition to linkage to Class II MHC genes, a large number of non-MHC genes distributed throughout the genome contribute to susceptibility to autoimmune diseases, and some of these genes, none of which has been definitively identified, are involved in multiple autoimmune diseases. Much further work is needed to clarify all the genetic factors that are involved in susceptibility to autoimmunity.

004

Fogo Selvagem: An Environmentally Triggered Form of Pemphigus Foliaceus

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Although Fogo Selvagem (FS) was originally described, and is still most frequently found in Brazil there are reports of other foci of endemic pemphigus foliaceus (PF) in Colombia and Tunisia. The clinical, histological and immunological features of FS are similar to those of the nonendemic form of PF seen in the U.S.A. and around the world. As in the nonendemic form of PF, FS is characterized by superficial subcorneal blisters and pathogenic autoantibodies that are specific for the ectodomain of dsg1. FS, however, shows several unique and remarkable features such as the geographic and temporal clustering of cases, the increased frequency of cases among young adults and children, the increased frequency of familial cases, and an association with certain distinct HLA-DR alleles.

We have recently described two settlements of Amerindian natives in Brazil that exhibit a high prevalence of FS, a Xavante Reservation located in the eastern region of the state of Mato Grosso and the Terena Reservation of Limao Verde. The Limao Verde reservation has a population of 1200 individuals with a prevalence of FS of 3.2% and an incidence of 1–4 new cases per year. We evaluated the presence of dsg1-specific autoantibodies in the sera of FS patients from the Limao Verde reservation and from a large group of normal donors from the U.S.A., Japan and Brazil using a highly sensitive and specific desmoglein1 (dsg1)-ELISA assay. The control sera from Brazil included samples from cities located at different distances from the Limao Verde reservation. Anti-dsg1 autoantibodies were absent in the control sera from the U.S.A. and Japan, which included samples from normal individuals and patients with other cutaneous autoimmune blistering diseases such as bullous pemphigoid, herpes gestationis and lupus erythematosus. Intriguingly, antidsg1 autoantibodies were detected not only in the sera of FS patients but also in the sera of several normal controls from the reservation and from Brazilian cities. The percentage of ELISA-positive sera among the normal control population was inversely related to the distance from the endemic focus of Limao Verde. In five FS cases followed in Limao Verde for several years, antidsg1 autoantibodies were present in blood samples obtained 1–5 years prior to the onset of the disease. However, the titers of antidsg1 antibodies increased several fold once the disease was clinically apparent. These results suggest that in an area endemic for FS, such as the Limao Verde reservation, certain members of the population become sensitized to an environmental antigen(s) producing antidsg1 autoantibodies that, in the course of several years, can lead to FS. The molecular mechanisms of antidsg1 formation and the putative environmental antigen(s) in FS remain to be determined. It is feasible that epidermal dsg1 and the environmental antigen(s) may share certain cross-reactive epitopes that are relevant to the immunopathogenesis of this disease.

006

Pemphigus and bullous pemphigoid

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Pemphigus and bullous pemphigoid are distinct autoimmune blistering skin diseases that are characterized by the presence of autoantibodies directed against specific adhesion molecules. This talk will compare and contrast the molecular mechanisms of blister formation of these two diseases, and demonstrate how knowledge of these pathophysiologic mechanisms can provide a rational therapeutic approach.

In pemphigus vulgaris, histologic examination shows intraepithelial blistering, and inflammatory cellular infiltration can be minimal or absent. Direct immunofluorescence shows IgG and variable C3 deposition on cell surfaces. Passive transfer of human IgG autoantibodies into neonatal mice precisely reproduces the disease, and seems to do so by down-regulating the adhesive function of the antigen. The binding of pemphigus antibodies does induce other events, such as activation of complement and plasminogen activator, but these do not seem to influence acantholysis significantly. Pemphigus antibodies can readily induce blistering in mice depleted of functional complement, or pretreated with high doses of corticosteroids, which effectively abolished plasminogen activator activity. The importance of desmoglein 3 in cellular adhesion was further demonstrated by genetically engineered mice with disruption of the desmoglein 3 gene. These mice demonstrated runting, due to intraoral lesions typical of pemphigus. These data establish that the goal of therapy in pemphigus must be directed towards reducing synthesis of autoantibodies, by systemic corticosteroids, alone or in conjunction with immunosuppressive drugs. Topical therapies have no role in management. Complications secondary to the use of very high dose corticosteroids contribute to the current mortality rate.

We employ the following treatment steps: (1) Prednisone at doses no greater than 1 mg/kg/day (2) prednisone plus a nonalkylating agent such as azathioprine or mycophenolate mofetil (3) prednisone plus an alkylating agent – cyclophosphamide or chlorambucil, and (4) prednisone plus an alkylating agent plus short-term plasmapheresis.Histologic examination in bullous pemphigoid shows subepidermal blistering, with large numbers of infiltrating polymorphonuclear cells. Direct immunofluorescence shows linear IgG and C3 deposition along the basement membrane. Indirect immunofluorescence shows circulating IgG BMZ autoantibodies.

Patients Sera recognize two hemidesmosomal protein antigens – the bullous pemphigoid antigen 1 (bpag1 or bp230 ag), and the 180 kda, bullous pemphigoid antigen 2 (bpag2 or bp180 ag). Unlike pemphigus antibody – induced cell detachment, in which antibody binding to the cell adhesion molecule directly induced cellular detachment, bullous pemphigoid antibodies induce a cascade of inflammatory events that are requisite for blister formation. Blistering occurs only after the sequence of antibody binding to the bp 180 ag, complement activation and polymorphonuclear cell infiltration. These data explain clinical observations about the disease and show why anti-inflammatory drugs have a role in treatment of bullous pemphigoid.

Prednisone is most useful, and is often rapidly effective at a dose of 0.5 mg/kg/day. In mild cases, anti-inflammatory drugs such as dapsone or tetracycline and niacinamide may have steroid-sparing effects. In cases of moderate severity, azathioprine 2–3 mg/kg or mycophenolate mofetil at a dose of 30 mg/kg/day can be very effective. More aggressive treatment with plasmapheresis and cyclophosphamide is rarely required, but when needed is highly effective.

007

Pemphigus: Drugs and Infectious Agents as Inducing Factors

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The onset and course of pemphigus depend on a variable interaction between predisposing and inducing factors. Genetic predisposition is known to be associated with human leukocyte antigens (HLA), in particular with DR4, 14; DQ 1,3 [1]. The genetic background alone, though essential, is not by itself sufficient to initiate the autoimmune response, as proven by the reports of pemphigus in only one of two monozygotic twins [2] and in only two of three siblings with identical predisposing haplotype [3]. The intervention of inducing or triggering factors seems to be crucial to set off the full-blown disease.

Even if in the majority of patients no inducing agent can be detected (*idiopathic pemphigus*), in several cases a meticulous clinical history discloses facilitating factors (induced or triggered pemphigus). *Induced pemphigus proper* refers to a condition where exogenous factors play a major role, so that the disease regresses after the inducing factor is eliminated, even without treatment. In *triggered pemphigus*, endogenous factors are more important and the inducing factors seem to only trigger, in a casual and non specific manner, a disimmune mechanism previously programmed and ready to be set off, so that, in spite of elimination of the inducing factor, the disease self-perpetuates [4].

Inducing factors are numerous and heterogeneous, but in most cases the induction is related to certain drugs (thiols, ACE-inhibitors, phenols, NSAIDs, interferons, and other cytokines) or some viruses (herpesviruses). The involvement of an inducing agent in the pathomechanisms leading to the outbreak of pemphigus is often suspected on the basis of circumstantial evidence, but sometimes it can be demonstrated with certainty [4–7].

As for certain drugs, their potential of provoking acantholytic changes has been confirmed by several experimental investigations [6]. In particular, a drug may provoke acantholysis by interfering with the keratinocyte membrane biochemistry (*biochemical acantholysis*) and/or with the immune balance, both cellular and humoral (*immunological acantholysis*) [5].

As for herpesviruses, frequently involved in pemphigus induction, but also for other occasional virus infections (e.g. cold, flu), the possibility exists that interferons and other cytokines, which the host produces as a consequence of the virus attack, overactivate the immune system leading to the antibody-mediated acantholytic autoimmune disease [8].

For practical purposes, avoiding or limiting the interaction of precipitating factors with the pemphigus-prone genetic background may be a useful precaution in the management of pemphigus patients, because it can improve the efficacy of conventional treatments, reduce risks of relapses and, in some cases (induced pemphigus proper), even result in a cure.

009

Do T Lymphocytes Play a Role in the Development of Pemphigus?

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Pemphigus is a cutaneous autoimmune disease characterized by intraepidermal blisters and circulating autoantibodies against desmosomal components, desmoglein-1 (Dsg1) and/or desmoglein-3 (Dsg3). Using a passive transfer animal model, we have demonstrated that purified autoantibodies against Dsg1 and Dsg3 can induce diseases similar to pemphigus foliaceus (PF) or pemphigus vulgaris (PV), respectively. These results indicate that anti-Dsg antibodies are directly involved in the tissue damage, which occurs in pemphigus. Since the production of antibodies in the T-dependent immune responses usually requires the assistance of antigen-specific T lymphocytes, it is proposed that T cells may play an essential role in the autoimmune reaction in patients with pemphigus by recognizing Dsg and regulating the secretion of harmful anti-Dsg antibodies. Using endemic PF, fogo selvagem (FS), as an example, we showed that T cells from these patients responded to Dsg1 in a dose dependent manner. This T cell reaction was specific to patients with FS because T cells from other patient groups, as well as normal individuals, failed to proliferate to Dsg1. Further, T cell lines and clones derived from FS patients were shown to specifically respond to Dsg1, but not to Dsg3 or BP180 antigens, suggesting that these T cells may be responsible for the autoimmune responses against Dsg1 in the disease. Characterization of these T cell lines and clones revealed that they are CD4+ memory T cells secreting a Th2-like cytokine profile, a subset of T cells supporting the production of antibodies. In summary, our data provide evidence that Dsg-specific T cells from patients with pemphigus may play a role in the development of the disease at a stage leading to the production of autoantibodies. Moreover, we demonstrated that the majority of FS T cell clones reacted with a 15 amino acid Dsg1 peptide that was predicted to be presented by FS associated MHC II molecules, HLA-DRB1*0102, 0401, 1402, and 1406. This information will allow us to identify the peptide that may trigger the FS autoimmune reaction and help to design a peptide antagonist that can inhibit the Dsg1-specific T cells in the near future.

011

The Importance of Non-Desmoglein Targets of Pemphigus Autoimmunity

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Most of organ-specific autoimmune diseases are associated with autoimmunity against cell-surface receptors. Acantholytic activity of pemphigus IgGs harbors pharmacologic effects on keratinocyte shape and adhesion. Approximately 85% of patients with pemphigus vulgaris (PV) and pemphigus foliaceus develop antibodies to acetylcholine receptors (AChRs) expressed on the cell membrane of epidermal keratinocytes. Classic studies showed that human keratinocytes express several muscarinic AChR subtypes as well as several types of heteromeric and homomeric nicotinic AchR channels. These cholinergic receptors regulate transmembrane Ca^{2+} flux and intracellular metabolism and have been implicated in mediating physiologic control of keratinocyte adhesion and motility. The immunopharmacologic action of pemphigus IgGs may therefore be mediated by alterations of physiologic control of keratinocyte shape and adhesion through a G protein coupled-metabotropic receptor and/or an ion channel. To identify the molecular structure of keratinocyte AChR(s) targeted by pemphigus antibodies, we performed blocking indirect immunofluorescence experiments. Preincubation of monkey esophagus with PV antibodies blocked specific staining of the keratinocyte cell membrane with rabbit antibody to a9 AChR, indicating that this first of its kind mixed muscarinic-and-nicotinic AChR is targeted by PV autoimmunity. In contrast to distinct *in vitro* acantholytic activity of antia9 antibody, its intraperitoneal administration to neonatal mice did not induce PV-like mucocutaneous changes. This prompted our search for other novel targets of PV autoimmunity. The PV IgG eluted from a 75-kDa keratinocyte protein band both stained epidermis in a PV-like pattern and induced acantholysis in keratinocyte monolayers. Screening of a keratinocyte cDNA library with this antibody identified clones carrying cDNA inserts encoding a novel molecule exhibiting ~40% similarity with annexin-2, named pemphaxin (PX). PX specifically binds acetylcholine, suggesting that it can be one of the keratinocyte cholinergic receptors targeted by PV antibodies. Preabsorption of PV sera with recombinant PX eliminated acantholytic activity. However, this antibody alone did not cause skin blisters *in vivo*. The lack of symptoms in neonatal mice injected with antia9 or anti-PX antibodies is not surprising, because the integrity of the epidermal barrier in higher species relies on more than a single molecule.

Therefore, we conclude that pemphigus is a complex process involving several antigen systems. To trigger pemphigus, anti-AchR antibody may be needed to functionally complement the effect of antidesmoglein antibodies. To test a hypothesis that cholinomimetics – the drugs that specifically bind to and activate AChRs – may counteract acantholytic effects of PV IgG, we treated neonatal mice with induced pemphigus using the cholinomimetic carbachol and obtained significant decrease of the extend of cutaneous acantholysis ($p < 0.05$). Currently, we are conducting a clinical trial of cholinomimetics in the treatment of patients with PV ort PF. The goal is to replace glucocorticoids with a well-tolerated acetylcholinesterase inhibitor, Mestinon (pyridostigmine bromide), given alone or in combination with topical application of a cream containing the cholinomimetic drug pilocarpine hydrochloride, Pilopine HS' Gel. Thus, our studies have re-defined the epitopes and mechanisms leading to blistering in pemphigus, and are expected to lead to the development of nonsteroidal therapy for pemphigus.

008

The Pathogenic Autoantibody Response of Animal Models in Pemphigus

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Pemphigus is a unique and interesting autoimmune disease, in which IgG autoantibodies play a pathogenic role and cause blister formation. The autoantibodies are directed against desmoglein (Dsg), an epidermal cell-cell adhesion molecule. Recently we have generated an active disease mouse model of pemphigus vulgaris (PV) with a unique approach using autoantigen knockout mice, in which self tolerance of the defective gene product is not acquired. We immunized Dsg3–/– mice with recombinant Dsg3 (rDsg3), the target antigen of PV. Then, splenocytes from these mice were adoptively transferred to Rag2–/– immunodeficient mice that express Dsg3. Similar to PV patients, the recipient mice stably produced pathogenic anti-Dsg3 IgG for over 6 months and caused blisters due to loss of cell-cell adhesion with the characteristic histology. This model was further characterized to investigate the cellular mechanism of tolerance loss against Dsg3. Purified T and B cells from Dsg3–/–, Dsg3+/–, and Dsg3+/+ mice were mixed with various combinations and transferred to Rag2–/– mice. The PV phenotype was observed only with a combination of Dsg3–/– T and Dsg3–/– B cells but not with the other combinations, suggesting that loss of tolerance against Dsg3 in both B and T cells is required for the development of the autoimmune state of PV. Furthermore, we have generated pathogenic anti-Dsg3 monoclonal antibodies from PV model mice. Our model is a valuable tool to dissect cellular and molecular mechanism of autoantibody production as well as to develop novel therapeutic strategies.

010

Other Antigens Recognized by Pemphigus Autoantibodies

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The major autoantigens for classic types of pemphigus are desmogleins 1/3 (Dsg1/Dsg3). However, the other proteins are also recognized by some types of pemphigus. For example, paraneoplastic pemphigus (PNP) sera react with various plakin family proteins, including plectin, desmoplakins I/II, BP230, envoplakin and periplakin, as well as an unknown 170 kDa protein, by either immunoprecipitation and immunoblotting. By a novel cDNA transfection methods using cDNAs of human desmocollins (Dsc) 1–3, we previously showed that IgA antibodies of subcorneal pustular dermatosis type IgA pemphigus react specifically with Dsc1 on the cell surfaces of COS-7 cells, which were transfected with eukaryotic expression vector containing human Dsc1 cDNA. However, the presence of IgG anti-Dsc autoantibodies is still controversial, and antibodies to Dsc2 and Dsc3 have not been clearly identified. Therefore, in the present study, we further analyzed autoantibodies against envoplakin, periplakin and Dsc1–3 in more details by various methods.

In the first study, using bacterial expression vectors containing polymerase chain reaction-amplified cDNAs, we prepared variously truncated recombinant glutathione-S-transferase-fusion proteins of envoplakin and periplakin, which presented N-terminal, central and C-terminal domains of each protein, as well as so-called C-terminal homologous domain of envoplakin. By immunoblotting using these 7 recombinant proteins, we demonstrated that most of the 26 PNP sera reacted very strongly with multiple recombinant proteins of envoplakin and periplakin, except for C-terminal homologous domain of eriplakin. We also examined the reactivity of other blistering diseases including pemphigus vulgaris, pemphigus foliaceus, and bullous pemphigoid with the 7 proteins, and found that a few of these non-PNP sera showed a weak reactivity with some of the recombinant proteins. Interestingly, some sera showed relatively strong reactivity with C-terminal homologous domain of periplakin, to which paraneoplastic pemphigus sera reacted less frequently. These results indicate that the immunoblotting using the recombinant proteins of envoplakin and periplakin should be a useful tool for the diagnosis of PNP. In addition, although non-PNP sera occasionally show a weak reactivity with envoplakin and periplakin, the pathogenecity and the mechanism of antibody production in these cases may be different from those in PNP.

Next, we have produced recombinant proteins containing entire extracellular domains of human Dsc1–3 by baculovirus-expression system, and subsequently established an enzyme-linked immunosorbent assays (ELISA) for both IgG and IgA antibodies using these recombinant Dscs. By this ELISA, none of the 45 sera of classic types of pemphigus showed IgG antibodies to any Dsc. In contrast, one atypical pemphigus serum showed both IgG and IgA antibodies to Dsc1, which were completely absorbed by incubation with Dsc1 baculoprotein. Furthermore, this ELISA detected IgA anti-Dsc3 antibodies and IgA antibodies to both Dsc2 and Dsc3 in one each of atypical pemphigus case. This reactivity was confirmed by the positive IgA immunofluorescence staining with human Dsc2 and Dsc3 expressed on COS-7 cells transfected with cDNA of each Dsc. These results suggest that, both IgG and IgA autoantibodies against all of Dsc1–3 are present in the sera of nonclassical types of pemphigus.

012

Pemphigus Vulgaris: The Pathogenic Activity of PV-FAB is Plakoglobin-Dependent

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With the aim to investigate the molecular mechanisms leading to blister formation in pemphigus vulgaris (PV), we recently developed an *in vitro* model based on long-term keratinocyte cultures of wild-type and plakoglobin knock-out ($PG^{-/-}$) mice, and uncovered a pivotal role for this plaque protein in lesion formation. Here we focused on the involvement of plakoglobin in the PV IgG-induced break-down of desmosome-mediated adhesion at the plasma membrane. Using immunofluorescence studies we found that binding of PV IgG to wild-type or $PG^{-/-}$ cells induced clustering of all investigated desmosomal proteins. We subsequently addressed whether this PV IgG-induced event is a by-stander phenomenon due to the bivalence of the antibody or is part of the pathdgenetic process. Using monovalent PV-Fab fragments on wild-type keratinocytes, clustering and break-down of the desmosomal organization concurrent with keratin retraction was similar than with bivalent PV IgG. In contrast, despite binding of PV-Fab to $PG^{-/-}$ cells, clustering was abrogated and keratin retraction did not occur in these cells. In summary, our dual *in vitro* model allowed to separate two as yet undescribed activities of the antibody. Namely, a strong cross-linking activity is exerted by bivalent PV IgG which is not required for lesion formation and is independent of plakoglobin. The second activity relays on plakoglobin, is independent of antibody valence and is pathogenic as it causes disruption of the entire desmosomal organization. Collectively our findings corroborate dependence of lesion formation on a plakoglobin-mediated cellular response, and thus attribute an important role to this plaque protein in maintaining the epithelial architecture. A better knowledge of these mechanisms will hopefully form the bases to develop alternative therapeutic strategies in profit of the PV patient.

013

The Role of Antigen Distribution in the Localization of Pemphigus Lesions

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Pemphigus is a group of autoimmune blistering disorders of the skin and mucous membranes resulting from loss of epithelial cell-cell adhesion due to circulating pathogenic autoantibodies. These antibodies are directed against several epithelial proteins, including the cadherins, desmoglein 1 (Dsg1) and desmoglein 3 (Dsg3). Desmogleins are transmembrane glycoproteins that function as adhesive components of desmosomes, the intercellular junctions that are critical for maintaining cell-cell adhesion, tissue integrity, and organ function. The two classic forms of pemphigus are pemphigus foliaceus (PF) and pemphigus vulgaris (PV). PF blisters occur in the superficial epidermis and spare the mucous membranes, although PF anti-Dsg1 antibodies have been shown to bind to the entire epidermis and to the superficial epithelia of oral mucosa. In contrast, PV patients develop antibodies against both Dsg1 and Dsg3. The hallmark pathogenic feature is acantholysis of epidermal cells as well as epithelial cells of the oral mucosa, and the blisters occurring within the basal and suprabasal layers. To correlate pemphigus antibody profiles with tissue distribution of desmogleins and explain blister localization in PF and PV, we used the neonatal mouse model of pemphigus by passive transfer of pemphigus IgG to normal and *DSG3* knockout mice (*DSG3*–/–). First we demonstrated by immunostaining on mouse tissues that the distributions of Dsg1 and Dsg3 are similar to that in human tissues. Dsg1 is expressed throughout the epidermis and the oral mucosa. In contrast, Dsg3 is found only in the basal layer of the epidermis, while it is expressed throughout the entire oral mucosa. To demonstrate the role of Dsg3 in limiting blister formation in PF, we injected mice with 1 mg of PF IgG. This low dosage (1/10 of the usual amount) of PF IgG caused small isolated blisters in *DSG3*+/– or +/+ mice but caused extensive blistering in *DSG3*–/– mice. Thus, animals devoid of Dsg3 are more susceptible to blister formation by anti-Dsg1 antibodies. We hypothesized that blisters occur in PF where Dsg1 is found without concomitant Dsg3, namely, in the superficial epidermis. As predicted, PF IgG only caused superficial epidermal acantholysis and no mucous membrane lesions in *DSG3*+/– or +/+ mice (n = 26), but in *DSG3*–/– mice resulted in superficial and deep epidermal acantholysis and marked acantholysis of tongue mucosa (n = 7). These data also clarify the recent observation that PV patients with exclusively oral lesions have only anti-Dsg3 antibodies, while patients with skin involvement have also anti-Dsg1 antibodies, indicating that both antibodies may be necessary to interfere with both desmogleins in the deep epidermis. We confirmed the pathogenicity of the anti-Dsg1 antibodies from PV sera in deep epidermis by showing extensive blistering with deep epidermal acantholysis in PV IgG injected *DSG3*–/– mice (n = 3). Furthermore, while PV sera containing anti-Dsg3 antibodies alone were inefficient at causing blister formation in mice (n = 8), anti-Dsg1 antibodies increased the pathogenicity of PV sera. These data suggest that pemphigus autoantibodies inhibit the adhesive function of desmoglein proteins and that either Dsg1 or Dsg3 alone is sufficient to maintain keratinocyte cell-cell adhesion.

015

Use of Plasmapheresis and Immunosuppression in the Treatment of Pemphigus Vulgaris

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Plasmapheresis can be used in immune and inflammatory conditions to remove antibody, antigen, antibody-antigen complexes, or a combination of the above. Plasmapheresis was first utilized in the treatment of auto-antibody mediated diseases in 1975, when it was applied to a patient with Goodpastures syndrome [1]. Three years later, it was adopted as a therapeutic approach in the treatment of pemphigus vulgaris [2]. In pemphigus, the rationale for plasmapheresis is to remove pathogenic antibodies to desmoglein I or III.

The only controlled study of the use of plasma exchange in pemphigus was published in 1988 [3]. This was a 40 patient randomized control trial, comparing patients receiving prednisolone alone vs. prednisolone plus plasma exchange. The study found no significant benefit, however, no immunosuppressive agent was used as an adjuvant. The absence of an administered immunosuppressive agent was a major flaw in this study, since pathogenic B-cells begin to synthesize new auto-antibodies soon after plasmapheresis. In fact, as quickly as three hours after plasmapheresis, auto-antibody levels may reach levels equivalent or exceeding those that existed prior to plasmapheresis. This phenomenon, referred to as the rebound phenomenon, is achieved through a negative feedback mechanism involving pathogenic B-cells activated by the sudden drop in circulating levels of auto-antibodies. Thus, the use of immunosuppressive agents is necessary to reduce this pathogenic B-cell response.

While there are no controlled studies on the use of plasmapheresis and immunosuppressive agents, there are numerous anecdotal reports [4–20]. Most recently, Marcusson published a case of pemphigus foliaceous successfully controlled with plasma exchange during a 10-year period[5]. The patient, when treated with plasma exchange, prednisone, and azathioprine, went into complete remission, thereby allowing discontinuation of systemic therapy. The patient is now maintained with plasma exchange on an 8–10 week basis. In a further series, Roujeau *et al* [13] published an uncontrolled trial on 10 patients showing that 8 of 10 had clinical improvement and that all patients had a decreased antibody level. In the December 2000 issue of the Journal of American Academy of Dermatology [21], 7 pemphigus patients treated with plasmapheresis were reviewed. Each treatment consisted of one volume exchanged (approximately 15 milliliters per kilogram). The replacement solution was a 5% albumin solution. As was the case in previous studies, in most patients plasmapheresis produced dramatic and immediate reductions in serum antibody titres. Furthermore, this was generally associated with significant clinical improvement. Our current recommendations are to follow plasmapheresis with IV cyclophosphamide (0.5% gm/m^2) to prevent the rebound phenomenon.

Plasma exchange is not without risk. Complications include nausea, vomiting, electrolyte disturbances, depletion of coagulation factors, hypotension or hypertension, and arrhythmia. The most severe reactions, hypotension and cardiac arrhythmia, emerge in roughly 25% of cases. Other serious reactions include CNS disturbances, dyspnea, abdominal pain and vomiting and chest pain. There are also limitations in the use of plasma exchange: firstly, it is not efficient in removing large amounts of antibody; secondly, it is clearly not specific for pathogenic antibodies, as all immunoglobulins are reduced; finally, in most cases plasmapheresis requires the use of replacement fluids. There is a significant higher risk when plasma is used as a replacement fluid as opposed to some other form of replacement fluid and colloid. When blood products are used as a component of replacement fluids an additional expense is introduced, as well as a possibility of transmissible disease. Furthermore, limited availability is problematic with some blood products.

A number of considerations should be made when considering the use of plasmapheresis in the treatment of pemphigus vulgaris. Plasmapheresis is not recommended for all patients, rather it should be restricted to the following situations – life threatening pemphigus, resistant disease, and persistent disease. It should also be considered in patients with unacceptable side-effects from therapy such as aseptic necrosis of joints induced by corticosteroid use. Patients with severe mucosal disease, leading to an inability to take oral medication, may benefit from plasmapheresis. Generally, plasmapheresis should be reserved for more resistant cases that have failed standard therapies or who are developing significant side-effects from the treatment. Plasmapheresis appears to be an effective modality for treating more aggressive forms of pemphigus and should be considered as an important addition to current therapies.

017

Novel Approaches to Therapy Based on Understanding Antigen Distribution

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Understanding the pathophysiology of pemphigus leads to a rationale approach to therapy. For example, in pemphigus foliaceus (PF) autoantibodies against desmoglein (dsg) 1 interfere with its adhesion function, causing acantholysis. However, since in some areas there are two dsg isoforms (1 and 3) (e.g. in the deep epidermis and throughout the mucous membranes), and in these areas the dsg 3 can compensate for antibody-induced loss of function of dsg 1, preventing acantholysis. Similarly, induction of expression of dsg 3 in the superficial epidermis prevents PF-induced blisters.

The present therapy of pemphigus utilizes corticosteroids and various immunosuppressive drugs, sometimes in combination with plasmapheresis. The rationale basis for immunosuppressive therapy and plasmapheresis is that they lower the titer of pathologic autoantibodies (along with all other antibodies). However, this cannot be the rationale for corticosteroids, because they act within days (when antibody titers are not yet lowered) and even act locally. We hypothesize that corticosteroids might induce transcription of dsg isoforms, providing protection from antidsg antibodies. Futhermore, innovative future therapies might be directed towards inducing compensating dsg isoforms.

014

"Combined Immunosuppressive Therapy in Pemphigus." Looking for Effectiveness and Safety

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Pemphigus is an autoimmune blistering disease that affects skin and/or mucose membranes. The prognosis of this disease has dramatically improved with the use of immunosuppressive therapy. However the actual morbidity and mortality is due to the side-effects of these immunosuppressive drugs mainly by the misuse of systemic corticosteroids. The goal in immunosuppressive therapy is to have the most effective and safest regimen for patients. At present time this goal can be only achieved by combination therapy of drugs.

The standard initial therapy in pemphigus patients is still systemic corticosteroids. However, the overdose and/or prolonged exposure of corticosteroids are the main culprit of the complications seen in patients receiving immunosuppresive therapy. Therefore, In this study we tried to find the drugs that at appropriate doses upon combination with corticosteroids can achieved the goal of immunosuppressive therapy:

Efficacy and safety Upon combination of drugs three therapeutics effects can be encountered. (1) additive (2) synergistic and (3) antagonistic. We used immunosuppressive agents that have effective inhibition on B Lymphocytes (immunologic cells producing the pathogenic antibodies in pemphigus) as well as on T lymphocytes (regulatory cells of the immune system) (1) An additive effect is the one in which the concept of "Corticosteroid Adjuvant Therapy" was originated. In this effect, the addition of the adjuvant drugs will allow the tapering down of the corticosteroid. This combination is not associated with a better therapeutic effect than that given by prednisone alone, but it will minimize the exposure to prednisone and thus the corticosteroid-side-effects. The combination of prednisone 1 mg/kg/day (ideal and not actual weight) with azathioprine (Imuran) 3–4 mg/kg day or prednisone 1 mg/kg/day with mycophenolate mofetil (Cellcept) 30–45 mg/kg/day has shown to be "additive" in pemphigus patients. (2) Synergistic effect is the one that ideally should be obtained in patients in whom corticosteroid therapy failed to control the disease. Thus the second drug is more effective (usually more toxic too) than corticosteroids. The combination of of prednisone 1 mg/kg/day and cyclophosphamide (Cytoxan) 2–3 mg/kg/day has a synergistic effect, and even higher effect was the addition of plasmapheresis. These combinations are the most effective but also more toxic than the additive regimens, thus they are reserved for severe cases or patients that failed additive therapy. (3) Antagonistic effect is the one that every single doctor is trying to avoid. Since the drugs share the mechanisms of action, upon combination the ultimate therapeutic effect gets diminished and the toxicity gets exponentially increased. The following combinations are antagonistic (a) two corticosteroids: prednisone with dexamethasone (b) two antimetabolites azathioprine with mycophenolate mofetil or (c) two alkylating agents cyclophosphamide with chlorambucil. Combined immunosuppressive therapy using drugs that have an effective inhibitory action on the synthesis of pathogenic antibodies is effective and safe in pemphigus patients.

016

Mechanism of Action of IVIg in Pemphigus

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Background Pemphigus vulgaris (PV) is a blistering skin disease mediated by autoantibodies to intercellular (IC) epidermal antigens. We evaluated the effectiveness of intravenous immunoglobulin (IVIg) for the control of active disease, and the mechanism of action of this agent.

Methods Six patients with active PV unresponsive to conventional therapy were treated with IVIg (400 mg/kg/day for 5 days) and concurrently given cyclophosphamide (100–150 mg/day). The primary end-points were healing of skin lesions, and changes in the level of IC antibodies and steroid dose. Two historical groups of patients treated conventionally with high doses of corticosteroids and cytotoxic drugs (n = 11) or by plasmapheresis (n = 11) served as controls.

Results Within 2 weeks of initiating IVIg, the activity of PV was controlled in all but one case and the extent of skin lesions was reduced on the average by 80%. Within 3 weeks, steroid doses were reduced by an average of 41%. The improvement was more rapid than that in control patients treated conventionally. Clinical improvement was associated with a rapid and selective decline in IC antibodies whose levels decreased by 72% within 1 week of initiating IVIg. The rapidity and extent of this decline were similar to that achieved by intensive plasmapheresis. The decline was not due to blocking the synthesis or the immunological activity of IC antibodies by IVIg, suggesting that it resulted from increased Ig catabolism.

Conclusions These results indicate that IVIg can effectively and rapidly control active PV and suggests a novel explanation for its mechanism of action. It is that it increases the catabolism of serum lgG, and that this results in a selective decrease in only pathogenic lgG antibodies as the level of normal antibodies is maintained by those present in the IVIg preparation.

018

Ancillary Care and Prevention of Complications in Pemphigus

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Prior to glucocorticoids (GCs), patients used to regularly die from pemphigus vulgaris. After GCs, patients have tremendous morbidity and some mortality related to therapy, but don't die from the disease itself. There are now more options for management of patients with PV, both in terms of GC-sparing agents and in preventing some of the more devastating side-effects. Clearly, early diagnosis and treatment of PV is important, and if patients don't respond or can't taper GCs, then adjunctive therapies must be utilized quickly and routinely. Patient compliance is also crucial to a good outcome of therapy.

Toxicities from GCs are related to higher doses and longer use, so early control of the disease is really one of the more critical issues in preventing side-effects. Early attention to a number of factors is important, including monitoring for weight gain, hypertension, hyperglycemia, glaucoma, hyperlipidemias, infections, and electrolyte abnormalities. Abnormalities in the blood pressure or labs should be carefully treated and followed.

Many advances have occurred relating to prevention of GC-induced bone loss. A baseline DEXA scan of spine and hip bone density is now considered quite important, since pre-existing osteopenia or osteoporosis necessitates prompt treatment, and monitoring of bone density can detect patients losing bone at an unacceptable rate. Much of the bone loss from GCs occurs in the first six months. There are many new therapies available to prevent loss of bone, and older patients are often routinely put on these treatments to prevent further loss of bone.

Patients on GCs who develop pain or decreased range of motion of hips or other joints should be promptly evaluated to rule out avascular necrosis. Early detection and treatment may prevent progression to disabling arthritis and the need for joint replacement. The rate of GC taper is also important. Although patients responding to GCs can be tapered fairly quickly at higher doses, it is clear that below 20 mg/day, prednisone should be tapered more slowly, and below 10 mg/day, patients often benefit from reductions of as little as 1 mg/week. This allows for determining the dose where lesions may recur, thus alleviating the need for rapid increases in GC dose with flares. Slow tapering at lower doses minimizes the development of the GC withdrawal syndrome, where patients feel symptoms of fatigue, achiness, and malaise related to the GC taper.

019

Cooperative Clinical Trials in PV: Where Are We?

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There is a complete absence of multicenter, randomized controlled trials to evaluate the therapies used in PV. This has led to large variations in treatment of PV from university to university and practioner to practioner. Reviews are based largely on case series and case reports, and there is little high quality evidence to guide therapeutic decisions. Without studies, it is difficult at times to get insurance companies to pay for potentially beneficial therapies. The difference between conventional and alternative medicine in regards to the treatment of PV is the desire to apply the scientific method and not to rely on anecdotes and theories in directing therapeutic choices. We have yet to proceed beyond that desire for scientific clinical studies, and it is clearly time for those truly interested in the treatment of PV to develop collaborative multicenter protocols, similar to what has been done in cutaneous lymphoma, scleroderma, and many other orphan diseases in internal medicine. There has been a lack of funding mechanisms for such an effort, and the cooperation required between centers for multicenter, clinical trials, is quite distinct from the competitive nature of basic science research.

In 1996, the Medical Dermatology Society (MDS) was formed, and one of the goals was to serve as a coordinating body for the development of multicenter cooperative studies in medical dermatology, taking advantage of the tools of modern electronic communication. After one two-hour session devoted to therapy of PV, it became clear that there was no standardization of treatment, and that each center and even each physician had evolved there own algorithm for management of patients with PV. Studies are difficult because of the relative rarity of PV, the heterogeneous clinical presentation, and the heterogeneous response to therapies.

It was determined, after extensive discussions with interested MDS members, collaboration with two statisticians, and discussion with experienced trialists in the rheumatology community, that a study that had a more uniform entry population would be the best initial study. To achieve this, it was decided to study patients who were in the maintenance phase of their disease, but steroid-dependent. Such patients often were unable to taper GCs below 15–40 mg prednisone a day without experiencing new skin lesions. Preliminary data indicated that one potential GC-sparing agent in maintenance phase disease was dapsone, and numerous anecdotes had been published over the years about the efficacy of dapsone in treating patients with PV. Study outcomes related to reduction of GC doses, and the relatively uniform entry population made a response relatively easy to assess. The study was initiated in 1997, with the support of Jacobus Pharmaceuticals and five centers have recruited 1/3rd of the needed recruitment, with just three centers recruiting most of those patients. It is clear the study could be easily completed if more centers were willing to participate. The issues have been bias, lack of time, and lack of a history/interest in doing these types of studies. There is a belief that dapsone either doesn't work or definitely works, and both views were shared during a poster presentation at the SID several years ago. Clearly, a study is the only way to sort out these disparate observations.

Most successful active centers in the PV-dapsone study have a dermatologist who is double boarded in medicine and dermatology, bringing the history of clinical trials that is so inculcated in the training of internists, and run drug study units. There is a need to develop more individuals in dermatology who want to participate in such studies, and the current trend of referring difficult management cases to academic medical centers (AMCs), would make the AMC the likely place for these studies to occur.

There are clearly many other studies that need to be done. The goal is to succeed at this focused, "easy" study, develop a cooperative network of centers, and then expand to the many questions that exist related to management of acute and chronic disease. What is the role of IVIG? Which immunosuppressive should we really be using, after the efficacy, cost, and side-effects issues are considered? Who should receive plasmapheresis? Doses maintenance with low-dose GCs after their disease is controlled alter the relapse rate?

There is a lot of work to do. The PV trial group meets annually at the AAD meeting, is happy to involve all interested centers, and is truly looking for collaborative interactions. Funding mechanisms to link centers should reflect the necessarily collaborative nature of such initiatives.

021

Peptide Based Vaccine Strategies

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Vaccination has been used for over 100 years to specifically induce or ameliorate immune responses n humans. Vaccination advantages over conventional disease treatment include that the targeting is selective, specific, and durable. In the case of pemphigus, development of a vaccine to down-regulate or eliminate pathogenic desmoglein responsive immune elements in patients, or prevent development of desmoglein specific responses in susceptible individuals would obviate the need for glucocorticoids and immunosuppressive regimens. The feasibility of a vaccine approach stems from advances in our understanding of the genetics of this disease, identification and sequencing of the major targets, and advances in our understanding of vaccine irnmunobiology,

Current strategies for vaccine development relevant to autoimmune diseases focus on the generation of regulatory T cells or immune deviation via: (1) immunization with peptides or altered peptide ligands; (2) use of MHC-peptide complexes with or without a cytotoxic fusion partner; and (3) induction of high-zone a tolerance using native peptide ligands. All 3 of these strategies are currently in development for other autoimmune processes including allergic rhinitis, rheumatoid arthritis, and multiple sclerosis.

The goal of vaccine immunotherapy is to anergize or delete disease-specific CD4+ T cells, The specificity of this response is obtained by employing peptides presented by the disease-associated MHC Class II molecules, in the case of pemphigus vulgaris, DR(3 1*0402. Identification of the relevant antigenic domains, or epitopes. that are important in the generation of an immune response, is critical in the development of a vaccine. Preliminary investigations by several investigators have identified B and T cell epitopes in the extracellular domain of desmoglein 3. We have continued and extended this work to more precisely map T cell epitopes in this region. Our preliminary results support previous studies by Wucherpfenning *et al* that identified T cell epitopes in desmoglein 3 that bind to MHC Class II molecules and are recognized by peripheral blood lymphocytes from patients with pernphigus vulgaris.

023

Gene Therapy to Treat Autoimmune Diseases

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CD4+ T cells have been implicated in the pathogenesis of several autoimmune diseases such as rheumatoid arthritis (RA), multiple (MS) and TID (IDDM). Autoantigen-specific T cells have tissue-specific homing properties, suggesting that these cells may be ideal vehicles for the local delivery of "immunoregulatory" products. We tested this hypothesis in animal models of autoimmune disease by using autoantigen-specific CD4+ T hybridomas, following gene transfer, as vehicles to deliver "immune-regulatory proteins" for the treatment animal models of human autoimmune diseases. Autoantigen-specific T.cells were transduced to express the interleukin (IL)- 12 antagonist, IL-12p4O, using retroviral vectors encoding IL-12p40 cDNA. Transfer of relevant autoantigen-specific IL-12p40 producing CD4+ T cells after immunization significantly inhibited the development of either CIA or EAE, while cells transduced with the vector control had no effect. Additionally, islet specific T cells, transduced to express IL-12 p40 blocked the adoptive transfer of IDDM into immunocompromised NOD mice. The development of CIA or EAE was inhibited as a result of local suppression of inflammatory autoimmune responses by the transduced autoantigen specific T cells. Detection of bioluminescent autoantigen-reactive T cell hybridomas following transfer into immunized mice demonstrated accumulation and retention in inflamed tissues. The beneficial effect of IL-12p40-transduced T cells required TCR specificity against the relevant autoantigen since MBP-specific IL-12p40-expressing T cells, that produced equivalent amounts of IL-12p40 as CII-specific IL-12p40-expressing T cells, were therapeutic in an EAE model, but had no effect in ameliorating CIA and vice versa. Thus, although MBP-specific T cells, that could ameliorate EAE were found to migrate into the inflamed joints of CIA mice, they were not retained there, and no therapeutic benefit on CIA was observed. These results indicated that the local delivery of IL-12p40 by autoantigen-specific T cells inhibited inflammatory autoimmune disease by suppressing the response at the site of inflammation. More recent studies have suggested that bone marrow derived dendritic cells, transduced to express immunoregulatory proteins, were also effective in ameliorating certain autoimmune diseases. Trafficking studies of these cells, using bioluminescence, revealed unexpected homing to inflamed tissues. We conclude that modifying antigen-specific T cells or bone marrow derived dendritic cells by retroviral transduction for expression of immunoregulatory proteins is a promising therapeutic strategy for the treatment of human inflammatory autoimmune diseases.

020

Immunological Mechanisms of Self-Tolerance

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It is widely accepted that the development of autoimmune disease involves a breakdown in the mechanisms that control self- vs. nonself discrimination. Thymic deletion of autoreactive T cells is the primary mechanism that leads to tolerance to self-antigens. However, some autoreactive T cells may escape thymic deletion or recognize antigens expressed only extrathymically. Studies over the past 10 years have led to the identification of a unique subset(s) of T cells whose primary role is to suppress the activation of autoreactive T cells that have escaped passive mechanisms of tolerance induction. One potent suppressor T cell population can be defined by the coexpression of CD4 and the Interleukin-2 receptor α-chain (CD25). CD4+ CD25+ T cells have been shown to be involved in the suppression of several animal models of autoimmune disease including gastritis, oophoritis, insulin-dependent diabetes, and inflammatory bowel disease. However, it has proven difficult to determine their mechanism of action, antigen specificity, or cellular targets. We have recently developed *in vitro* model systems that mimic the function of these cells *in vivo*. CD4+ CD25+ T cells are nonresponsive to stimulation by antigen or mitogens; when coculutred with CD4+ CD25- T cells, the CD4+ CD25+ T cells suppress proliferation and effector cytokine production by a cytokine-independent, cell contact-dependent mechanism. CD4+ CD25+ T cells with identical properties have also been described in human peripheral blood. Augmentation of the function of these CD4+ CD25+ suppressor T cells may represent a therapeutic option for the treatment of autoimmune diseases.

022

Immunoablative Therapy of Refractory Pemphigus

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Pemphigus encompasses a group of autoimmune mucocutaneous blistering diseases characterized by two features: a) disruption of the cell-cell adhesion of stratified squamous epithelia and (b) the presence of pathogenic IgG autoantibodies reacting against desmosomal adhesion molecules. The three major subsets of the disease are pemphigus vulgaris (PV), pemphigus foliaceus (PF), and paraneoplastic pemphigus (PNP). We have previously demonstrated that high-dose cyclophosphamide (CY) without bone marrow transplantation (BMT) can induce durable treatment-free remissions in a variety of severe autoimmune disorders including aplastic anemia, systemic lupus erythematosus, and autoimmune hemolytic anemias. We now report the results of high-dose Cy (50 mg/kg/day for 4 consecutive days) in 6 patients with refractory PV. Eligible patients had persistent disease activity despite treatment with mycophenolate mofetil (MMF) and/or azathoprine (aza) and were dependent on corticosteroids. Patients had a median age of 33.5 (range, 27–47) years, a median disease duration of 3.5 (range, 2–5) years, and a median prednisone dosage of 50 (range, 30–60) mg/day. All patients were refractory to aza; 5 were refractory to MMF. High-dose Cy was well tolerated; common toxicities included reversible alopecia, transient nausea/vomiting, febrile neutropenia (2 patients). The median time to a neutrophil count of 0.5×10^9/L was 11.5 (range, 11–18) days; the median number of packed red cell transfusions was 2 (range, 0–4) units and the median number of platelet transfusions was 1 (range, 0–2). Complete remission (CR) was defined as: resolution of all skin lesions and reduction of circulating pemphigus antibody levels to $\leq 1:20$. Partial remission (PR) was defined as: improvement in skin lesion and reduction of pemphigus antibody levels to $\leq 1:80$. Four patients have achieved a CR and 2 patients are in a PR with a median follow-up of 6 (range, 1–30) months. The median prednisone dosage after therapy in 2.5 (range 2.5–10) mg/day. Additional follow-up and a larger number of patients are needed; however, these preliminary data suggest that high-dose Cy can produce durable complete remissions in patients with refractory PV.

P001

Pemphigus Vulgaris (PV): The Cleveland Clinic Experience 1987–2000

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The purpose of the study was to assess the clinical and histological profile and treatment outcomes in a consecutive group of patients with PV followed at one center. A retrospective chart analysis with telephone follow-up on 56 PV patients seen at the CCF between 1987 and 2000 was performed. The gender of patients was 68% female and the mean age of onset of PV was 48 years. The areas of involvement: 56 (100%) oral, 38 (67.9%) skin, 16 (28.6%) genital, 15 (26.8%) nasal, < 25% pharyngeal, ocular, laryngeal, anal, or esophageal involvement (in descending order). 63 (96.9%) of 65 biopsies in 53 patients were consistent with PV. 43 (81.1%) of 53 direct immunofluorescence tests performed in 46 patients were consistent with PV. 45 (90.0%) of the 50 indirect immunofluorescence tests performed were positive for PV. 44 (78.6%) achieved complete remission (CR) with a median duration of 17 months. The medications used at onset of CR were prednisone in 30 (68.2%), dapsone in 10 (22.7%), azathioprine in 8 (18.2%), and no treatment in 5 (11.4%), topical corticosteroids, cyclophosphamide, oral methyl prednisolone, tetracycline, niacinamide, and mycophenolate (in descending order). The median time needed to achieve CR after the first visit was 9 months. The majority of PV patients were females. Middle age was the time at which the disease most often presented. The oral cavity was involved in all cases over the course of the disease. Histologic examination of biopsy material confirmed the diagnosis of PV most reliably. CR was achieved in the majority of patients using combinations of medications. Drugs associated with CR most frequently were prednisone, dapsone and azathioprine either alone or in combination.

P002

Levels of IgG, IgG1 and IgG4 Antibodies to Desmoglein 3 in Sera of Three Patients with Pemphigus Vulgaris Undergoing Pulse Intravenous Methylprednisolone Therapy

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It is known that patients with pemphigus vulgaris (PV) respond to oral immunosuppressive agents before titers of their pemphigus antibodies start to decrease. Moreover, IgG1 and IgG4 pemphigus antibodies predominate in sera of patients with this disease.

The aim of this study was to evaluate the levels of serum IgG, IgG1 and IgG4 antibodies to desmoglein 3 in patients with PV undergoing pulse intravenous methylprednisolone therapy.

Three patients were administered 3 g of methylprednisolone IV over 3–6 days. Their sera were checked for the levels of IgG, IgGI and IgG4 antibodies a day before starting a pulse and a day after completing the pulse with indirect immunofluorescence on normal human skin, and desmoglein 3 Elisa (MBL) modified to evaluate not only IgG but also IgGI and IgG4 antibodies. Despite the rapid cessation of a formation of new blisters and healing of already established lesions, there was no decrease of levels of serum IgG, IgG1 and IgG4 antidesmoglein 3 antibodies.

Therefore, it seems that antidesmoglein 3 antibodies alone might not be sufficient to maintain a disease process in PV.

P003

Immunological Follow-Up of Patients with Pemphigus Vulgaris

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There is major interest in defining immunological markers that can be applied to monitor the activity of pemphigus vulgaris (PV). We thus sought to correlate the clinical activity of PV with T helper (Th) cell and auto-antibody (Ab) reactivity against desmoglein 3 (Dsg3), the major autoantigen of PV.

Reactivity of Ab subtypes against Dsg3 was analysed by immunoblot and ELISA analysis utilizing baculovirus-derived recombinant Dsg3. The cell reactivity against Dsg3 was quantitated by ELISPOT analysis.

A total of 41 patients with PV were examined by immunoblot analysis. In active PV, Dsg3-reactive IgG1 was detected in 20/33 (60.6%), IgG4 in 29/33 (87.9%), IgA in 21/33 (63.6%) and 1gE in 4/33 (12.1%) sera. Sera from patients with remittent PV contained Dsg3-reactive IgGl in 6/8 *(75%)* and IgG4 in 5/8 (63%) but not Dsg3-reactive IgA and 1gE. By ELISA, sera from patients with active disease contained titers of Dsg3-reactive IgG4 that exceeded those of IgG1 and IgA by a factor of at least 2. By ELISPOT assay, three patients with active oral PV had 3.8 ± 1.4 Dsg3-reactive Th1 cells and 9.1 ± 2.1 Th2 cells/10^5 PBL while 0/3 PV patients in remission had detectable autoreactive Th cells/10^5 PBL. Our study suggests that, in addition to quantitating total IgG reactivity to Dsg3, the titers of Ab subtypes may be helpful therapeutic and/or prognostic markers in PV. Quantitation of autoreactive Thi and Th2 cells may represent an additional highly sensitive parameter of disease activity: and may provide further insight into the role that autoreactive TH cells play in the pathogenesis of PV.

P004

Pemphigus Erythematosus: An Attempt to Redefine the Clinical and Immunopathological Features

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Diagnostic criteria for Pemphigus erythematosus (PE, Senear–Usher syndrome) are controversial. PE was originally described as a variant of pemphigus with additional features of lupus erythematosus. Other associated diseases included thymoma, myasthenia gravis and rheumatoid arthritis. By direct immunofluorescence (DIF) PE typically shows intercellular substance (ICS) and basement membrane zone (BMZ) staining. Its diagnosis is often based on the localization of eruptions in seborrheic and/or malar areas and currently PE is frequently regarded a localized early form of pemphigus foliaceus (PF).

In this report we describe two patients with erythematous erosive skin lesions without mucosal involvement. The first patient with 11 years' medical history of seropositive rheumatoid arthritis showed generalized erosive lesions with crusts whereas the second patient with an unremarkable medical history displayed a single erosive lesion with crusts confined to the forehead. Lesional biopsies showed superficial acantholysis; DIF testing revealed an ICS staining pattern with IgG and C3 in both patients. In addition, a prominent granular deposition of C3 along the BMZ was present. Indirect immunofluorescence (IIF) performed on monkey esophagus found circulating anti-ICS antibodies with a titer of 320 and 80, respectively; using normal human "salt split skin" as a substrate both patients' sera contained circulating anti-BMZ IgG antibodies that bound to the epidermal side. By ELISA and by Westernblot desmoglein-1 (Dsg-1) specific antibodies were detected in the first patient's serum. In contrast, desmoglein-3 (Dsg-3) specific antibodies were present in the serum of the second patient. In addition, both sera contained antibodies targeting a 230-kDa band comigrating with the bullous pemphigoid antigen-1 (BPAg-1). Laboratory studies revealed a positive rheumatoid factor and elevated antinuclear antibodies in the former but not in the latter patient. Despite the differences in the extent of skin involvement, in autoantibody reactivity against desmogleins and in associated diseases, we believe that both patients qualify as PE because they show similar superficial primary lesions without involvement of mucous membranes and both display a combined ICS and BMZ staining pattern by DIF.

Thus, PE can occur as a generalized as well as a localized disease and does not represent simply a localized form of PF. In addition, we propose that PE is not necessarily associated with other autoimmune diseases and the PE sera not only target Dsg-1 but can also target Dsg-3. The significance of the antiBPAg-1 antibodies in both patients' sera escapes us at the present time; while they may simply represent an innocent bystander phenomenon it is tempting to speculate that they serve as a trigger of the antidesmoglein mediated disease. Larger studies are warranted to investigate if the presence of anti-BPAg-1 antibodies is of regular occurrence in PE.

P005

IgA Pemphigus. Report of Five Cases with Different Therapy Approach

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IgA pemphigus is relatively newly described, rare, autoimmune-mediated blistering disease characterized by intraepidermal neutrophilic pustules, intercellular IgA deposits, and circulating IgA antibodies in some cases.

In two year period five female patients with IgA pemphigus presented to our Department. Patients were aged between 30 and 77 years and presented with different clinical pictures – two of them presented with pruritic erythematous patches of their forehead, scalp and intertriginous areas which were partly covered with yellowish crusts, other two patients had vesicles, erosions and crusts in periumbilical and presternal region. One patient had vesiculopustules on her trunk and in the scalp. In histopathology there was finding of intraepidermal blister in the upper epidermis with acantholitic cells in two of them and without acantholitic cells in others. Lymphocytic spongiosis was also present. In direct immunofluorescence in all patients IgA intercellularly was detected, in two of them there was also finding of C3 in the basement membrane zone, and in one patient IgA was detected intercellularly and in the basement membrane zone. In three patients, indirect immunofluorescence was positive for IgA intercellularly. In four patients therapy with prednisolone in a dose of 1 mg/kg body weight was introduced. In two of them remision was achieved with this therapy, in one patient azathioprine was introduced as adjuvant therapy, along with corticosteroid dose reduction (because of steroid diabetes in a patient), and in other one we had to introduce Dapsone in a dose of 150 mg daily, because of poore response to prednisolone. In fifth patient we introduced Dapsone as a drug of choise, which resulted in considerable improvement in 10 days.

We conclude that IgA pemphigus is relatively benign type of pemphigus disease with specific histopathological and immunofluorescent findings, with different clinical presentations as well as with different possibilities of therapy approach.

P006

Pemphigus Foliaceus as a Model of Polygenic Disease

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We previously described a silent single nucleotide polymorphism (SNP) of the desmoglein 1 gene which consists of a T to C transition at position 809. To investigate the role of genetic background in pemphigus foliaceus and to ask whether PF, like other autoimmune diseases, is expressed as a complex trait, we simultaneously examined the role of major histocompatibility complex (MHC) class II polymorphism and SNP(809) in PF susceptibility.

Thirty-one Caucasian French patients and 84 healthy Caucasian French controls were studied by PCR-RFLP for SNP(809) genotyping and by PCR-SSO and PCR-SSP for DRB1 and DQB1 typing.

This analysis confirmed involvement of DRB1*04 ($p = 0.01$) and DRB1*14 ($p = 0.04$) generics in disease susceptibility and individualized DRB1*0102 ($p = 0.04$), DRB1*0402 ($p = 0.02$), DRB1*0406 (0.003), and DRB 1*1404 ($p = 8.10^{-4}$) as susceptibility MHC class II alleles in French PF patients. Homozygous C/C(809) genotype was also found associated with the disease ($p = 0.03$). Furthermore, patients with both DRB1*04 and C/C(809) had a very significant risk to develope PF ($p = 1.10^{-4}$) and comparison, by logistic regression, of susceptibility given by both risk factors showed a significant interaction between DRB1*04 and C/C(809).

DRBl *04 and C/C(809) are significant risk factors to PF and interact to enhance disease susceptibility. PE therefore constitutes another demonstrative example of the role of epistatic interaction of individual genes in autoimmune diseases susceptibility.

P007

The Pathogenic Study of ECI-2 of Pemphigus Vulgaris Antigen

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Purpose Pemphigus Vulgaris autoantigen (PVA) is a transmembrane glycoprotein, which is called desmoglein 3 (Dsg3). It has five extracellular domains (ECI to EC5). The purpose is to test whether EC1-2 is immunogenic domain and whether its antibody is pathogenic by establishing neonatal mouse model of PV.

Methods A segment of the human Dsg3 cDNA (about 1100 bp in the amino terminus, including EG1-2) was subcloned into glutathione S-transferase (GST) gene by *pGEX-4T-l* expression vector, and the recombinant vector was used to generate GST fusion protein (FP) containing the ECI-2 segment of Dsg3. The FP was expressed in *E.coli. JMIO9* and was purified by glutathione affinity chromatography. It was confirmed by nucleotide sequence analysis and immunoblotting with anti-GST antibody. We also tested PV patients by immunoblotting analysis with purified EC1-2 FP. New Zealand white rabbits were immunized with the EC1-2 FP. IgG fraction was got from the antisera of rabbit by precipitation with caprilic acid, followed by DEAE-52 ion-exchange chromatography. Then it dialyzed agaist phosphate-buffered saline (PBS, pH7.4) and concentrated with Polyethylene glycol 20000 to 40mg/mI. Purified lgG fraction was injected subcutaneosly into the upper back skin of neonatal BALB\c mice (<24h of age). The lgG dose was 200pl each time, two doses 5h apart, about lOmg/g body weight. Neonatal mice were examined 16h after injection. Skin was studied by light microscopy, electronic microscopy and direct immunofluorescence (DIF). Serum was assayed by indirect immunofluorescence (IIF).

Results Immunoblotting analysis of EC1-2 FP with 22 PV patients' sera demonstrated that 17 PV patients were positive. By DIF, the skin of mice showed lgG antiserum deposition on the cell surface of acantholytic cells. By IIF, using normal neonatal mouse skin as substrate, the titer of lgG was 40-80. Histologically, we can see stratum corneum detached and acantholysis, but no blisters in the epidermis by light microscopy. Electronic microscopy examination confirmed that wider intercellular spaces (105) occurred in the regions of the cell surface.

Conclusions These results show that EC1-2 in the amino terminus of Dsg3 is immunogenic in PV and its antisera is pathogenic. The anitbody of EC1-2 FP can induce a neonatal mouse model of PV which gives a tool of studying autoimmune disease.

P008

Direct Immunofluorescence of Skin, Oral Mucosae and Esophagus in Pemphigus Vulgaris Patients in Remission

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Objective To compare the results of skin, oral mucosae and esophagus direct immunofluorescence (DIF) tests of patients with pemphigus vulgaris in the remission stage of treatment.

Design Cross-sectional study. Setting: Tertiary care center.

Methods Female or male adult patients with history of confirmed pemphigus vulgaris in remission stage (absence of skin or mucosal lesions for more than 6 months, with <20 mgs. prednisone /day) , with informed consent had three biopsies: skin, oral mucosae, and esophageal mucosae. Esophagus biopsies were obtained by endoscopic means. Tissues were tested with a standard method of direct immunoflourescence.

Results 8 female and 2 male patients, 46 (S.D.: 12.2) years of mean age and 11 (S.D: 6.2) months mean time under remission were included. 4 patients were not taking already prednisone. Nine had objective esophageal abnormalities when endoscopy was performed. DIF was positive in 7 esophagus biopsies, 4 oral mucosae biopsies and only 3 skin biopsies. Sensitivities of DlFskin test and of DIF oral mucosae test were 43% and 57% as compared to DIF esophagus mucosae test.

Conclusions A negative DIF esophageal mucosae test may be useful when the decision to stop treatment becomes an issue.

P009

Kininogens-Kallikreins-Kinin System in Plasma of Brazilian Patients with Pemphigus Foliaceus

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Objective Pemphigus foliaceus (PF) is an autoimmune bullous disease affecting the skin caused by IgG4 autoantibodies against desmoglein. Considering that are few studies involved kallilcrein-kinin patterns in PF, we evaluated activation of kinins in this condition.

Methods Fifteen patients (11 men and 4 women) presenting active PF with nikolsky sign positive, 15 controls subjects were studied. The concentration of total kininogen (TKg), low molecular weight kininogen(LKg) and was determined by ELISA(J Reumathol., *25 : 1*–4,1988).The activity of plasma kallikrein and tissue kallikrein were evaluated upon selective substrates.

Results The results are shown in the table and indicate the median values of kininogens and kallikreins parameters, we observed decreased kininogens in plasma of PF and impaired values of tissue kallikrein and plasma kallikrein of PF patients *compared with controls*.

Conclusion The impaired levels of kallikrein and a decreased kininogens suggested a possible activation of kinin system and participation of acantholysis observed in PF.

P010

Dendritic Cells in Endemic Pemphigus Foliaceus (EPF)

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To elucidate the pathophysiology of EPF, dendritic cells (DC) were measured in skin biopsies (lesional skin) from 22 EPF patients and in normal thoracic sun-protected skin of a non perilesional area in 13 of them. Controls consisted of normal thoracic skin from 8 cadavers and from 12 women submitted to breast plastic surgery. DC were identified with anti-CDIa and quantified by morphometry. Epidermal DC numbers in lesion [60.18 DC/mm^2, 5.00 DC/mm basement membrane (BM), 3.55 DC/mm stratum corneum (SC)] and normal skin (28.45 DC/mm^2, *2.50* DC/mmBM, 2.87 DC/mmSC) of EPF patients were similar to those for the plastic surgery (72 35 DC/mm^2, 4.53 DC/mmBM, 4.42 DC/mmSC) and cadaver controls (47.15 CD/mm^2, 2.53 CD/mmBM, 2.42 CD/mmSC). Dermal DC number in lesions (0.98 DC/mmBM) of EPF patients was similar to the plastic surgery control (0.48 DC/mmBM), but higher than in the cadaver controls (0.13 DC/mmBM, $p < 0.05$). The epidermal DC/mmMB/dermal DC/mmMB ratio was lower in lesional EPF skin (5.72) than in controls (9.22, $p < 0.05$), confirming the higher DC number in dermis in the former. In the same patient, the amounts of epidermal and dermal DC were higher in EPF lesional skin (61.50 DC/mm^2, 5.49 DC/mmBM, 6.64 DC/mmSC, 0.86 dermal DC/mmBM) than in normal skin (28.45 DC/mm^2, 2.50 DC/mmBM, 2.87 DC/mmSC, 0.04 dermal DC/mmBM). A direct association was found between dermal DC/mmMB in lesional skin of EPF patients and titration of serum antibodies by IFI ($r = 0.4729$, $p < 0.O5$), confirming that dermal DC could play an important role in EPF pathogenesis. We may propose that DC may be in transit through the dermis towards the regional lymph nodes, stimulating T lymphocytes to produce autoantibodies.

P011

Detection of Pemphigus Autoantibodies by Immunoblot in Tunisian Patients

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Introduction Pemphigus is a rare organ-specific autoimmune blistering disease involving skin and mucous membrane. Autoantibodies are pathogenic and are directed against desmosomes adhesion molecules . Pemphigus foliaceus (PF) antigen is desmoglein 1 (Dsg 1) and Pemphigus vulgaris (PV) antigen is desmoglein 3 (Dsg3). We looked in this study for the different autoantigens recognized by autoantibodies with immunoblot method in Tunisian pemphigus patients.

Patients and methods

Patients 43 with PF, 10 male (mean age 52 years) and 33 female (mean age 35 years), 35 with PV, 6 male (mean age 50 years) and 29 female (mean age 46 years).

Methods Immunoblot using human epidermal extracts for detecting IgG class and subclasses of different autoantibodies.

Results Anti-Dsg 1 and anti-Dsg 3 were found in PF patients in 65% (28/43) and 2% (1/43), but in PV patients they were found in 11,4% (4/35) and 45,7% (16/35)

Conclusion Most of the PF patients are young women. This data was previously reported in Tunisian epidemiological studies. Anti-Dsg 1 characterize Tunisian PF patients and anti Dsg 3 characterize Tunisian PV Patients. Updated January 15, 2000.