A VALIDATION OF ELISA TEST FOR THE DETECTION OF DESMOGLEIN 1 & DESMOGLEIN 3 ANTIBODIES AS A DIAGNOSTIC TEST OF PEMPHIGUS

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A

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CERTIFICATE

This is to certify that the dissertation titled **'A Validation Of Elisa Test For Detection Of Desmoglein 1 & 3 Antibodies As A Diagnostic Test Of Pemphigus'** is the bonafide work of **Dr. Teena Mathew**, in fulfillment of rules and regulations for the **M.D., Branch XII A, Dermatology, Venereology and Leprosy** Examination of **The Tamil Nadu Dr. M.G.R. University**, to be held in 2007.

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CONTENTS

INTRODUCTION

Pemphigus is the commonest autoimmune vesiculobullous disorder on the Indian subcontinent.[1] A study on mortality among inpatients in dermatology from India found pemphigus vulgaris to be the commonest disorder to cause death.[2] Pemphigus is an autoimmune disease that results in blistering of the skin and oral cavity, which if left untreated is almost always fatal. It is caused by autoantibodies directed against cell-surface antigens on keratinocytes, which when targeted lose their cellular adhesion properties and separate from one another to form blisters within the epidermis.[3] It is classified into two major subtypes: pemphigus vulgaris (PV) and pemphigus foliaceus (PF).[4] Differences in the particular antigens targeted by the antibodies and in the distribution of these antigens in the different regions of the body and in the separate layers of the epidermis result in different clinical manifestations of the disease.[3] The diagnosis of pemphigus rests upon the demonstration of these autoantibodies by immunofluorescence techniques whilst the differentiation of PV and PF relies upon the combination of clinical and histological features. Although Western immunoblotting and immunoprecipitation can be used to identify the target antigen in cases of pemphigus, these are both time consuming qualitative techniques which are impractical for routine screening of large number of serum samples.[5] Pemphigus autoantibodies are mainly directed against the desmosomal adhesion molecules, desmoglein 1 (Dsg 1) and desmoglein 3 (Dsg 3). The

production of recombinant Dsg 1 and Dsg 3 antigens has made possible the development of enzyme linked immunosorbent assays (ELISAs).[6] Recently Dsg 1 and Dsg 3 ELISAs have been shown to provide a simple, sensitive and highly specific assay for the diagnosis of patients with PV and PF.[7] Since precise diagnosis of these two diseases is important for determining treatment plans and prognosis, sensitive and specific testing for the pathogenic autoantibodies found in PV and PF would be helpful in the clinical management and laboratory investigation of these two severe autoimmune diseases.[6]

As there is a paucity of studies from India,[8,9] the present study is an attempt to validate ELISA estimation of desmoglein antibodies as a diagnostic test for pemphigus and to study the prevalence of desmoglein antibodies in the various clinical types of pemphigus in Indian patients.

AIMS OF THE STUDY

1) To validate ELISA estimation of Dsg 1 and Dsg 3 antibodies in pemphigus as a diagnostic test in Indian patients.

2) To study the correlation of Dsg 1 and Dsg 3 antibody titres with
   a) mucosal and cutaneous involvement
   b) severity of disease.

3) To investigate the prevalence of Dsg 1 and Dsg 3 antibodies in the different pemphigus variants.

REVIEW OF LITERATURE

Introduction:

Pemphigus is an autoimmune blistering disease targeting skin and mucous membranes. It is divided into two major subtypes: PV and PF. Autoantibodies in pemphigus play a primary pathogenic role in initiating blister formation. Currently, the diagnosis of pemphigus largely relies on immunoflourescence testing utilising direct immunoflourescence (DIF) staining and indirect immunoflourescence (IIF). Despite the different antigens involved in each disease the cell staining pattern using DIF or IIF is virtually identical making it difficult to distinguish between PV and PF. Immunoblotting and immunoprecipitation studies have demonstrated the target molecules of pemphigus to be a 130-kDa glycoprotein in PV and a 160-kDa glycoprotein in PF.[6]

History:

Pemphigus is derived from the Greek term, *pemphix*, meaning blister or bubble.[10] De Sauvages in 1760 coined the word 'pemphigus'.[11] In 1943 Civatte reported histopathological findings in various types of pemphigus and coined the term acantholysis. In 1964 Beutner and Jordan demonstrated that autoantibodies are directed against the squamous epithelial cell surface in pemphigus. In 1982 Anhalt et al constructed an animal model for pemphigus by passive transfer of immunoglobulins (IgG) from patients to neonatal mice.[12]

Definition:

The term 'pemphigus' refers to a group of autoimmune blistering diseases of skin and mucous membranes that are characterized histologically by intraepidermal blisters due to acantholysis and immuopathologically by in vivo bound and circulating IgG directed against the cell surface of keratinocytes.[13]

Demographics and Incidence:

The mean age of onset of pemphigus in India has been reported to be 36.9 years and 39.5 years in different studies, whereas a higher mean age of onset of 52.7 years has been reported from United Kingdom (UK).[14,15] In India no sex predilection was observed.[15] In the UK the commonest subtypes are PV and PF with equal prevalence (44.4%), but in India PV is the most frequent (79.4%), while PF is uncommon (7.6%) and with equal prevalence of the other subtypes.[14] The exact incidence of disease and prevalence of PV compared with that of PF depend very much on the population studied. The highest incidence of pemphigus is among Jews of Ashkenazi origin.[16]

Etiology:

The predisposition to pemphigus is linked to genetic factors. Certain major histocompatibility complex (MHC) class II genotypes, in particular alleles of HLA-DRB1*04 and DRB1*14 subtypes are common in patients with PV across racial barriers.[10] In India a haplotype related to DSG3*TCCTC is

significantly associated with PV as it is found in 20% of patients vs 6% controls (p=0.002).[17] Pemphigus may also occur in patients with other disorders characterised by immunological disturbances.[18] Viral DNA has been detected in some skin biopsies or peripheral blood mononuclear cells from pemphigus patients.[19,20] Endemic pemphigus foliaceous, also known as fogo selvagem, common in rural parts of South America is probably triggered by an environmental factor.[21] Drugs may exacerbate or induce pemphigus.[10] A distinctive form of pemphigus has been described in association with a variety of underlying neoplasms.[22]

Types: [10]

The different types of pemphigus is shown in Table 1.

Pathophysiology of pemphigus:

PV and PF are characterized by intraepidermal blister formation due to loss of cell adhesion of keratinocytes and immunopathologically by the findings of in vivo bound and circulating IgG directed against the cell surface of keratinocytes.[6]

Pathogenicity of autoantibodies in pemphigus:

Autoantibodies in pemphigus play a primary pathogenic role in initiating blister formation[12] Disease activity in patients often correlates with anti-cell surface antibody titre as determined by IIF.[23] Also neonates of mothers with PV may have transient disease due to maternal IgG that crosses the placenta.

As maternal antibody is catabolised, the disease subsides.[24] In skin organ culture IgG alone without complement or inflammatory cells from patients with pemphigus can induce loss of cell-cell adhesion, with the same histology as seen in patients. Passive transfer of IgG from patients sera to normal mice results in loss of cell-cell adhesion and blisters with typical histological findings.[12,25] However between 40 and 71% of immediate relatives of pemphigus patients have been found to be carriers of low titre PV autoantibodies.[26] Anti-intercellular substance (ICS) antibodies can be produced in several clinical conditions in which there is damage to the epidermis.[27] Antibodies that mimic or that may give in vitro deposition in stratified squamous epithelium in the absence of pemphigus have been reported in bullous pemphigoid, cicatricial pemphigoid, burns, toxic epidermal necrolysis, penicillin allergy and systemic lupus erythematosus.[28] The release of epidermal antigens caused by physical injury or biochemical insults may permit exposure of these antigens to the immune system with resultant production of anti-ICS antibodies. However in such cases these antibodies are only transiently present.[27]

Proposed mechanisms of acantholysis:

The mechanisms whereby these pemphigus antibodies induce acantholysis is an area of active investigation.[29]

Organ and cell culture studies have suggested that binding of pemphigus autoantibodies to the keratinocyte cell surface causes release of a protease which in turn causes acantholysis.[30,31,32] The identity of this proteolytic enzyme is in dispute. Some studies suggest that it is plasminogen activator that, once released by keratinocytes activates, plasminogen to plasmin which in turn causes acantholysis.[33]

The role of complement in blister formation is controversial. Pemphigus sera are capable of causing acantholysis in skin organ culture and detachment of keratinocytes in cell culture without complement.[26,30] However complement does enhance pemphigus antibody mediated detachment of cultured epidermal cells, probably by damage to cell membranes.[34,35]

Recent studies have also proposed a novel explanation for acantholysis in pemphigus, based on the basal cell shrinkage hypothesis.[36]

There is abundant evidence from various studies that desmogleins and anti-desmoglein antibodies are directly involved in causing blisters in PV and PF. Patients with PF have anti-Dsg1 antibodies[6,37] and patients with PV have anti-Dsg 3 antibodies with greater than 50% having, in addition, anti-Dsg1 antibodies.[6,38] Patients with PV limited to mucous membranes usually have only anti-Dsg3 antibodies[6,39,40] whereas, PV patients who develop extensive skin involvement in addition to oral involvement develop anti-Dsg1 antibodies in addition to anti-Dsg3 antibodies.[6,39,41] Passive transfer studies in neonatal mice confirm the findings in humans that both anti-Dsg 3 and anti-Dsg 1 antibodies are necessary for efficient blister formation in skin. In neonatal

mouse model of pemphigus, PV sera with anti-Dsg 3 alone are much less efficient at causing skin blisters than those with anti-Dsg 3 and anti-Dsg 1. Furthermore, addition of small amounts of anti-Dsg 1 antibodies from PF sera dramatically enhances the formation of PV skin blisters caused by PV sera containing only anti-Dsg 3 antibodies.[42] Inactivation of Dsg 3 by genetic means causes lesions in mice that are strikingly similar to those seen in pemphigus patients with anti-Dsg 3 antibodies.[43] Also pathogenic activity can be adsorbed from PF and PV sera by the extracellular domains of Dsg 1 and Dsg 3 respectively.[44] Dsg 3^{null} mice can be immunised with Dsg 3 to produce anti-Dsg 3 antibodies that cause typical lesions when transferred to Dsg 3+/+ mice.[45]

However Nguyen et al[46] propose that antibodies against keratinocyte antigens other than desmoglein 1 and 3 can induce pemphigus vulgaris like lesions. Dsg 3 lacking mice were injected with PV IgGs that did not cross react with the 160 kDa Dsg 1 or its 45 kDa immunoreactive fragment and that showed no reactivity with recombinant Dsg 1. These PV IgGs caused gross skin blisters with PV like suprabasal acantholysis and stained perilesional epidermis in a fish net like pattern indicating that the PV phenotype can be induced without anti-Dsg3 antibody. The anti-Dsg1 antibody was also not required as its presence in PV IgG does not alter the PV like phenotype in skin organ cultures and because pemphigus foliaceous IgGs produce a distinct phenotype in Dsg 3^{null} mice.

Countering the theory proposed by Nguyen et al, Stanley et al[47] contend that all passive transfer experiments that resulted in blisters were performed with Dsg 3^{null} mice which already have altered adhesion and that there is no evidence that non Dsg antibodies produce pemphigus lesions in normal mice or humans. Amagai et al[48] have opined that although other antibodies may be involved, there is little to validate this alternative theory and that in fact it has been clearly shown that mouse and human anti-Dsg antibodies alone cause typical blisters of pemphigus.

There have been recent discoveries of two new human keratinocyte molecules targeted by disease-causing PV IgGs namely (a) the α9 acetylcholine receptor with dual muscarinic and nicotinic pharmacology and (b) pemphaxin a novel annexin that can act as a acetylcholine receptor. [49,50]

It has been proposed that acantholysis in pemphigus is mediated by at least two complementary pathways, namely, (1) anticholinergic receptor antibody that weakens intercellular adhesions between keratinocytes via inactivation of the cholinergic receptor mediated physiologic control of cadherin (Dsg) expression and/or function that causes dyshesion, cell detachment and rounding up or acantholysis and (2) antibodies to adhesion molecules that prevent formation of new desmosomes in acantholytic keratinocytes by blocking the extracellular domain of desmosomal cadherins that mediate homophilic adhesion. These separate pathophysiological pathways targeting 2 different types of keratinocyte cell surface molecules (i.e

cholinergic receptor and desmoglein) may explain high affinity and low affinity binding of pemphigus IgGs to keratinocytes.[51]

Stanley et al[48] further opine that though some interesting and significant observations about cholinergic recepors in keratinocytes and their adhesion have been made there is no convincing evidence for their role in the pathogenesis of pemphigus and that the significance of non desmoglein antibodies to the pathophysiology of pemphigus remains to be proved.[48]

Distribution, structure and function of desmosomes: (Fig. 1)

Desmoglein, a cadherin type of adhesion molecule is found in desmosomes. Desmosomes are cell–cell complexes found primarily in epithelial tissues but also in the meninges, the dendritic reticulum cells of lymph node follicles and the myocardium. They represent the major intercellular adhesion mechanism in both follicular and interfollicular epidermis, anchoring keratin intermediate filaments to the cell membrane and bridging adjacent keratinocytes and allowing them to withstand trauma. Initially described as discontinuous button like structures of epithelia, desmosomes are now also recognized as signaling intermediates composed of an emerging and expanding network of tissue specific membrane and membrane-cytoskeletal linker molecules.[52] The basic function of the desmosome is to attach the keratin cytoskeleton of one cell to the cytoskeleton of adjacent cell. There must be intermediate proteins to do this, because keratins do not cross the cell membrane. In the desmosome the multiple proteins that compose the

structure can be clumped into 3 functional groups (1) the keratin filaments that link to (2) the “plakin” proteins which are found just under the plasma membrane and link to (3) the transmembrane proteins, desmoglein. These desmogleins then hook into desmoglein/plakin/keratins in the adjacent cell and complete the anchorage of both cells’ cytoskeletons to each other (keratin-plakin-desmoglein-plakin-keratin).[53]

Desmogleins and their expression :

Desmosomal cadherins are of two types, desmogleins and desmocollin, both of which occur as 3 isoforms, Dsg1, Dsg 2, and Dsg 3 and Dsc 1, Dsc 2, and Dsc 3.[54]

Desmoglein 3, a 130–kd transmembrane glycoprotein and desmoglein1, a 160-kd glycoprotein of the cadherin family, mediate weak calcium dependent homophilic cell- cell adhesion of epidermal keratinocytes. In the skin, Dsg 3 is expressed in the lower part of the epidermis mainly in the basal and immediate suprabasal layers, while Dsg 1 is expressed throughout the epidermis, but more intensely in the superficial layers. In mucous membranes Dsg 3 is highly expressed throughout the mucosa while expression of Dsg 1 is much less than in the skin.[55] Desmoglein 2 is expressed in all desmosome possessing tissues including simple epithelia and myocardium whereas Dsg 1 and Dsg 3 are usually restricted to stratified squamous epithelia where blister formation is found in pemphigus.[56,57] Since either desmoglein can mediate adhesion, the reason for their differential distribution is not known.[58]

Desmogleins are targeted in skin-blistering diseases such as pemphigus, staphylococcal scalded skin syndrome and bullous impetigo.[59,60]

Desmogleins in the various clinical forms of pemphigus: (Table 2)

The distinct forms of pemphigus are determined by which specific desmoglein is targeted and whether additional protein antigens are also involved. Although there are three immunologic variants there are many clinical variants. In paraneoplastic pemphigus a much broader range of desmosomal proteins are targeted because the plakin proteins are involved.

In pemphigus foliaceus blistering occurs in and around the granular cell layer of the epidermis These patients have antibodies against desmoglein 1. This desmoglein is expressed mainly in the upper layers of the epidermis and antibodies binding to it cause acantholysis only where it is primarily expressed. Desmoglein 1 is present in the oral mucosa but mucosal lesions never occur because there is co expression of Dsg 3 in the oral epithelium and this is able to keep the cells from detaching. Pemphigus vulgaris is usually characterized by oral lesions and progression to cutaneous lesions. In PV, antibodies impair the adhesive function of Dsg 3 and cause acantholysis in the oral cavity. As PV progresses many but not all patients will experience cutaneous disease. This seems to be caused by spread of the antibody response and development of antibodies against both Dsg 1 and 3.[53]

Intramolecular epitope spreading in pemphigus vulgaris:

At the early stage of the disease (mucosal PV) patients display only autoimmunity to Dsg 3 and develop mucosal blisters, while at the later stages of the disease (mucocutaneous PV) patients exhibit non-cross reactive autoimmunity to both Dsg 3 and Dsg 1 and acquire cutaneous as well as mucosal blisters. At these two disease stages, Dsg3 autoantibodies exhibit different tissue binding patterns and pathogenic activities, suggesting that they may recognize distinct epitopes.[61] Salato et al[61] tested this hypothesis and demonstrated that autoantibodies from the majority of mucosal PV patients target epitopes at the COOH-terminal portion of the Dsg 3 ectodomain. Interestingly only antibodies against the Dsg 3 NH2-terminal epitope are able to bind to human skin. They also discovered that the intramolecular epitope spreading from Dsg 3 (87-566) to Dsg 3 (1-88) is a critical step that precedes the intermolecular epitope spreading from Dsg 3 to Dsg 1. During disease transition this mechanism dictates the development of Dsg autoantibodies that recognize human skin and lead to expression of cutaneous PV lesions.[61]

Desmoglein compensation hypothesis: (Fig. 2)

Pemphigus foliaceus sera contain only anti desmoglein 1 antibodies and cause blisters by interfering with the function of Dsg 1, where there is no Dsg 3 to compensate. In the superficial epidermis of skin in early pemphigus vulgaris when patients produce only anti Dsg 3 antibodies, separations develop deep in the mucous membrane where Dsg 1 will not compensate for

loss of Dsg 3 mediated adhesion. Later in the course of the disease when, the patient's serum contains both anti-Dsg 1 and anti-Dsg 3 antibodies the function of both Dsgs is compromised and blisters occur in the skin and mucous membranes.[62]

Desmoglein compensation also explains why neonatal pemphigus foliaceus is so unusual even though the antidesmoglein 1 antibodies cross the placenta. In neonatal skin as opposed to adult skin, desmoglein 3 is co-expressed with desmoglein 1 in the superficial epidermis.[63]

Recently described experiments that explain blister localization in PF on the basis of the Dsg compensation hypothesis also suggest that anti-Dsg antibodies cause disease by inhibiting Dsg-dependent adhesion. The validity of this hypothesis has been confirmed in experiments with PV and PF antibodies in normal and Dsg 3^{null} mice. The Dsg compensation hypothesis is incompatible with the protease theory of blister formation. If protease were the major cause of blister formation in pemphigus then binding of autoantibodies to either Dsg 1 or Dsg 3 will not cause protease release and blister formation and prevent functional compensation of one by the other.[62]

Clinical features of pemphigus variants:

Pemphigus Vulgaris:

Pemphigus vulgaris, the most common type of pemphigus, predominates in middle-aged and elderly Jewish patients and is characterized by formation of flaccid blisters and/or erosions. Neonates born to mothers with

active PV may have similar lesions during the first few months of life.[62] Mucosal dominant PV is characterized clinically by mucosal erosions mainly in the oral cavity with minimal skin involvement. Mucocutaneous PV is characterized by extensive skin blisters and erosions in addition to the mucosal lesions. Cutaneous type PV exists as a rare phenotype of pemphigus. This type is clinically characterized by PF-like scaly crusted erosions on the skin without mucosal involvement, although the skin lesions can be polymorphic, including tense vesicles or blisters in an annular arrangement, or eczematous lesions.[64]

Pemphigus vegetans is a variant of pemphigus vulgaris. It has been subdivided into two groups, the Neumann and the Hallopeau type. The Neumann type is characterised by vegetating lesions on the flexures. The Hallopeau type is a relatively benign form which may start as vesicles and pustules which erode and heal with vegetations. Extensive fissuring of the tongue called 'cerebriform tongue' has been classically decribed in pemphigus vegetans by Premlatha et al.[65]

Pemphigus foliaceus:

Pemphigus foliaceus, is a disease of the middle-aged and elderly but does not preferentially affect Jews. The most characteristic lesions of PF are scaling and crusted plaques on the trunk. In contrast to PV, oral lesions are almost never seen in PF. Intact blisters are also uncommon.[62]

Pemphigus erythematosus is a variant of PF with erythematous scaly lesions over the nose and cheeks in a butterfly distribution simulating cutaneous lupus erythematosus or seborrhoeic dermatitis. Sunlight may exacerbate the disease. Oral lesions are rare.[10]

Pemphigus herpetiformis is a variant of pemphigus foliaceus. The clinical picture is variable often with coalescent annular or gyrate vesiculopustular lesions.[66]

Intercellular IgA dermatosis:

Two types are distinguished based on the level of pustule formation and IgA deposition: the subcorneal pustular dermatosis type and intraepidermal neutrophilic type.[67] Patients with both types have flaccid vesicles or pustules arising on either erythematous or normal skin. The lesions may be pruritic and show a circinate or annular configuration with central clearing and evolve to crusted or scaly erythematous macules. The sites of predilection are the axillae and groins.

Induced pemphigus:

The clinical features of drug induced pemphigus may be atypical. The initial manifestation may be a non specific erythematous rash. The full-blown disease usually presents as scaling and crusting with superficial vesicles and blisters. Some cases may present with pemphigus erythematosus-like picture with crusting and scaling on the butterfly area of the face. Rarely some cases present with classical pemphigus vulgaris. Oral mucosa is involved in 50% of cases.[68]

Paraneoplastic pemphigus :

Paraneoplastic pemphigus (PNP) constitutes a distinctive syndrome characterized by extensive recalcitrant oral erosions and polymorphic skin lesions, that often occur in patients with known neoplasms (especially B-cell lymphomas, leukemias, thymomas or Castleman tumors).[62]

The transition between PV and PF is a rare medical event.[69,70,71] The transition from PF to PV is less common than that of PV to PF. The mechanism of transition remains elusive. One possible mechanism is epitope-spreading phenomenon which is considered as a primary autoimmune or inflammatory process that causes tissue damage by exposing immunologically hidden protein to the immune system thereby evoking a secondary autoimmune response.[72]

Diagnosis:

Cytodiagnosis:

Cytological examination using a Tzanck preparation is useful for the rapid demonstration of acantholytic epidermal keratinocytes in the blisters of pemphigus. A smear is taken from the underside of the roof and from the base of an early freshly opened bulla. Giemsa staining is done with subsequent rinsing and air drying.[73] Since acantholytic keratinocytes are occasionally seen in various non acantholytic vesiculobullous or pustular diseases as a result of secondary acantholysis, cytologic examination represents merely a preliminary test and should not supplant histological examination.[28]

Histopathology:

Early blisters, preferably small ones, should be selected for biopsy. If no recent blister is available an old one may be moved into the neighbouring skin by gentle vertical pressure with a finger.

Pemphigus vulgaris:

The earliest feature may either be eosinophilic spongiosis or more commonly spongiosis in the lower epidermis. Acantholysis leads first to the formation of clefts and then to blisters in a predominantly suprabasal location. The basal keratinocytes though separated from one another remain attached to the dermis like a “row of tombstones”. The blister roof consists of the remaining intact squamous epithelium. Within the blister cavity are acantholytic keratinocytes. Cutaneous type PV has suprabasal acantholysis as found in PV. Superficial blistering is occasionally observed.[28]

Pemphigus vegetans:

In the Neumann type, the early lesions consist of bullae and denuded areas that have the same histologic picture as that of pemphigus vulgaris. As the lesions age there is formation of villi and verrucous epidermal hyperplasia. In the Hallopeau type the early lesions consist of pustules arising on normal skin with acantholysis and formation of small clefts, many in a suprabasal location. The clefts are filled with numerous eosinophils and degenerated acantholytic epidermal cells. Early lesions may reveal more eosinophilic abscesses than in Neumann type.[28]

Pemphigus foliaceus:

The earliest change consists of acantholysis in the upper dermis,within or adjacent to the granular layer, leading to a subcorneal blister in some instances. The number of acantholytic keratinocytes is usually small. In the setting of a subcorneal blister, dyskeratotic granular keratinocytes are diagnostic.[28]

Pemphigus herpetiformis:

There are diverse histopathological patterns: intraepidermal and subcorneal microabscesses, eosinophilic spongiosis or superficial bullae with scant acantholytic cells.[66]

Pemphigus erythematosus:

The light microscopic features are identical to those of pemphigus foliaceous.[28]

IgA pemphigus:

Two patterns are observed that parallel the clinical presentation. In the first there are subcorneal vesiculopustules or pustules with minimal acantholysis. In the second, intraepidermal vesiculopustules or pustules which contain small to moderate number of neutrophils.[28]

Drug induced pemphigus:

The findings in early lesions are non specific, consisting of spongiosis, parakeratosis and a variable dermal infiltrate. Well developed lesions are essentially identical to those of pemphigus foliaceous or pemphigus vulgaris. Eosinophilic spongiosis may be prominent.[28]

Paraneoplastic pemphigus:

The principal findings are suprabasal acantholysis with, in addition, dyskeratosis. Characteristically there is also a vacuolar interface dermatitis with lichenoid inflammation. [28]

Direct immunofluorescence:

Currently the diagnosis of pemphigus largely relies on immunofluorescence testing utilizing both DIF and IIF. DIF testing is a very reliable and sensitive diagnostic test for Pemphigus, in that it demonstrates IgG in the squamous intercellular/cell surface areas in upto 95% of cases, including early cases and those with very few lesions, and in up to100% of cases with active disease.[74] It may remain positive, often for many years after the disease has subsided. Negative DIF findings when patient is in remission may be a good prognostic indicator. A study on immunofluorescence in pemphigus from North India has demonstrated the value of DIF for a definitive diagnosis of pemphigus. However, the authors also felt it important to appreciate that immunofluorescence is not a substitute for histopathology, but rather complementary to it.[15]

The edge of a blister with intact surrounding normal skin or uninvolved skin adjacent to a blister is taken for biopsy. The tissue may be snap frozen or transported in Michel's medium.[28] (Table 3)

Indirect immunofluorescence:

Indirect immunofluorescence is a two step procedure used to identify circulating autoantibodies to cutaneous or mucosal structures in patients serum.[75] Reproducible results can be obtained only if the substrate is appropriately selected with respect to the nature of the circulating antibodies. The best results are obtained by using normal human skin from unexposed sites. The other substrates used are monkey oesophagus, guinea pig lip and guinea pig oesophagus. In case of paraneoplastic pemphigus rat bladder epithelium is found to be an excellent substrate.[76] With serial dilutions of the patients serum, the highest dilution that results in fluorescence is taken as the titre. Although the titre generally correlates with disease activity, a single titre cannot be used as a basis for adjusting the drug dosage. Patients in remission but with persistent antibodies are likely to develop a relapse on cessation of therapy. A two fold rise in the titre may indicate an impending relapse. Continued negative titres for more than one month are good prognostic indicators of prolonged remission.[75]

Immunoblotting and Immunoprecipitation:

Although Western immunoblotting and immunoprecipitation can be used to identify the target antigen in cases of pemphigus these are both time consuming, qualitative techniques, impractical for routine screening of large samples.[5]

Immunoblotting and immunoprecipitation studies have demonstrated that the target molecules of pemphigus are, a 130-kDa glycoprotein in PV and a 160 kDa glycoprotein in PF.[6] Hashimoto et al[77] studied 18 cases of pemphigus characterized by diagnostic difficulty and suggested that immunoblot analysis for pemphigus antigens is a reliable tool for the diagnosis of pemphigus.[77]

Desmoglein assay:

The desmoglein proteins Dsg 3 and Dsg 1 have been identified as the antigens in PV and PF respectively. cDNA cloning of the 130 kDa glycoprotein and the 160 kDa glycoprotein revealed them to be members of the desmoglein subfamily of the cadherin supergene family. For PV the autoantigen was determined to be Dsg 3, whereas the PF autoantigen was found to be Dsg 1. Ishii et al[6] produced recombinant pemphigus antigens using a baculovirus expression system, PV Ig for PV antigen and PF Ig for PF antigen. These molecules were designed as a chimeric molecule which contains the entire extracellular domain of Dsg 3 and Dsg 1 fused with the constant region of human IgG1. Further, utilizing the recombinant Dsg 1 and Dsg 3 they developed a quantitative, sensitive and specific ELISA for the detection of antibodies directed against these antigens.[6]

The demonstration that large quantities of extracellular domain of PV antigen with the proper conformation can be produced by baculovirus has several important implications. From a clinical point of view ELISA using PV

Ig-Sf9 as an antigen source would be a sensitive and specific diagnostic tool. Scores of this ELISA should correlate even better with disease activity than currently used immunoflourescence tests with normal skin because IgG titres can only be measured against the pathogenic extracellular domain of PV antigen. Furthermore as a new type of treatment, antigen specific plasmapheresis with a PVIg-Sf9 column could be developed which could specifically remove pathogenic IgG against PV antigens from patient's circulation with minimal side effects. From a biological point of view this recombinant protein would be valuable to study adhesion function of PV antigen in cultured keratinocytes and also to do fine epitope mapping of PV antigen.[44]

Usefulness of desmoglein ELISA as a diagnostic test:

The first report of ELISA for diagnosis in pemphigus found it to be highly sensitive and specific. Ishii et al[6] studied 49 PV patients and 46 PF patients. The sensitivity and specificity of Dsg 3 ELISA were 94% and 96%, the sensitivity and specificity of Dsg1 ELISA were 96% and 96% respectively.

Lenz et al[78] in a study from Vienna produced recombinant Dsg (rDsg) 3 protein in their laboratory and validated the test among their patients and expanded the published results by testing additional diseases groups including other bullous dermatoses or autoimmune connective tissue diseases. They found a sensitivity of 93% which increased to 98% when IIF negative serum

samples were excluded indicating that both tests have a similar sensitivity. They also established a specificity of 93%.

Amagai et al[7] further evaluated the practical application of these ELISA tests for the serological diagnosis of PV and PF with a large number of serum samples collected from four different dermatological institutions in Japan. They used PF, PV and normal sera to analyse the sensitivity and specificity using ROC curves. The cut-off values for achieving highest sensitivity and specificity were determined to be 11.0 for Dsg 1 and 10.0 for Dsg 3 ELISA. However `with these cut-off values a small number of sera obtained from controls fell into the positive zone. They set up a grey zone which was above the cut-off value and below the kits cut-off of 20.0. They then determined the sensitivity and specificity of Dsg1 to be 97.9% and 98.9% respectively and Dsg 3 to be 97.5% and 97.8% respectively.[7]

Harman et al[5] have critically evaluated two ELISA's for the detection of antibodies to the major pemphigus antigens, demoglein 1 and 3. They found that when serum samples taken from untreated cases were examined both the Dsg 1 and Dsg 3 ELISA showed a sensitivity of 100% for the diagnosis of PV and PF. When patients on systemic treatment were included, the sensitivity dropped to 95% in PV and 92% in PF but the figures compared favourably to the overall sensitivity of IIF which was 79% in PV and 84% in PF. The high sensitivity of ELISA was matched by a high specificity of 98% or more. Dsg 3 autoantibodies were not detected in any of the PF subjects

whereas Dsg 1 autoantibodies were detected in 60% of PV subjects. Using a combination of Dsg 1 and Dsg 3 ELISA , PV and PF could be distinguished.

Bhol K et al[79] have also evaluated the use of recombinant pemphigus vulgaris antigen in development of ELISA and immunoblot assays to detect pemphigus vulgaris autoantibodies and found ELISA and immunoblot titres in all patients to be higher than with conventionally used IIF assay. They concluded that ELISA and immunoblotting are superior to IIF in evaluating antibody levels in pemphigus patients and that ELISA is more practical and preferable than immunoblot in routine clinical use.[79]

A recent study from India evaluated Dsg ELISA in Indian patients with pemphigus vulgaris. Sharma et al[8] studied twenty seven active PV patients and 26 controls. They found the sensitivity and specificity of ELISA for anti-Dsg 1 in the diagnosis of PV to be 96.3% and 92.3%, respectively. For anti-Dsg 3 they were 85.2% and 100%, respectively. [8]

Amagai et al[7] propose that Dsg 3 positivity irrespective of Dsg 1 is diagnostic of PV, and Dsg 1 positivity with negative Dsg 3 is diagnostic of PF(Table 4). In their study of 81 PV and 48 PF sera, serodiagnosis by ELISA matched the clinical diagnosis in 86.8 % of PV and PF cases. However these criteria applied only to patients with active disease. They observed that sera from some PV patients in remission showed reactivity only against Dsg 1 and they speculate that these patients could have had both Dsg 1 and 3 autoantibodies when the disease was active, but as the disease subsided only anti Dsg 1 antibodies stayed positive.[7]

Severity of cutaneous and oral pemphigus in relation to Dsg 1 and Dsg 3 antibody titres:

The apparent correlation of pemphigus antibody titres with severity of disease was first suggested in early studies on the specificity of the pemphigus antibody.[80] This initial impression has been confirmed repeatedly in several series of patients by studying antibody titre by IIF in subsequent years.

Ishii et al[6] demonstrated that PV patients with skin involvement tend to have higher anti-Dsg 1 IgG titres than those with predominant oral lesions.[6]

Harman et al[81] in a study of 104 patients have found that there was a clear relationship between the clinical presentation of disease and the Dsg autoantibody profile.[81]

Kumar et al[9] attempted to correlate the extent of skin and mucosal involvement with independent values of Dsg 1 and Dsg 3 ELISA. They used a simple arbitrary scoring system to grade the severity of skin or mucosal disease. Although a wide range of values were obtained the general trend was that Dsg 3 levels were higher in patients with extensive oral mucosal involvement than those with mild or no mucosal involvement. There was a direct relationship between severity of skin involvement and levels of Dsg 1 antibodies. Forty four patients were analysed using ELISA. A statistically significant correlation was seen between increase in Dsg 3 antibody titres with severity of oral involvement, and in DsG 1 titres with severity of skin disease in

both PV and PF patients. For Dsg 1 ELISA, 5 of 6 PF patients (83.3%) and 23 of 38 (60.52%) PV patients were above cut off values. For Dsg 3 ELISA, 31 of 38 (81.57%) PV patients and 1 of 6 (16.6%) PF patients exceeded the cut off value.[9]

Ding et al[40] showed that mucosal and mucocutaneous pemphigus vulgaris show distinct antibody profiles.

Sharma et al[8] found that though the different morphological types could not be differentiated on the basis of antibody profile, they were able to demonstrate a direct correlation between anti-Dsg 3 titres and severity of oral disease and also between anti–Dsg 1 titres and severity of cutaneous disease. Amagai et al[39] showed that there was a difference in antibody profile among mucosal dominant PV, mucocutaneous PV and PF and that the clinical phenotype is defined by the antibody profile.

However Jamora et al[82] have reported that though in general, patients with PV and only oral lesions have anti-bodies against Dsg 3, and those with skin lesions have, in addition, antibodies to Dsg 1, their results did not support the hypothesis that the sole determinant of lesion location in PV is the profile of autoantibodies against Dsg. This was because a significant minority of patients did not have the PV phenotype predicted by their antibody profile.[82]
Despite the overall relationship between Dsg 1 antibodies and skin severity and between Dsg 3 antibodies and oral severity, Harman et al[81] found a wide range of values within each severity score. They speculate that since pemphigus sera target multiple epitopes on both Dsg 1 and Dsg 3 molecules,

it is possible that cases with minimal disease but high antibody levels may have a high proportion of non pathogenic antibodies either by virtue of their subclass or perhaps because they bind to epitopes that do not result in disease triggering. An alternative explanation they propose is that treatment may have dampened pathogenic mechanisms triggered by antibody binding in these cases. In cases with low ELISA values but active disease, they suggest it is possible that the serum contains pathogenic antibodies to non desmoglein molecules as demonstrated in other studies of pemphigus sera or to epitopes on the intracellular domain of Dsg 1 or Dsg 3 which would be undetectable to ELISA.[81]

Harman et al[83] suggest that patients who are Dsg 3+/Dsg 1- at presentation will not necessarily develop Dsg 1 antibodies while Dsg 3+/Dsg 1+ patients are at risk of developing a more severe phenotype with extensive cutaneous ulceration.[83]

Desmoglein ELISA to monitor disease activity:

Recombinant ELISAs have been shown to be useful for monitoring disease activity.[6,7] Ishii et al[6] showed that Dsg 1 and Dsg 3 ELISA scores showed parallel fluctuation with disease activity along the time course. Utilisation of ELISA seemed to be superior to IIF to assess antibody level, as routine IIF missed some increases in the level of specific antibody that were picked by ELISA.[6]

Amagai et al[7] also used ELISA to quantify individual levels of antibodies against Dsg 1 and Dsg 3 and examined their correlation with the progression of disease activity. In three of the patients studied, the respective Dsg 1 and Dsg 3 ELISA scores showed parallel fluctuation with disease activity along the time course. These observations suggest that ELISA's will be a valuable tool to measure disease activity. ELISA scores may also be useful to plan tapering schedules of corticosteroids and to predict flares or relapses by detecting increase in antibodies before clinical evidence of disease flares are noticed.[7]

However in some patients high antibody index values in ELISA did not necessarily parallel disease activity. Cheng SW et al[84] have shown that index values obtained with appropriately diluted high titre sera do reflect disease activity. They suggest that in practical terms when diagnostic laboratories receive high titre sera, dilutions of 1:1600 in Dsg 1 or Dsg 3 ELISA should be used and if the index value is still higher than 120, further dilutions may be required. This is because ELISA is based on an enzyme reaction sera containing high titre antibodies and can reach a plateau or saturation level and thus yielding scores or index values which are artefactually lower than they should be.[84]

Numerous exceptions have been noted in the relationship of disease activity to antibody titre and several authors remain unconvinced of the clinical usefulness of sequential titre as a guide to therapy. Prolonged elevation of titre after clinical improvement, a lag period in the fall of titre by six to eight weeks

and failure of the titre to fluctuate with the clinical course, are some of the problems encountered in observing serial titres clinically. If a direct relationship of sequential antibody titre to disease activity exists in a predictable manner, this would indeed be a valuable clinical guide in the management of pemphigus. Though a rough correlation of high disease activity with high titre and low disease activity with low titres has been demonstrated in literature, it is the two fold change in titre that is meaningful and the timing of such a change that is clinically important.[80]

Desmoglein profiles of the rarer variants of pemphigus :

In cutaneous pemphigus both anti-Dsg 1 and anti-Dsg 3 IgG antibodies are detected with a tendency towards higher titre of anti-Dsg 1 IgG than of anti-Dsg 3 IgG.[64]

The target antigen for pemphigus herpetiformis is most often Dsg 1. In some cases the autoantibodies are directed to multiple epidermal antigens or to Dsg 3.[66,85]

Anti-Dsg 1 antibodies have been detected in pemphigus erythematosus.[86]

Desmogleins are recognized by IgA antibodies in a few patients with IgA pemphigus.[54]

Anti-Dsg 1 antibodies have been reported in drug induced pemphigus. ELISA scores for anti-desmoglein 1 antibodies also revealed a surprisingly rapid decline when the drug was discontinued.[87] Korman et al[88] have reported patients with drug-induced pemphigus foliaceus having

circulating autoantibodies that are directed against the pemphigus foliaceus antigen complex and a patient with drug-induced pemphigus vulgaris who had circulating autoantibodies that were directed against the pemphigus vulgaris antigen complex.

Patients with paraneoplastic pemphigus raise antibodies against multiple antigens including members of the plakin family as well as desmogleins.[89,90] Studies have shown that the association between clinical phenotype and anti-desmoglein autoantibody profile in PNP is not as clear as that in classic pemphigus. [91]

Advantages of ELISA

1) A major advantage of ELISA is the selective detection of antibodies against extracellular and conformational epitopes of desmoglein which include most pathogenic antibodies in patients with PV. In contrast conventional IIF testing may also identify a variety of antibodies against protein of the extracellular matrix. Indirect immunoflourescence may also fail to distinguish between antibodies directed against the intracellular portion of Dsg 3, antibodies against Dsg 1 or desmocollins or other ICS constituents.
2) Determination of antibody titres by IIF analysis requires serial dilutions of serial samples, is subjective, and requires an experienced examiner. A potential advantage of ELISA over IIF is that the process can be fully automated and that an objective optical density value is obtained at a single dilution of 1:10000.

3) ELISA allows estimation of pemphigus antibody levels similar to IIF. This aspect may be of importance as antibody can parallel clinical course.

Dsg 3 ELISA studies have shown that increase in ELISA values occurred earlier and were more pronounced when compared to IIF titres. This may reflect a higher sensitivity of ELISA in specifically detecting pathogenic anti-Dsg 3 antibodies which may be helpful in monitoring the clinical course and for adjustment/discontinuation of immunosuppressive therapy. Thus far the most reliable indicator of PV remission seems to be a negative DIF result requiring repeated skin biopsies. Further investigation will determine whether a negative Dsg 3 ELISA obtained during clinical remission is a sensitive indicator to predict if disease will stay in remission following cessation of therapy.[78]

4) Desmoglein ELISA compared with IIF does not require a skilled observer and is a simple test to perform. Moreover it is able to differentiate PV from PF.[92]

5) In cases where IIF was negative, nuclear or cytoplasmic fluorescence most likely masked and interfered with the detection of intercellular antibodies, and ELISA is particularly useful in this subset of patients. Also in one of the cases where IIF had been negative, it was later positive after a lag period of five months, suggesting that ELISA is more sensitive in the early stages of the disease.[92]

Limitations of ELISA:

Limitations of ELISA have come to light as a result of comparing results with IIF.

1. The first limitation is that ELISAs are not quantitative at high antibody titre. The probable explanation for the plateau in ELISA which results at a high antibody titre is that a limited amount of Dsg 1 and 3 antigen is coated to the ELISA plates and that this becomes saturated in the presence of high levels of Dsg 1 or 3 antibodies.
2. The second limitation is that there is a wide variation in the results of ELISA in sera that have identical levels of antibodies by IIF assays. The cause for this variation is not known. Possibly it reflects the presence of antibodies against intercellular antigens other than Dsg 1 and 3 in patients with pemphigus.[93]

The significance of the presence of PV-IgG in the sera of healthy family members and the reason why these carriers do not develop clinical symptoms of the disease are unknown. One possible explanation is that the total quantity of pathogenic PV-IgG in the sera of carriers is low and therefore is usually significant for binding the antigen sites and causing acantholysis but that exposure to other factors, such as infections or drugs, may trigger onset of the disease. Another possibility is a difference in the circulating PV-IgG

subclasses between the affected patients and their clinically unaffected but antibody positive relatives.[94]

Production of autoantibodies in PV is polyclonal and in PV patients with active disease most autoantibodies are of the IgG4 subclass. Patients in remission have mainly autoantibodies of the IgG1 subtype while healthy relatives of PV patients and healthy carriers of PV prevalent HLA class II alleles appear to have low levels of Dsg 3 reactive IgG.[95]

Veldman C et al[96] suggest that immunological tolerance against Dsg 3, the autoantigen of PV may be, at least partly, mediated by Dsg 3-specific type 1 T regulatory cells. This provides an explanation as to why B cell tolerance against Dsg 3 exists in healthy individuals who carry autoaggressive T cells reactive to Dsg 3 epitopes identical to those recognized by T cells from the PV patients. Thus, Dsg 3-responsive type 1 T regulatory cells may represent an ideal tool to therapeutically restore Dsg 3-specific immune tolerance in PV.[98]

There are several reports which describe the coexistence of features of both PV and PF in the same patient or the transformation of PF to PV and vice versa on an extended period of time.[71,97] Sami et al[98] suggest that in PF,for those patients who do not respond to conventional therapy in a reasonable time serological evaluation for both antidesmoglein 1 and 3 antibodies may provide an identification of this group of patients.

Spaeth et al[99] have studied IgG, IgA and IgE autoantibodies against the ectodomain of desmoglein 3 in active pemphigus vulgaris and conclude that by immunoblot analysis utilising a baculovirus –encoded recombinant form of Dsg 3 encompassing its entire extracellular domain autoantibodies of the IgG4, IgG1, IgA and IgE subclasses were detected in both acute onset and chronic active PV while IgG1 and IgG4 were the only subtypes of autoantibodies present in sera of patients in remission. The detection of Dsg 3 reactive IgA and occasionally IgE in active PV was a novel finding which could be exploited as a predictive and prognostic marker.[99]

Patients with bullous diseases present physicians with formidable challenges. Precise diagnoses are usually possible using a combination of clinical, histological, immunohistological and immunochemical criteria. Advancements in our understanding of the mechanisms that regulate formation of anti-Dsg antibodies may facilitate development of more specific interventions. ELISAs using the recombinant pemphigus antigens when used together provide a sensitive, specific and quantitative diagnostic tool for the evaluation of patients with pemphigus. This new diagnostic tool will be useful not only for clinical application but also for the more precise characterization of the antibody response in patients with pemphigus and for understanding fundamental immunopathologic mechanisms of pemphigus. The challenge for the future is to develop more specific less toxic therapies based on better understanding of the immunoregulatory mechanisms that allow autoantibody production.[62]

MATERIALS AND METHODS

Study Design : Cross sectional study

Setting: The study was conducted in the Outpatient Department of Dermatology, Venereology and Leprosy, Christian Medical College, a tertiary care hospital in South India

Duration of study: The study was conducted between March 2005 and July 2006.

Study population: All patients with acquired immunobullous disorders, diseases targeting desmogleins or having conditions where antibodies mimic or show in vitro deposition in stratified squamous epithelium in the absence of pemphigus were eligible for inclusion into the study.

Inclusion criteria:

Patients :

All consecutive patients with pemphigus who consented for the study and in whom the diagnosis had been confirmed by histopathology and direct immunoflourescence were included.

Controls:

1) Patients with acquired immunobullous disorders other than pemphigus (eg. bullous pemphigoid, epidermolysis bullosa aquisita or chronic bullous dermatosis of childhood) who consented for the study and who had been diagnosed by clinical features, histopathology and DIF were included as in other studies.

2) Patients with diseases targeting desmogleins like bullous impetigo or staphylococcal scalded skin syndrome (SSSS) or having conditions where antibodies mimic or show in vitro deposition in stratified squamous epithelium in the absence of pemphigus like toxic epidermal necrolysis (TEN), who consented for the study and who had been diagnosed by clinical features, gram stain, histopathology or DIF as appropriate were included.

Exclusion criteria:

Patients

Patients not willing for the study.

Controls

Patients not willing for the study

Methodology:

Patients

All pemphigus patients who conformed to the inclusion criteria were examined by the principal investigator after written informed consent was taken (annexure I). The details regarding duration of disease, sites of involvement, duration and type of treatment, relapses and extent of lesions were recorded in a proforma (annexure II). Details of diagnostic tests were recorded. Patients were categorized into the various clinical types based on the clinical features, histopathology and DIF. The severity of lesions were assessed clinically based on the scoring system used by Harman et al.[81] Skin lesions and oral lesions were scored separately and recorded.

Oral lesions were scored as

0 - Quiescent

1 - Minor activity (upto 3 erosions)

2 - Moderate activity (4 -10 erosions or generalized desquamative gingivitis)

3 - Severe (>10 discrete erosions or extensive confluent erosions or generalized desquamative gingivitis with discrete erosions at other oral sites)

Skin lesions were scored as

0 - Quiescent

1 - Minor activity (< 5 discrete lesions)

2 - Moderate activity (5-20 discrete lesions)

3 - Severe (> 20 discrete lesions or extensive confluent areas of eroded skin.)

Patients with pemphigus were also divided into 3 categories : acute onset, chronic active and remittent as by Spaeth et al.[99] The same criteria was applied to cutaneous lesions as well.

Acute onset was defined as the de novo development of blisters or erosions on previously unaffected mucosal or cutaneous surfaces. None of the patients would have received immunosuppressive therapy at the time the serum was obtained.

Chronic active disease was defined as expansion/persistence of existing blisters or erosions on mucosal or cutaneous surfaces. Patient could have already received immunosuppressive therapy.

Patients with remittent disease had not experienced mucosal or cutaneous blisters/erosions during the two or more months prior to study

Sera were obtained from pemphigus patients when the diagnosis was established and patients were scored.

Controls:

Thirty six consecutive controls who conformed to the inclusion criteria were examined by the principal investigator after written informed consent was taken (annexure I). The details regarding duration of disease, sites of involvement, duration and type of treatment were recorded in a proforma (annexure III). Details of diagnostic tests were recorded. Sera were obtained when the diagnosis was established.

All sera were coded and the investigator performing the ELISAs was blinded. Sera were stored at -70°C until assays were performed. The ELISAs were performed using kits purchased from Medical and Biological laboratories Co.Ltd., Nagoya, Japan.

Principle of the test:

The test measures, by ELISA, the Dsg antibodies present in the serum. Sera are added to separate microwells coated with Dsg 1 or Dsg 3 allowing

anti-Dsg 1 or anti-Dsg 3 antibodies respectively to react with the immobilized antigen (sample incubation). After wash, to remove any unbound serum proteins, horseradish peroxidase conjugated anti-human IgG monoclonal antibody is added and incubated (conjugate incubation). Following another washing step, the peroxidase substrate is added and incubated for an additional period of time (substrate incubation). Acid solution is then added to each well to terminate the enzyme reaction and to stabilize the color development. The assay can be quantified by measuring the reaction photometrically.

Brief assay procedure :

(Sample incubation) Add 100µl of diluted sample to each well of the microwell plate (20-25° C) 60 min

↓

Wash

↓

(Conjugate incubation) Add 100µl of conjugate solution to each well (20-25° C) 60 min

↓

Wash

↓

(Substrate incubation) Add 100µl of substrate to each well (20-25° C) 30 min

↓

Add 100µl of stop solution to each well

↓

Read absorbance and interpret result.

$$\text{Unit value(U/ml)} = \frac{(\text{Optical density(OD) of tested serum} - \text{OD of negative control})}{(\text{OD of positive control} - \text{OD of negative control})} \times 100$$

Both positive and negative controls provided in the kit were included in every plate.

For all analysis the cut –off values given in the kit were taken as standard.

The cut-off values were applied to patients and controls to determine the sensitivity and specificity of ELISA estimation of Dsg antibodies in pemphigus. Dsg 1 values were determined from PF patients and Dsg 3 values were determined from mucocutaneous, cutaneous and mucosal PV patients. The sensitivity and specificity of ELISA estimation of Dsg 1 and 3 antibodies in differentiating PV and PF using criteria proposed by Amagai et al were also studied.[7]

We also generated cut off values for the local population as the kit was manufactured elsewhere. The Receiver-operating-characteristic (ROC) analysis was performed to determine cut-off values for Dsg 1 and Dsg 3 ELISAs. The sensitivity and specificity were plotted when different scores were used as cut-off values and the one which gave the highest score for the sum of sensitivity (%) and specificity (%) was determined to be the cut off value for the population studied. For this analysis, data obtained with PF sera and controls were used for Dsg 1 ELISA and data obtained with PV sera and controls were used for Dsg 3 ELISA .[7]

Correlation of Dsg 1 and Dsg 3 titres with mucosal and cutaneous involvement, category of disease, duration of disease, number of relapses and severity of disease were investigated. The prevalence of Dsg antibodies in the various clinical variants of pemphigus included in the study were also investigated.

The study was approved by the institutional research committee.

Sample size:

The sample size was calculated using the formula $\frac{1.96^2 \times (p.q)}{d^2}$

A minimum of 36 patients and 36 controls were required to validate ELISA estimation of Dsg 1 and Dsg 3 antibodies as a diagnostic test for pemphigus with a sensitivity and specificity of 90% and confidence intervals of 80-100.

Statistical analysis:

The statistical analysis was performed using SPSS 11.0 for Windows. The optimum cut off values of Desmoglein 1 and 3 ELISA for the population studied were determined using the ROC (Receiver Operating Characteristic) Curve approach.

Associations between categorical variables were analyzed using the Chi-square tests.

Relationships between continuous variables were assessed using Pearson's product moment correlation with scatter plots as diagrammatic representation of the correlation. Relationship between continuous and categorical variables were analysed using 't'- tests and error bars were provided for a graphical representation to see how the means of the continuous variables were different across different levels of the categorical variables.

RESULTS

Study population:

Distribution of cases and controls in the study population:

Seventy five patients were enrolled into the study between March 2005 and July 2006. There were 39 (52%) pemphigus patients and 36 (48%) controls.

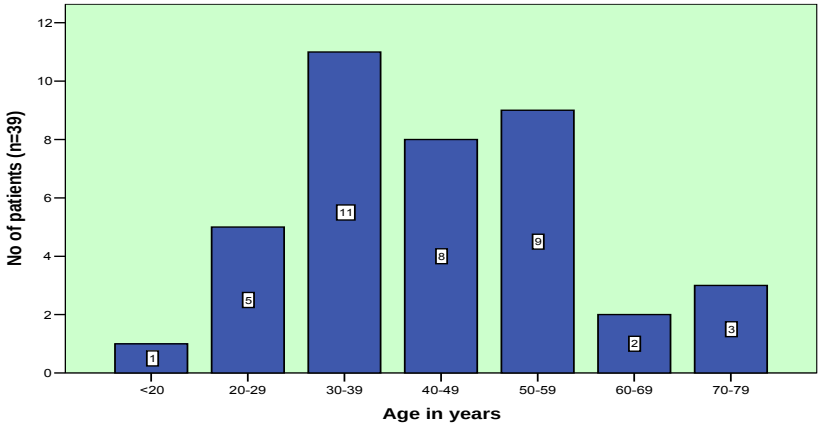
Age and Sex distribution:

The mean age of the study population was 37 years with a SD of ± 20.68. The oldest being 75 years and the youngest being 1 year old. There were 36 males (48%) and 39 females (52%). Male to female ratio was 1:1.08.

Profile of patients with pemphigus:

Age distribution:

Figure 3: Age distribution of patients with pemphigus



The mean age of pemphigus patients was 43.21 years with a SD of ± 13.89. The youngest was 17 years and the oldest was 73 years.(Fig.3) The majority of patients were in the age group of 30 – 59 years.

Gender distribution of patients:

There were 23 (58.97%) females and 16 (41.03%) males. The male to female ratio was 1: 1.43.

Duration of lesions:

The mean duration of lesions at presentation was 21.96 months with a SD of ±23.34. The range was 0.6 to 108 months.

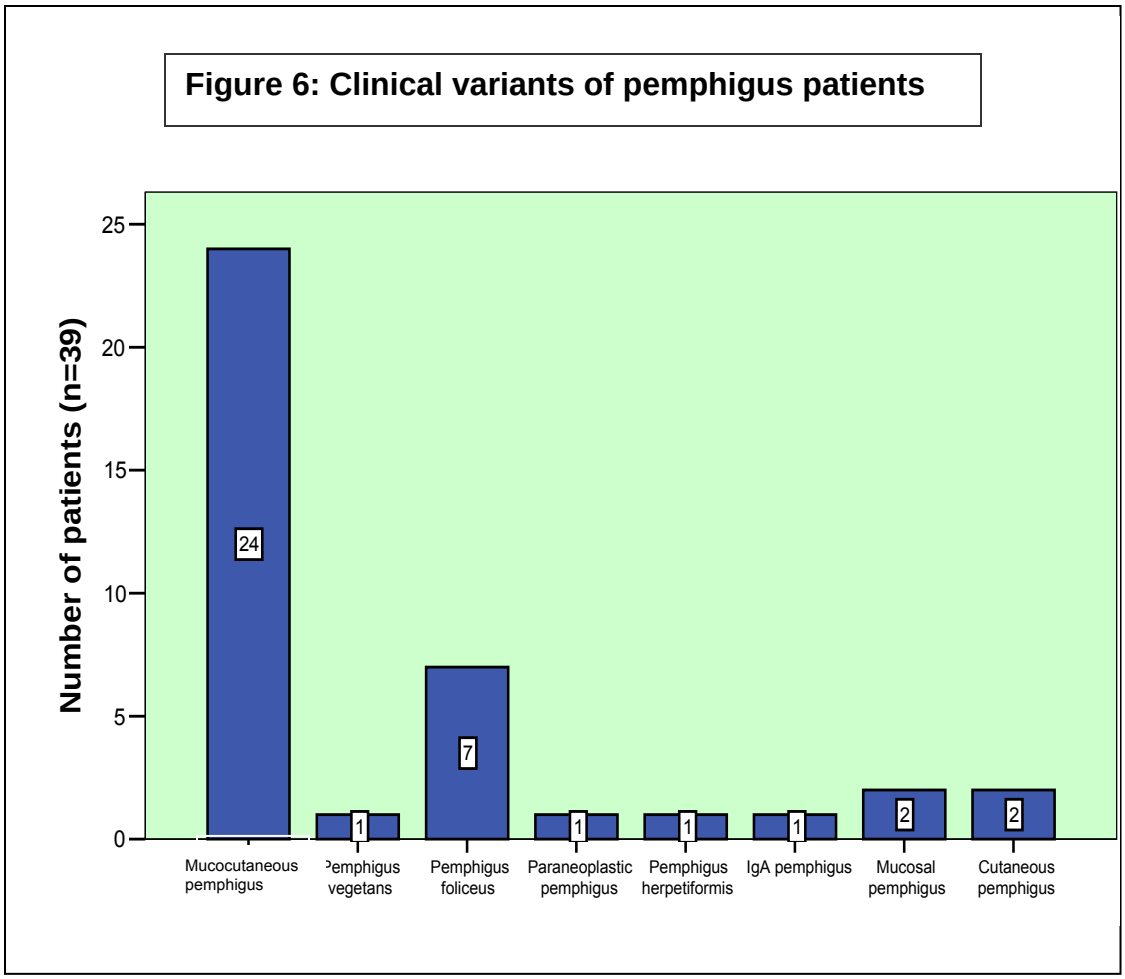
Presence of oral and skin lesions:

Of the 39 patients, 32 (82.05%) had skin lesions and 27 (69.23%) had oral lesions at presentation. Twenty patients (51.2%) had both oral and skin lesions.

Distribution of acute onset and chronic active patients:

There were 8 (20.51%) patients with acute onset disease and 31 (79.48%) patients with chronic active disease. There were no patients with remittent disease.

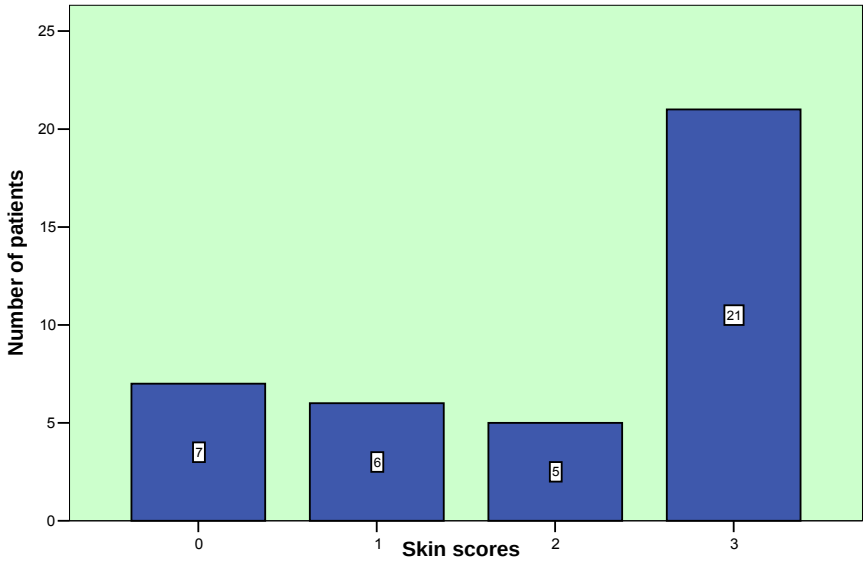
Clinical variants of pemphigus patients



Among the 39 pemphigus patients, there were 24 patients with mucocutaneous PV and 2 patients each with cutaneous pemphigus and mucosal pemphigus. Seven patients had PF. One patient each had pemphigus vegetans, pemphigus herpetiformis, IgA pemphigus and paraneoplastic pemphigus. (Fig. 4,5,6,7,8).

Severity scoring of skin lesions

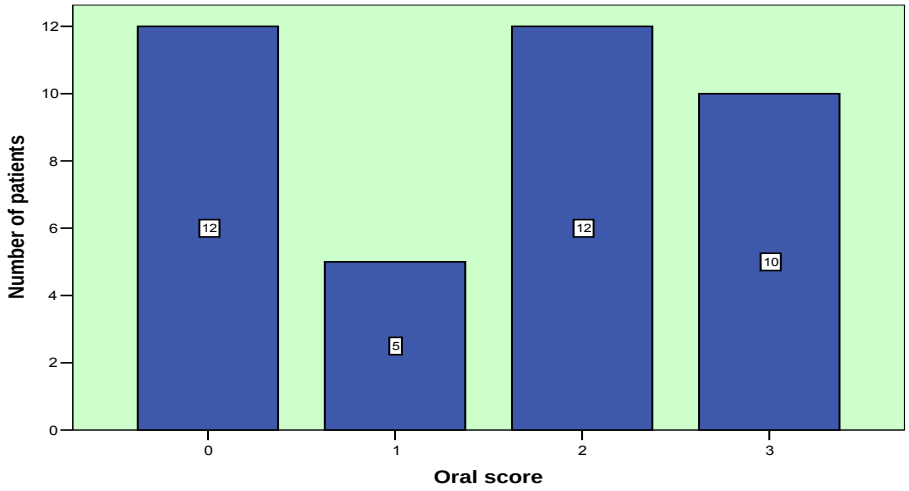
Figure 9: Severity scores of pemphigus patients with skin lesions



Seven patients did not have any active disease in the skin. Twenty one patients had the maximum skin severity score of three. (Fig.9)

Severity scoring of oral lesions

Figure 12: Severity scores of pemphigus patients with oral lesions



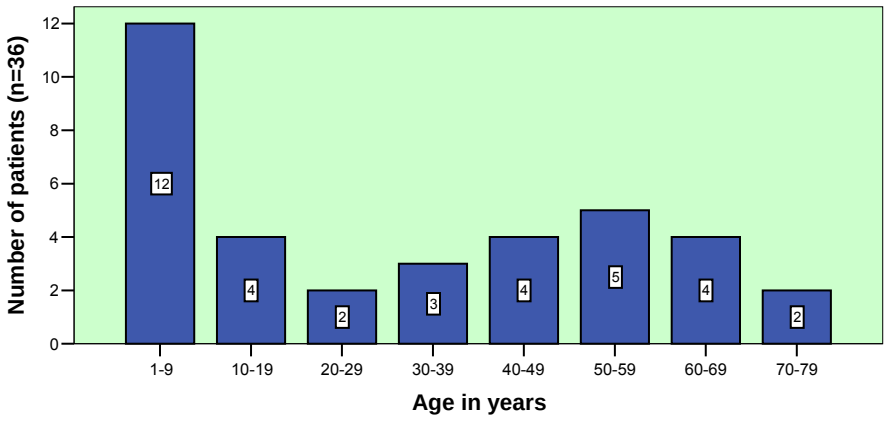
Twelve patients did not have active disease of the oral mucosa. Five, twelve and ten patients had an oral severity score of one, two and three respectively. (Fig.12).

In all patients the diagnosis was based on clinical features, histopathology and DIF findings.(Fig. 10,11,13,14)

Controls:

Age distribution of controls:

Figure 15: Age distribution of controls



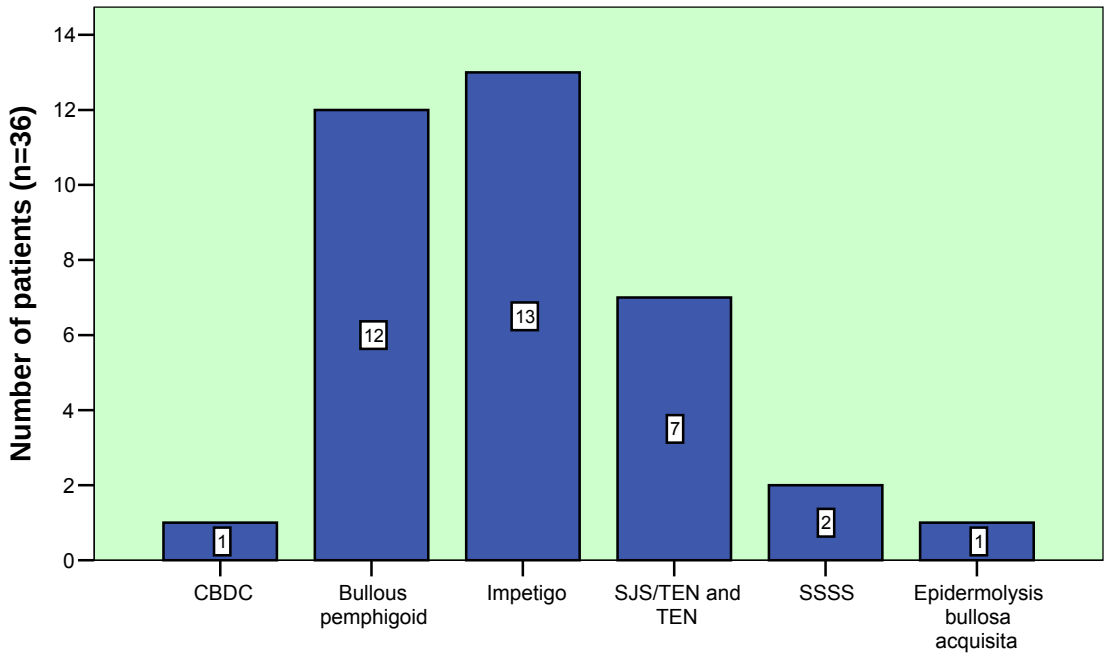
The mean age of controls was 30.33 years with a SD of ± 24.63. The range was 1 to 75 years. (Fig.15)

Gender distribution

There were 16 (44.44%) males and 20 (55.56%) females. The male to female ratio was 1:1.25

Clinical profile of controls

Figure 18: Clinical profile of controls



CBDC - Chronic bullous dermatosis of childhood

SSSS - Staphylococcal scalded skin syndrome

SJS - Stevens Johnson syndrome

TEN - Toxic epidermal necrolysis

Among the controls nine patients had bullous pemphigoid, one patient each had chronic bullous dermatosis of childhood and epidermolysis bullosa acquisita. There were 13 patients with bullous impetigo, 7 patients with SJS /TEN or TEN and 2 patients with SSSS. (Fig.16,17,18)

Validity of ELISA estimation of desmoglein 1 and 3 antibodies in the diagnosis of pemphigus. (Fig 19,20,21)

Table 5: Sensitivity and specificity of ELISA estimation of Dsg 3

Diagnosis	Desmoglein positive	Desmoglein negative	Total
Pemphigus Vulgaris	23	5	28
Control	0	36	36

The sensitivity of the test was determined to be 82.14% and the specificity was 100%. (Table 5)

Table 6: Sensitivity and specificity of ELISA estimation of Dsg 1

Diagnosis	Desmoglein positive	Desmoglein negative	Total
Pemphigus foliaceous	7	0	7
Control	1	35	36

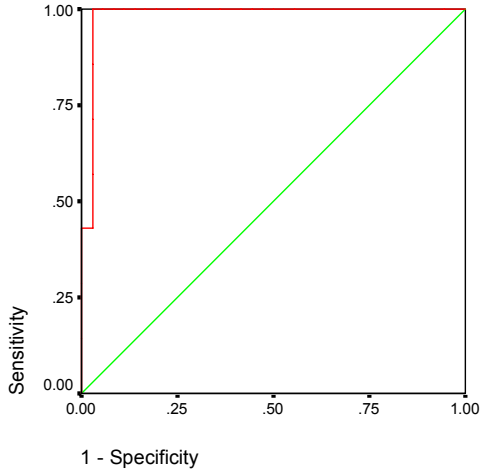
The sensitivity of the test was determined to be 100% and the specificity was 97.2%. (Table 6)

Application of diagnostic criteria for pemphigus vulgaris and pemphigus foliaceous proposed by Amagai et al (Table 4)

All of the 7 PF sera were positive against Dsg1 but negative for Dsg 3.Of the 28 PV sera 23 were positive against DsG 3 (82.14%). Applying these values to the diagnostic criteria proposed by Amagai et al[7] the serodiagnosis was consistent with the diagnosis in all (100%) of PF patients and (82.14%) of the PV patients.

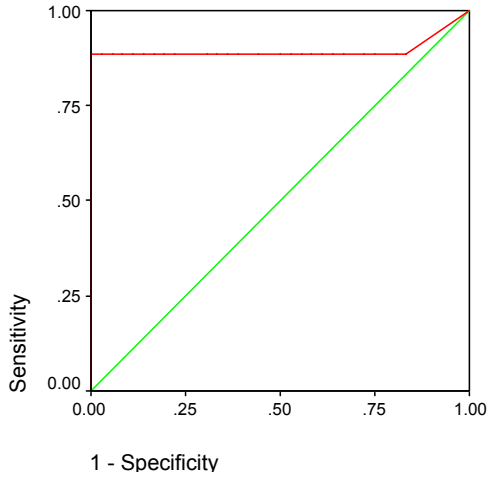
Determination of desmoglein 1 and 3 cut-off for local population

Figure 22: ROC curve for desmoglein 1



The ROC analysis using PF sera and normal control for Dsg 1 ELISA determined a cut-off value of 17.79 with a sensitivity of 100% and specificity of 97.22%.(Fig. 22)

Figure 23: ROC curve for desmoglein 3



The ROC analysis using PV sera and normal control for Dsg 3 ELISA determined a cut-off value of 28.91 with a sensitivity of 88.46% and specificity of 100%. (Fig. 23)

Prevalence of desmoglein 1 and 3 in patients with mucosal, cutaneous and mucocutaneous involvement

Table 7: Prevalence of desmoglein 3 in patients with oral lesions

Oral lesions	Dsg 3 positive	Dsg 3 negative	Total
Present	25	2	27
Absent	1	11	12
Total	26	13	39

Dsg 3 was positive in 25 (92.5 %) of the 27 patients with oral lesions. Dsg 3 was positive in one patient who did not have oral lesions. (Table 7)

Table 8: Prevalence of desmoglein 1 in patients with skin lesions

Skin lesions	Dsg 1 positive	Dsg1 negative	Total
Present	25	7	32
Absent	2	5	7
Total	27	12	39

Dsg 1 was positive in 25(78.12%) of the 32 patients with skin lesions. Dsg 1 was positive in 2 patients without skin lesions. (Table 8)

Table 9: Prevalence of desmoglein 1 and 3 in patients with oral and skin lesions

Skin and Oral Lesions	Dsg 1 and 3 positive	Dsg1 and/or 3 negative	Total
Present	14	6 *	20
Absent	2	17	19
Total	16	23	39

* 6 Dsg 1 negative

Dsg 1 and 3 were positive in 14 (70%) of the 20 patients with oral and skin lesions. (Table 9)

Correlation of desmoglein 1 and 3 titres with oral and skin lesions

Table 10: Correlation of desmoglein 1and 3 titres with skin lesions

Skin lesion	No of patients	Dsg 1 U/ml Mean ± SD	Dsg 3 U/ml Mean ± SD
Present	32	88.21 ± 80.05	120.58 ± 98.64
Absent	7	16.18 ± 20.18	114.56 ± 107.84
p value		0.025	0.886

The mean Dsg 1 titres of patients with skin lesions were significantly higher than those of patients without skin lesions.(p=0.025).There was no such correlation between oral lesions and Dsg 1 titres.(p=0.886) (Table 10)

Table 11: Correlation of desmoglein 1 and 3 titres with oral lesions

Oral lesion	No of patients	Dsg 3 U/ml Mean ± SD	Dsg 1 U/ml Mean ± SD
Present	27	166.83 ± 79.98	63.35 ± 71.54
Absent	12	13.01 ± 28.20	102.14 ± 88.07
p value		0.000	0.154

The mean Dsg 3 titres of patients with oral lesions were significantly higher than those of patients without oral lesions(p=0.000).There was no such correlation between skin lesions and Dsg 3 titres(p=0.886). (Table 11)

Correlation of desmoglein titres with acute onset and chronic active disease.

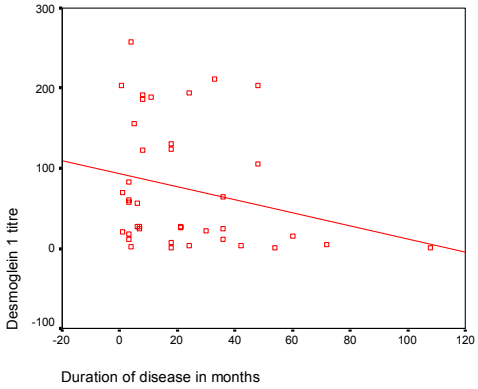
Table 12: Correlation of desmoglein titres with acute onset and chronic active disease

Category	Acute onset	Chronic active	P value
Dsg 1	55.14 ± 85.34	80.48 ± 76.55	0.313
Dsg 3	129.54 ± 111.58	116.91 ± 97.20	0.626

In the acute onset disease, the Dsg 1 mean value was 55.14 with SD ± 85.34 when compared to a mean value of 80.48 with SD ± 76.55 in chronic active disease(p = 0 .313). The Dsg 3 mean value in acute onset disease was 129.54 with a SD ± 111.58 while in chronic active disease the mean value was 116.91 with a SD ± 97.20(p value = 0.626). There was no significant difference in Dsg 1 or Dsg 3 titres of patients with acute onset and chronic active disease. (Table 12)

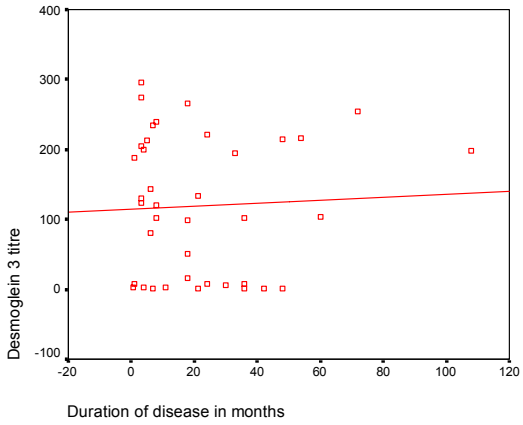
Correlation of Desmoglein titres with duration of disease

Figure 24: Correlation of desmoglein 1 titres with duration of disease



Using the Pearsons correlation coefficient to correlate Dsg 1 titres with duration of disease r value was found to be -0.242 and p value was 0.137. (Fig. 24)

Figure 25: Correlation of desmoglein 3 titres with duration of disease



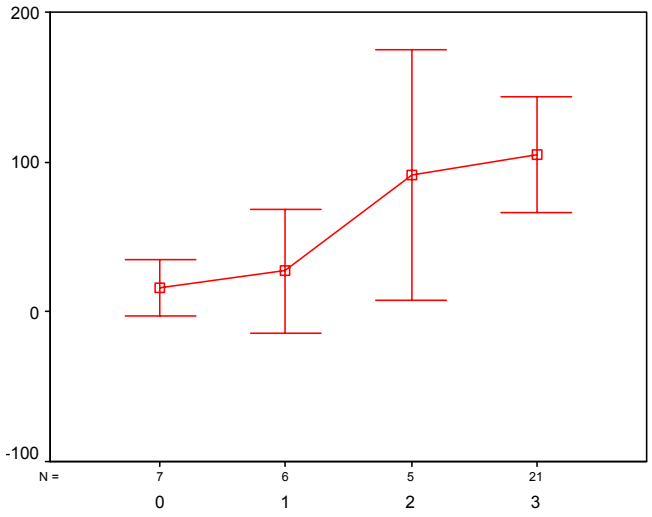
Using the Pearsons correlation coefficient to correlate Dsg 3 titres with duration of disease r value was found to be 0.048 and p value was .773 (Fig 25).There was no significant change in Dsg 1 or 3 antibody titres with respect to duration of disease.

Correlation of skin severity scores with desmoglein 1 titres

Table 13: Skin severity scores and mean desmoglein 1 titres

Score	No of patients	Mean titre(U/ml)	Standard deviation
0	7	16.18	20.18
1	6	27.34	39.28
2	5	91.65	67.31
3	21	104.79	85.07

Figure 26: Skin severity scores and mean desmoglein 1 titres with 95% confidence intervals



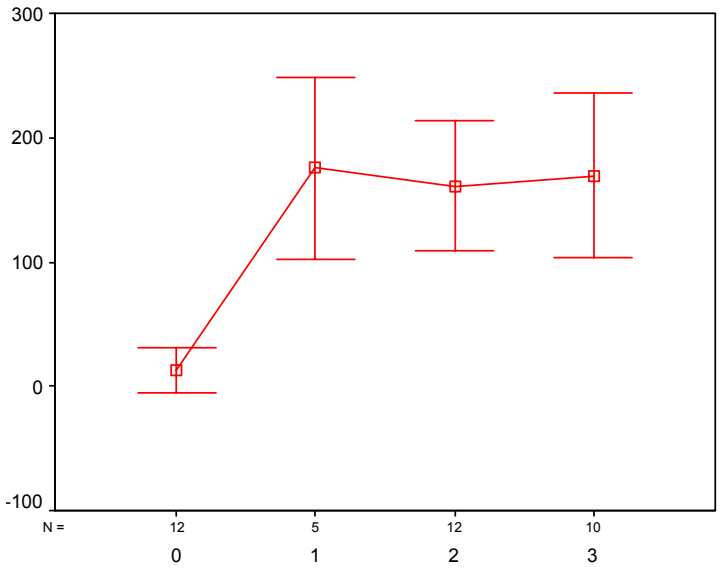
When Dsg 1 titres were correlated with severity of disease there was a trend to increasing titres with increasing severity of disease for Dsg 1. (Fig. 26)

Correlation of oral severity scores with desmoglein 3 titres

Table 14: Oral severity scores and mean desmoglein 3 titres

Score	No of patients	Mean titre (U/ml)	Standard deviation
0	12	13.01	28.20
1	5	173.59	58.89
2	12	161.03	81.91
3	10	169.40	92.98

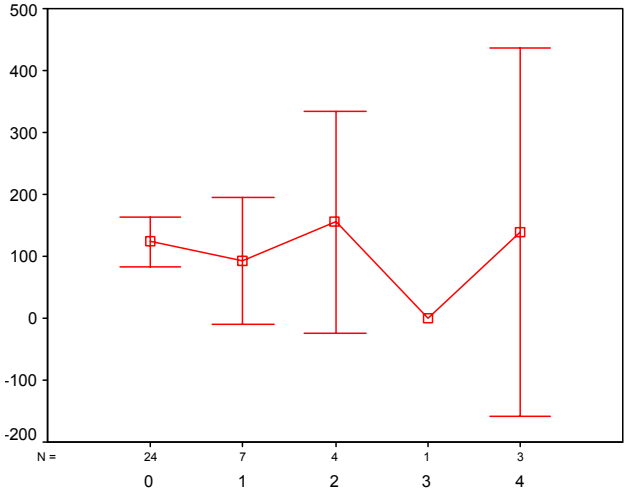
Figure 27: Oral severity scores and mean desmoglein 3 titres with 95% confidence intervals



Patients with oral lesions had higher titres than those without oral lesions. However there was no trend to increasing titres with increasing severity of disease. (Fig. 27)

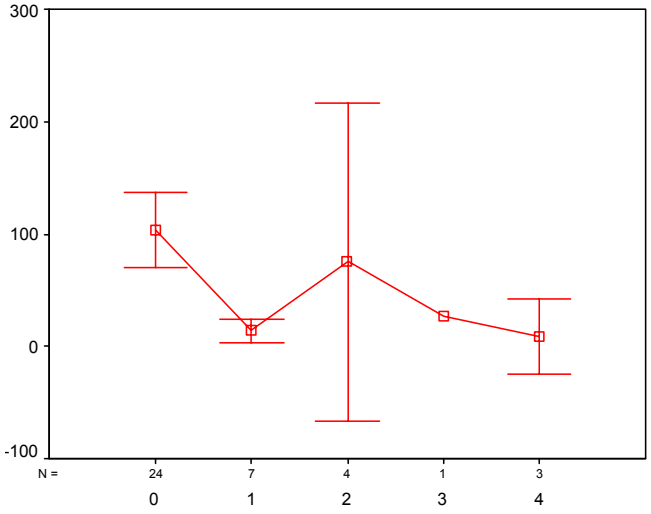
Correlation of Desmoglein 1 and 3 titres with prior number of relapses.

Figure 28: Desmoglein 3 titres and prior number of relapses



There was no correlation between Desmoglein 3 titres and number of prior relapses (Fig. 28)

Figure 29: Desmoglein 1 titres and prior number of relapses



There was no correlation between Desmoglein 1 titres and number of prior relapses. (Fig.29)

DISCUSSION

Pemphigus is a life threatening blistering skin disease in which patients' autoantibodies are directed against desmosomal glycoproteins, Dsg 1 and Dsg 3. Rapid and accurate diagnosis is of utmost importance to enable early initiation of therapy. DIF and IIF studies are subjective, require experienced examiners and are expensive. IIF may detect a variety of antibodies against proteins of the extracellular matrix. Although Western immunoblotting and immunoprecipitation can be used to identify the target antigen in cases of pemphigus, these are time consuming, qualitative techniques, and impractical for routine screening of large numbers of serum samples. Dsg 1 and Dsg 3 ELISAs have been shown to provide a sensitive and highly specific assay for the diagnosis of PV and PF.[7] Desmoglein ELISAs do not require a skilled observer and is a simple test to perform. Moreover it is able to differentiate PV from PF.[92] Also it selectively detects antibodies against extracellular and conformational epitopes of desmoglein which include the most pathogenic antibodies in patients with PV.[78] The primary aim of the study was to validate ELISA estimation of Dsg1 and Dsg3 antibodies in pemphigus as a diagnostic test in Indian patients as there is a paucity of studies from India.

Our study over a period of 16 months included 39 patients with pemphigus and 36 controls. The different clinical types of pemphigus were diagnosed based on clinical features, histopathology and DIF. There were 2

patients with oral pemphigus, 24 patients with mucocutaneous PV and 2 patients with cutaneous pemphigus. Seven patients had PF. One patient each had pemphigus vegetans, pemphigus herpetiformis, IgA pemphigus and paraneoplastic pemphigus. Among the controls, nine patients had bullous pemphigoid, one patient each had chronic bullous dermatosis of childhood and epidermolysis bullosa acquisita. There were 13 patients with bullous impetigo, 2 patients with SSSS and 7 patients had Stevens Johnson syndrome-Toxic epidermal necrolysis overlap or TEN.

The mean age of pemphigus patients in our study was 43.21 years. This was close to the mean age of 36.9 and 39.5 years previously reported in Indian studies whereas a higher mean age of onset has been reported from the UK.[14,15] The male to female ratio in our study was 1:1.43. A study has noted no sex predilection in Indian patients.[15]

Among the clinical variants in our study there was a predominance of PV patients which is consistent with other studies showing pemphigus vulgaris to be the most frequent subtype in India while pemphigus foliaceous was uncommon.[14]

The sensitivity and specificity of Dsg 1 ELISA was determined to be 100% and 97.2% respectively. Dsg 3 ELISA had a sensitivity and specificity of 82.14% and 100% respectively. This is similar to the sensitivity and specificity reported in studies from India and abroad.[5,7,78] Sharma et al[8] in a study from India on 27 patients with PV reported a sensitivity and specificity of 96.3% and 92.3% for Dsg 1 and 85.2% and 100% for Dsg 3 respectively. Ishii et al[6]

have reported a sensitivity and specificity of 94% and 96% for Dsg 3 ELISA and 96% and 96% for Dsg 1 ELISA respectively. Lenz et al[78] have reported both sensitivity and specificity of 93% for Dsg 3 in IIF positive patients. Amagai et al[7] determined the sensitivity and specificity of Dsg1 to be 97.9% and 98.9% respectively and Dsg 3 to be 97.5% and 97.8% respectively. Harman et al[5] have also reported similar results. Our results validate ELISA estimation of Dsg 1 and 3 antibodies in pemphigus to be a sensitive and specific test. False positivity was detected only in one HIV infected patient with SJS-TEN overlap. The polyclonal stimulation of antibodies in HIV may have resulted in false positivity though further studies are needed to elucidate this. All the other controls were negative for Dsg 1 and Dsg 3. In our study ELISA values were false negative in 2 pemphigus patients. One of them had IgA pemphigus and therefore IgG ELISA detected by the kit was found to be negative. The other was a patient with oral pemphigus of 3 years duration, who at the time of study had desquamative gingivitis.

When the diagnostic criteria for PV and PF proposed by Amagai et al[7] was applied to this study group, the serodiagnosis matched the diagnosis in all (100%) PF patients and 23 of the 28 (82.14%) PV patients. This was similar to the 86.8 % for PV and PF obtained by Amagai et al.[7] The ability to differentiate PV from PF is a potential advantage of ELISA over DIF and IIF. As in the study by Amagai et al[7] the criteria was applied only to active cases.

We also generated cut-off values for Dsg 1 and Dsg 3 ELISAs for the local population using the receiver-operating-characteristic (ROC) analysis, as the Dsg 1 and Dsg3 ELISA kits were manufactured elsewhere with appropriate cut off values for that population. The sensitivity and specificity were plotted when different scores were used for cut-off values and the one which gave the highest score for the sum of sensitivity (%) and specificity (%) was determined to be the cut off value to be used. For this analysis as in other studies data obtained with PF sera and control were used for Dsg 1 ELISA and data obtained with PV sera and control were used for Dsg 3 ELISA. This data will be useful for future studies as it will increase the sensitivity of the test. The cut-off values were not used in this study to avoid using it on the same population from which it was generated.

Patients with oral lesions had significantly higher Dsg 3 titres when compared to patients without oral lesions. There was no correlation between skin lesions and Dsg 3 titres. Similarly patients with skin lesions had higher Dsg 1 titres than those without skin lesions. There was no correlation between Dsg 1 titres and oral lesions. Sharma et al[8] were also able to demonstrate a direct correlation between anti-Dsg 3 titres and severity of oral disease, and also between anti –Dsg 1 titres and severity of cutaneous disease. Though Kumar et al[9] obtained a wide range of values, Dsg 3 levels were higher in patients with extensive oral mucosal involvement than those with mild or no mucosal involvement while severity of skin involvement had a direct relationship with levels of Dsg 1 antibodies. Amagai et al[39] and Ding et al[40]

have shown a difference in the antibody profile among mucosal dominant PV, mucocutaneous PV and PF. However Sharma et al[8] and Jamora et al[82] found that the antibody profile did not correlate with the different morphological types. Sharma et al[8] suggest that other factors such as genetic and racial influences or the effect of some minor antigens yet to be characterized may be considered to explain the differences.

When severity of disease graded between 0 to 3 separately for oral lesions and skin lesions was compared with antibody titres there was a trend for increase in Dsg 1 titres with increasing severity of skin disease. However though patients with oral disease had titres significantly higher than those without the disease, there did not seem to be much difference in titres between varying severity of disease. Amagai et al,[7] Ishii et al[6] and Kumar et al[9] have found parallel fluctuation of titres with disease activity. Harman et al[81] found a wide range of values within each severity scores. They speculate that since pemphigus sera target multiple epitopes on both Dsg 1 and 3 molecules, it is possible that cases with minimal disease but high antibody levels may have a high proportion of non pathogenic antibodies either by virtue of their subclass or perhaps because they bind to epitopes that do not result in disease triggering. An alternative explanation they propose is that treatment may have dampened pathogenic mechanisms triggered by antibody binding in these cases. In cases of low ELISA values but active disease they suggest it is possible that the serum contains pathogenic antibodies to non desmoglein

molecules or to epitopes on the intracellular domain of Dsg 1 or 3 which would be undetectable to ELISA.[81]

There was no significant difference in titres of patient with acute onset and chronic active disease suggesting that treatment had not modified antibody levels. However there was only a small group of 8 patients with acute onset disease as compared to 31 patients with chronic active disease. Also all patients including the chronic active group of patients had active disease at the time sera was taken and therefore presumably had high titres of disease despite treatment.

There was no significant relationship between duration of disease and ELISA titres. However it is to be noted that though there was a wide range in the duration of disease (0.6 to 108 months) all patients had active disease at the time of study.

We also analysed whether patients who have had multiple relapses had titres higher than those without. There seemed to be no significant difference in titres suggesting that patients with higher titres did not have more relapses. Others factors like rapid tapering and discontinuation of medication which might have caused a relapse were not studied.

Dsg 3 was positive in 23 (82.14%) of the 28 patients with PV, while Dsg 1 was positive in 18 (64.28%) of the 28. Dsg 1 was positive and Dsg 3 was negative in all patients with PF.

Two PV patients in our study had only cutaneous lesions. They had no past history of oral lesions. Histopathology showed suprabasal bullae and DIF showed fishnet fluorescence for IgG rimming keratinocytes. They were diagnosed to have cutaneous pemphigus. While both patients had positive Dsg 1 both had negative Dsg 3. A similar study by Sharma et al[8] have reported six patients with cutaneous pemphigus.[8] All of them had elevated Dsg1 and four had positive Dsg 3. Yoshida et al[64] studied 4 patients with cutaneous pemphigus and they have noted that during the course of treatment anti-Dsg 3 IgG decreased more quickly than anti-Dsg1. One of our patients one had received oral steroids prior to presentation and this may explain why Dsg 3 was negative.

Our study also included one patient with paraneoplastic pemphigus. He had an associated Castleman's tumor. He presented with intractable stomatitis, ocular and genital lesions. Histopathology showed a suprabasal bulla and DIF showed fishnet fluorescence for IgG and linear deposition for IgG and C3 along the basement membrane. Both Dsg 1 and 3 were elevated. Pateints with PNP raise antibodies against multiple antigens including desmoplakin I and II, BPAg1, envoplakin, periplakin, HD1/plectin as well as desmogleins.[89,90] Studies have shown that the association between clinical phenotype and anti-desmoglein autoantibody profile in PNP is not as clear as that in classic pemphigus. [91]

One of our patients had pemphigus herpetiformis. She presented with intensely pruritic herpetiform lesions over the trunk and extremites without

oral involvement. Histopathology revealed an intraepidermal bulla and DIF showed fishnet fluorescence for IgG rimming the keratinocytes. Dsg 1 and 3 were positive. A study on a large series of patients with pemphigus herpetiformis showed that the main target autoantigen is Dsg 1 and that Dsg 3 is detected in a few cases. [85]

The patient with IgA pemphigus presented with flaccid annular vesicles over the face, abdomen, elbows and legs. Histopathology showed an intraepidermal bulla. DIF showing fishnet fluorescence for IgA established the diagnosis. As expected, IgG antibodies to Dsg 1 and 3 were detected to be negative in this patient. This was consistent with previous reports of absence of antibodies to Dsg 1 and Dsg 3 using ELISA for IgG antibodies. Hashimoto et al[54] have demonstrated IgA antibodies to desmogleins in patients with IgA pemphigus using a new ELISA for detecting IgA antibodies.

Patients with bullous disorders present physicians with formidable challenges. Precise diagnosis is important for determining treatment plans and for prognostication. Sensitive and specific testing for the pathogenic autoantibodies found in PV and PF would be helpful in the clinical management and laboratory investigation.

Dsg 1 and Dsg 3 ELISAs evaluated in this study were highly specific and sensitive technique for diagnosing pemphigus. Moreover it is possible to differentiate PV from PF. This new diagnostic tool will be useful in dermatology

clinics to make a rapid and accurate diagnosis especially where facilities for histopathology, DIF or IIF are not available.

ELISA scores may be useful to plan tapering schedules of corticosteroids and to predict flares or relapses by detecting increase in antibody titres before clinical evidence of disease flares are noticed. Further prospective studies are needed to confirm this. Studies monitoring titres in patients in remission will provide useful data regarding correlation of titres with disease activity. Further studies are needed to determine whether the distribution of lesions affects antibody titres, due to the differential distribution of desmoglein in the body.

CONCLUSIONS

1. There were a total of 75 patients in the study consisting of 39 patients and 36 controls. The pemphigus patients had a mean age of 43.21 years and the male to female ratio was 1:1.43. Among the clinical variants of pemphigus there was a predominance of PV patients as seen in other studies.
2. ELISA estimation of Dsg 1 antibodies in pemphigus had a sensitivity and specificity of 100% and 97.2% respectively. Dsg 3 estimation had a sensitivity and specificity of 82.14% and 100% respectively. This is similar to the sensitivity and specificity reported from other studies.
3. The cut off values with the highest sum of sensitivity and specificity for Dsg 1 and Dsg 3 ELISAs in the population studied were 17.79 and 28.91 respectively.
4. Dsg 3 was positive in 23 (82.14%) of the 28 patients with PV, while Dsg 1 was postitive in 18 (64.28%) of the 28. Dsg 1 was posititve and Dsg 3 was negative in all patients with PF.
5. Using ELISA for Dsg 1 and Dsg 3 antibodies, it is possible to differentiate PV from PF.
6. There was a significant correlation between the presence of mucosal disease and positive Dsg 3 antibodies, and the presence of cutaneous disease and positive Dsg 1 antibodies.

7. There was a trend for increase in Dsg 1 titres with increasing skin severity scores but no such trend was seen between increasing oral severity scores and Dsg 3 antibody titres.
8. There was no significant difference in Dsg titres between patients with acute onset and chronic active disease.
9. There was no correlation between Dsg titres and duration of disease.
10. There was no correlation between Dsg titres and number of relapses.

LIMITATIONS OF THE STUDY

1) Due to financial constraints serial dilutions were not done for patients with high titres of antibodies to avoid the plateau effect.
2) Follow up titres were not available to correlate Dsg titres with severity of disease.
3) The ELISA kit used detected only IgG antibodies.
4) Severity of genital and conjunctival lesions were not scored.
5) There were no patients with remittent disease.
6) There were only 8 patients who had not received immunosuppressive therapy prior to Dsg estimation.
7) There was only one patient in each of the rarer variants of pemphigus in our study. This was insufficient to reach any significant conclusion.

SUMMARY

Pemphigus is an uncommon but formidable blistering disease. The current diagnostic tests are time consuming, require experienced examiners and are expensive. Recently ELISA estimation of Dsg antibodies has been shown to be a highly sensitive and specific test.

A cross sectional study was conducted between March 2005 and July 2006. The study looked at the validity of ELISA estimation of Dsg 1 and Dsg 3 antibodies in pemphigus as a diagnostic test. The ability to differentiate PV from PF was also studied. The correlation of desmoglein titres with mucosal and cutaneous disease, with severity of disease and prevalence of Dsg 1 and 3 antibodies in the various clinical variants were also studied.

There were a total of 39 patients and 36 controls. In all patients, the diagnosis was based on clinical examination, histopathology and DIF findings. The mean age of the pemphigus patients was 43.21 years and the male to female ratio was 1:1.43. The clinical profile of patients was similar to that previously reported.

We were able to validate ELISA estimation of Dsg antibodies as a diagnostic test for pemphigus with a sensitivity and specificity of 100% and 97.2% for Dsg 1 and sensitivity and specificity of 82.14% and 100% for Dsg 3

respectively. Serodiagnosis matched the diagnosis in 100% of PF patients and 82.14% of PV patients. There was a significant correlation between positive Dsg 1 antibodies and presence of skin lesions and positive Dsg 3 antibodies and presence of mucosal lesions. There was a trend for increase in Dsg 1 titres with increase in severity of cutaneous disease but this was not seen between Dsg 3 and mucosal disease. Prior treatment with steroids did not seem to modify titres when lesions were active. However there was no correlation between Dsg titres and duration of disease or between Dsg titres and number of prior relapses. Dsg levels can be a useful adjunct in the diagnosis of the rarer variants of pemphigus.

Our study validates ELISA estimation of Dsg antibodies as a sensitive and specific test for the diagnosis of pemphigus in Indian patients. It will be particularly useful as a rapid and economical test in dermatology clinics especially where histopathology and DIF are not available.

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ANNEXURE I

INFORMED CONSENT DOCUMENT

It has been explained to me that the study involves my examination.

The necessity of investigations as well as need for regular follow up has been explained to me. I also understand that the information I divulge is confidential and shall be used for study purposes only. Thereafter I give my informed consent for the same.

Date: Signature of the patient/guardian

CMCH, Vellore.

ANNEXURE II

PROFORMA FOR PATIENTS

S.No: Date:

Name:

Hospital No:

Age:

Sex:

Address:

Diagnosis:

Category:

Duration of disease:

Presenting complaints:

Type of lesion	Duration	Site
Blisters\Erosions on skin		
Oral lesions		
Others		

Past History Yes/No

Oral lesions

Skin lesions

Treatment taken

	Maximum dose	Current dose	Duration
Steroids			
Oral			
Injection			
Topical			
Other immunosuppressants			
Azathioprine			
Cyclophosphamide			
Cyclosporin			
Mycophenolate mofetil			
Others			

Prior Relapses (Yes/No)

No	Date	Skin	Oral	Dose of steroid at relapse

NO	DATE	ORAL SCORE	SKIN SCORE	DsG1	DsG3

Investigations

1. Tzanck
2. Histology
3 . DIF

ANNEXURE III

PROFORMA FOR CONTROLS

S.No Date:

Name:

Hospital No

Age:

Sex

Address:

Diagnosis:

Duration of disease

Presenting complaints

Type of lesion	Duration	Site

Blisters\Erosions on skin

Oral lesions

Others

Treatment taken

	Maximum dose	Current dose	Duration
Steroids			
Oral			
Injection			
Topical			
Other immunosuppressants			
Azathioprine			
Cyclophosphamide			
Cyclosporin			
Mycophenolate mofetil			
Others			

Investigations

- Gram stain
- Histopathology
- DIF
- Dsg 1
- Dsg 3

GLOSSARY TO THE MASTER TABLE

AGE-Age in years

SEX: 1=Male, 2=female

GROUP: 1=Patients, 2=Controls

DIAGN -Diagnosis: 1=Pemphigus vulgaris, 2=Pemphigus vegetans, 3=Pemphigus foliaceous, 4=Paraneoplastic pemphigus, 5=Pemphigus herpetiformis, 6=Chronic bullous dermatosis of childhood, 7=IgA pemphigus, 8=Oral pemphigus, 9=Bullous pemphigoid,

10=Impetigo, 11=Stevens Johnson syndrome- toxic epidermal necrolysis (TEN)overlap / TEN, 12=Staphylococcal scalded skin syndrome, 13=Epidermolysis bullosa acquisita.

CATE-Category: 1=Acute onset, 2=Chronic active, 3 =Remittent.

DUR_SK-Duration of lesions in months

For the following: 0= No, 1=Yes:

PRIOR_S - Prior skin lesions

PRIOR_O - Prior oral lesions

SKIN_LE - Skin lesions at present

ORAL_LE - Oral lesions at present

OTHERS - Others sites involved: 0=No, 1=Genital, 2=Genital and conjunctival, 3=Conjunctival

For the following: 0= No, 1=Yes:

HEAD - Lesions on the head

TRUNK - Lesions on the trunk

UL - Lesions on the upper limbs

LL - Lesions on the lower limbs

CURENT - Current treatment

STEROI-Steroids

ORAL-Oral steroids

O_DO-Dose of oral steroids:0=No, 1=High>60mg, 2=Moderate=10-60,

3=Low<10mg

INJ-Injectable steroids: 0=No, 1=Yes

I_DO- Dose of injectable steroids in mg;0=No, 1=High dose, 2=Low dose

TOP-Topical steroids: 0=No, 1=Yes

AZA-Azathioprine: 0=No, 1=Yes

A_DO-Dose of Azathioprine in mg: 0=No, 1=50 mg, 2=100 mg

CYP-Cyclophosphamide: 0=No, 1=Yes

CYP_DO-Cyclphospamide dose in mg; 0=No, 1=50 mg, 2=100mg

CYS-Cyclosporine: 0=No, 1=Yes

CYS_DO-Cyclosporine dose in mg:0=No

MMF-Mycophenolate moefetil: 0=No, 1=Yes

M_DO-Myocphenolate dose: 0=No,1=1gm

OTH-Others: 0=No, 1=Dapsone, 2=Methotrexate

0_DO-Dose of othes in mg: 0=No, 1=25mg, 2=50mg

PAST_R-Past treatment: 0=No, 1=Yes

STEROI_1- Past treatment with steroids: 0=No, 1=Yes

ORAL_1- Past treatment with oral steroids: 0=No, 1=Yes

O_DO_1- Maximum dose of past treatment wih oral steroids in mg: 0=No,1=High >60 mg,2=Moderate 10-60, 3=Low <10mg

O_DU- Duration of past treatment withoral steroids in months

INJ_1-Past treatment with injectable steroids: 0=No, 1=Yes

I_DO_1- Maximum dose of past treatment wih injectable steroids dose in mg:0=No,1=High,2=Low

I_DU-Duration of past treatment with injectable steroids in months

TOP_1- Past treatment with topical steroids: 0=No, 1=Yes

T-DU-Duration of past treatment with topical steroids in months

AZA_1- Past treatment with Azathioprine: 0=No, 1=Yes

A_DO_1- Maximum dose of past treatment wih Azathioprine in mg:0=No, 1=50 mg, 2=100 mg, 3=150mg

A_DU-Duration of past treatment with Azathioprine in months

CYP_1- Past treatment with Cyclophosphamide: 0=No, 1=Yes

CYP_DO_1- Maximum dose of past treatment wih cyclphospamide in mg: 0=No,1=50mg, 2=700mg

CYP_DU-Duration of past treatment with cyclophosphamide in months

CYS_1- Past treatment with cyclosporine: 0=No, 1=Yes

CYS_DO_1- Maximum dose of past treatment wih Cyclosporine in mg: 0=No

CYS_DU-Duration of past treatment with cyclosporine in months

MMF_1-Mycophenolate moefetil: 0=No, 1=Yes

M_DO-1- Maximum dose of past treatment wih Myocphenolate in mg:1=1500 mg

M_DU-Duration of past treatment with mycophenolate moefetil in months

OTH_1-Past treatment with other drugs: 1=Dapsone, 2=IV IG, 3=MTX,

4=Ayurvedic, 5=Pyridostigmine, 6=Antibiotics, 7=Uknown, 8=ART

0_DO_1- Maximum dose of past treatment wih others in mg: 1=<50, 2=50, 3=>50.

O_DU-Duration of past treatment with others in months

PRIR_Re-Pior relapses: 0=No, 1=1, 2=2, 3=3, 4=>3

NUM-Number of prior relapses

R1-Date of first relapse (dd/mm/yy)

SKIN_1-Skin lesions at first relapse: 0=No, 1=Yes

ORAL_1-Oral lesions at first relapse: 0=No, 1=Yes

DS-S1-Dose of steroid at first relapse in mg

R2-Date of second relapse (dd/mm/yy)

SKIN_2-Skin lesions at second relapse: 0=No, 1=Yes

ORAL_2-Oral lesions at second relapse: 0=No, 1=Yes

DS-S2-Dose of steroid at second relapse in mg

R3-Date of third relapse (dd/mm/yy)

SKIN_3-Skin lesions at third relapse: 0=No, 1=Yes

ORAL_3-Oral lesions at third relapse: 0=No, 1=Yes

DS-S3-Dose of steroid at third relapse in mg

R4-Date of fourth relapse (dd/mm/yy)

SKIN_4-Skin lesions at fourth relapse: 0=No, 1=Yes

ORAL_4-Oral lesions at fourth relapse: 0=No, 1=Yes

DS-S4-Dose of steroid at fourth relapse in mg

V1-Date of visit

OS1-Oral score

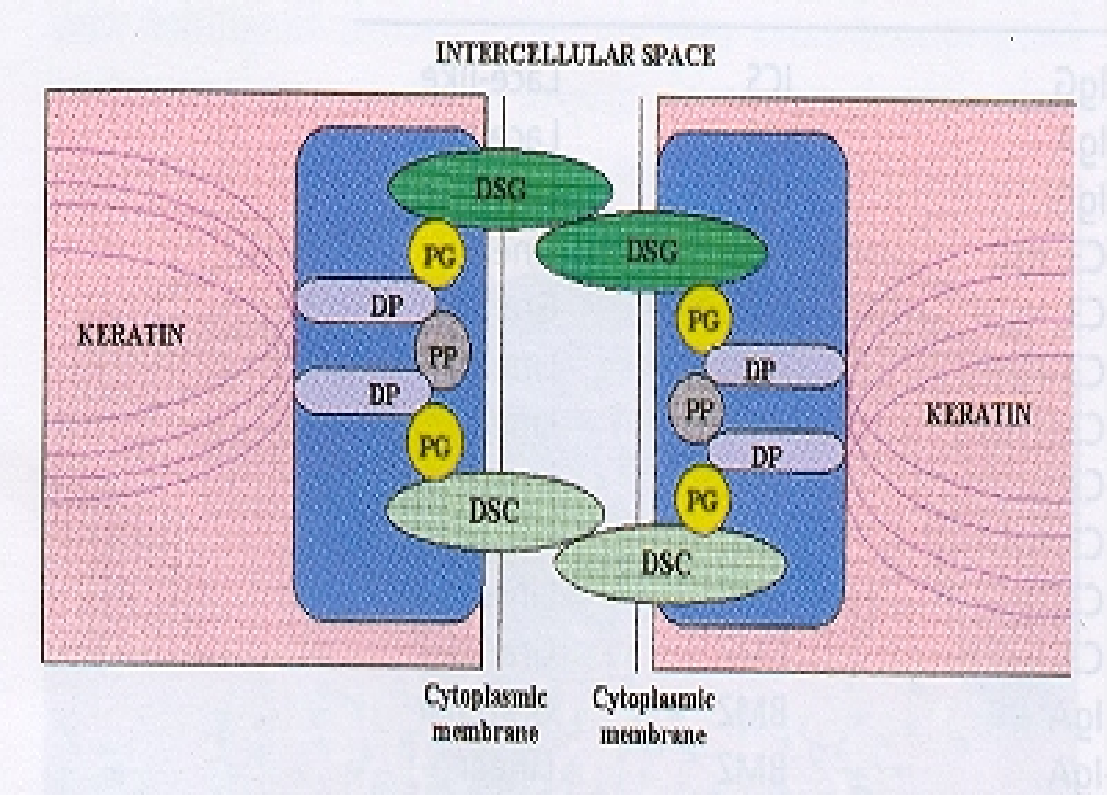
SS1-Skin score

V1DSG1-Dsg 1 titre

V1DSG3-Dsg 3 titre

BIOPSY –Biopsy consistent with: 1=Pemphigus vulgaris, 2=Pemphigus vegetans, 3=Pemphigus foliaceous, 4=Paraneoplastic pemphigus, 5=Bullous pemphigoid, 6=Chronic bullous dermatosis of childhood, 7=Stevens Johnson syndrome-Toxic epidermal necrolysis overlap/Toxic epidermal necrolysis, 8=Oral pemphigus, 9=Subepidemal bullous disorder, 10=IgA pemphigus, 11=Pemphigus herpetiformis.

DIF-Direct immuofluorescence findings: 1=Fishnet fluorescence for IgG rimming epidermal keratinocytes, 2=1+linear fluorescence for IgG along basement membrane, 3=Fishnet fluorescence for IgA rimming keratinocytes, 4=Linear fluorescence for IgG and c3 along basement membrane, 5=Linear fluorescence for IgA along the basement membrane, 6=Negative



Dsg-Desmoglein Dsc-Desmocollin PG-Plakoglobin

DP-Desmoplakin PP-plakophilin

Fig. 1: Structure of desmosomes: The desmosome complex includes desmogleins and desmocolllins as transmembrane components, and plakoglobin, plakophilin and desmoplakin as cytoplasmic components.[28]

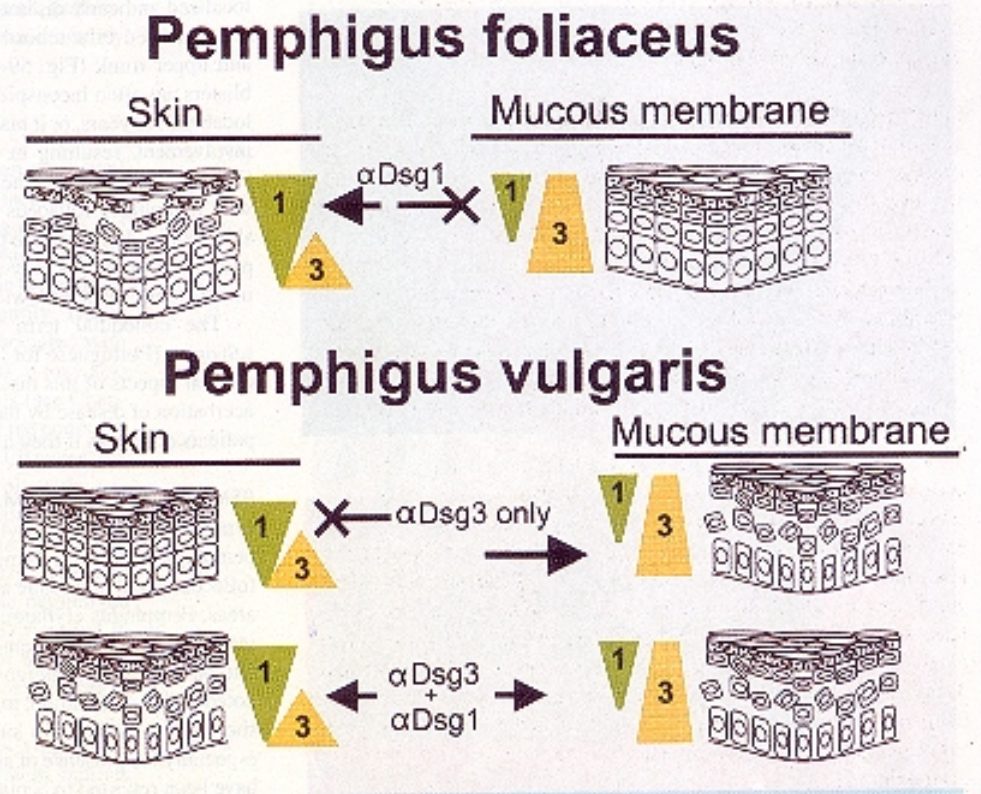


Fig. 2: Desmoglein compensation hypothesis.Triangles represent the distribution of Desmoglein (Dsg) 1 and Dsg 3 in skin and mucous membrane. Anti-Dsg 1 antibodies in pemphigus foliaceous cause acantholysis only in the superficial epidermis of skin. In the deep epidermis and in mucous membranes, Dsg 3 compensates for antibody induced loss of function of Dsg 1. In early pemphigus vulgaris antibodies are present only against Dsg 3, which cause blisters only in the deep mucous membrane where Dsg 3 is present without compensatory Dsg 1. However in mucocutaneous pemphigus antibodies against both Dsg 1 and Dsg 3 are present and blisters form both in mucous membrane and skin. The blister is deep probably because antibodies diffuse from the dermis and interfere first with the function of desmosomes at the base of the epidermis.[13]

Table 1: Clinical Variants of Pemphigus[10]

Pemphigus vulgaris
Variant :Pemphigus vegetans
Pemphigus foliaceous
Variant : Pemphigus herpetiformis
Variant : Pemphigus erythematosus
Induced Pemphigus
Intercellular IgA dermatosis
Paraneoplastic pemphigus

Table 2: Target antigens in Pemphigus[28]

Diseases	Auto-antibodies	Antigens	Location of Antigens
Pemphigus vulgaris			
Mucosal mainly	IgG	Desmoglein 3 (130 kD)	Desmosomes
Mucocutaneous	IgG	Desmoglein 3 (130 kD) Desmoglein 1 (160 kD)	
Pemphigus foliaceus	IgG	Desmoglein 1 (160 kD)	Desmosomes
Paraneoplastic pemphigus	IgG	Desmoglein 1 (160 kD) Desmoglein 3 (130 kD) Desmoplakin I (250 kD) Envoplakin (210 kD) Periplakin (190 kD) Plectin (500 kD) BPAG1 (230 kD)[a] γ-Catenin (plakoglobin- 82 kD)	Desmosomes or hemidesmosomes
Drug-induced pemphigus	IgG	Desmoglein 3 (130 kD) Desmoglein 1 (160 kD)	Desmosomes
IgA pemphigus			
SPD type	IgA	Desmocollin 1 (110/100 kD)	Desmosomes
IEN type	IgA	Desmoglein 1 (160 kD) Desmoglein 3 (130 kD)	

[a]BPAG1, bullous pemphigoid antigen.

Table 3: Direct immunofluorescence testing in pemphigus[28]

Dermatosis	Principal immunoreactant	Site	Pattern
Pemphigus,all variants except	IgG	ICS	Lace-like
IgA pemphigus	IgA	ICS	Lace-like
Paraneoplastic Pemphigus	IgG	ICS	Lace-like
	C3, IgG	BMZ	Linear
	C3, IgG	BMZ	Granular

ICS – Squamous intercellular substance

BMZ- Basement membrane zone

Table 4: Diagnostic criteria for PV and PF

Amagai et al[82] have proposed a diagnostic criteria for PV and PF as below:

	Desmoglein 1	**Desmoglein 3**
PV	+/-	+
PF	+	-

Legends to photos

Fig. 4 Flaccid bulla of pemphigus vulgaris.

Fig. 5 Extensive erosions of pemphigus vulgaris.

Fig. 7 Superficial erosions of pemphigus foliaceus.

Fig. 8 Herpetiform grouping of lesions in pemphigus herpetiformis.

Fig. 10 Suprabasal bulla in an oral biopsy specimen x 25.

Fig. 11 Subcorneal blister of pemphigus foliaceus x 20

Fig. 13 DIF showing fishnet fluorescence rimming basal keratinocytes

Fig. 14 DIF showing fishnet fluorescence rimming the full thickness of the epidermis

Fig. 16 Erosions in a patient with bullous pemphigoid

Fig. 17 Bulla in toxic epidermal necrolysis

Fig. 19 Desmoglein ELISA kit

Fig. 20 Equipment used for desmoglein ELISA test

Fig. 21 Positive and negative desmoglein 1 and desmoglein 3