The Role of Subclass Switching in the Pathogenesis of Endemic Pemphigus Foliaceus

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Endemic pemphigus foliaceus, like the sporadic form seen in the developed world, is mediated by IgG antibodies to desmoglein-1. We studied an endemic focus in Limao Verde, Brazil, where disease prevalence is 3.4%. We previously detected IgG antibodies to desmoglein-1 in 97% of patients, but also in 55% of normal subjects in the endemic focus, with progressively lower levels in normal subjects in surrounding areas. An environmental trigger is hypothesized to explain these and other findings. In this study we sought to determine if patients and enzyme-linked-immunosorbent-assay-positive normal subjects in Limao Verde differ in IgG subclass response to desmoglein-1. We developed a sensitive and specific subclass enzyme-linked immunosorbent assay using recombinant desmoglein-1 and standardized the assay to enable comparability between the four subclasses. We found that normal subjects have an IgG_1 and IgG_4 response, whereas patients have similar levels of IgG_1 but a mean 19.3-fold higher IgG_4 response. Patients in remission have a weak IgG_4 response, and a 74.3-fold higher IgG_4 response is associated with active disease. Finally, in five patients in whom we had blood samples from both before and after the onset of clinical disease, a mean 103.08-fold rise in IgG_4 was associated with onset of clinical disease, but only a mean 3.45-fold rise in IgG_1. These results suggest that the early antibody response in normal subjects living in the endemic area and in patients before the onset of clinical disease is mainly IgG_1. Acquisition of an IgG_4 response is a key step in the development of clinical disease. *Key words: autoantibodies/autoimmunity/desmoglein/fogo selvagem/IgG_4. J Invest Dermatol 120:000–000, 2003*

Endemic pemphigus foliaceus is found mainly in rural areas in Brazil (Diaz *et al*, 1989a; 1989b) as well as in Colombia and Tunisia (Robledo *et al*, 1988; Morini *et al*, 1993). Like the sporadic form seen in the developed world, it is mediated by IgG antibodies to desmoglein-1 (dsg1), an epidermal adhesion molecule (Stanley *et al*, 1986). Antibody binding leads to loss of intercellular adhesion and subsequent blister formation. Affinity-purified antibodies to dsg1 have been shown to mediate disease when passively transferred into mice (Ding *et al*, 1999), and preabsorption of antidsg1 antibodies has been shown to prevent disease in the mouse model (Amagai *et al*, 1995).

We have recently identified a new focus of endemic pemphigus foliaceus in Limao Verde, Brazil, where the prevalence of disease is 3.4% (a total of 31 patients in a population of 916) (Hans-Filho *et al*, 1996). Using a highly sensitive (97%) and specific (98%) enzyme-linked immunosorbent assay (ELISA) (Warren *et al*, 2000), 30 of 31 patients had detectable IgG antibodies to dsg1. We also identified IgG antibodies to dsg1 in 55% of normal subjects living in this endemic focus, and at lower levels in normal subjects living in nearby areas. Antibodies to dsg1 were also detected in patients with endemic pemphigus up to 4 y before the onset of clinical disease, with a marked increase in antibodies to dsg1 (as measured in an ELISA for total IgG) associated with onset of clinical disease. An environmental trigger factor has been proposed to explain these and other findings (Diaz *et al*, 1989b; Hans-Filho *et al*, 1996; Warren *et al*, 2000). These findings also raise the issue of why the normal subjects with antibodies to dsg1 did not develop clinical disease.

In this study we therefore sought to determine if patients and normal subjects differ in terms of their IgG subclass response to dsg1.

METHODS

Sources of sera We obtained sera from all 31 patients with endemic pemphigus foliaceus in Limao Verde, Brazil. For five patients we also obtained serum samples from before the onset of clinical disease. The patients consisted of 13 women and 18 men. Their age range was 7–71 y, with a mean age of 36 y. The diagnosis of endemic pemphigus was made by a dermatologist on the basis of clinical examination, biopsy, direct immunofluorescence, and indirect immunofluorescence. We also selected serum samples from 31 normal subjects living in Limao Verde from a larger group of 93 that were previously tested (Warren *et al*, 2000) who had a strong total IgG antibody response to dsg1. They were of comparable age and sex to the patients, with 13 women and 18 men. The age range was 5–85 y and the mean age was 38 y. We also selected serum samples from 10 normal subjects living in Limao Verde who had previously been identified as having undetectable levels of antibodies to dsg1, and 41 normal subjects from the U.S.A. The latter were randomly selected healthy

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Abbreviation: dsg, desmoglein; TBS-Ca^{2+}, Tris-buffered saline with calium.

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blood donors, identified only by a code. All normal subjects from Brazil were examined by a dermatologist at the time of blood drawing. Blood samples from Limao Verde were shipped to the nearby city of Campo Grande, where the serum was separated and stored at −20°C. The samples were shipped frozen to the U.S.A. where all immunologic testing was done. The study had the approval of FUNAI, a body representing the interests of the Indians of Limao Verde, as well as institutional approval of the University of North Carolina, Chapel Hill.

ELISA The complete extracellular domain of dsg1 was produced in the baculovirus system (Ding *et al*, 1997). A polyhistidine tag at the carboxy end enabled purification on a nickel-NTA affinity column (Qiagen, Chatsworth, CA). Protein was eluted from the column with 150 mM imidazole in Tris-buffered saline with calcium (TBS-Ca^{2+}), pH 8.0. The imidazole was removed by dialysis against TBS-Ca^{2+} pH 7.2 for 48 h using 25 kDa cellulose ester dialysis bags (Spectrum, Houston, TX, USA). The presence of the 66 kDa extracellular domain was confirmed by immunoblot assay using rabbit antidsg1 antibodies. The average yield of protein was 20 μg per ml of crude supernatant.

Dsg1 in TBS-Ca^{2+}, 0.2% bovine serum albumin (BSA) was coated onto nickel-NTA ELISA plates (Qiagen) at 1.1 μg per well and incubated overnight at 4°C. Plates were washed five times with TBS-Ca^{2+} pH 7.2, 0.05% Tween-20, and the patient or normal subject sera were added in duplicate at a dilution of 1:50 in TBS-Ca^{2+}, 0.05%Tween, 0.2% BSA. Wells were incubated for 1 h and washed as above. Each serum sample was incubated with horseradish-peroxidase-conjugated mouse monoclonal antibody specific for human IgG_1, IgG_2, IgG_3, and IgG_4 (Zymed Laboratories, San Francisco, CA), for 1 h. Following a wash step, 200 μl of O-phenylenediamine in phosphate−citrate−perborate buffer (Sigma, St. Louis MO) was added for color development and 2 N H_2SO_4 was used to stop the reaction after 10 min.The optical density (OD) was read at 492 nm using an ELISA plate reader (Biorad, Hercules, CA).

The dilutions of the secondary antibodies were determined as follows. Elisa plates were coated for 1 h with 14 ng of purified IgG_1, IgG_2, IgG_3, and IgG_4 (Sigma) in bicarbonate buffer pH 9.6. Plates were blocked with 0.2% BSA (Sigma) in phosphate-buffered saline (PBS) and washed in PBS, 0.05% Tween-20, and the horseradish-peroxidase-conjugated monoclonal mouse antihuman IgG subclass antibodies (Zymed) were added. The dilutions of the secondary antibodies were adjusted so that each gave the same OD result with its own subclass standard.

The same positive and negative controls were used in duplicate on every ELISA plate, and incubated with all four secondary antibodies on every plate.

Statistics Duplicate ELISA results were averaged. A cut-off value for each subclass ELISA was established as a value 3 SD above the mean of the 41 normal donors from the U.S.A. Several sera generated negative values after subtraction of background and correction for plate-to-plate variability, as their OD value was below the negative control. An ELISA index value for each subclass was obtained for each patient or normal subject as follows: (mean test result−mean subclass negative control) ÷ (mean subclass positive control−mean subclass negative control) × subclass correction factor × 100.

The subclass correction factor was determined for each subclass as the mean of all the positive controls for that subclass on all plates minus the mean of the negative controls, divided by the cut-off for that subclass assay. The reason for the use of this correction factor was to maintain the comparability of the different subclass results to each other. As a result, any index value over 1 was considered positive. Groups of patients and normal subjects were compared using the Van der Waerden nonparametric procedure. For comparing patients before and after the onset of disease, the sine rank test was used.

RESULTS

Characterization of the dsg1 subclass ELISA assays We tested 41 normal donors from the U.S.A. One control serum out of 41 fell just above the cut-off for the IgG_2 and IgG_4 assays, with values of 1.44 and 1.003, respectively. Two control sera fell just above the cut-off for IgG_1, with values of 1.29 and 1.27. No sera fell above the cut-off for the IgG_3 assay. Specificity for the assays was therefore as follows: IgG_1 was 95%, IgG_2 was 97.5%, IgG_3 was 100%, and IgG_4 was 97.5%. To confirm the specificity of each monoclonal secondary antibody for its own immunoglobulin subclass, plates were coated with standard concentrations of all four purified IgG subclasses. Each subclass assay was tested on plates coated with its own purified immunoglobulin subclass and all the others. No cross-reactivity was seen. The mean OD value for wells in which reactivity was expected was 1.47, whereas the mean OD value for wells in which no reactivity was expected was 0.016. Antibodies were detected in 28 of 31 patients in one or more subclasses, giving the assay a sensitivity of 90%. All three patients with a negative test were in clinical remission at the time of testing.

IgG subclass response to dsg1 in patients with endemic pemphigus foliaceus We tested all 31 patients with endemic pemphigus in Limao Verde. The humoral immune response was predominantly IgG_1 and IgG_4 (see **Fig 1**, *gray bars*). We detected IgG_1 subclass antibodies to dsg1 in 25 of 31 patients (mean index value 5.99), and IgG_4 subclass antibodies in 24 out of 31 (mean index value 111.42). The mean index value for IgG_2 was just above the cut-off (1.62 units) and the mean value for IgG_3 was just below the cut-off (0.16 units).

IgG subclass response to dsg1 in normal subjects living in the endemic area We tested 31 normal subjects from Limao Verde who had previously been identified (Warren *et al*, 2000) to have a high level of IgG antibodies to dsg1 by ELISA and immunoprecipitation (see **Fig 1**, *black bars*). We detected IgG_1 subclass antibodies to dsg1 in 28 of 31 normal subjects, with a mean index value of 5.84. This was not significantly different from IgG_1 in the patients (mean index value 5.99, $p = 0.53$). We detected low levels of IgG_4 subclass antibodies to dsg1 in 17 of 31 normal subjects. The mean index value was 5.76, which was 19.3-fold lower than the mean index value in the patients (index value 111.42). Patients and normal subjects were significantly different in the IgG_4 assay ($p = 0.004$). The mean index values for IgG_2 and IgG_3 were both below the cut-off (mean index values 0.79, 0.09, respectively) and there were no significant differences between the patients and normal subjects ($p = 0.18$ for IgG_2, $p = 0.83$ for IgG_3). We also tested a separate group of 10 normal subjects from Limao Verde who had previously been identified to have no IgG antibodies to dsg1 by ELISA and

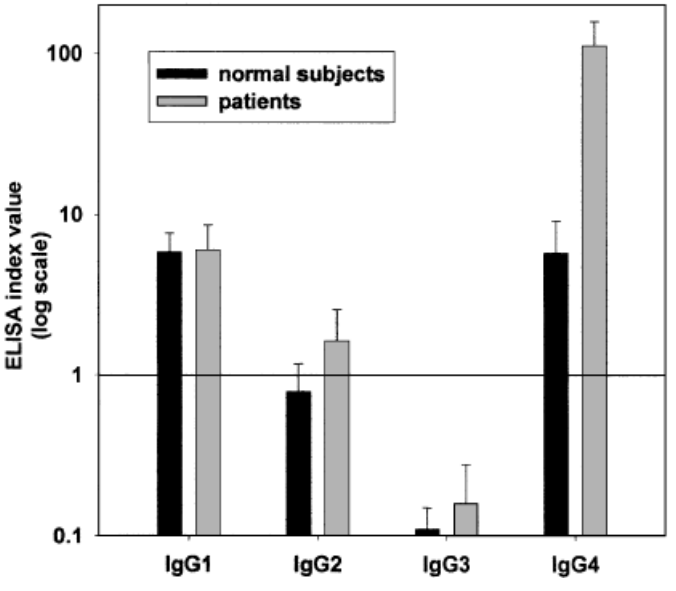

Figure 1. Mean IgG subclass values in 31 patients and 31 ELISA-positive normal subjects from Limao Verde. Antibodies to dsg1 in each subclass were measured by ELISA. Each assay was standardized so as to enable comparability between subclasses. A group of 41 normal subjects from the U.S.A. was used to determine the normal ranges for each subclass (see *Methods*). All 31 patients in Limao Verde (*gray bars*) were compared to a group of 31 age and sex matched normal subjects (*black bars*) from the endemic area who were previously confirmed to have an antibody response to dsg1. *Bars* indicate mean ± SEM.

immunoprecipitation (Warren *et al*, 2000). No antibodies in any IgG subclass were detected except for two normal subjects whose IgG_4 OD value was just above the cut-off (index values 2.19 and 1.77) (data not shown).

Levels of IgG_4 correlate closely with disease activity and remission Information about the presence of active disease or remission at the time of the blood sample was available for all patients. The level of IgG_4 correlated closely with disease activity and remission. A positive IgG_4 test was associated with active disease in 22 out of 23 patients. A negative IgG_4 test was associated with remission in six of eight patients. These correlations were not seen with IgG_1, which was positive in active disease in 20 of 23 patients but also remained positive in remission in five of eight patients. Mean levels of IgG_4 (**Fig 2**) were 74.3-fold higher in patients with active disease (index value 149.47) than in those in remission (mean index value 2.01), and these differences were significantly different ($p = 0.0006$). This marked trend was not seen with IgG_1 where the mean value in active disease was 7.01 and the mean value in remission was 3.08, giving a 2.2-fold increase associated with active disease. The difference in IgG_1 was not significant at the 5% level ($p = 0.086$). There was also a significant difference between remission and active disease for IgG_2 ($p = 0.009$). Only 11 of 23 patients with active disease had a weakly positive IgG_2 response, however, and the mean IgG_2 response was only just above the cut-off (2.04). For IgG_3 there was again a statistically significant difference between active disease and remission ($p = 0.024$); however, only one patient with active disease had a weak positive result (1.18), and the mean values were below the cut-off for both active disease and remission.

In five patients with endemic pemphigus, from whom blood samples were available before and after the onset of clinical disease, a marked rise in antidsg1 antibodies of IgG_4 subclass was observed with onset of the disease We tested five patients from Limao Verde from whom blood samples were available from 1 to 4 y before the onset of clinical disease, as well as after the onset of clinical disease (**Fig 3**). In three of five, anti-dsg-1 antibodies of IgG_1 subclass were detectable 1–4 y before the onset of the disease. Onset of the disease was associated with a mean increase in IgG_1 OD value of 3.45-fold ($p = 0.15$). In contrast IgG_4 antibodies were detectable in only two of five before the onset of disease, and there was a marked rise in levels of IgG_4 antibodies in four of five patients with a mean 103.08-fold rise in ELISA value for IgG_4 after onset of disease ($p = 0.03$).

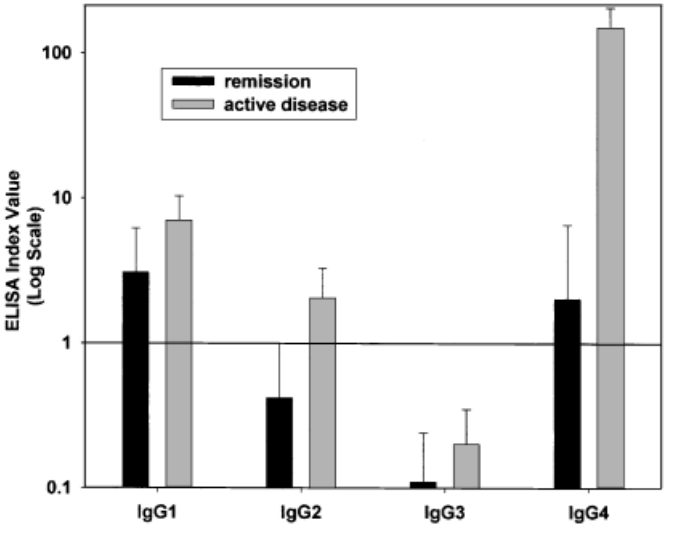

Figure 2. Mean IgG subclass values for eight patients in remission and 23 patients with active disease. Patients with endemic pemphigus were divided into a group of eight in clinical remission (*black bars*) and 23 with active disease (*gray bars*) and assayed using the subclass ELISA for reactivity to dsg1. *Bars* indicate mean ± SEM.

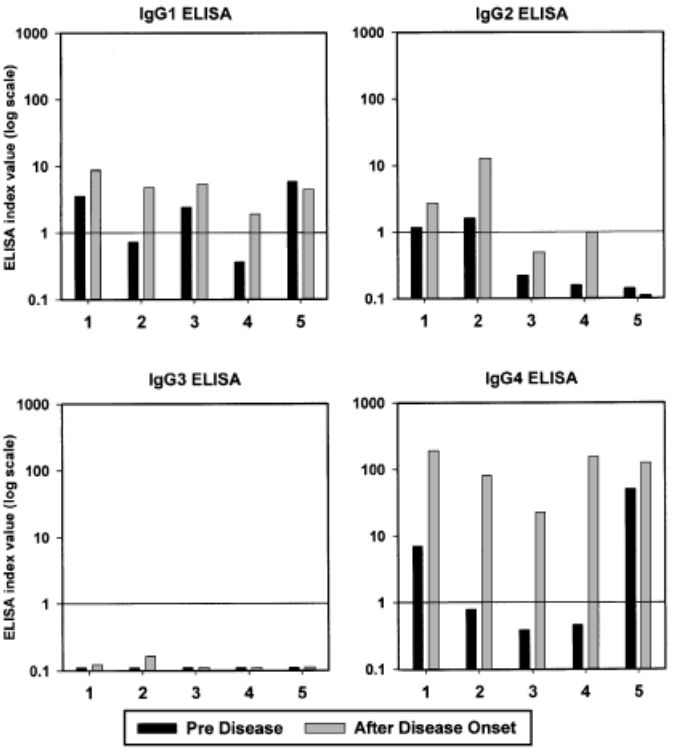

Figure 3. IgG subclass values in five patients before and after the onset of clinical disease. In five patients *(*numbered 1–5*)* blood samples were available up to 4 y before the onset of clinical disease, as well as after the onset of clinical disease. Levels of antibodies to dsg1 in the IgG subclasses were compared both before (*black bars*) and after (*gray bars*) the onset of clinical disease, using the subclass ELISA for dsg1.

DISCUSSION

It has been well documented that patients with pemphigus have a pathogenic antibody response to dsg1 (Rock *et al*, 1989; Amagai *et al*, 1995; Ding *et al*, 1999). We have previously shown that antibodies to dsg1 are also detectable in normal subjects living in the endemic area, and in patients 1–4 y before the onset of clinical disease (Warren *et al*, 2000). In this study we show that normal subjects and patients from Limao Verde differ mainly in terms of their IgG_4 subclass response to dsg1 (**Fig 1**). Patients in remission differ from patients with active disease mainly in terms of their IgG_4 response (**Fig 2**). In the five patients from whom we had blood samples both before and after the onset of clinical disease (**Fig 3**), the immune response before onset of disease again differs from that after onset of disease mainly in terms of IgG_4.

These results suggest that progression from a preclinical to a clinical phase of the disease, and also the transition from disease in remission to active disease, is closely associated with subclass switching from IgG_1 to IgG_4. These results also suggest that antibodies to dsg1 of IgG_1 subclass alone are incapable of triggering disease. Preliminary studies (Lin *et al*, unpublished observations) suggest that IgG_1 subclass antibodies from a normal subject who has only an IgG_1 response are incapable of mediating disease at 15 mg per kg in the mouse passive transfer model. In contrast IgG_4 antibodies from a patient were capable of mediating disease at 1.5 mg per kg, a concentration 10 times lower.

This study raises the issue of susceptibility genes for pemphigus. If we assume that both patients and normal subjects living i n the endemic area are exposed to the putative environmental trigger (Robledo *et al*, 1988; Diaz *et al*, 1989b; Warren *et al*, 2000),

possibly patients are able to make a much stronger pathogenic IgG_4 response because of the presence of susceptibility gene(s). HLA susceptibility genes for endemic pemphigus have already been identified in Limao Verde and elsewhere in Brazil, namely HLA DRB1-0102, 0404, 1402 (Moraes *et al*, 1991; Cerna *et al*, 1993; Moraes *et al*, 1997). These alleles all share a consensus sequence LLEQRRAA at position 67–74 in the third hypervariable domain of the DRB1 gene. These alleles confer a high relative risk of disease and are seen at lower levels in normal subjects from endemic areas (Moraes *et al*, 1997). It is possible that exposure to the unknown environmental trigger is enough for over half of those living in the endemic area to produce a nonpathogenic antibody response (Warren *et al*, 2000). The HLA susceptibility alleles would then be required for production of a pathogenic IgG_4 response and the development of disease. Circumstantial support for this model comes from the finding that the ELISA-positive normal subjects do not have an increase above the population level in the HLA susceptibility alleles (Diaz, Warren *et al*, unpublished), suggesting that the susceptibility alleles are not involved in the production of the initial, nonpathogenic antibody response, but rather play a role in the transition to a pathogenic antibody response.

Additional support for this model comes from the animal models of myasthenia gravis and multiple sclerosis (Piddlesden *et al*, 1993; Karachunski *et al*, 1995; Drachman *et al*, 1998; Yang *et al*, 1998; Ichikawa *et al*, 1999). Drachman *et al* (1998) and Yang *et al* (1998) immunized two groups of congenic mice with purified acetylcholine receptor. The two groups of mice differ only in a three amino acid portion of the MHC $I\text{-}A^{-b}$. One group is capable of producing a pathogenic antibody response of subclass IgG_2b resulting in clinical disease, whereas the other group produces a nonpathogenic antibody response without accentuation of IgG_2b subclass, and does not develop clinical disease. Interestingly the two groups of mice also responded to different epitopes on the acetylcholine receptor. Ichikawa *et al* (1999) immunized NOD mice with myelin oligodendrocyte protein, and identified a subgroup with a severe multiple-sclerosis-like disease and a subgroup with mild or no disease. Both subgroups had an antibody response to myelin oligodendrocyte protein, and mean total IgG against myelin oligodendrocyte protein was similar in the two subgroups; however, the subgroup with severe disease had a predominantly IgG_2b response that was not seen in the other subgroup.

Similar findings have also been recorded in pemphigus vulgaris (Jones *et al*, 1988; Bhol *et al*, 1994; Hertl and Riechers, 1999; Kricheli *et al*, 2000). Kricheli *et al* (2000) identified antibodies to dsg3 in the serum of 91% of patients by immunoblot, and also in 49% of those related to patients. The distribution of IgG_1 through IgG_3 was similar in patients and relatives. IgG_4 was seen in 62% of the patients but fewer than 2% of the relatives and none of the normal subjects. Bhol *et al* (1994), using an immunoblot assay, found a predominantly IgG_1 response to dsg3 in relatives of patients and patients in remission, with patients with clinical disease having an IgG_1 and IgG_4 response. Our group has previously identified IgG_4 as being the predominant antibody subclass in endemic pemphigus foliaceus, and also capable of reproducing the disease after passive transfer into mice (Rock *et al*, 1989). In addition IgG_1 has been detected early in the course of clinical disease, with IgG_4 being detected throughout the course of clinical disease (dos Santos, 1996). This study is the first to elucidate the difference between the antibody response in normal subjects living in an endemic area and patients, the role of subclass switching in the pathogenesis of the disease, and the association of IgG subclasses with active disease *versus* remission.

Our study leaves open the possibility that the pathogenic IgG_4 antibodies recognize a different epitope to the nonpathogenic IgG_1 antibodies. This issue may be amenable to study using domain-swapped molecules. Alternatively IgG_4 may have a still unknown effector function that is not present in IgG_1, or a higher *in vivo* binding affinity for dsg1. IgG_4 is not thought to activate complement: previous studies (Rock *et al*, 1990; Espana *et al*, 1997) have suggested that complement is not essential for blister formation, and that antibodies to dsg1 may cause blisters by binding to dsg1 rather than by activating proteolytic enzymes or an inflammatory cascade. Nevertheless components of complement are usually deposited in lesional biopsies and pemphigus foliaceus sera have been shown to be capable of activating complement (Hashimoto *et al*, 1982; Kawana *et al*, 1988).

This study also makes it possible to identify a subgroup of normal subjects, namely those with a raised level of IgG_4 antibodies to dsg1, who may be at a higher risk of developing clinical disease. In addition these results suggest that the level of IgG_4 as measured in a subclass ELISA may be the most sensitive indicator of clinical activity in patients with endemic pemphigus, and possibly in other related forms of pemphigus. In conclusion, the dsg1 subclass ELISA should prove to be a powerful tool in dissecting stages in the pathogenesis of this autoimmune disease.

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