

Correlation of peptide specificity and IgG subclass with pathogenic and nonpathogenic autoantibodies in pemphigus vulgaris: A model for autoimmunity

(pathogenic autoantibodies)

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ABSTRACT Pemphigus vulgaris (PV) is a rare, potentially fatal, autoimmune disease that affects the skin and mucous membranes. The PV antigen (PVA) has been characterized as desmoglein 3. PV patients carry HLA-DR4- or HLA-DR6-bearing extended haplotypes. We recently demonstrated that patients with active disease have high titers of PV autoantibodies of the IgG1 and IgG4 subclasses. Patients in remission, healthy unaffected relatives, and some MHC-matched normal individuals have low levels of PV autoantibodies, which are IgG1 only. Furthermore, intraperitoneal injection of IgG from patients with active disease caused clinical disease in mice, but IgG from patients in remission, healthy relatives, or MHC-matched normal individuals did not. We prepared 12 peptides of 30 amino acids each (peptides Bos 1–12) spanning the extracellular domain of PVA. Patients with active disease recognize peptides Bos 1 and Bos 6 with high titers of IgG1 and IgG4 autoantibodies. Patients in remission have IgG1 autoantibodies to peptide Bos 1 only, in statistically significantly lower titers ($P < 0.01$). They no longer have IgG4 subclass autoantibodies to peptide Bos 6. Healthy relatives and normal unrelated individuals have low levels of only IgG1 autoantibodies that recognize only Bos 1. *In vitro* studies indicate that Bos 6-specific IgG and, to a lesser extent, Bos 1-specific IgG can cause acantholysis. Our data suggest that Bos 6-specific IgG4 is probably the main acantholytic autoantibody, while Bos 1-specific IgG4 may act as a facilitator or enhancer of the process. In this study we illustrate some of the paradigms that demonstrate the interactions between the MHC, subclass of autoantibodies, and peptide specificities of the autoantibodies in the autoimmune process. Thus, PV provides an important model to study the pathogenesis of autoimmunity.

Pemphigus vulgaris (PV) is a rare, autoimmune, potentially fatal, blistering disease that affects the skin and mucous membranes (1). It is characterized by the presence of an autoantibody that is directed against the 130-kDa antigen in the intercellular cement substance (ICS) now characterized as desmoglein 3 (1). The binding of autoantibody to the epidermal keratinocytes causes a loss of intercellular adhesions resulting in a process called acantholysis. The production of clinically indistinguishable disease in neonatal mice by the intraperitoneal injection of IgG from PV patients and the presence of transient PV in neonates born to mothers with PV clearly demonstrate that the final clinical manifestations of the disease are the direct consequence of high titers of the autoantibody (2). Additional evidence comes from studies in which explants of human skin demonstrated *in vitro* acanthol-

ysis when cultured in medium enriched with IgG from sera of PV patients (3).

In recent studies, we have shown that Ashkenazi Jewish patients with PV have a highly statistically significant association with the following extended haplotypes: (HLA-B38, -SC21, -DR4, -DQ3) and (HLA-B35, -SC31, -DR4, -DQ3) or a portion of them (4). Non-Jewish Caucasian patients carry the following extended haplotypes: (HLA-B35, -SC31, -DR4, -DQ3) or (HLA-B55, -SB45, -DR6, -DQ1) or portions of them (5). The development of a highly sensitive immunoblot assay (6) helped demonstrate a strong linkage between the presence of the PV autoantibody and the MHC class II genes with a lod score of 9.82 (7). In subsequent studies, we have shown that in PV patients with active disease, the high titer autoantibody is of the IgG1 and IgG4 subclasses. These are referred to as PV pathogenic autoantibodies. Patients in prolonged remission, healthy unaffected relatives, and normal controls have low titers of the IgG1 subclass autoantibody to the 130-kDa epidermal protein (8).

The PV antigen (PVA) is a transmembrane glycoprotein that has been cloned and sequenced (9). It has been shown that the PV autoantibodies bind to fusion proteins corresponding to the first and second extracellular domains of the recombinant PVA (10). The purpose of this study was to address the following questions: (i) do DR4- and DR6-associated sera recognize the same or different peptides in the PVA, (ii) do IgG1 and IgG4 subclass autoantibodies (pathogenic and nonpathogenic) recognize the same or different peptides in the PVA, and (iii) what is the peptide specificity of the pathogenic autoantibody?

MATERIALS AND METHODS

Sera. Four groups of sera were studied. Sera from 21 patients with active disease; 19 patients in clinical remission, defined as absence of clinical disease and treatment for three years; 18 healthy unaffected relatives; and 4 normal unrelated controls were studied. Of these sera, one was DR6/DQ5 and three were DR4/DQ8, and all four sera contained low levels of IgG1 autoantibodies to 130-kDa PVA on the immunoblot assay. A panel of 136 serum samples from randomly selected, unrelated normal individuals was tested to determine the frequency of binding to peptides in the PVA in the ELISA.

Peptides. On the basis of the published sequence of PVA, 12 synthetic peptides of 30 amino acids each were prepared (11). These peptides were used for the ELISA and to isolate peptide-specific autoantibodies. These peptides are called Bos (for Boston) 1–12 and their initial and terminal amino acid numbers and sequences are presented in Fig. 1. An immuno-

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Abbreviations: PV, pemphigus vulgaris; PVA, PV antigen.

BOS 1 50-79 **E W V K F A K P C R E G E D N S K R N P I A K I T S D Y Q A**
 BOS 2 80-109 T Q K I T Y R I S G V G I D Q P P F G I F V V D K N T G D I
 BOS 3 110-139 N I T A I V D R E E T P S F L I T C R A L N A Q G L D V E K
 BOS 4 140-169 P L I L T V K I L D T N D N P P V F S Q Q I F M G E I E E N
 BOS 5 170-199 S A S N S L V M I L N A T D A D E P N H L N S K I A F K I V
 BOS 6 200-229 **S Q E P A G T P M F L L S R N T G E V R T L T N S L D R E Q**
 BOS 7 230-259 A S S Y R L V V S G A D K D G E G L S T Q C E C N I K V K D
 BOS 8 269-298 R D S Q Y S A R I E E N I L S S E L L R F Q V T D L D E E Y
 BOS 9 311-340 G N E G N W F E I Q T D P R T N E G I L K V V K A L D Y E Q
 BOS 10 361-390 S R Y R V Q S T P V T I Q V I N V R E G I A F R P A S K T F
 BOS 11 394-423 Q K G I S S K K L V D Y I L G T Y Q A I D E D T N K A A S N
 BOS 12 439-468 D S K T A E I K F V K N M N R D S T F I V N K T I T A E V L

Extracellular Domain EC₁ begins at 51 and ends at 157 EC₃ begins at 270 and ends at 384
 EC₂ begins at 158 and ends at 269 EC₄ begins at 385 and ends at 493

FIG. 1. Amino acid sequences of PVA peptides Bos 1–12. For each peptide, the position of the initial and terminal amino acids is given along with the amino acid sequence, presented in the one-letter code. The sequences of the two peptides, Bos 1 and Bos 6, recognized by autoantibodies of PV patients are shown in boldface. The locations of the extracellular domains of PVA are at the bottom.

blot assay using normal human epidermal lysate as substrate was performed as described (6).

ELISA. An ELISA was designed on a pattern recently described (12, 13). Wells of microtiter plates were coated with 5 μ g of each peptide and then incubated with 100 μ l of serially diluted test sample. Specific binding was detected by a colorimetric change after the addition of subclass-specific mouse monoclonal antibodies and horseradish peroxidase-conjugated anti-mouse IgG. Thus, the titers of autoantibody were determined by using logarithmic dilutions. Peptide-specific autoantibodies were isolated by passing serum over a column containing peptide-conjugated Sepharose 4B. Bound antibodies were eluted with 0.1 M glycine-HCl buffer, pH 2.5 and immediately neutralized with Tris. This peptide-specific autoantibody was tested in the ELISA. IgG subclass specificity of the test autoantibody was determined by using monoclonal anti-IgG1, -IgG2, -IgG3, and -IgG4 antibodies (monoclonal antibodies HP6001, HP6014, HP6050, and HP6025; Sigma).

Peptide-Specificity Determination by Cross Adsorption. Sera from all four groups were similarly studied. For example, serum known to bind to Bos 6 and Bos 1 from a patient with active disease was first serially adsorbed with peptide Bos 1, and then the antibody titer to Bos 6 was determined by the ELISA and binding in the immunoblot assay. Similarly, sera depleted of binding to peptide Bos 1 by serial adsorption were then tested against the entire panel of sera in the ELISA and the immunoblot assay. For patients in remission, healthy relatives, and healthy, unrelated, normal controls, serial adsorption of sera with Bos 1 was followed by testing the binding of the sera against the entire panel of peptides in the ELISA and in the immunoblot assay.

Relation Between MHC and Binding of Autoantibody to PVA Peptides. Sera from eight DR4-bearing patients and eight DR6-bearing patients were tested in the ELISA against the panel of 12 synthetic peptides to identify any MHC class II-related differences in the PV autoantibodies. Sera from eight DR4-bearing healthy, unaffected relatives and eight DR6-bearing healthy, unaffected relatives were similarly tested.

Indirect Immunofluorescence Assay. The standard indirect immunofluorescence assay (14) using monkey esophagus was used to compare the staining pattern of autoantibodies binding to different PVA peptides. Normal human serum and serum from a patient with bullous pemphigoid were used as controls.

Pathogenicity Assay. *In vivo* pathogenicity was determined by intraperitoneal injection of the test reagents into neonatal BALB/c mice as previously described (2). Mice were injected with IgG from: (i) normal controls, (ii) patients with active disease, (iii) patients in remission, and (iv) antibody-carrying, healthy relatives. *In vitro* pathogenicity was determined by the presence of acantholysis in normal human skin explants grown in medium containing test reagents as described (3). Test reagents included the following: (i) IgG from patients with active disease, (ii) Bos 1-specific IgG from healthy, unaffected relatives, (iii) Bos 1-specific IgG from PV patients, and (iv) Bos 6-specific IgG from PV patients. Negative controls for the

experiment included the following: (i) Bos 4-specific IgG from PV patients, (ii) normal human serum, and (iii) serum from a patient with bullous pemphigoid.

Statistical Method. Data were analyzed by using the non-parametric Wilcoxon signed-rank test (15) for paired observations and the Mann-Whitney nonparametric *U* test for unpaired observations, with an appropriate adjustment to the significance level for multiple comparisons (16).

RESULTS

Correlation of IgG Subclasses and Peptide Specificity of Autoantibodies. When using the ELISA to determine the binding of various subclasses of IgG to various PVA peptides, we observed the following (data summarized in Table 1 and Fig. 2): (i) In patients with active disease, titers of IgG1 and IgG4 autoantibodies against peptide Bos 1 are higher than those against peptide Bos 6 ($P < 0.01$). The titer of IgG1 autoantibody binding to Bos 1 is higher than the titer of IgG4 ($P < 0.01$). However, there is no statistically significant difference between the antibody titers of Bos 6-specific IgG1 and Bos 6-specific IgG4 in patients with active disease. (ii) While the titers of Bos 1-specific IgG1 remain elevated in patients in remission, the titers are significantly lower when compared with patients with active disease ($P < 0.01$). We have not detected an IgG4 response to Bos 6 in these patients. In patients in remission, the titers of Bos 1-specific IgG1 autoantibody are not significantly different from the titers in unaffected healthy relatives. (iii) Healthy relatives of PV patients demonstrate only an IgG1 response to peptide Bos 1. No binding of IgG4 to peptide Bos 1 or Bos 6 is observed. (iv) Unrelated normal controls bind only to peptide Bos 1 with IgG1 autoantibodies in low titers similar to those of healthy relatives. No binding to peptide Bos 6 is observed. There were no differences in the selective binding to any other Bos peptides between the test sera in the four groups studied and a panel of 136 normal control sera.

Table 1. Antibody response to Bos 1 and Bos 6 antigens

Group	Number tested	Antigen peptide	Antibody response	
			IgG1	IgG4
Patients with active disease	21	Bos 1	25905 \pm 4201	7009 \pm 835
		Bos 6	2400 \pm 482	1924 \pm 511
Patients in prolonged remission	19	Bos 1	4716 \pm 865	547 \pm 123
		Bos 6	Negative	Negative
Healthy relatives of patients	18	Bos 1	1255 \pm 276	Negative
		Bos 6	Negative	Negative

Values shown are mean \pm SEM of IgG1 and IgG4 titers.

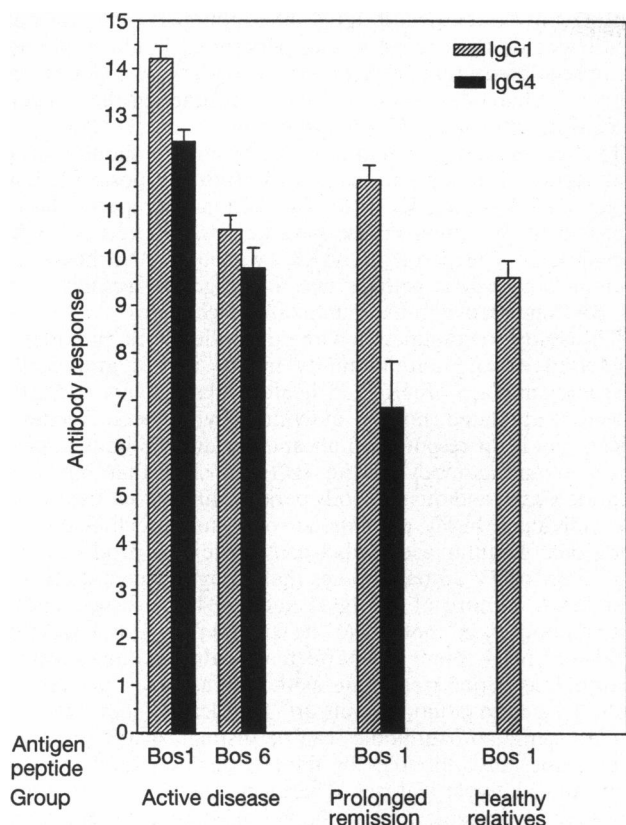


FIG. 2. Antibody response to the peptides Bos 1 and Bos 6, expressed as mean \pm SEM of \log_{10} titer values. The IgG1 and IgG4 titers to Bos 1 and Bos 6 in the active disease group are elevated over those in remission ($P < 0.01$). The difference between IgG1 and IgG4 binding to Bos 1 during active disease is significant ($P < 0.01$), whereas IgG1 and IgG4 binding to Bos 6 show no significant difference. IgG1 and IgG4 responses to Bos 6 were negative during remission. There is no significant difference in IgG1 binding to Bos 1 between the remission group and their healthy relatives.

Thus, the IgG4 autoantibody is present in active disease at considerably higher levels than in remission. Bos 6-specific IgG4 autoantibody is present only during active disease. Though Bos 1-specific IgG4 is present in patients in remission, the levels are significantly lower. The level of Bos 1-specific IgG1 in patients in remission is identical to that of healthy, unaffected relatives. These data support the hypothesis that

the principal pathogenic autoantibody is Bos 6-specific IgG4. It is possible that the high titers of Bos 1-specific IgG4 during active disease may play a facilitatory role.

Cross-Adsorption Studies. When sera from patients in remission, healthy relatives, and unrelated, normal controls are adsorbed with peptide Bos 1, no binding to any other peptide within the PVA is observed in the ELISA and immunoblot assay. When the sera from PV patients with active disease are first adsorbed with peptide Bos 1, there is still binding to peptide Bos 6 and vice versa. When these sera are first adsorbed with both peptide Bos 1 and Bos 6 and then used in an immunoblot assay, a very faint band at 130 kDa is still seen. Fifty-six percent of the sera from 136 unrelated, Caucasian normal controls also demonstrated exactly the same level of binding in the ELISA. When these test sera were adsorbed with these peptides, the band was completely eliminated. It is possible that there is considerable similarity between some of PVA peptides and either common viral proteins or other ubiquitous antigens to which many individuals make low levels of antibodies.

Correlation of Autoantibody Specificities with MHC. PV autoantibodies from both DR4- and DR6-bearing patients bind to peptides Bos 1 and Bos 6 and do not selectively bind to the other Bos peptides. Antibodies from healthy, unaffected relatives bearing either DR4 or DR6 bind only to Bos 1. Thus there does not appear to be any difference in the binding pattern of sera on the basis of MHC class II differences.

Indirect Immunofluorescence Studies. A fish-net pattern of staining indistinguishable from that obtained with serum from patients with active disease is seen with IgG from PV patients and with Bos 1- and Bos 6-specific IgG (Fig. 3). Staining with Bos 1-specific IgG from patients in remission was somewhat similar to that from healthy relatives and from unrelated antibody-positive controls, but with much less intensity and in very low titers. No staining was observed with Bos 1-specific IgG isolated from serum of healthy relatives, unrelated, antibody-positive controls or normal human serum, whereas the positive control serum from a patient with bullous pemphigoid showed typical basement membrane zone staining (Fig. 3).

Pathogenicity Assay. *In vivo* experiments with neonatal BALB/c mice demonstrated that when IgG from patients with active disease was injected intraperitoneally, clinical disease and binding of autoantibody to mouse intercellular cement substance were observed. Injection of IgG from patients in prolonged remission, from healthy relatives, and from normal (antibody-negative) controls did not produce any clinical disease. The results of an *in vitro* acantholysis assay demon-

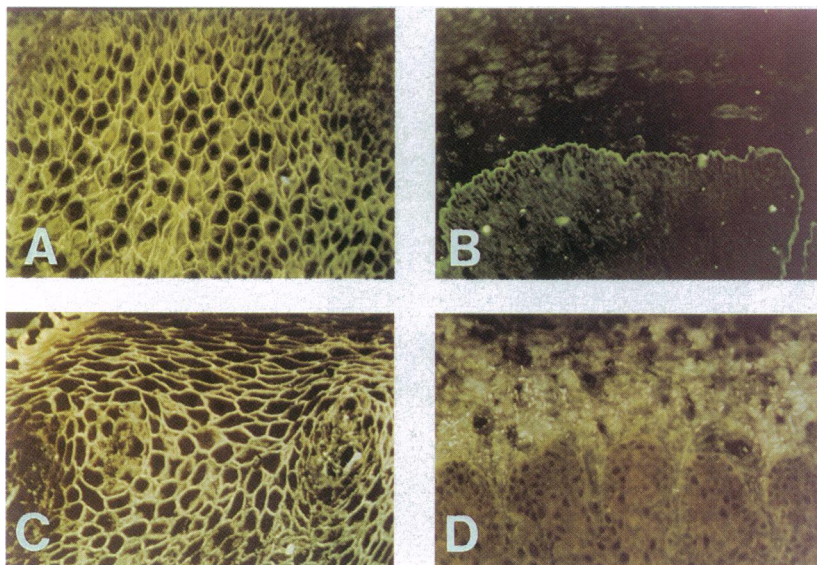


FIG. 3. Indirect immunofluorescence assay using monkey esophagus with various fractions of test sera. (A) Intercellular staining of epithelium with IgG specific for peptide Bos 6 from serum of PV patient with active disease. (B) Basement membrane zone staining of epithelium with serum from patient with bullous pemphigoid. (C) Intercellular staining of epithelium with total IgG from serum of PV patient with active disease. (D) Lack of intercellular staining of epithelium with IgG specific for peptide Bos 1 from healthy relative of PV patient.

strate that acantholysis, often indistinguishable from clinical PV, is seen in normal human-skin explants cultured in medium containing total IgG, Bos 6-specific IgG, and Bos 1-specific IgG from PV patients with active disease. The acantholysis observed in explants grown in the presence of Bos 6-specific IgG was uniform, consistent, suprabasilar, and indistinguishable from that observed in the presence of total IgG from PV patients. Acantholysis observed with Bos 1-specific IgG was focal, inconsistent, and not uniform. No acantholysis is observed with Bos 1-specific IgG from serum of healthy relatives, with serum from bullous pemphigoid patients, or with normal human serum (Fig. 4). These results suggest that high titers of Bos 1-specific IgG and Bos 6-specific IgG are acantholytic and therefore pathogenic. Low levels of Bos 1-specific IgG are nonacantholytic and nonpathogenic.

DISCUSSION

This report is a further extension of our earlier studies demonstrating that there are pathogenic and nonpathogenic autoantibodies in PV. The pathogenic autoantibodies cause disease in mice and produce acantholysis *in vitro* (2, 3). PV patients with active disease have high levels of Bos 1- and Bos 6-specific autoantibodies of the IgG1 and IgG4 subclasses. Once in prolonged remission, Bos 6-specific IgG1 and IgG4 autoantibodies disappear, whereas Bos 1-specific IgG1 and IgG4 autoantibodies continue to persist in low titers. Healthy, unaffected relatives and unrelated controls have low titers of Bos 1-specific IgG1 only. Therefore, it appears that IgG4 that binds to peptide Bos 6 plays a more critical role in causing acantholysis than the IgG that binds to peptide Bos 1. Perhaps Bos 6-specific IgG4 is the primary initiator while Bos 1-specific IgG4 is a facilitator of the acantholytic process. It appears that Bos 1-specific IgG1 does not cause disease, although it is present in high titers and can bind to the intercellular cement substance in the epidermis.

Nonetheless, levels of IgG1 and IgG4 antibodies specific for peptide Bos 1 are higher than the levels of IgG1 and IgG4 antibodies specific for peptide Bos 6 during active disease and seem to persist during remission. The probable cause is that prior to the initiation of clinical PV, the immune system is primed with peptide Bos 1. Once acantholysis occurs, there is a greater release of Bos 1, resulting in a significant anamnestic response. In the course of time, with clinical improvement,

pathogenic Bos 6-specific IgG4 autoantibodies decrease but Bos 1-specific IgG1 persists. This persistence could be due to an upregulated immune response, secondary to a persistent antigenic challenge resulting from the leakage of the antigen as cells die during epidermal migration.

In a recent study, we demonstrated by an immunoblot assay that serum of patients in prolonged remission possesses low levels of PVA-specific IgG1 (8). The data in this report indicate that patients in remission also have detectable levels of PVA-specific IgG4. The reason that no IgG4 was observed in the earlier immunoblot assay is perhaps due to the greater sensitivity of ELISA compared with the immunoblot technique.

This study, in conjunction with our earlier work, provides a model to explain autoimmunity in PV and is graphically presented in Fig. 5. Unaffected, healthy relatives and randomly selected, unrelated, normal individuals with specific genetic backgrounds, in response to an undetermined stimulus, produce an autoantibody of the IgG1 subclass that binds to peptide Bos 1, and this antibody persists throughout the life of the individual. In PV patients, at some time preclinically, an additional stimulus occurs that causes them to produce very high titers of PV autoantibodies that recognize peptide Bos 1 and Bos 6 and are of the IgG1 and IgG4 subclasses. These autoantibodies, in contrast to the low titers of Bos 1-specific IgG1 and IgG4 found in unaffected, healthy relatives, cause acantholysis. Upon treatment, as the disease goes into remission, these autoantibody levels are significantly reduced.

Pathogenic autoantibodies can be distinguished from nonpathogenic autoantibodies by their antigenic-specificity patterns and idiotypic features. Therefore, the origin and regulation of pathogenic autoantibodies may be different from natural or nonpathogenic autoantibodies (17). Some autoimmune diseases appear to be associated with restricted antibody responses, as manifested by oligoclonal banding of IgG in extravascular fluids such as the cerebral spinal fluid of multiple sclerosis patients (18) and synovial fluid of rheumatoid arthritis patients (19). These appear to be locally produced antibodies, but the nature of the antigenic specificity is not always clear. Some autoimmune diseases may be associated with restricted serum antibody responses as manifested by a cross-reactive idio-type, as shown with rheumatoid factors and anti-DNA autoantibodies in lupus patients (20). Restricted idiotypes have been shown to correlate with oligoclonal banding of

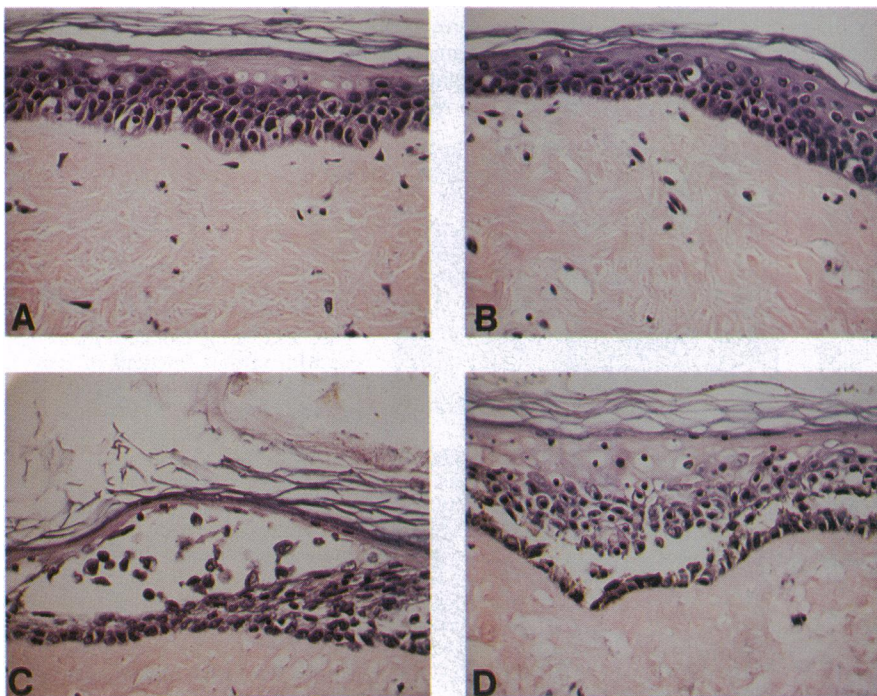


FIG. 4. Acantholysis assay of normal human skin incubated with the following reagents: (A) normal human serum; (B) Bos 1-specific IgG from healthy relative of PV patient; (C) Bos 6-specific IgG from PV patient with active disease; and (D) Bos 1-specific IgG from PV patient with active disease.

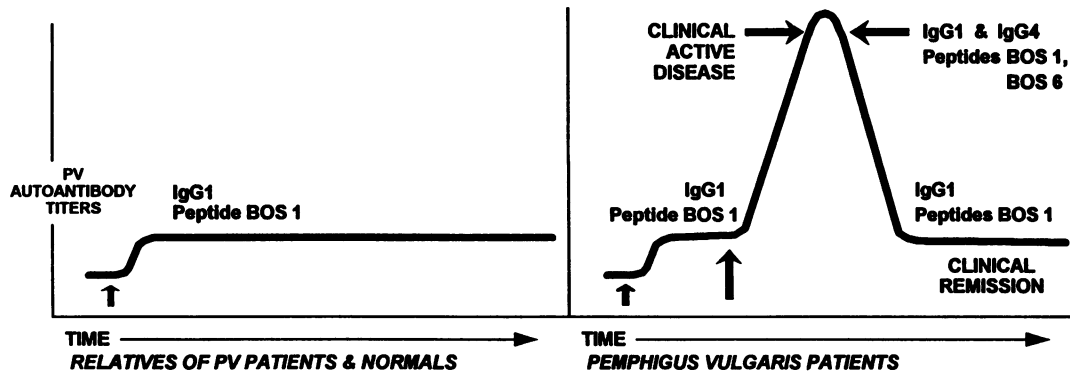


FIG. 5. Proposed hypothesis for pathogenesis of the autoimmune response in PV.

anti-DNA antibodies in sera from systemic lupus erythematosus patients (21). Restricted antibody responses are generally thought to arise by activation of one or a few closely related B-cell clones. Some workers believe that after activation and proliferation of B-cells, somatic mutation gives rise to autoantibodies, and that this process is driven by antigen selection (22). It is also known that normal individuals make anti-DNA antibodies. However, this "natural antibody" is generally a polyclonal IgM (23).

Recent studies on pemphigus foliaceus (PF) or fovo selvagem have provided valuable information in the characterization of the pathogenic IgG4 PF autoantibody (24). Both IgG1 and IgG4 exhibit oligoclonal banding, but the distribution of these bands in the pH gradient differs for these two subclasses. Whereas the IgG4 oligoclonal bands are distributed throughout the IgG4 pH range, IgG1 banding appears to be concentrated in the more basic region of the IgG1 pH range. These authors suggest that the IgG1 autoantibodies have undergone selective somatic mutation by a negatively charged autoantigen. The wide distribution of IgG4 banding suggests that this response may have followed the IgG1 response and has not undergone selective mutation (24).

Our data raise an important question of how the immune system recognizes Bos 6 after its sensitization to Bos 1. The various T-cell determinants and epitopes from a single protein are not recognized with equal efficiency, and there appears to be a hierarchy in the ability of any of these epitopes to be recognized by T cells (25). In the mouse model of multiple sclerosis called experimental allergic encephalomyelitis, the disease is induced by immunization with myelin basic protein (26). While the immune system is primed with one peptide, cryptic determinants later become immunogenic and cause chronic disease. This phenomenon of "determinant spreading" could account for priming to additional determinants on self-antigens, thus amplifying and broadening the autoimmune response (26–28). They could also account for the relapses and remission noted during the clinical course of PV in some patients. In studying the sequential sera samples of PV patients with multiple relapses and remissions, we will determine if there is a phenomenon of determinant spreading with the PV antigen.

The data presented in this study demonstrate important interactions between the MHC class II molecules, subclass of IgG autoantibodies, and the peptides to which the autoantibody binds. By studying patients during the active phase of the disease process and while in remission and in healthy, unaffected relatives, a hypothesis for the generation of the pathogenic process is presented. In this manner, PV provides a model which permits us to study the various processes and the sequence of events that eventually result in clinical autoimmune disease in humans.

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