Linkage of Pemphigus Vulgaris Antibody to the Major Histocompatibility Complex in Healthy Relatives of Patients

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Summary

Pemphigus vulgaris (PV) is an autoimmune disease caused by high concentrations of antibody to an epidermal cadherin. The disease is associated with two kinds of HLA-DR4, DQ8 haplotypes dominantly distributed among Jewish patients, and these plus DR6, DQ5 haplotypes in non-Jewish patients. Low levels of the PV antibody were found in 48% of a total of 120 asymptomatic parents, children, and siblings of 31 patients, thus exhibiting dominant inheritance. The inheritance of these low levels of antibody in asymptomatic relatives was linked to the major histocompatibility complex with a highly significant logarithm of the odds score of 9.07, almost always to a DR4 or DR6 haplotype of the patient. Disease appears to occur in susceptible individuals with low levels of antibody when a second factor, either environmental or genetic, induces high levels, sufficient to produce blisters.

In contrast to diseases like sickle cell anemia, where all genetically susceptible individuals have the disease and carriers are easily detected, the genetics of autoimmune diseases are far less clear. Although many autoimmune diseases show MHC associations, genetically susceptible persons may not have the disease and carriers are usually not detectable. This phenomenon has been called incomplete penetrance. Another problem is extensive linkage disequilibrium within the human MHC. In our experience, and that of others, many MHC-associated diseases are marked by fixed or extended haplotypes with conserved DNA in independent examples, over at least the HLA-B-DR, DQ interval, that form the basis of the linkage disequilibrium, making localization of a specific susceptibility gene particularly difficult (1).

Our previous studies demonstrated that in patients with the rare autoimmune blistering skin disease pemphigus vulgaris (PV),[1] caused by an antibody to a skin cadherin (2–4), three conserved or extended MHC haplotypes or their class II segments are disease associated (5, 6). We found that susceptibility in Ashkenazi Jewish patients was associated with the extended haplotype [HLA-B38, SC21, DR4, DQ8], and the possible extended haplotype HLA-B35, SC31, DR4, DQ8, or (unusually) DR4, DQ8-containing segments of these haplotypes with a variety of other HLA-B and complotype markers. These findings helped explain the DR4 increase (7) and the DR4, DQ8 association among Jewish PV patients (8). Of non-Jewish PV patients (6), some had HLA-DR4, DQ8 haplotypes, as in the Jewish patients, but also, consistent with the known increase in HLA-B55(22) (9) and HLA-DR6, DQ1 (10), many had HLA-B55, SB45, DR14(6), DQ5(1) or its presumed DR14, DQ5 segment.

Clues to the mode of inheritance of these disorders are the distribution of MHC alleles in populations (11) or among multiple affected siblings (12). Thus, the inheritance of MHC-associated susceptibility to type I diabetes is essentially recessive (13, 14), whereas that for ankylosing spondylitis is dominant (11). From the analysis of the distribution of DR4, DQ8 on MHC haplotypes among Jewish patients with PV, we inferred that the MHC susceptibility gene operated in a dominant fashion (5).

If there is a dominantly expressed gene specifying autoantibody production in PV patients, autoantibody might also be present, although at low levels, insufficient to cause symptoms, in relatives of patients who share the susceptibility haplotype. Furthermore, a fraction of normal persons from such populations who carry one of these haplotypes might also have the PV antibody.

In the present study, we have approached the problem directly and tested the serum of close relatives of PV patients

[1] *Abbreviation used in this paper:* PV, pemphigus vulgaris.

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for the presence of low levels of antibody, using a recently developed sensitive Western blotting procedure. We found that about half of the healthy immediate relatives of patients carry low levels of this antibody and that the presence of antibody is genetically linked to one of the HLA-DR4 or DR6 haplotypes, the same as occur in patients.

Materials and Methods

We studied 174 members of 31 families of patients with PV. The criteria for diagnosis and MHC types of most of these were published earlier (5, 6). For the present study, serum was obtained from clotted venous blood by centrifugation and analyzed for antibody to skin epithelial surface antigens.

Serological typing of PBL for HLA-A, B, C, DR, and DQ was by microlymphocytotoxicity (15). Fresh frozen EDTA plasma was used to type the MHC-encoded complement proteins C4 (16), BF (17), and C2 (18). Complotypes are given as BF, C2, C4A, C4B types, for example, SC31 is *BF*S, C2*C, C4A*3, C4B*1*.

Serum samples from the study and control subjects were tested for antibody to the 130-kD (keratinocytes) (4) and 105-kD (COLO 16 tumor cell line) (19) antigens characteristic of PV by a Western blot method (20). Immunoblots were produced by incubating subject serum with detergent-solubilized normal skin cells or squamous carcinoma cells after electrophoresis in polyacrylamide gel in the presence of SDS. Two modifications of the method described previously (20) were introduced to increase sensitivity. Cell extracts were passed through a Sepharose 4B column previously coupled to normal human serum to avoid nonspecific binding by serum proteins and transferred after electrophoresis to PVDF membranes (Bio-Rad Laboratories, Richmond, CA). The membranes were overlaid with an IBI enzygraphic web (Eastman Kodak, New Haven, CT) to detect bound antibody by the peroxidase reaction. Immunoblots were performed and interpreted blindly. PV antibody levels were estimated in all available members of 10 families by 1:5 and then doubling dilution, and the titer was assigned as the last immunoblot-positive dilution.

Sera from 74 control healthy individuals who carried [HLA-B38, SC21, DR4] (n = 8); HLA-B35, SC31, DR4 (n = 10); DR4 in general (n = 21); HLA-B55, SB45, DR6 (n = 10); DR6 in general (n = 2); or none of these markers (n = 23) were studied by immunoblot in addition to 29 spouses of patients for a total of 103 control subjects.

Serum samples were also tested for PV antibody by immunofluorescence using rhesus monkey esophagus as substrate (2). By immunofluorescence, 30 of 31 patient samples were positive. Of 29 spouses of patients or carriers of antibody, three (10%) were positive for the autoantibody by immunoblot and 2 of 13 (15%) by immunofluorescence (one of these was also positive by immunoblot). Only 7 of 62 (11%) of first-degree relatives' sera were positive by immunofluorescence, but, of these, six were positive by immunoblot. This, coupled with the negativity of one patient sample by this technique, suggested that immunofluorescence, although useful for detecting the high levels of antibody in PV patient sera, was not sensitive enough to regularly detect low levels of antibody in relatives. On blind specific testing of patients' sera diluted serially, it appeared that samples with titers below ~700 were sometimes negative and below 200 were usually negative by immunofluorescence. Thus, immunofluorescence was one to two orders of magnitude less sensitive than the immunoblot assay. Therefore, the presence or absence of PV antibody was assigned by immunoblot.

To determine formally whether the presence of the antibody was MHC linked, linkage analysis (21) was carried out between the presence of antibody detected by immunoblot and the MHC. One family, with two antibody-positive spouses, was considered to be uninformative for linkage. If a patient was heterozygous for HLA-DR4 or DR6 and some other HLA-DR, the assumption was made that the antibody was linked to the DR4 or DR6 haplotype and the family was treated as phase known. Families of patients homozy-

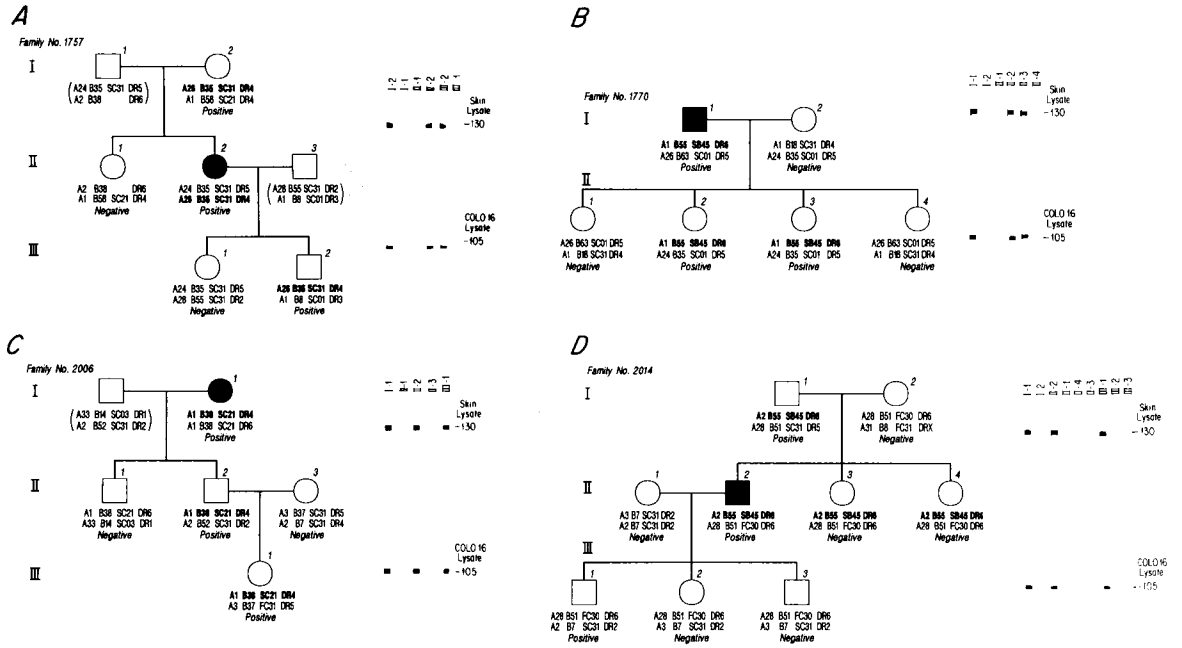

Figure 1. Four families with a proband with PV (*filled symbols*). Males are shown as squares, females as circles. The two MHC haplotypes of each person and the presence (positive) or absence (negative) of PV antibody are shown below each symbol. The haplotypes linked to the presence of antibody are shown in bold. To the right of each family are shown the immunoblots for PV antibody.

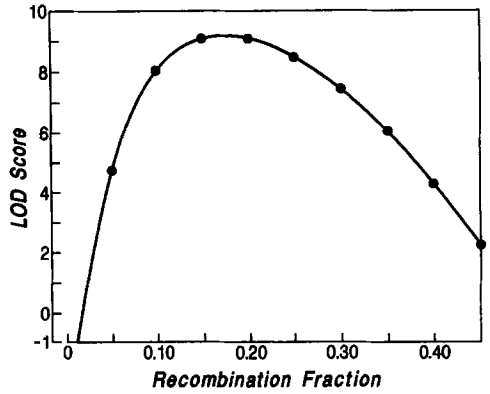

Figure 2. A plot of the cumulative LOD scores in the 30 informative families at recombination fractions from 0 to 0.45. It is clear that the maximal LOD score is a little over 9 at an apparent recombination fraction of 0.15–0.20.

gous for HLA-DR4 or DR6 or DR4/DR6 heterozygotes were analyzed by standard linkage analysis.

Results

All patients (31/31) had PV antibody by immunoblot, as expected. Of serum samples from 120 parents, children, and siblings of patients, 58 or 48% were positive by immunoblot, very close to the 50% expected for autosomal dominant inheritance of the PV antibody. Patient's antibody titers ranged from 640 to 5,120 (arithmetic mean, 1,728), whereas relatives' antibody titers ranged from 10 to 80 with a mean of 20.

Fig. 1 shows the inheritance pattern of PV antibody in four illustrative families. In family 1757 (Fig. 1 *A*), it is seen that of the patient's immediate relatives, her mother, and one of her two children were positive for the PV antibody. These individuals shared the MHC haplotype HLA-A26, B35, SC31, DR4 with the patient. Her other relatives, who did not share this haplotype, were also antibody negative. Thus, in this family, there were four informative meioses and no recombinants. Similarly, in family 1770 (Fig. 1 *B*), there was linkage between the antibody and HLA-B55, SB45, DR6 with no recombination. Family 2006 (Fig. 1 *C*) shows linkage without recombination between the antibody and HLA-A1, B38, SC21, DR4. On the other hand, in family 2014 (Fig. 1 *D*), although individual I-1 appears to have transmitted the antibody in linkage with the putative susceptibility haplotype HLA-A2, B55, SB45, DR6 to the patient II-2, his sisters (II-3 and II-4) received the haplotype but did not have the antibody and were thus apparent recombinants. His two children (III-2 and III-3) were nonrecombinants who received the nonsusceptibility haplotype A28, B51, FC30, DR5 and did not have antibody. His third child (III-1), on the other hand, received the same haplotype but unexpectedly had the antibody and was thus a recombinant.

Of 117 informative meioses in all informative families, 100 were nonrecombinant for the presence of PV antibody and the MHC, and 17 (14.5%) were recombinant. In six of the subjects with the latter, the antibody was present but not the linked patient haplotype, although four of the six had a nonpatient DR4 or DR6 haplotype. In the 11 remaining subjects with recombination, the patient haplotype was present but no PV antibody was detected. There was no clear age-related difference with reference to failure to have antibody despite the presence of the patient's putative susceptibility haplotype. One of eight such parents (12.5%), 3 of 15 sibs (20.0%), 6 of 33 children (18.2%), and one of five (20%) grandchildren of patients were of this kind.

On formal testing (Fig. 2) of all informative families for linkage, the maximum logarithm of the odds (LOD) score for linkage between the presence of antibody and the MHC was 9.07 (highly significant). The most likely recombination fraction, or maximal θ, was 0.15–0.20.

Table 1 gives the haplotypes clearly segregating with the presence of antibody in each of 30 families with a PV patient. These are virtually exclusively those with HLA-DR4, DQ8 and DR6, DQ5 that were assumed earlier to be the dominantly expressed susceptibility haplotypes (5, 6). There were four exceptions. Patients 1699 and 1727 carried SC31, HLA-DR4, DQ8 haplotypes on their other chromosomes, and patient 1689 carried HLA-B55, SB45, DR6, DQ5, making the identification of the susceptibility haplotype ambiguous from class II genes or extended haplotypes alone. Only in patient 1749, in whom the antibody-linked haplotype was with DR5 and the guessed susceptibility haplotype was HLA-A26, B35, SC31, DR4, DQ8, was the guess truly incorrect. All the other 24 patient's haplotypes were correctly guessed, and the haplotypes in common listed here are the same as shown previously (5, 6).

Of 29 spouses of patients or carriers of antibody, three (10%) were positive for the autoantibody by immunoblot. One of these was a 91-yr-old man with myasthenia gravis, a condition known to be associated in some patients with the presence of PV antibody (22), and two were in family 1797. Of the latter, one carried HLA-B35, SC31, DR4, and the other carried [HLA-B38, SC21, DR4]. Serum from the latter was also positive for the pemphigus antibody by immunofluorescence assay, as was that of another spouse with no DR4 or DR6 haplotype in family 1719. These results suggested that some normal individuals with these haplotypes (and even perhaps some without the haplotypes) but no family history of PV might carry low levels of the PV antibody. However, of the remaining 74 serum samples, including seven with [HLA-B38, SC21, DR4] and nine with HLA-B35, SC31, DR4 from healthy controls, none were positive by immunoblot.

Discussion

In autoimmune diseases characterized by autoantibodies, several genetic factors, including the MHC, in addition to environmental factors, may be important in pathogenesis. However, since the methods for detection of autoantibodies have been neither very sensitive nor highly specific, it had not been possible to study the genetics of autoantibodies and their relationship to the MHC. We have used a very sensitive and specific Western blot assay that can detect 10–100 pg of the anticadherin (pemphigus) autoantibody. By these means,

Table 1. *MHC Haplotypes Linked to Autoantibody in Families*

Jewish families						Non-Jewish families					
Family no.	HLA A	HLA B	Comp.	HLA DR	HLA DQ	Family no.	HLA A	HLA B	Comp.	HLA DR	HLA DQ
1799	1	58	SC21	4	8	1770	1	55	SB45	6	5
1716	2	27	SC21	4	8	2014	2	55	SB45	6	5
2006	1	38	SC21	4	8	1696	30	55	SB45	6	5
1789	26	38	SC21	4	8	2016	26	38	SC21	6	5
1778	26	38	SC21	4	8	1774	24	49	SC01	6	5
2005	26	38	SC21	4	8	1739	1	8	FC31	6	5
2370	26	38	SC21	4	8	1945	24	51	SC43	6	5
1906	26	38	SC21	4	8	1727*	2	37	FC31	6	5
1807	26	38	SC21	4	8	1740	2	44	SC30	6	5
1757	26	35	SC31	4	8	1689*	26	38	SC21	4	8
1773	26	35	SC31	4	8	1688	3	7	SC31	4	8
1946	26	35	SC31	4	8	1719	23	45	S1C2 (1,17)	4	8
1796	2	44	SC31	4	8	1699*	2	44	SC30	4	8
1763	3	14	SC2 (1,2)	4	8	1750	1	51	SC32	4	8
1749*	1	35	SC31	5	3	2406	1	8	FC31	5	3

Elements of known PV susceptibility haplotypes are underlined. Patients 2006 and 2406 were not reported earlier; all others were reported (5, 6).
* These haplotypes were incorrectly guessed previously (5, 6) to be nonsusceptibility haplotypes; see text.

we found many individuals within families of PV patients who were positive for low levels of the autoantibody. The study demonstrates that the inheritance of this autoantibody is consistent with dominant Mendelian genetics. It has been known from work done by others and by us that there are MHC class II genes that are associated with PV (5, 6, 10, 23–25), but the relationship of these alleles or extended haplotypes to the presence or absence of autoantibody was unknown. The distribution of homozygotes and heterozygotes for HLA-DR4 in Jewish patients with PV was consistent with dominant but not recessive inheritance (5, 6).

Formal linkage analysis provides strong evidence for linkage between the MHC and the presence of PV antibody in relatives' families with odds of $>10^9$ to 1 for linkage (10^3 is evidence for linkage at $p = 0.05$). Supposed recombinants were of two kinds, those in which a bearer of a suspected susceptibility haplotype or an arbitrarily assigned MHC haplotype failed to have the antibody (11/17 of instances), and those in which an offspring who did not inherit that haplotype nevertheless had antibody (6/17). Although both situations could have arisen from recombination between the MHC and the putative immune response gene, other explanations are at least as likely. The first kind of "recombinant" could be due to a failure of penetrance, or to lack of sensitivity of the autoantibody detection method. The unexpected presence of the antibody could be due to lack of specificity of the detection assay (false positivity) or to inheritance of a susceptibility haplotype from the unaffected parent whose antibody response is impenetrant. Nonparenthood (for which we had no evidence) could also confuse the picture.

The current findings redefine the concept of penetrance, at least as it is applied to PV. In the sense of specifying the presence of antibody, penetrance of the MHC susceptibility gene is nearly complete even though penetrance for the disease is much lower. Since multiple cases of PV in the same family are rare, neither the presence of the PV-associated MHC nor low levels of antibody suggest impending disease in an unaffected relative.

There was a significant difference between the amounts or titers of the autoantibody (anticadherin) in the patients and the unaffected relatives. Although PV is a rare disease, the asymptomatic presence of low levels of nondisease-producing PV antibody could be several-fold more common, particularly in subjects who carry the specific MHC haplotypes known to be PV associated. Our findings are consistent with these predictions. Of 103 control subjects enriched in specific susceptibility-type MHC haplotypes, only three individuals who were unrelated members of the nuclear families of PV patients were found to carry low levels of PV antibody. Other individuals with the same extended haplotype without disease, who were from other households, did not have detectable anticadherin autoantibody. One positive elderly man without a specific susceptibility-type haplotype had myasthenia gravis, known to be occasionally associated with PV

antibody (22). The other two positive individuals were spouses of PV patients or PV antibody carriers and both carried susceptibility-type MHC haplotypes. This raises the question of a common household environmental factor such as a virus being involved in the production of low levels of PV antibody in MHC-susceptible persons. We postulate that either a non-MHC gene or an environmental factor acts occasionally in the individual with low levels of pemphigus antibody to increase production and this results in disease.

Thus, our results suggest that the production of high levels of PV antibody, and thus the disease PV, is a two-step process. The first involves the MHC with or without an environmental factor and leads to the presence of low levels of antibody. The second involves either an environmental factor and/or a non-MHC gene and results in high levels of the antibody.

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References

1. Awdeh, Z.L., D. Raum, E.J. Yunis, and C.A. Alper. 1983. Extended HLA/complement allele haplotypes: evidence for T/t-like complex in man. *Proc. Natl. Acad. Sci. USA.* 80:259.
2. Beutner, E.H., R.E. Jordan, and T.P. Chorzelski. 1968. The immunopathology of pemphigus and bullous pemphigoid. *J. Invest. Dermatol.* 51:63.
3. Anhalt, G.J., R.S. Labib, J.J. Voorhees, T.F. Beals, and L.A. Diaz. 1982. Induction of pemphigus in neonatal mice by passive transfer of IgG from patients with the disease. *N. Engl. J. Med.* 306:1189.
4. Amagai, M., V. Klaus-Kovtun, and J.R. Stanley. 1991. Autoantibodies against a novel epithelial cadherin in pemphigus vulgaris, a disease of cell adhesion. *Cell.* 67:869.
5. Ahmed, A.R., E.J. Yunis, K. Khatri, R. Wagner, G. Notani, Z. Awdeh, and C.A. Alper. 1990. Major histocompatibility complex haplotype studies in Ashkenazi Jewish patients with pemphigus vulgaris. *Proc. Natl. Acad. Sci. USA.* 87:7658.
6. Ahmed, A.R., R. Wagner, K. Khatri, G. Notani, Z. Awdeh, C.A. Alper, and E.J. Yunis. 1991. Major histocompatibility complex haplotypes and class II genes in non-Jewish patients with pemphigus vulgaris. *Proc. Natl. Acad. Sci. USA.* 88:5056.
7. Park, M.S., P.I. Terasaki, A.R. Ahmed, and J.L. Tiwari. 1979. HLA-DRW4 in 91% of Jewish pemphigus vulgaris patients. *Lancet.* ii:441.
8. Brautbar, C., M. Moscovitz, T. Livshits, S. Haim, S. Hacham-Zadeh, H.A. Cohen, H.A. Sharon, and D. Nelken. 1985. HLA-DRW4 in pemphigus vulgaris patients in Israel. *Tissue Antigens.* 16:238.
9. Zervas, J., A. Tosca, I. Apostolakis, and A. Varelzidis. 1979. HLA and pemphigus. *Br. J. Dermatol.* 101:357.
10. Szafer, F., C. Brautbar, E. Tzfoni, G. Frankel, L. Sherman, I. Cohen, S. Hacham-Zadeh, W. Aberer, G. Tappeiner, K. Holubar, L. Steinman, and A. Friedmann. 1987. Detection of disease-specific restriction fragment length polymorphisms in pemphigus vulgaris linked to the *DQw1* and *DQw3* alleles of the *HLA-D* region. *Proc. Natl. Acad. Sci. USA.* 84:6542.
11. Thomson, G., and W. Bodmer. 1977. The genetic analysis of HLA and disease associations. *In* HLA and Disease. J. Dausset and A. Svejgaard, editors. Munksgaard International Publishers Ltd., Copenhagen. 84–93.
12. Penrose, L.S. 1935. The detection of autosomal linkage in data which consists of pairs of brothers and sisters of unspecified parentage. *Ann. Eugenics.* 6:133.
13. Rubinstein, P., F. Ginsberg-Fellner, and C. Falk. 1981. Genetics of type I diabetes mellitus: a single, recessive predisposition gene mapping between *HLA-B* and *GLO. Am. J. Hum. Genet.* 33:865.
14. Raum, D., Z. Awdeh, and C.A. Alper. 1981. BF types and the mode of inheritance of insulin-dependent diabetes mellitus (IDDM). *Immunogenetics.* 12:59.
15. Zachary, A.A., and G.A. Teresi. 1990. ASHI Laboratory Manual. 2nd Ed. American Society for Histocompatibility and Immunogenetics, New York. 195–201.
16. Awdeh, Z.L., and C.A. Alper. 1980. Inherited structural polymorphism of the fourth component of human complement. *Proc. Natl. Acad. Sci. USA.* 77:3576.
17. Alper, C.A., T. Boenisch, and L. Watson. 1972. Genetic polymorphism in human glycine-rich β-glycoprotein. *J. Exp. Med.* 135:68.
18. Alper, C.A. 1976. Inherited structural polymorphism in human C2: evidence for genetic linkage between *C2* and *Bf. J. Exp. Med.* 144:1111.
19. Acosta, E., and L. Ivanyi. 1985. Identification of pemphigus-like antigens expressed by SCaBER cells. *Br. J. Dermatol.* 112:157.
20. Mirza, N.M., A. Mohimen, and A.R. Ahmed. 1990. Detection of pemphigus vulgaris antigen on COLO and SCaBER tumor cell lines by the immunoblot technique. *Arch. Dermatol. Res.* 282:418.
21. Cavalli-Sforza, L.L., and W. Bodmer. 1971. The Genetics of Human Populations. Freeman Publications, San Francisco. 870 pp.
22. Kaplan, R.P., and J.P. Callen. 1983. Pemphigus associated dis-

eases and induced pemphigus. *In* Clinics in Dermatology. J.B. Lippincott Company, Hagerstown, MD. 42–54.
23. Sinha, A.A., C. Brautbar, F. Szafer, A. Friedmann, E. Tzfoni, J.A. Todd, L. Steinman, and H.O. McDevitt. 1988. A newly characterized HLA DQ_β allele associated with pemphigus vulgaris. *Science (Wash. DC).* 239:1026.
24. Todd, J.A., H. Acha-Orbea, J.I. Bell, N. Chao, Z. Fronek, C.O. Jacob, M. McDermott, A.A. Sinha, L. Timmerman, L. Steinman, and H.O. McDevitt. 1988. A molecular basis for MHC class II-associated autoimmunity. *Science (Wash. DC).* 240:1003.
25. Scharf, S.J., A. Friedmann, C. Brautbar, F. Szafer, L. Steinman, G. Horn, U. Gyllensten, and H.A. Erlich. 1988. HLA class II allelic variation and susceptibility to pemphigus vulgaris. *Proc. Natl. Acad. Sci. USA.* 85:3504.