Acta Derm Venereol (Stockh) 1989; 69: 424–428

Local Involvement of Antigen-presenting Cells and Activated T Cells in Perilesional and Clinically Uninvolved Skin in Pemphigus Vulgaris

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Biopsies obtained from both the perilesional areas and clinically uninvolved skin of patients with pemphigus vulgaris (PV) were studied for antigen-presenting cell and lymphocyte phenotype and/or activation phenotype using monoclonal antibodies in avidin–biotin-peroxidase complex staining. Perilesional PV skin contained CD4+ and CD8+ T lymphocytes as the predominant cell type, but cells with a potential antigen-presenting function displaying CD11b phenotype of monocyte/macrophages and, in particular, CD1 phenotype of Langerhans cells were also present. The number of mononuclear inflammatory cells was greater in perilesional than in clinically uninvolved PV skin, and so were the proportions of CD4+, CD8+, CD25+, Ia+ cells ($p<0.01$), and CD1+ Langerhans cells and transferrin receptor positive cells ($p<0.05$). These findings confirm and extend earlier observations on local involvement of immunocompetent cells in PV. ***Key words: Cell-mediated immunity; Immunohistopathology.***

(Accepted April 24, 1989.)

Acta Derm Venereol (Stockh) 1989; 69: 424–428.

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Pemphigus vulgaris (PV) is a rare autoimmune disease, with the skin and mucous membrane involvement displaying characteristic superficial flaccid intra-epidermal blisters, predominantly of suprabasal localization (1). Acantholysis, which leads to the loss of epidermal cell-to-cell adhesion, is associated with the presence of IgG class autoantibody against an intercellular epidermal antigen, possibly a desmosomal protein (2). This autoantibody synthesis may be T-cell-dependent and is at least associated with HLA-DR4, particularly Dw10 (3) and possibly also with DRw6a subtype (4), suggesting that third hypervariable region sequences are of critical importance in susceptibility to PV (4).

Considering the role of HLA antigens in T cell recognition phenomena such as epitope selection and major histocompatibility complex restriction, such findings stress the need for studies of immunocompetent lymphocytes and accessory antigen presenting cells (APC) (5, 6) in PV. Although there has been widespread interest in humoral immunity in PV, studies on the involvement of cell-mediated immunity in general, and on the eventual local skin involvement, in particular, have been few. Recently, Nestor et al. (7) reported on immunohistochemical analysis of the differentiation phenotype of lymphocytes in PV skin. We confirm their findings and extend them to an analysis of lymphocyte differentiation and activation markers of perilesional and clinically uninvolved PV skin trying to ascertain whether APC-lymphocyte involvement is restricted to lesional skin or reflects a more universal involvement of the immune system.

MATERIALS AND METHODS

Patients and biopsies

Specimens for immunohistochemical analysis were obtained by punch biopsy both of perilesional skin (area immediately adjacent to the fully developed PV blister) and of clinically uninvolved skin non-adjacent to lesions of 3 male and 4 female patients, 41–54 years of age (mean 47 years) from the Department of Dermatology, Moscow Medical Stomatological Institute, USSR. The duration of the disease in these patients varied between 2 1/2 and 5 months (mean 3 1/2 months). At the time of the study, none of the patients had any other disease or medication. Perilesional biopsies were taken from the chest (3 patients), back (2 patients), abdominal skin or gluteal area. The study was performed before the initiation of corticosteroid treatment.

The diagnosis of PV in all these patients was confirmed clinically, histologically, cytologically and by means of immunofluorescence techniques. Direct IF was performed using fluorescein isothiocyanate-labelled goat antihuman IgG (Hyland Laboratories, Calif., USA) diluted 1 : 10 with phosphate-buffered saline containing 2% Tween-80. The IF specimens were examined with a Leitz Ortholux fluorescence micro-

scope equipped with a Xenon arc using a Schott BG12 excited filter and a Leitz K 530 barrier filter. All the specimens examined showed strong characteristic intercellular staining. The titres of intercellular autoantibodies evaluated by indirect IF using monkey esophagus as substrate, varied in these patients between 1 : 160 and 1 : 320. The biopsies for immunohistochemical study were frozen in liquid nitrogen and stored at −70°C until transported to Finland in liquid isopentane/dry ice at −50°C.

Immunohistochemistry

For the immunostaining, 6 µm cryostat sections were prepared and fixed in cold (+4°C) acetone for 5 min. After washing in phosphate-buffered saline (PBS; 0.1 M, pH 7.3) the sections were stained with monoclonal antibodies using the ABC method (8). Briefly, the endogenous peroxidase activity was blocked with 0.3% H_2O_2 for 15 min. To avoid loss of immunoreactivity of the lymphocyte markers used, methanol was not utilized. Non-specific binding sites were blocked by incubation in 1 : 20 normal horse serum for 20 min. After washing in PBS, the sections were treated with 1) mouse monoclonal antibody (Table I); 2) biotinylated horse anti-mouse IgG (1 : 200); and 3) ABC complexes. The ABC kit was purchased from Vector Laboratories (Burlingame, Cal., USA). Finally, the peroxidase-binding sites were revealed with 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma Chemical Co., St Louis, Mo, USA) 50 mg/150 ml PBS and 0.003% H_2O_2 for 6 min. Between each step the slides were rinsed twice in PBS for 5 min. After the DAB reaction the slides were washed, counterstained with haematoxylin, dehydrated in a graded ethanol series, cleared in xylene and mounted.

To test the specificity of immunohistochemical staining, the following controls were prepared: 1) omission of the primary monoclonal antibodies in the staining sequence; and 2) use of inappropriate biotinylated goat anti-rabbit IgG antibodies in the second stage. The slides were also stained for endogenous peroxidase using the DAB reaction only.

Evaluation of the results

The brown stain caused by the peroxidase reaction was visible under a light microscope. The cells in the dermis were counted from 6 µm tissue sections using an ocular counting square (20×20 squares) and an oil immersion objective (×1 000 magnification). No statements are given as to epidermal Langerhans cells, which because of their long dendritic processes are difficult to count in CD1 staining. All calculations are expressed as a percentage of all mononuclear cells in the microscopic fields calculated, because it is impossible to discern inflammatory mononuclear cells from various structural resident cells based on haematoxylin counterstain in immunoperoxidase stained specimens. At least 200 cells were calculated for each differential count. When the proportion of peroxidase-positive cells was less than 1% of all mononuclear cells in situ, no attempt was made to get a more exact number than that based on the difficulties caused by sample error in such cases. For statistical calculations, such values were given a value of 0.5%. The mean ± the standard error of the mean was used to express dispersion. The significance of the differences between mean values was tested using Wilcoxon's rank sum test. Linear regression analysis was used to study the relationship between two variables. Interobserver (four investigators) coefficient of variation (%) was calculated as $(SD \times 100)/\bar{x}$. Counting could not be done blind because perilesional areas and clinically uninvolved PV skin were easily distinguishable on histological grounds alone.

RESULTS

The staining controls were found to be negative. Staining with the DAB-H_2O_2 alone was also negative,

Table I. *Characteristics of monoclonal antibodies used*

Monoclonal antibody	CD class	Cell surface antigen recognized	Specificity
OKT3	CD3	Part of the antigen receptor	Total T cells
OKT4	CD4	gp 56–62	Inducer/helper T cells
OKT6	CD1	gp 45	Langerhans' cells, cortical thymocytes
OKT8	CD8	gp 32–33	Suppressor/cytotoxic T cells
OKT9	–	Transferrin receptor	Proliferating cells, e.g. activated lymphocytes
OKM1	CD11b	gp 24	Monocytes
OKIa	–	MHC class II antigen	Activated T cells, B cells, macrophages, monocytes null cells, Langerhans' cells
OKT26	CD25	Interleukin-2 receptor	Activated and functionally mature T cells, suppressor T cells, cytotoxic killer T cells, activated B cells
Tac[a]	CD25	Interleukin-2 receptor	

[a] From Dakopatts, Copenhagen, Denmark. All other monoclonal antibodies were purchased from Ortho Diagnostic System Inc., Raritan, NJ, USA.

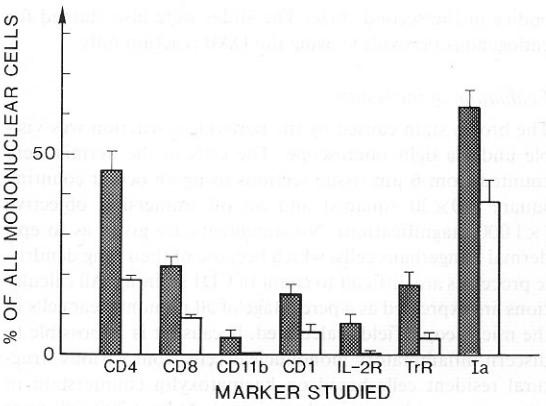

Fig. 1. The proportion of various inflammatory cell subsets in subepidermal infiltrates in PV skin. ■, Perilesional biopsies; □, clinically uninvolved skin. Values are means ± SE. The difference in the proportions of CD11b+ monocytes was not significant, but there was a difference in the proportion of CD1+ Langerhans' cells and transferrin receptor positive cells ($p<0.05$). The difference in the proportion of CD4+, CD8+, interleukin-2 receptor positive and Ia+ cells was particularly large ($p<0.01$). (Wilcoxon's test for unpaired samples).

a result which shows the successful inhibition of endogenous peroxidase. The interobserver coefficient of variation was less than 10% for all markers studied.

Perilesional PV skin

Immunoperoxidase analysis of perilesional PV skin showed that most of the local inflammatory cells were CD4+ or CD8+ T lymphocytes (Fig. 1). The CD4/CD8 ratio was approximately two, varying in individual patients from 1.2 to 3. In addition to T lymphocytes, some CD11b+ monocytes and, even more so, CD1+ Langerhans' cells were found *in situ* in PV skin (Fig. 2). Interestingly, the proportion of CD3+ 'total' T lymphocytes was lower than the sum of CD4+ and CD8+ T cells. T cells also showed interleukin-2 receptor as a sign of their activated state (Fig. 3). Almost 20% of all local mononuclear cells in the perilesional PV skin expressed transferrin receptor as a further sign of local lymphocyte activation, but transferrin receptor expression is naturally not confined to activated lymphocytes only. The correlation between the proportion of CD1+ cells and transferrin receptor positive activated cells was not significant. The relationship between the proportion of CD1+ cells and any of the other lymphocyte activation markers (Ia or interleukin-2 receptor) was not significant either. As has been described in other diseases (9, 10), OKIa or MHC locus II antigen expression was a particularly sensitive indicator of active involvement of local immigrant and resident cells.

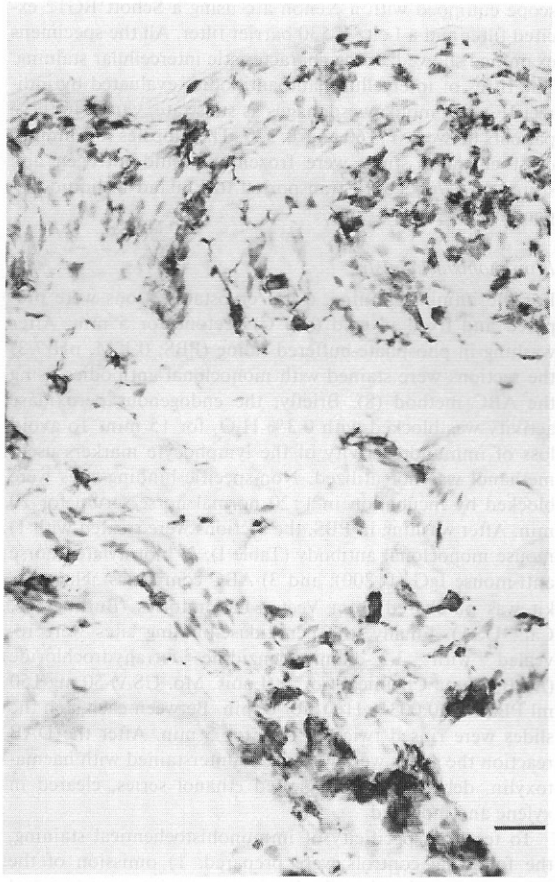

Fig. 2. CD1+ Langerhans' cells in perilesional PV epidermis and dermis. ABC staining with haematoxylin counterstaining. Original magnification, ×480. Bar=20 μm.

Clinically uninvolved skin

In contrast to perilesional PV skin, clinically uninvolved PV skin showed only slight signs of subclinical, histopathological involvement. Most of the local mononuclear cells seemed to be resident fibroblasts and endothelial cells, but some belonged to the T cell series. Further, the local CD4+/CD8+ ratio was now approximately 2, but unlike perilesional PV skin, the signs of T cell activation in the form of interleukin-2 receptor expression were lacking. Moreover, a slight expression of transferrin receptor and of OKIa may indicate local activity, but in the absence of double labelling studies, no definite conclusion can be drawn

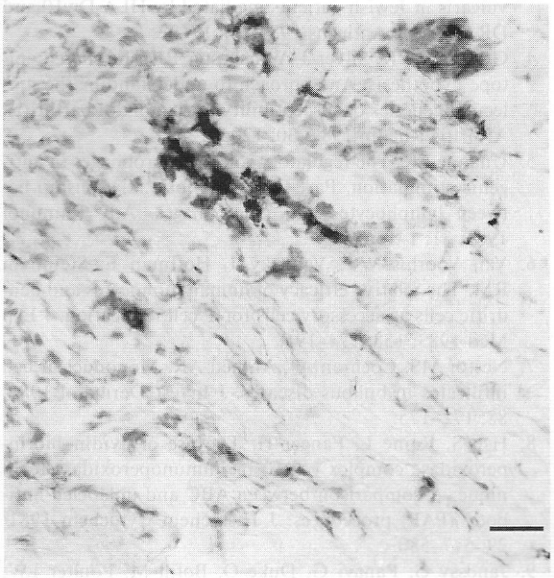

Fig. 3. Interleukin-2 receptor positive lymphocytes in dermis in perilesional PV skin. ABC staining utilizing monoclonal OKT26 antibodies. Haematoxylin counterstaining. Original magnification, ×480. Bar=20 μm.

about the possible presence of these activation markers on T cells. Interestingly, there were also few cells of the mononuclear phagocyte lineage in clinically uninvolved PV skin as assessed by CD11b expression. However, about 5% of all local mononuclear cells were CD1+ Langerhans' cells. The immunohistopathological results are summarized in Fig. 1.

DISCUSSION

In agreement with the study of Nestor et al. (7), phenotyping of the immunocompetent cells *in situ* in perilesional PV skin disclosed that CD4+ T lymphocytes were more frequent than CD8+ T lymphocytes, the mean CD4/CD8 being approximately 2. An earlier study failed to demonstrate any significant alterations in the relative percentages of T cells expressing inducer/helper (Leu-3) or suppressor/cytotoxic (Leu-2) phenotype in the peripheral blood of patients with active PV, compared with healthy controls or those with inactive disease (11). Although contradictory findings have also been reported (12, 13), it may be that the local disease process is not reflected in the CD4/CD8 ratio and that, at least in some PV patients, disarranged lymphocyte homing may be a random process with no selection for CD4+ or CD8+ phenotypes. We were therefore interested in establishing whether or not the T lymphocytes show signs of active involvement in the local pathomechanisms.

The specific immune activation of T lymphocytes requires accessory antigen presenting cells (APC) (5). Monocyte/macrophages have been regarded as important in this context (5) but, more recently, dendritic cells, often contaminating monocyte/macrophage preparations, have been cited as the most potent APCs (6). Mononuclear phagocytes, but in particular CD1+ Langerhans' cells, were relatively frequent in perilesional PV skin. However, CD1+ cells we observed in the dermis are not necessarily Langerhans' cells, since by definition only cells containing Birbeck granules can be considered Langerhans cells. As an extension to the work reported by Nestor et al. (7), we observed that both the intensity of cellular response and the proportion of CD1+ cells, for instance, were more intensive in perilesional than in clinically uninvolved PV skin. However, none of the correlations between the proportion of CD1+ cells on the one hand and the various lymphocyte activation markers studied on the other was significant, suggesting that also other factors are involved in the local lymphocyte activation.

Lymphocyte activation marker profile assay was used to assess any active T lymphocyte involvement. Upon activation, resting T lymphocytes pass the restriction point in the G1 phase of the cell cycle. Intense mRNA and protein synthesis lead to the expression of various activation markers such as interleukin-2 receptor. Such activation marker-positive lymphoid cells were found in perilesional (but not in clinically uninvolved) PV skin, suggesting local lesionally restricted T cell activation. Although interleukin-2 receptor is also known to be synthesized for secretion (14), soluble extracellular interleukin-2 receptor, if present at all, was not visualized as specific background staining.

It is, at least theoretically, possible that interleukin-2 receptor loses its immunoreactivity upon interleukin-2 binding. Therefore, transferrin receptor staining (15) was also applied in the present study. In fact, the proportion of transferrin positive cells was larger in both perilesional and clinically uninvolved skin than was that of the interleukin-2 receptor. However, this does not mean that interleukin-2 receptor–ligand complexes were found *in situ*, because it is difficult to explain why some cells came to be saturated to the degree of invisibility whereas others stained strongly.

According to our study, local T-cell activation seems to be restricted to a subpopulation of T cells as far as markers indicating lymphocyte cycling (interleukin-2 receptor, transferrin receptor) are concerned. T-cell activation is also implied by the relatively low proportion of CD3+ cells compared with the proportions of CD4+ and CD8+ cells. CD3 is known to be internalized upon T-cell activation (16). Alternatively, some of the CD3+ lymphocytes may be double marker (CD4+, CD8+) positive. However, we can conclude that some of the T cells in the diseased skin are actively involved in local pathomechanisms in PV.

The cellular infiltrate in clinically uninvolved PV skin was relatively sparse compared with the dense infiltration in the perilesional specimens. There might be differences in the stationary cells in perilesional and uninvolved PV skin. However, it is impossible to make any definite statements on this, because none of the markers used is totally specific for immigrant or stationary cells. The differentiation phenotypes and lymphocyte density in normal skin have been earlier reported by Nestor and co-workers (7). However, they did not study lymphocyte activation markers or CD1 expression, so the proportions of such cells in normal skin are not known. Therefore, it is impossible to be sure whether the presence of a few immunocompetent/accessory cells and occasional Ia or transferrin receptor positive cells indicates immunopathological involvement of clinically uninvolved PV skin, or whether similar cells would be found in normal healthy skin as well.

REFERENCES

1. Lever WF, Schaumburg-Lever G. Pemphigus. In: Lever WF, Schaumburg-Lever G, eds. Histopathology of the skin, 6th edn. Philadelphia: J B Lippincott, 1983; 104–113.
2. Jones JCR, Arnn J, Staehelin LA, Goldman RD. Human autoantibodies against desmosomes: Possible causative factors in pemphigus. Proc Natl Acad Sci USA 1984; 81: 2781–2785.
3. Amar A, Rubinstein N, Hacham-Zadeh S, Cohen O, Cohen T, Brautbar C. Is predisposition to pemphigus vulgaris in Jewish patients mediated by HLA-Dw10 and DR4? Tissue Antigens 1984; 23: 17–22.
4. Gregersen PK, Silver J, Winchester PJ. The shared epitope hypothesis. An approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. Arthritis Rheum 1987; 30: 1205–1213.
5. Unanue ER. The regulatory role of macrophages in antigenic stimulation. Part two: Symbiotic relationship between lymphocytes and macrophages. Adv Immunol 1981; 31: 1–136.
6. Van Voorhis WC, Valinsky J, Hoffman E, Steinman RM. The relative efficacy of human monocytes and dendritic cells as accessory cells for T cell replication. J Exp Med 1983; 158: 174–191.
7. Nestor MS, Cochran AJ, Ahmed AR. Mononuclear cell infiltrates in bullous diseases. J Invest Dermatol 1987; 88: 172–175.
8. Hsu S, Raine L, Fanger H. The use of avidin–biotin-peroxidase complex (ABC) in immunoperoxidase technique: A comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem 1981; 29: 577–580.
9. Janossy G, Panayi G, Duke O, Bofill M, Poulter LW, Goldstein G. Rheumatoid arthritis: A disease of T-lymphocyte/macrophage immunoregulation. Lancet 1981; ii: 839–842.
10. Hanafusa T, Pujol-Borrell R, Chiovato L, Russell RCG, Doniach D, Bottazzo GF. Aberrant expression of HLA-DR antigen on thyrocytes in Grave's disease: Relevance for autoimmunity. Lancet 1983; ii: 1111–1115.
11. Ahmed AR, Murahata RI, Schroff RW, Stevens R, Saxon AS. Production of pemphigus antibody in vitro and analysis of T-cell subsets. J Clin Immunol 1983; 3: 241–252.
12. Berger CL, Kung P, Goldstein G, DePietro W, Takezaki S, Chu A, Fithian E, Edelson RL. Use of orthoclone monoclonal antibodies in the study of selected dermatologic conditions. Int J Immunopharmacol 1981; 3: 275–282.
13. Mashkilleyson N. T- and B-lymphocytes in pemphigus vulgaris: A clinico-immunological follow-up study of 50 patients with pemphigus vulgaris. Acta Derm Venereol (Stockh) 1987; 67: 218–224.
14. Robb RJ, Kutny RM. Structure–function relationships for the IL 2-receptor system. IV. Analysis of the sequence and ligand-binding properties of soluble Tac protein. J Immunol 1987; 139: 855–862.
15. Trowbridge IS, Omary MB. Human cell surface glycoprotein related to cell proliferation is the receptor for transferrin. Proc Natl Acad Sci USA 1981; 78: 3034–3043.
16. Alcover A, Ramarli D, Richardson NE, Chang H-C, Reinherz EL. Functional and molecular aspects of human T lymphocyte activation via T3-Ti and T11 pathways. Immunol Rev 1987; 95: 5–36.