THE ROLE OF AUTOANTIGENS AND AUTOANTIBODIES IN THE PATHOGENESIS AND DIAGNOSTICS OF AUTOIMMUNE DERMATOLOGIC DISEASES

PhD Dissertation

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1. Husz Sándor, Altmayer Anita, Kiss Mária, **Molnár Katalin**, de Jong Marcelus C.J.M.: Epidermolysis bullosa acquisita.
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2. Husz Sándor, Kiss Mária, Marczinovits Ilona, Tóth Gábor, **Molnár Katalin,** Molnár János, Dobozy Attila: Detection of bullous pemphigoid antibodies by means of synthetic peptides as antigenic epitopes.
   Acta. Microbiol. Immunol. Hung. 43, 339-343 (1996)

3. Kiss Mária, Husz Sándor, **Molnár Katalin**: Identification of different circulating autoantibodies in patients with bullous pemphigoid and pemphigus vulgaris by means of immunoblotting.
   Acta Microbiol. Immunol. Hung. 43, 115-123 (1996)

4. **Molnár Katalin**, Kiss Mária, Pokorny Gyula, Kovács László, Dobozy Attila: Monitoring of antineutrophil cytoplasmic antibodies (ANCA) in patients with immunological disorders (Abstract)
   J. Invest. Dermatol. 107, 492 (1996)

5. Kiss Mária, Husz Sándor, Marczinovits Ilona, Molnár János, **Molnár Katalin**, Tóth Gábor, Dobozy Attila: Development of an ELISA system for the rapid diagnosis of bullous pemphigoid. (Abstract)
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6. **Molnár Katalin**, Kiss Mária, Husz Sándor, Dobozy Attila: Autoantitest vizsgálatok lupus erythematosusban.
   Bőrgyógy. Vener. Szle. 72, 49-54 (1996)

7. **Molnár Katalin**, Kiss Mária, Husz Sándor, Marczinovits Ilona, Molnár János, Tóth K. Gábor, Dobozy Attila: Development of a system for the detection of circulating antibodies against desmosomal proteins in patients with pemphigus. (Abstract)
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8. **Molnár Katalin,** Kemény Lajos, Korom Irma, Dobozy Attila: Panniculitis in dermatomyositis: report of two cases
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9. Robert M Vodegel, Kiss Mária, Marcelus CJM de Jong, Hendri H Pas, Altmayer Anita, **Molnár Katalin**, Husz Sándor, JB Van der Meer, Jonkman MF: The use of skin substrates deficient in basement membrane molecules for the diagnosis of subepidermal autoimmune bullous disease.
   Eur. J. Dermatol. 2: 83-5(1998)

10. Mehravaran Mehrdad, Morvay Márta, **Molnár Katalin**, Oláh Judit, Korom Irma, Husz Sándor, Dobozy Attila: Juvenile pemphigus foliaceus
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11. Kovács László, Szabó János, **Molnár Katalin**, Kovács Attila, Pokorny Gyula: Antineutrophil cytoplasmic antibodies and other immunologic abnormalities in patients with habitual abortion
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12. Bata-Csörgő Zsuzsanna, Husz Sándor, Földes Márta, Korom Irma, **Molnár Katalin**, Morvay Márta, Dobozy Attila: Scleromyxedema.
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13. **Molnár Katalin,** Kemény Lajos, Korom Irma, Dobozy Attila: Panniculitisszel járó dermatomyositis: 2 eset ismertetés.
    Bőrgyógy. Vener. Szle.75, 229 (1999)

14. **Molnár Katalin**, Gerrit van der Steege, Marcel F. Jonkman, Kiss Mária, Husz Sándor, Jan B.van der Meer, Hendri H. Pas, Dobozy Attila: A second type XVII collagen mRNA transcript in human keratinocytes.(Abstract)
    J. Invest. Dermatol.113, 439 (1999)

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16. Husz Sándor, Kiss Mária, **Molnár Katalin**, Marczinovits Ilona, Molnár Katalin, Tóth K. Gábor, Dobozy Attila: Development of a system for detection of circulating antibodies against hemidesmosomal proteins in patients with bullous pemphigoid
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17. **Katalin Molnár**, László Kovács, Mária Kiss, Sándor Husz, Attila Dobozy, Gyula Pokorny: Anti-neutrophil cytoplasmic antibodies in patients with systemic lupus erythematosus (Submitted to Clinical and Experimental Dermatology)

I. INVESTIGATION OF AUTOANTIBODIES IN PATIENTS WITH SYSTEMIC AUTOIMMUNE DISEASES

I/1. INTRODUCTION

Systemic autoimmune diseases

Autoimmune diseases are disorders, in which the characteristic process is the chronic inflammation destroying self structures (cells, organs). Depending on the target specificity (specific organs or common cell structures) of this process, there are organ specific or systemic autoimmune diseases. Overlap between the two groups is common. In the organ specific autoimmune disease (such as bullous skin diseases, insulin-dependent diabetes mellitus) antibodies are produced against special cells or cell structures. Whereas in case of systemic autoimmune disease (as the systemic lupus erythematosus, rheumatoid arthritis), the antibody response is directed against cells or cell structures, that present in not only one, but several organs.

The importance and diagnostic role of autoantibody detection in autoimmune skin diseases have been well known for a long time (1, 2). Those are necessary to establish the subtypes and activity of a certain disease (3). Systemic lupus erythematosus (SLE) and its group (subacut cutaneus lupus erythematosus (SCLE), discoid lupus erythematosus (DLE)) may be the most characteristic ones of the non-organspecific autoimmune diseases, where firstly the autoantigen-autoantibody reaction has been detected (4). With this method (so called LE test) the antinuclear antibodies (ANA) can be detected, and now it is - together with the autoantibody detection against extractable nuclear antigens (ENA) - used as a screening method at the suspicion of autoimmune disease (5, 6). Since then, numerous autoantibodies have been detected and characterised, and their number have been continuously increasing: anti-dsDNA, -ssDNA, -SSA, -SSB, -U1-RNP, -Sm, -Scl-70, -Jo1 and anti-cardiolipin antibodies.

The anti-double-stranded (ds) DNA antibodies are sensitive and specific markers for SLE (7). The presence of anti-dsDNA antibodies in SLE has been included as one of the criteria for disease classification by the American Rheumatism Association. The Sm and U1-RNP (ribonucleoprotein) are non-histon glycoproteins which play a role in the maturation process of the messenger RNA. The anti-Sm antibodies are highly specific, but less sensitive in SLE, while anti-U1RNP antibodies occur in the other diseases of the lupus erythematosus group (8)

and mixed connective tissue disease (MCTD). The SSA cellular antigens are RNA-protein complexes, that are localised not only to the nucleus but also at the cytoplasm and plasma membrane and play a role in cell proliferation. The anti-SSA antibodies can be detected most frequently in SLE, SCLE and neonatal LE (9, 10). The anti-SSB autoantibodies occur less frequently, mostly at anti-SSA positive cases (11). The anti-cardiolipin antibodies together with VDRL and lupus anticoagulant (LAC) are anti-phospholipid antibodies. Thus they may occur in patients with SLE complicated with vasculitis and patient with idiopathic habitual abortion (12).

The anti-neutrophil cytoplasmic antibodies (ANCA) constitute a family of antibodies against various components of the cytoplasm of neutrophil granulocytes (13). The antigen in the immunofluorescent cytoplasmic staining (cANCA) is a serine proteinase-3 (PR-3) (14). Perinuclear ANCA (pANCA) bind predominantly to myeloperoxydase (MPO) and occasionally to elastase, lysosyme, catepsin G and lactoferrin, localised in the neutrophil primary granules (15). Originally they were detected in systemic vasculitis, but since then they have been described in several other diseases (chronic inflammatory bowel diseases, systemic autoimmune diseases) (16). Numerous studies have been published in this topic involving more potentially „ANCA associated diseases". We did some research work in this field, detecting ANCA in SLE and habitual abortion.

In the last decade essential development has been observed and more and more precise and quick methods can be used to detect those autoantibodies (enzyme-linked immunosorbent assay (ELISA), pinELISA, IF), which facilitate easier diagnosis. In the first half of this section we report our examination of the presence and diagnostic significance of the „classical" and later known autoantibodies, respectively. Later, we reported two cases as clinical „usefulness" of autoantibody detection.

I/2. AIMS

Our aims were to identify serum autoantibody panel in LE patients and to establish their special pattern to the clinical status and disease activity.

Our aims were to identify the ANCAs and to analyse their occurrence and clinical significance in SLE and habitual abortion.

I/3. METHODS

Immunofluorescence studies

ANA detection was performed with indirect immunofluorescence using rat liver as substrate.

Isolation of ENA and detection by counter-immunoelectrophoresis

Acetone-precipitated protein extract of new-born calf thymus nuclei (extractable nuclear antigens; ENA) served as antigen substrate, prepared by Sönnichsen et al. (17). It contains many different nuclear antigens.

This is another important screening test for the detection of ENAs. This method involves electrophoresis of antigens and antibodies towards each other. Autoantibodies from patients' sera are identified by formation of specific precipitation lines with their antigens using electrophoresis in agarose gel. This method was especially important when purified autoantigens were not available.

Detection of autoantibodies by ELISA kits

ANCAs were measured with ELISA technique (Shield Diagnostics; Dundee,UK) The antigens were PR3 for cANCA and MPO for pANCA.

ELISA was used for detection of anti-DNA, -SSA, -SSB, -Sm, -U1-RNP (Epignost; Leonding/Linz, Austria) and anti-cardiolipin (aCL) (Shield Diagnostics; Dundee, UK).

Detection of LAC

LAC was investigated with the measurement of activated partial thromboplasin time.

In vitro growth of normal and scleromyxedema fibroblasts

Fibroblasts from normal skin and from the skin of a scleromyxedema patient were grown in vitro up to 3 passages then plated into plates. Three days later, the media were changed by adding either normal human serum or scleromyxedema serum to the cultures. After 24-hour incubation, [3H] thymidine was added to the plates. Cells were harvested 16 hours later, and reactivity was measured with liquid scintillation counter.

I/4. RESULTS and DISCUSSION

I/4/A Detection of autoantibodies in systemic lupus erythematosus

The prevalence of autoantibodies, their specificity and sensitivity had already been investigated. Our goal was to establish, whether those data can be applied for our SLE patients. The autoantibody profile of patients with different LE diseases and the clinical importance of these investigation were discussed. We have chosen a representative panel of 40 patients with lupus erythematosus (18 Discoid LE, 6 Subacut Cutan LE, 16 SLE) and detected the ANA, anti-ENA, -DNA, -SSA, -SSB, -Sm, -U1RNP antibodies.

Results can be seen on figures (Fig. 1-3.). At the SLE patients the most frequently detected antibodies were ANA and ENA (13/16, 81%; 11/16, 69%, respectively). Anti-DNA and anti-SSA antibodies could be detected at half of the cases (8/16, 7/16). At patients with SCLE the ANA and anti-ENA could be shown in half of the cases (3/6, 3/6), while the anti-SSA antibody was observed in the highest ratio (5/6, 83%). We could detect these autoantibodies with the lowest frequency at patients with DLE. Only the ANA was present at less than half of the patients (8/18, 44%). The anti-SSB, -Sm, -U1-RNP were detected only in low percentage at all three cases.

The screening of autoantibodies may help in the diagnosis and to assess the activity and prognosis of the disease, but for this goal the exact antibody panel should be known. Patients showing only anti-SSA and/or anti-SSB positivity seemed to have better prognosis, the onset of the disease was later, the renal involvement was rare. Progression of the disease and complications might occur if not only anti-SSA positivity could be detected but anti-Sm or anti-DNA also (7).

We could not detect anti-cardiolipin antibodies at most of the patients. 4 patients with DLE, 1 with SCLE and 1 with SLE had borderline level anti-cardiolipin antibodies, 1 patient with DLE, and two with SLE had moderately high anti-cardiolipin antibody level (Table 1). One of the patients with SLE had had habitual abortion.

Numbers of cases

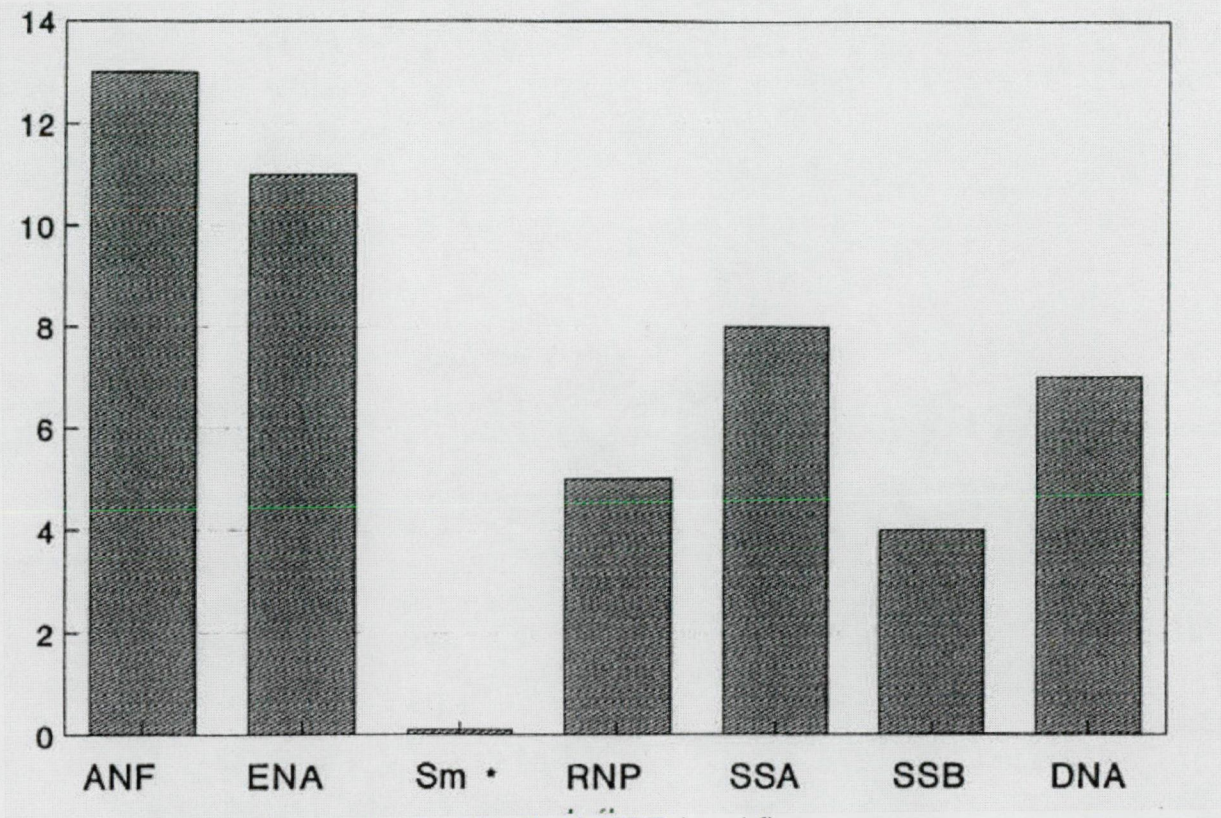

Figure 1.: The prevalence of autoantibodies in SLE (n = 16)

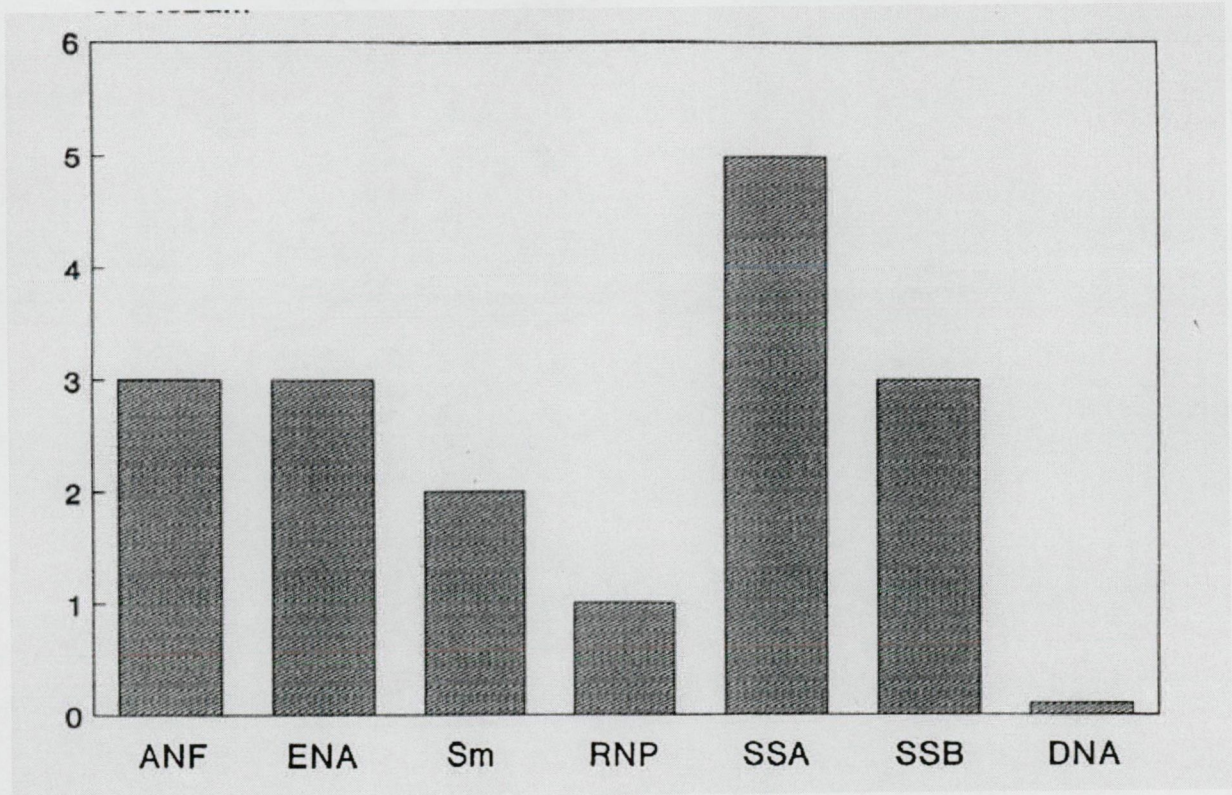

Figure 2.: The prevalence of autoantibodies in SCLE (n = 6)

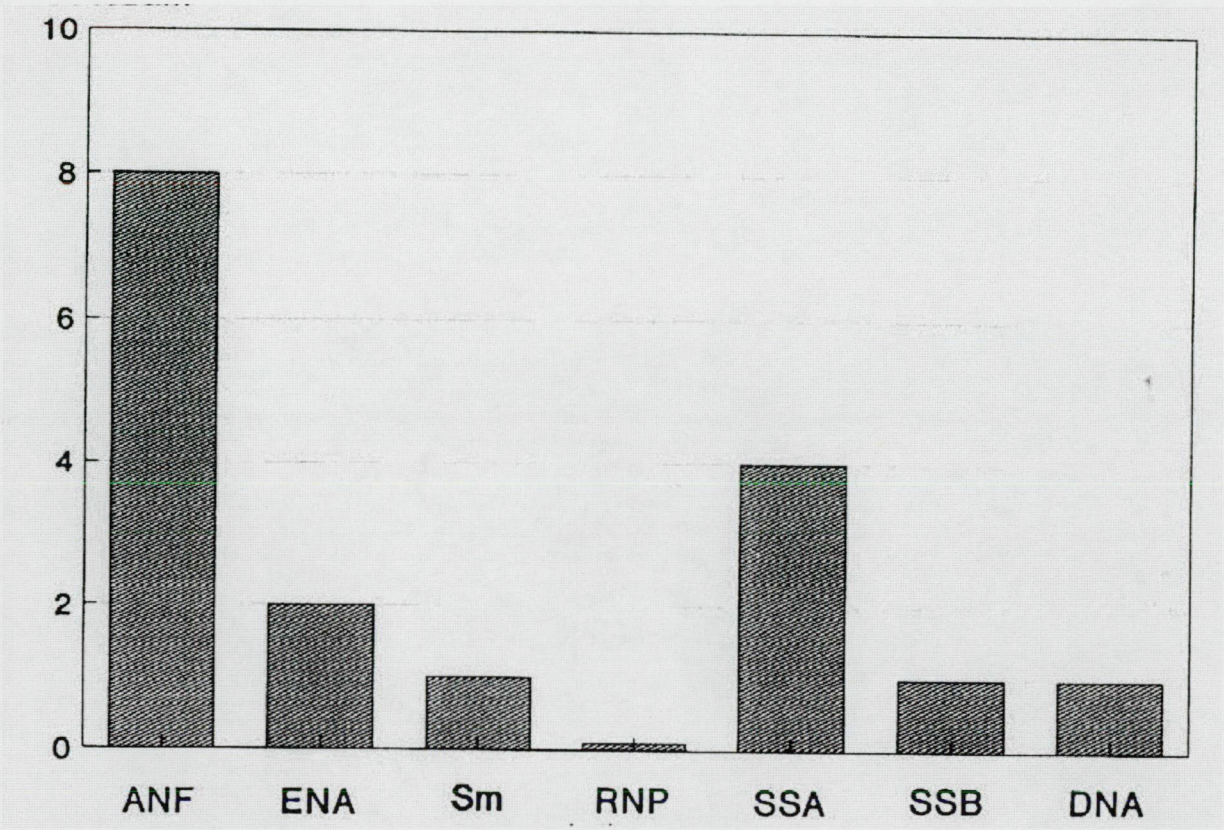

Figure 3.: The prevalence of autoantibodies in DLE (n = 18)

Table 1.: Serum anti-cardiolipin antibodies in patients with SLE (n= 40)

Anti-cardiolipin IgG Units/ml	**Values**	**Patients**
< 10	Negative	13 SLE 13 DLE 5 SCLE
10 - 15	Borderline	1 SLE 4 DLE 1 SCLE
15 - 60	Moderately high	2 SLE 1 DLE

I/4/B Detection of ANCAs in systemic lupus erythematosus and habitual abortion

Detection of ANCAs in systemic lupus erythematosus

After the well-known antibodies, we examined relatively new but intensively researched antibodies (c and pANCA), which were first detected in systemic vasculitis. Later, their diagnostic relevance in other diseases was investigated. Several data have already existed about ANCA detection at SLE patients, but they were controversial. In this study, we determined the prevalence of ANCA in SLE patients and examined whether there is a correlation between ANCA positivity and disease activity, the positivity for other autoantibodies and organ involvement. In the second study we examined whether the presence of ANCA, similarly to antiphospholipid antibodies or ANA was associated with idiopathic habitual abortion (18, 19).

41 patient with SLE were examined. The two control groups included 15 patients with systemic vasculitis and 12 healthy persons. In the *SLE group*, elevated levels of cANCA were detected in 3 (3/41, 7.3%) and pANCA in 10 SLE patients (10/41, 24.4%). (Fig. 4 a, b) The cANCA levels were in the low positive range (2-6 U/l) in all three SLE patients. Similarly, the antibody levels were only mildly elevated in all 10 pANCA positive SLE patients.

Our findings are consistent with previous studies in which the concentrations of ANCAs are quite similar in the SLE patients and the *normal control group* (mean cANCA concentration: 1.0 U/l vs. 0.7 U/l; mean pANCA concentration: 5.1 U/l vs. 3.7 U/l). Of the 15 patients with *systemic vasculitis*, 7 cANCA positives and 5 pANCA positives were found. (Fig 4 a, b) In this group elevated values for cANCA were significantly more frequent than in the SLE group (7/15 versus 3/41; p=0.0021), while the prevalences of pANCA were not different in the two groups (5/15 versus 10/41; p= 0.51). In the vasculitis control group, the mean levels of cANCA (10.5 U/l) and pANCA (15.5 U/l) were essentially higher than those in the SLE patients and the healthy controls (vasculitis/SLE: cANCA: 10.5 U/l vs. 1.0 U/l, p=0.035; pANCA: 15.5 U/l vs. 5.1 U/l, p=0.035; vasculitis/normal controls: cANCA: 10.5 U/l vs. 0.7 U /l, p=0.07; pANCA: 15.5 U/l vs. 3.7 U/l, p=0.044).

We examined the frequencies of various organ involvements manifest at the time of sampling. There were no statistically significant differences in the occurrences of symptoms between the ANCA positive and ANCA negative patients. Other autoantibodies, such as anti-DNA, -SSA, -SSB, -Sm, -U1RNP, -cardiolipin antibodies and lupus anticoagulant were detected, but we

did not find significant correlation between the observed autoantibody positivities and the ANCA positivity.

Existing reports concerning the presence of *cANCA* in SLE patients is similarly controversial. Several authors found anti-PR3 antibodies in SLE patients, while, in contrast, several authors reported on the absolute absence of cANCA in SLE (16-20). As far as the presence and prevalence of *anti-MPO* antibodies in SLE patients is concerned, Schnabel et al. did not detect anti-MPO antibodies in patients with SLE. Other studies report low percentage (1,4%) of the occurrence of anti-MPO antibodies in SLE patients while higher frequencies, up to 21%, have also been detected (20).

Controversial data can be found about the connection between ANCA and other autoantibodies. Spronk et al. found that levels of anti-dsDNA were similar in patients with or without ANCA positivity (22). Lawton et al. detected anti-dsDNA in 16 of the 18 SLE patients (89%), who also proved to be positive for anti-lactoferrin antibody and this prevalence was higher than that for the whole SLE group (23). It is thought that ANCA, through inducing the release of reactive oxygen species in the vascular wall, may induce the release of DNA and thus it may play a role both in anti-DNA production and in the inflammatory response.

Since the clinical picture of SLE is varied, it is of important clinical necessity to answer the question whether the presence of ANCA may identify a subgroup of SLE patients. There are several different data about the organ involvements in ANCA positive SLE patients. Some authors observed more frequent occurrence of serositis in ANCA positive patients, while in other studies the arthritis or neurological involvement was found to be more common in these cases (22-25).

Based on our data, it seemed reasonable for us to compare the prevalence of ANCA in SLE patients with and without vasculitis, however, in this study we were not able to detect close correlation either between the ANCA positivity and vascular manifestations or other organ involvements.

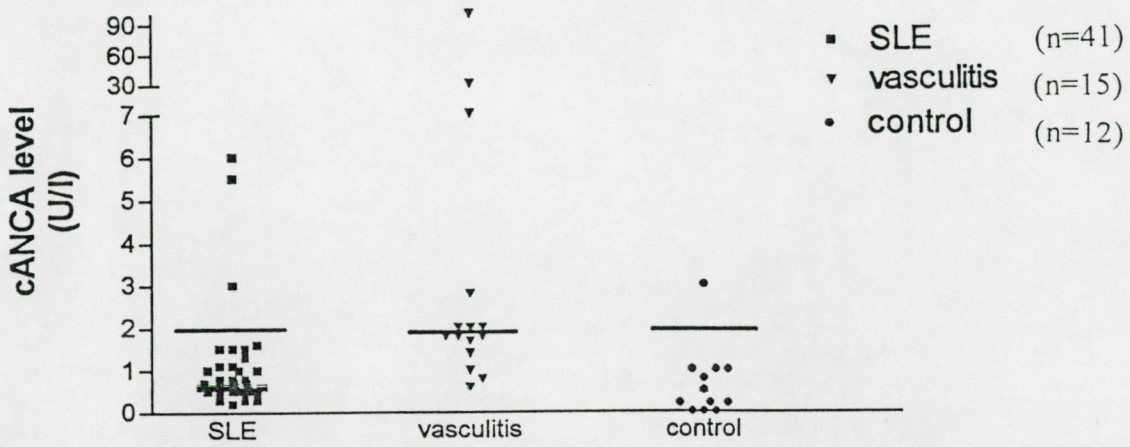

Figure 4 a: cANCA levels in the patient groups. Values below the lines are normal.

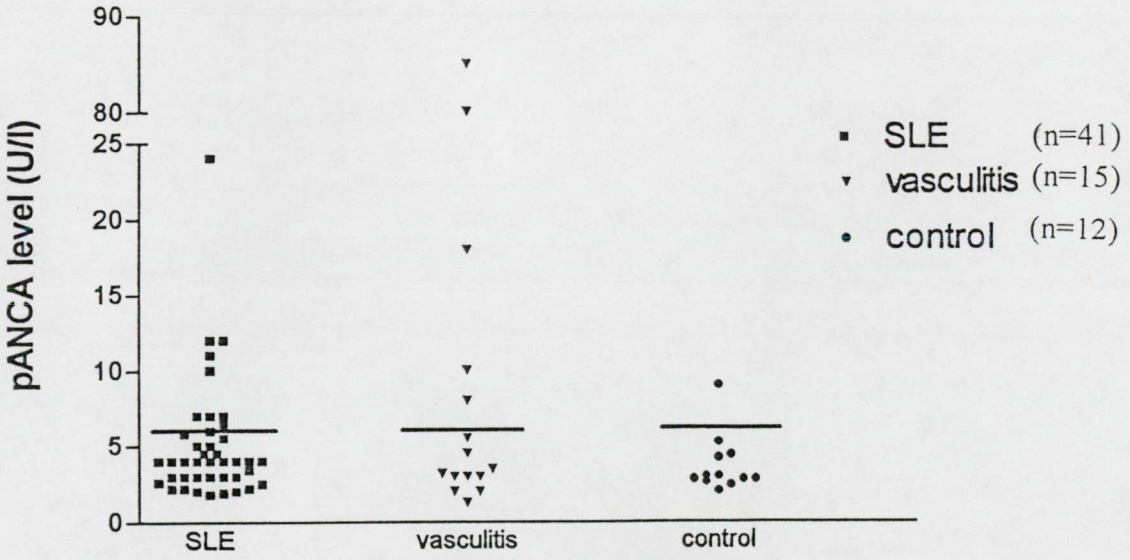

Figure 4 b: pANCA levels in the patient groups. Values below the lines are normal.

Detection of ANCAs in habitual abortion

In systemic lupus erythematosus an increased rate of spontaneous abortions may be observed even before the onset of the classical symptoms of the disease (26). Therefore, we started a study of women with idiopathic recurrent spontaneous abortion with the aim of investigating a wide spectrum of immunoserologic markers occurring in patients with SLE. Systemic vasculitis constitutes another group of systemic autoimmune diseases, where ANCAs are useful tools in the diagnosis (23). As far as we know, they have not been investigated so far in the context of habitual abortion. We therefore examined their prevalence in our patients. 59 women with a history of at least two consecutive spontaneous abortions in pregnancies from the same partner (average age: 30,47 years) and 25 healthy women were involved in the study. The frequencies of anti-DNA, anti-SSA, anti-SSB, anti-Sm, anti-U1RNP, anti-CL IgG autoantibodies; the LAC; LE cell; ANA; and decreased complement-3 levels were detected. Among the 59 patients with recurrent abortion 28 proved positive for at least one of the examined laboratory variables. Although aCL and LAC occurred more frequently in habitual aborters, and markedly elevated levels of aCL were found only in the study group (in five patients), no significant differences were observed in the prevalences of examined serologic variables between the two groups (27, 28). pANCA was found in 2/59 examined patients but in none of the controls, while cANCA was found in 6/59 examined patients but none in the control patients ($p = 0.09$) (Fig. 5).

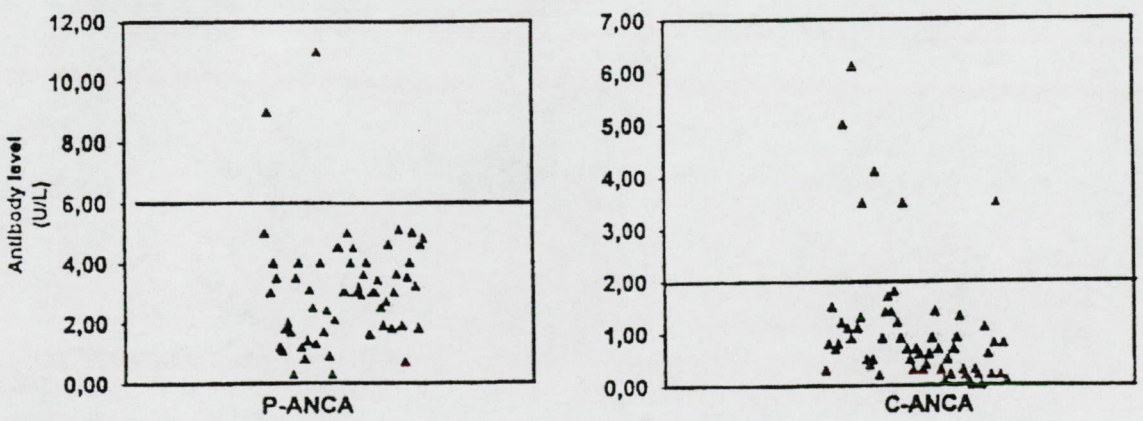

Figure 5.: Distribution of pANCA and cANCA in patients with recurrent abortion. Values below the lines are negative.

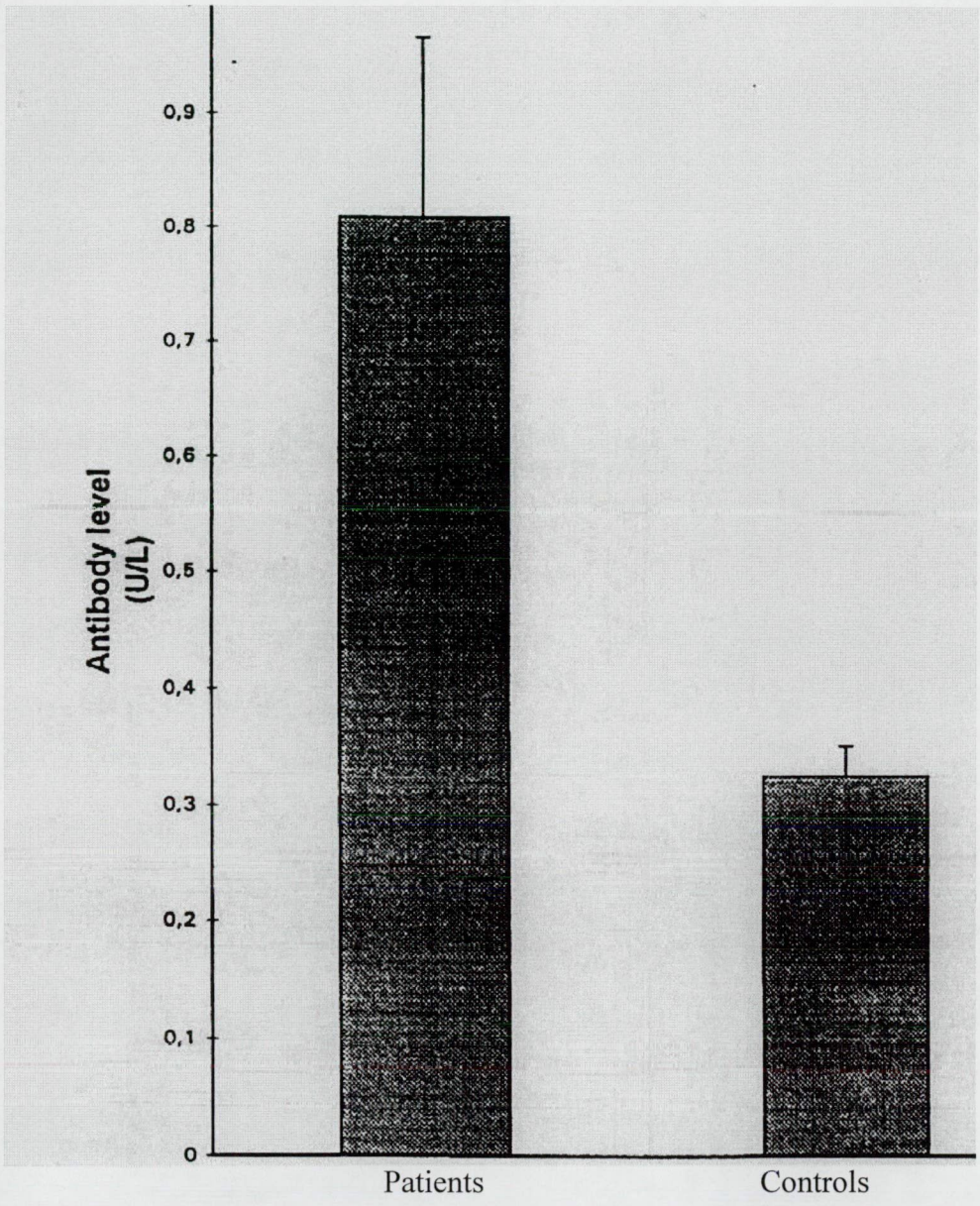

Figure 6.: Comparison of cANCA levels in patients with habitual abortion and in healthy controls. Bars show mean +/- SE. P=0.028

However, when the levels of cANCA in the two groups were compared, statistically higher values were revealed in the habitual abortion patients than in the healthy persons (p= 0.028) (Fig. 6.). The ANCA levels in the study patients were lower than those observed in patients with active systemic vasculitis and similar to the levels described in studies about patients with SLE. The higher prevalence and level of cANCA raise the possibility that this autoantibody may in some way be associated with recurrent abortion.

I/4/C The importance of autoantibody investigation in clinical work: case reports (Dermatomyositis with panniculitis and scleromyxedema)

Here we report two cases, where the laboratory findings, which were the subject of earlier research work, were used in everyday clinical diagnosis.

Dermatomyositis (DM) with panniculitis

Clinically manifest panniculitis which is an inflammatory disorder of the fatty tissue is rarely found in dermatomyositis, but microscopic panniculitis has frequently been observed in DM (29). Here we report 2 cases involving clinically manifest panniculitis, discuss the diagnostic significance of our findings and the reviewed literature. A *54-year-old female* was presented with 6-month history of proximal muscle weakness, low-grade fever and a 15 kg weight loss. Some excavated, red papules were situated on an indurated basis on the lower parts of the arms. No anti-SSA, anti-SSB, anti-Sm or anti-dsDNA antibodies or other antinuclear antibodies were found. No internal malignancy was found. A *57-year-old woman* was presented with a history of rash on the face, tiredness, joint pain, low-grade fever, proximal muscle weakness and dysphagia. There were red, warm, indurated plaques and nodules on the buttocks, the left thigh and the sacral region. ANA (nucleolar type, 1:32), examined on mouse liver substrate were positive; anti-dsDNS, -SSA, -SSB antibodies were negative. A tumor search proved to be negative. In different studies various percentages of malignancy (6-60%) in adult patients with DM were reported (30). Among the reported patients, neoplasm was detected in only one case. All patients responded well to prednisolone and in some cases to methotrexate therapy, exception of one patient who needed i.v. immunoglobulins (31). Our own data and the literature review suggest that panniculitis present in DM may be a positive prognostic sign, since nearly all DM patients with panniculitis exhibited good clinical response to the therapy, and internal malignancy was detected in only one case. We propose that DM accompanied by panniculitis may comprise a distinct subset of DM (Table 2).

Table 2.: The table summarizes the clinical and therapeutical data of the cases have been published till now in the literature and our two patients.

Case/sex/age	Reference	Occurrence of DM relative to that of panniculitis	Localization	Calcification	Malignancy	Therapy	Response
1/F/3	6	simultaneously	arm	no	no	1 mg/kg/d prednisolone methotrexate	yes
2/M/8	7	simultaneously	buttocks,thighs	no	no	2 mg/kg/d prednisolone	yes
3/M/51	8	14 ms after	buttocks	no	rhabdomyo-sarcoma	50 mg/d prednisolone	transient
4/F/58	9	2 ws after	thighs, abd., buttocks	no	no	60 mg/d prednisolone	yes
5/F/24	10	3 ms after	arms	no	no	60 mg/d prednisolone 3x2.5 mg/w methotrexate	yes
6/M/10	11	1 y before	buttocks, thigh	no	no	2 mg/kg/d prednisolone	yes
7/F/42	12	10 ms after	butt., thigh, arm, abd.	no	no	1 mg/kg/d prednisolone	yes
8/M/42	13	5 ys before	buttocks, hands	no	no	2 mg/kg monthly iv. immunoglobulins (5x)	yes
9/F/65	14	simultaneously	buttocks, thighs, lower legs	yes	no	60 mg/d prednisolone 1000 mg methylprednisolone (1 occasion)	yes
10/F/54	present	simultaneously	arms	no	no	1 mg/kg/d prednisolone 2 mg/kg/d azathioprin	yes
11/F/57	present	2 ys before	buttocks, thighs	no	no	1 mg/kg/d methylprednisolone 3 mg/kg/d cyclosporine A	yes

Scleromyxedema

Scleromyxedema is a sclerotic variant of papular mucinosis in which lichenoid papules and scleroderma-like features are present (32). We describe a patient with scleromyxedema with IgG type lambda chain paraprotein, a systemic sclerosis-like illness and myositis. The patient's serum contained anti-Scl-70 antibodies, characteristic of scleroderma. Electromyography showed signs of acute myositis and creatine phosphokinase (CPK) level was elevated. It was published, that serum from patients with scleromyxedema enhance the proliferation of normal fibroblasts in vitro (33). To see whether fibroblasts from lesioned skin would respond similarly to normal fibroblasts to scleromyxedema serum, fibroblasts were derived from the lesioned skin of the patient. Both normal and scleredema fibroblasts responded with higher proliferation to *normal serum* in a dose-dependent manner. Lesioned fibroblasts, however, seemed to respond more readily to lower concentrations of the normal serum and in fact, grew at a much higher rate than normal fibroblasts even without serum.

	Incorporation of /3H/ thymidine (dpm/3x10^4)	
	Normal fibroblasts	Scleromyxedema fibroblasts
Without serum	524 ± 174	2,455 ± 192
5% normal serum	3,074 ± 319	14,587 ± 2,720
10% normal serum	8,281 ± 2,517	32,385 ± 1,332
20% normal serum	37,886 ± 3,815	39,000 ± 1,634
5% scleromyxedema serum	30,280 ± 3,793	11,464 ± 928
10% scleromyxedema serum	46,158 ± 736	25,260 ± 1,823
20% scleromyxedema serum	51,176 ± 3,614	32,578 ± 2,758

Figure 7.: In vitro growth of normal and scleroderma fibroblasts based on (3H) thymidine incorporation

Our *patient's serum* had growth enhancing effect on normal fibroblasts, however the lesioned fibroblasts did not respond with higher proliferation to the patient's serum than to normal serum (Fig. 7). These data suggest the existence of an altered growth regulation in dermal fibroblasts of Scleromyxedema. The patient was treated with cyclosporin with improvement (34).

I/5. CONCLUSIONS

A/ We investigated the autoantibody panel of patients with SLE, SCLE and DLE. ANA, anti-ENA, anti-DNA, -SSA, -SSB, -Sm, -U1-RNP were examined. ANA, anti-ENA and anti-SSA could be detected most frequently, the other five only in several cases. We conclude, that the knowledge of the autoantigen panel in separate cases may reflect to the prognosis, but for general statements further clinical and laboratory research work would be necessary.

B/ Our goal was to identify subgroups of patients with different clinical presentation in SLE. On the basis of ANCA positivity, no specific SLE subgroups could be identified. ANCAs (especially pANCA) could be detected in higher frequencies in SLE patients than in healthy controls, but there was no difference in the levels of autoantibodies. In our experience, the routine examination of ANCA in SLE patients is unnecessary, as the results do not furnish any additional data concerning the diagnosis and prognosis.

C/ The immunological mechanism of pregnancy loss in habitual aborters with antiphospholipid and antinuclear antibodies have not been fully clarified. To clear up this patomechanism we examined the possible association of ANCAs with recurrent miscarriage. pANCA was found in 2/59 examined patients but in none of the controls, while cANCA was found in 6/59 examined patients, but non of the controls. Statistically higher cANCA level was revealed in the habitual abortion patients than in healthy persons ($p=0,028$). The questions whether the presence of ANCA especially of cANCA has a specific role in habitual abortion, and whether it is of clinical importance in reproductive immunology must be answered after the follow up of our patients and further examinations.

D/ We examined two cases of DM with panniculitis and reviewed similar cases in the literature. We found, that the panniculitis occurring in DM may be a good prognostic sign. We propose that DM accompanied by panniculitis may comprise a distinct subset of DM.

We have published a case with typical sign of scleromyxedema. The scleromyxedema serum was found to have growth enhancing effect on normal fibroblasts. The existence of an altered growth regulatory mechanism in scleromyxedema fibroblasts was supported.

II. INVESTIGATION OF ANTIBODIES IN AUTOIMMUNE BULLOUS DERMATOSES

II/1. INTRODUCTION

Blistering skin diseases form a clinically and pathologically very heterogenous group. In the autoimmune blistering skin diseases autoantibodies are directed towards the connecting molecules of the epidermis (desmosomes), the basal membrane zone (BMZ), (hemidesmosomes) and dermis (collagen type VII). The desmosomes are punctate membrane domains less than 1 μm in diameter, containing adhesion molecules as structural elements; these provide adhesion between adjacent cells and link their intermediate filaments (35). Desmosomes consist of transmembrane proteins, proteins of desmoglea, and proteins of the desmosomal plaque. The main transmembrane glycoprotein components are desmogleins. Desmogleins (Dsg) are members of the cadherin supergene family (Ca^{++}-dependent cell adhesion molecules). To date, three desmoglein isoforms (Dsg 1, 2, 3) have been isolated. Dsg1 and Dsg3 share approximately 60% primary structural homology.

The hemidesmosomes are attachment structures in the epidermal basement zone which assure the stable connection of epidermis and dermis (36). They link the intermediate filament network of the basal epithelial cells to the anchoring filaments of the underlying basement membrane. Binding of the autoantibody to the connecting molecule disturbs its important function leading to blister formation.

Depending on the depth of the antigen-antibody reaction (epidermis, BMZ, dermis) two main group were formed: the pemphigus group and pemphigoid group.

The pemphigus group belongs to the intraepidermal blistering diseases. All forms of pemphigus are characterised by a loss of normal epidermal cell-cell adhesion (acantholysis) and by the presence of pathogenic IgG autoantibodies directed against cellular adhesion molecules. Blisters result from damage to the desmosomes. *Pemphigus vulgaris* (PV) is the most common form of pemphigus in Europe. It has a chronic disease course and affects glabrous skin and mucous membranes. The PV patients have IgG autoantibodies against the PV antigen, the ectodomain of Dsg3. It implicates both sexes equally in the age of forty and fifty. *Pemphigus foliaceus* (PF) can be divided in five subsets: idiopathic, endemic, drug - induced, pemphigus erythematosus and IgA pemphigus. They possess similar symptoms and immunologic features but appear in special circumstances. Their most common primary

lesion is a superficial vesicopustule. Mucosal involvement does not occur. The autoantigen is the Dsg1. *Paraneoplastic pemphigus* (PNP) is also a blistering disorder, that arises exclusively in the context of known or occult neoplasms. Both intraepidermal and subepidermal blister occur. The course of the disease may be rapidly progressive or may resolve depending on benign or malignant neoplasm. In PNP autoantibodies are directed against desmosomal and hemidesmosomal components such as desmoplakins I, II and bullous pemphigoid antigen 1 (37).

In acquired blistering skin diseases many of these adhesion molecules become targets of autoantibodies. Numerous projects have recently been designed to determine the relationship between antibodies targeting cellular adhesion molecules and the pathophysiology of the bullous diseases. It is already known, that pemphigus autoantibodies play a direct role in the pathogenesis of the disease (38).

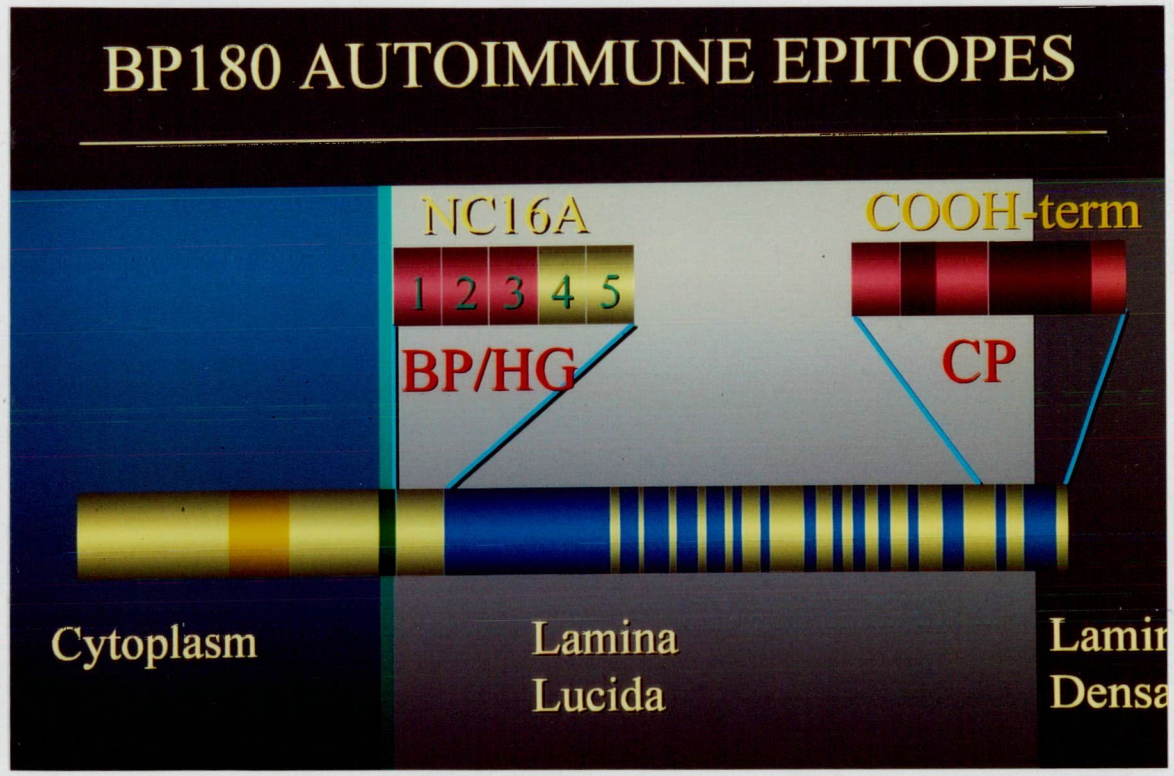

Figure 8.: The position, structure and antigenic epitopes of BP180 molecule

Bullous pemphigoid is a subepidermal blistering disease of elderly people, however, the condition can occur in the under-forties and occasionally in children, too. The autoantibody response is directed against a hemidesmosomal plaque protein, the so called major antigen (BP230) and the minor antigen (BP180) (42). This second molecule -one of the best studied components of the hemidesmosome- is the transmembrane glycoprotein, also known as type

XVII collagen. Electron-microscopical and biochemical analysis confirmed the predicted trimeric conformation (39, 40). The molecule has a distinct shape; a globular head, located in the cytoplasm, and a central rod to which a flexible tail is attached sticking out into the extracellular matrix. The central rod corresponds in length to the larger collagen domain (COL15) which leaves the flexible tail to consist of the fourteen remaining collagen domains (COL1 to COL14) interrupted by non-collagenous hinge regions (41) (Fig. 8).

Epidermolysis bullosa acquisita is an uncommon blistering disorder characterised by cicatrising lesions located predominantly in areas of trauma, with blisters occurring beneath the lamina densa. These patients have IgG autoantibodies reactive against various epitopes within the NC1 domain of the anchoring fibrils, which are composed of type VII collagen and the most dominant epitopes are found in fibronectin-like repeats within the molecule (43).

The pathogenic role of these autoantibodies has already been established (38). Because of these essential importance, the antigen molecules and their epitopes of the autoimmune bullous diseases have been investigated very intensively in the last decade. The main antigenic epitopes and the complementary DNA (cDNA) sequences of the antigen molecules have already been known, so it is possible to predict and synthesise antigenic epitopes (44, 45). The diagnosis of the bullous skin diseases is based on the results of histology, DIF, IIF. Besides this time-consuming classical methods, easier ones are under development (46).

II/2. AIMS

We tested and compared different methods (IIF, immunoblotting, ELISA) to detect bullous dermatoses (PV, PF, BP) and developed ELISA system using synthetic antigenic epitopes and recombinant antigens.

We have analysed BP180 mRNA expression in normal human keratinocytes.

Use of newly improved ELISA technique in the quick diagnostics; case reports: pemphigus foliaceus and epidermolysis bullosa acquisita.

II/3. METHODS

Immunofluorescence studies

Direct immunofluorescence (DIF)

The standard technique was used. Each specimen was sectioned into 5 μm sections on a cryostat. Specimens were stained for IgG , IgA, IgM and C3 by incubation of the sections with monospecific FITC-labelled antibodies.

Indirect immunofluorescence (IIF)

All serum samples were examined by using cryostat sections of rabbit organs or in the cases of bullous dermatoses rabbit oesophagus, normal human skin and salt-split skin were used as substrates. Sera were routinely diluted 1:32 in phosphate-buffered saline (PBS). The sections were incubated with the patients' serum and then examined for the presence of circulating autoantibodies by using anti-human FITC–labelled immunoglobulins.

Fluorescent Overlay Antigen Mapping (FOAM)

This is a kind of developed immunofluorescent method to differentiate the bullous pemphigoid from epidermolysis bullosa acquisita, antigen mapping with overlaying immunofluorescent dyes. The histological specimen was incubated with antihuman-IgG/FITC (green coloured reaction), and anti-collagen VII (red coloured reaction, rhodamin). The overlay of fluorescence of FITC-green and rhodamin-red show the double, yellow basalmembran reaction (47).

Demonstration of anti-epidermal antibodies by means of Western blot technique (immunoblotting)

Immunoblotting studies were performed according to Hashimoto et al. (48) with slight modifications. Normal human skin was obtained from patients undergoing plastic surgery. The skin pieces were incubated in a mixture of 1 M NaCl, 1 mM EDTA and 10 μM phenylmethylsulphonyl-fluoride (PMSF) at 4 °C for 72 hours. The epidermis was then easily separated from the dermis and homogenised in a solution containing 31.2 mM TRIS-HCl, 2% SDS, 1 mM PMSF, 2 mM EDTA and 0.1 M dithiotreitol. After a 24-hour incubation at 4 °C, the homogenizate was centrifuged at 15 000 g and the supernatants were stored at –70 °C until

needed. The constituent proteins of the epidermal extracts were separated by Sodium-Dodecyl-Sulphate-Polyacrylamide-Gel-Electrophoresis (SDS-PAGE) with 6% separating gel and then transferred to nitro-cellulose membrane before probing the test sera. All sera were used to probe immunoblots at a dilution of 1:40. Specific binding by the sera was detected by using peroxidase-linked class-specific second antibodies (goat antihuman IgG and IgA) and visualised with diamino-benzidine.

Cell culture

Primary human keratinocytes were cultured in serum free keratinocyte culture medium (BioWhittaker Europe SPRL, Belgium). The squamous cell carcinoma line UMSCC-22B, derived from a tumour of the hypopharynx, was cultured in DMEM supplemented with 5% foetal calf serum (FCS), 2 mM L-glutamin, 100 U/ml penicillin and 100 µg/ml streptomycin.

In silico elongation of the known BP180 3'-UTR mRNA sequence

Internet BLAST-analysis searches against the EMBL/GenBank human EST database were initially carried out with the 4669 bps human BP180 mRNA sequence (GenBank # M91669). With these results, and the results of subsequent BLAST searches with identified EST's, a hypothetical sequence of 877 bases could be added 3' to the M91669 sequence.

Isolation of mRNA

Cells were grown until subconfluence and then harvested by extraction with 1ml/15cm^2 TRIZOL reagent (Life Technologies BV, Breda, Holland). The RNA was isolated according to the manufacturers protocol and the poly (A^+) mRNA was purified from the total RNA using the mRNA Isolation Kit (Boehringer, Mannheim, Germany).

RNA probes

Four digoxigenin(DIG)-labelled RNA probes were synthesised using the SP6/T7 RNA Labelling kit according to the manufacturers protocol (Boehringer Mannheim, Germany).

DNA fragments were generated by PCR on partial cDNA clones coding for the cytoplasmic domain of BP180 (probe 1), the extracellular domain of BP180 (probe 3) or human genomic DNA (probe 4). The PCR-products were subcloned into vector pCRII (TOPO^{TM} TA Cloning kit, Invitrogen, Groningen, The Netherlands). For probe 2 a cDNA fragment containing bp

1771 to 2553 was subcloned from plasmid p213 into vector pSPT19. DIG-labelled antisense RNA probes were then generated by in vitro run-off transcription on linearized plasmid DNA using either T7 or SP6 polymerase. The final probes were complementary to BP180 mRNA sequences at the following positions: probe 1, nt 304-741; probe 2, nt 1771-2553; probe 3, nt 3679-4131; probe 4, nt 4649-5101.

Northern Blotting

RNA samples were electrophoretically separated on an 18% formaldehyde-1% agarose gel and transferred to a positively charged Nylon Membrane filter (Boehringer, Mannheim, Germany). The RNA was covalently immobilised to the filter by a 5 minutes 254 nm UV exposure. The filters were then hybridised with the DIG-labelled probes and all incubation and washing procedures were done according to the protocol for DIG-labelled Northern blotting (Boehringer, Mannheim, Germany). Chemiluminescence was detected by exposing the filters to Kodak Safety Film ARD for 60 min.

Peptide synthesis

The antigenic epitope for the PV antigens, Dsg1 and 3 were chosen using PeptideStucture software. The antigenic epitope for Dsg1 is part of the extracellular (EII) domain located between two Ca+ binding sequences. The antigenic epitope for Dsg3 located in the extracellular (EI) domain and is part of the Bos1 sequence. The chosen epitopic fragments were as follows:

DSG1	LVPRGSRGSDRDGGADGMSA	(**Dsg1**, 238-251)
DSG3/PVA1	LVPRGSPCRQGGDNSKRNP	(**Dsg3**, 57-69)
DSG3/PVA3	LVPRGSRNNGGYLMIDSK	(**Dsg3**, 429-440)

The sequences of the BP antigens were from Swiss-Prot and TrEMBL data banks and were analysed with the WISCONSIN Package, Version 8 (Genetic Computer Group, Madison, USA) by means of the programs PeptideStructure and Plotstructure. Three of the fragments were chosen according to their reactivities with patients' sera in an ELISA system for further investigations. The chosen epitopic fragments were as follows:

BP1 WTQEPQPQPLEEKWQHRVVEQIP (**BPAG1**, AC Q03001; 1814-1834)

BP2 RSILPYGDSMDRIEKDRLQMAP (**BPAG2**, AC Q02802; 507-528)

BP3 GELSSRNTGHLHPTPRSPLLR (**BPAG1**, AC Q03001; 1793-1813)

Peptide sequences were synthesised by a solid-phase technique, utilising Boc chemistry. Side-chain protecting groups were as follows: Arg (Tos), Asp (OcHex), Glu(OcHex), His(Z), Ser(Bzl), Thr(Bzl), Tyr(2BrZ) and Lys(2CIZ). The peptide chains were elongated on a p-methylbenzhydrylamine resin (0.48 mmol/g) and the syntheses were carried out with an ABI 430A automatic machine, with certain minor modifications in the standard protocol. Couplings were performed with dicyclohexylcarbodiimide, with the exceptions of Asn and Gln, which were incorporated as their 9-hydroxybenzotriazole esters. Amino acid incorporation was monitored with the ninhydrin test. The completed peptide resins were treated with liquid hydrogen fluoride (HF)/dimethyl sulfide/p-cresol/p-thiocresol (86:6:4:2, vol/vol), at 0 ^{0}C, for 1 h. The HF was then removed and the resulting free peptides were solubilized in 10% aqueous acetic acid, filtered and lyophilized. The crude peptides were purified by reverse-phase HPLC on a Lichrosorb RP-18 10 μ column (16x250 mm). The solvent system used was as follows: 0.1% trifluoroacetic acid (TFA) in water (**A**), 0.1% TFA, 80% acetonitrile in water (B), gradient: 0% B for 15 min, then 0% →15% B in 60 min, flow 3.5 ml/min, detection at 226 nm.

The appropriate fractions were pooled and lyophilized. The purified peptides were characterised with a Finnigan TSQ 7000 tandem quadrupole mass spectrometer equipped with an electrospray ion source. Peptide purities were above 97% (HPLC) and the measured Mw values were in all cases in good agreement with the calculated values.

Plasmid constructions

Nucleotide sequences coding for the synthetic peptide epitopes which exhibited high reactivity with patients' sera were chemically synthesised in vitro. Taking into account codon usage in E. coli, the degrees of substitution in the DNA sequences of BP1, BP2 and BP3 were 16%, 29% and 35%, respectively. These synthetic DNA sequences were inserted through the BamHI site to the glutathione S-transferase (GST) gene of the fusion-expression plasmid pGEX-4T-2 (Pharmacia) in frame. As a result of the cloning strategy, a dipeptide of GS (one letter code) was inserted between GST and the particular epitope sequence. Besides the GST-

monomer epitope fusion constructions, fusion variants carrying tandem dimer and multimer blocks of homologous and heterologous epitope units were constructed. In the case of monomer fusion expression constructions, a stop codon is at the end of the inserted epitopes, followed by SalI for the BP180 and the EcoRI restriction enzyme site for the other constructions. In the homologous or heterologous dimer and multimer constructions, DNA blocks encoding the peptides without stop codons are linked to one another by BamHI and BglII restriction sites in frame through a sequence coding for a PPRS tetrapeptide sequence. The identity of the inserted DNA blocks and the flanking plasmid regions was controlled by sequencing.

Expression, isolation and characterisation of recombinant fusion products

Recombinant plasmids were expressed and purified according to Marczinovits et al (49) and as described briefly below.

E. coli DH5α cells harbouring the different fusion-expression plasmid constructions were grown in LB medium and induced with 0.5 mM isopropyl beta-D-thiogalactopyranoside for 3 h. Bacteria were harvested by centrifugation, then treated with lysozyme and disrupted by sonication. Following the above method, more than 90% of the recombinant fusion products were in the soluble fraction of the cell lysate and were isolated directly from this fraction with the help of a Glutathione Sepharose 4B affinity gel (Pharmacia) in the presence of 1% Triton X-100. The fusion proteins were eluted with reduced glutathione according to the instruction manual (Pharmacia). The recombinant fusion products were analysed by electrophoresis in 12.5% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) under reducing conditions.

II/4. RESULTS and DISCUSSION

II/4/A Usefulness of antigenic epitopes in the diagnosis of pemphigus and pemphigoid groups

The tools for the diagnosis of the autoimmune bullous disorders have developed very intensively in the last decade. The difficult DIF, IIF methods were completed by the time-consuming immunoblot and the latest ELISA which use synthetic antigenic epitopes (50). Our studies were directed to improve and to test these modern techniques.

At the beginning we determined the target antigen specificity of the sera of patients with BP and PV by immunoblotting methods. These results were correlated with the immunofluorescence picture and the Western blot patterns of the patients with BP were correlated with certain clinical data. Western immunoblotting proved to be a more sensitive assay than IIF for the identification of different antibodies. In 60% of the 20 BP patients and 60% of the 15 PV patients with negative IIF results, molecular weight specific antigen-antibody reactions were demonstrated by Western blotting on salt-split epidermis extracts.

To continue our research we determined the target antigen specificity of the sera of patients with pemphigus by Western blot method, and demonstrated the circulating autoantibodies against Dsg1 and Dsg3 by means of an ELISA technique with synthetic antigenic epitopes and recombinant fusion peptides. Our aim was to develop this ELISA system containing synthetic antigenic epitopes and recombinant fusion peptides which help dermatologists to differentiate the pemphigus group from the pemphigoid group. Thirty two patients (16 males and 16 females) with PV were examined. The immunoblotting yielded 130 kD protein band in 13 of 32 patients (45%) with PV. By means of ELISA the sera of these patients showed positivity against type DSG3/PVA3 epitope in 7 cases (21%) against type DSG3/PVA1 epitope in 16 cases (51%). Positivity was detected against the epitope of Dsg1 in further 11 cases (34%). This might exist because of the high level of homology between the Dsg1 and Dsg3. The serum of the PF patient showed no positivity with immunoblotting, while with ELISA technique there were positive reaction not only against the Dsg1-epitope but also the Dsg3 type DSG3/PVA1 epitope.

The ELISA method was improved using new fusion peptides. Monomer, dimer and heteromultimer forms of the Dsg1 and Dsg3 epitopes were applied. The monomer epitopes gave positive reaction in 34-50% of the cases, the dimer forms in 38-47% of the cases, while

the heteromultimers reached 65% positivity. These epitopes were not developed further to received higher sensitivity (Fig. 9).

On the basis of our results, we may state, that the sensitivity of ELISA is compareble to that of immunoblotting, or perhaps ELISA more sensitive in these cases. So next to or instead of the sensitive, but quite difficult immunoblotting the use of the ELISA system may provide a new possibility for the detection of autoantibodies of patients with PV and PF. It seams reasonable that the different dsg molecules are highly glycosilated in their native immunogenic state, and the porkaryota E. coli systems are not able to produce glycosilated epitopes. An eucaryota system should be needed for the production of recombinant constructs of desmogleins.

For the next step we addressed the question whether the demonstration of circulating antibodies against the BP180 and BP230 antigens is possible by means of an ELISA technique with antigenic synthetic peptides. IIF, immunoblot and ELISA techniques were compared. Thirty-four patients with proved BP were investigated (17 females and 17 males). (8/34, 22%) sera of patients with BP gave positivity by means of IIF and (21/34, 61%) sera by means of immunoblotting assay. The ELISA technique using synthetic antigenic epitopes revealed serum positivity in (23/34, 68%) of the patients with BP. The epitope of the BP180 antigen gave higher positivity (19/34, 56%) than that of the BP230 protein (17/34, 50%) by ELISA technique respective to the immunoblotting method (51).

In the latest study the question was addressed again of whether it is possible to demonstrate circulating antibodies against BP autoantigens (BPAG1 and BPAG2) by means of an ELISA system, using not only simple synthetic antigenic epitopes but recombinant antigens containing only short antigenic epitopes of the two BP antigens fused to a GST partner. With the help of the programs PeptideStructure and Plotstructure, antigenic epitopes of BP antigens were predicted, chemically synthesised and screened by using sera of 43 proved BP patients. The coding sequences of the best antigenic epitopes were then chemically synthesised and inserted as monomer and homo- or hetero-oligomer forms into fusion-expression plasmids (PGEX-4T, Pharmacia) in frame to the C-terminus of glutathione-S-transferase. Fusion products were expressed and purified from E. coli cells by affinity chromatography. The recombinant proteins were used for the detection of antibodies in the sera of 43 BP patients and of 60 controls (including 30 healthy persons, 22 subjects with pemphigus vulgaris and 8 subjects with other bullous dermatoses). 72% of the BP patients had autoantibodies against at

least one of the monomer fusion proteins, 77% had them against at least one of the homo-oligomer products, and 79% had them against at least one of the hetero-oligomers. Use of the homo- and hetero-oligomers of the recombinant fusion peptides increased the sensitivity of the disease-specific antibody detection. When a mixture of the best recombinant fusion proteins was used, the sensitivity of the ELISA assays in the case of BP patients was 0.90 (Fig. 10). We consider this kind of investigation as one that offers the possibility of determining the antibodies in patients with BP. It is possible to diagnose BP in a relatively simple way by means of an ELISA system, and highly equipped laboratory is not necessary.

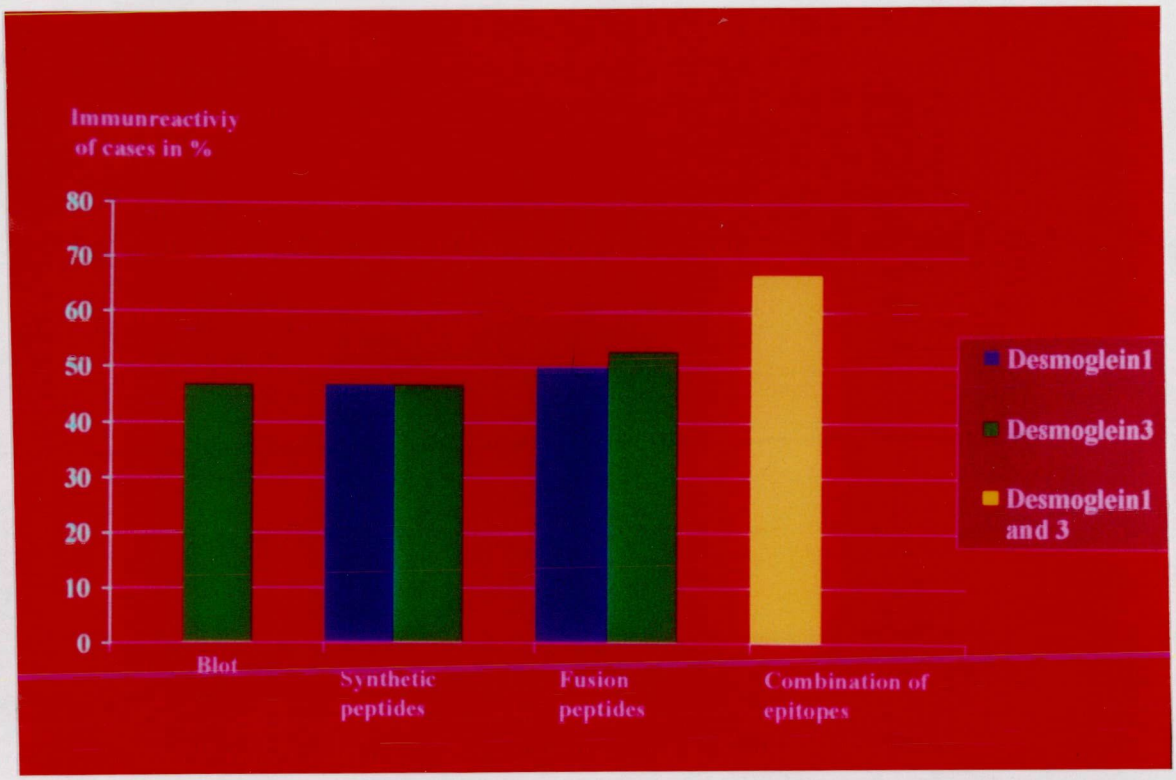

Figure 9.: Possibility of detection of disease-specific antibodies in pemphigus vulgaris (n = 32)

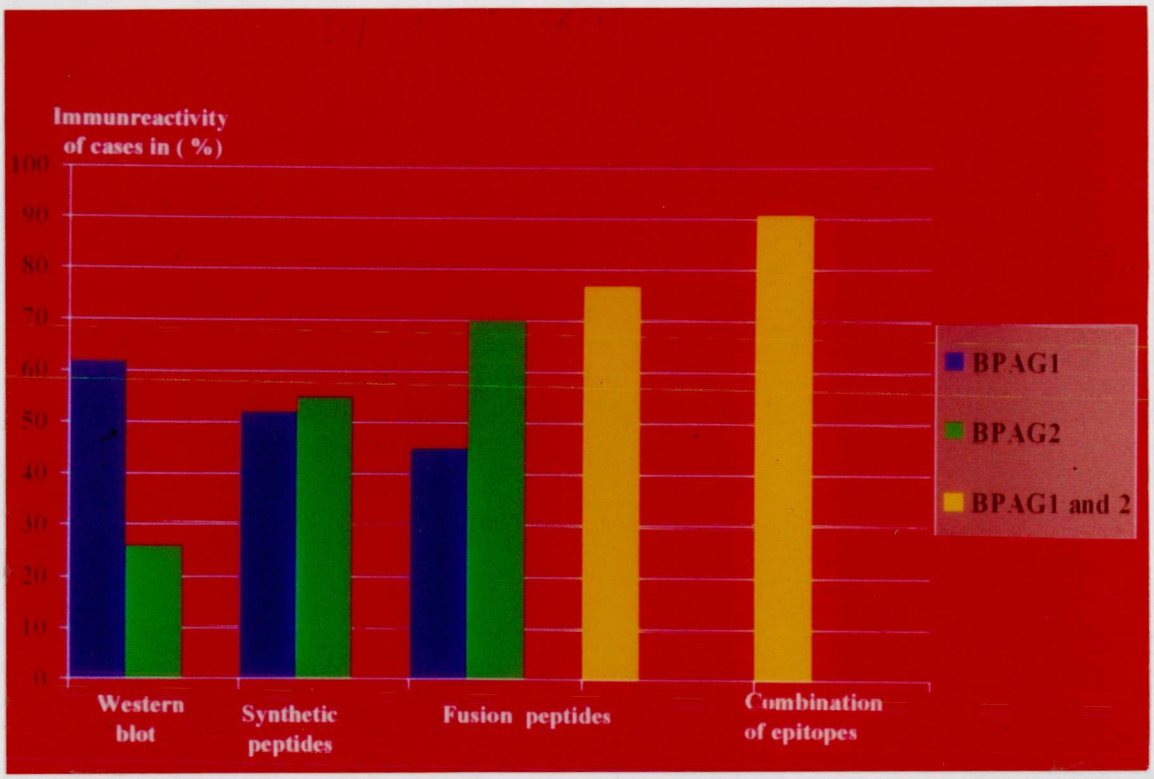

Figure 10.: Possibility of detection of disease-specific antibodies in bullous pemphigoid (n=34)

II/4/B Detection of two types XVII. collagen mRNA transcripts in human keratinocytes

The BP180 molecule is an autoantigen in the autoimmune-mediated disorders bullous pemphigoid, cicatrizal pemphigoid and pemphigoid gestationis (52-54). Because of the heterogenity of BP180 in terms of tissue distribution, cellular localisation, protein product and its disregulation in neolasms we have reinvestigated the expression of BP180 mRNA (41, 55). We have analysed the BP180 mRNA expression in normal human keratinocytes. Here we report the presence in normal keratinocytes of two COL17A1 transcripts which differ in length by 0.6 kb.

Two mRNAs were detected on Northern blot with RNA probes directed to sequences coding for intracellular and extracellular fragments of BP180 (5,2 kb and 5,6 kb mRNAs). In normal keratinocytes the level of the smaller transcript was 5-15% of that of the larger transcript whereas in a squamous cell carcinoma cell line this ratio was inverted, the smaller mRNA being three times more abundant than the larger mRNA. For further characterisation, the Northern blot was repeated but with three probes complementary to intracellular and extracellular protein coding sequences (probes 1-3) and one probe complementary to a part of the hypothetical 3'-untranslated region (UTR) sequence (probe 4). We constructed a hypothetical 3'UTR sequence by BLAST homology search alignments using the GenBank human EST's database at the NCBI site in Bethesda. Starting with the end of the mRNA sequence as published by Giudice (56), ending with nucleotide 4669, we found a series of overlapping cDNA fragments by which we could add 877 nucleotides to the 3' (pronounced three-prime) side. Three out of twenty BLAST identified cDNAs contained a 610 bps deletion in this new 3'UTR sequence (Fig. 11). Northern blot analysis with a probe complementary to this deleted sequence showed only binding to the larger mRNA (Fig. 12). The deletion of 610 nucleotides in the smaller mRNA was verified by RT-PCR and sequencing. Genomic PCR showed the new sequence to be an extension of exon 56 of the COL17A1 gene which suggests that the second mRNA is generated by differential splicing. The homology between the sequences of the BP180 mRNA 3'UTR in human and mouse was elucidated (57) (Fig. 13). Because of the high level homology between those sequences, there may be an evolutionary pressure to conserve these specific stretches. However, their function remains unknown.

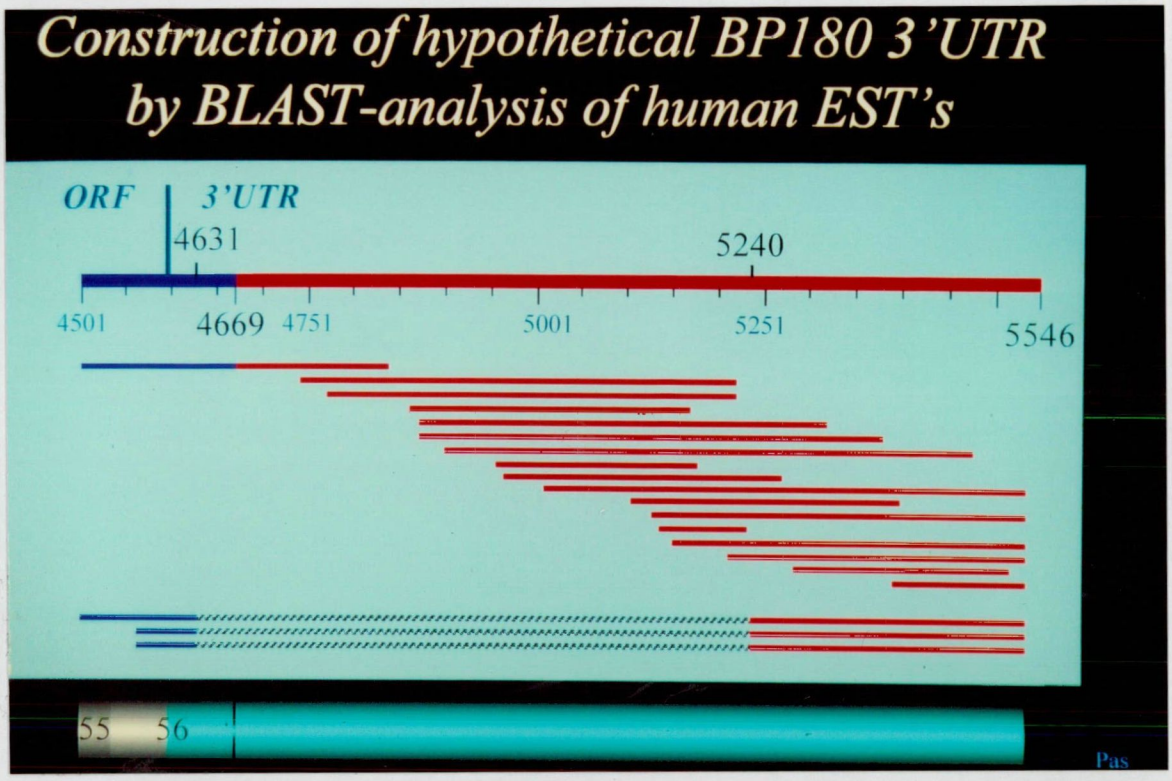

Figure 11.: Overlapping EST clones identified by BLAST-searches which combine to an 877 bps extension of the known 3'UTR of the BP180 mRNA. The top of the figure shows the 3'-end of BP180 mRNA, starting at residue 4501 (numbering according to Giudice et al.), which was elongated by using sequence information from the twenty overlapping clones described underneath. Three clones, numbers 18-20, were found to contain a 610 bps deletion if aligned with the new sequence. In grey, end of M91669 sequence; in blue, new hypothetical 3'UTR.

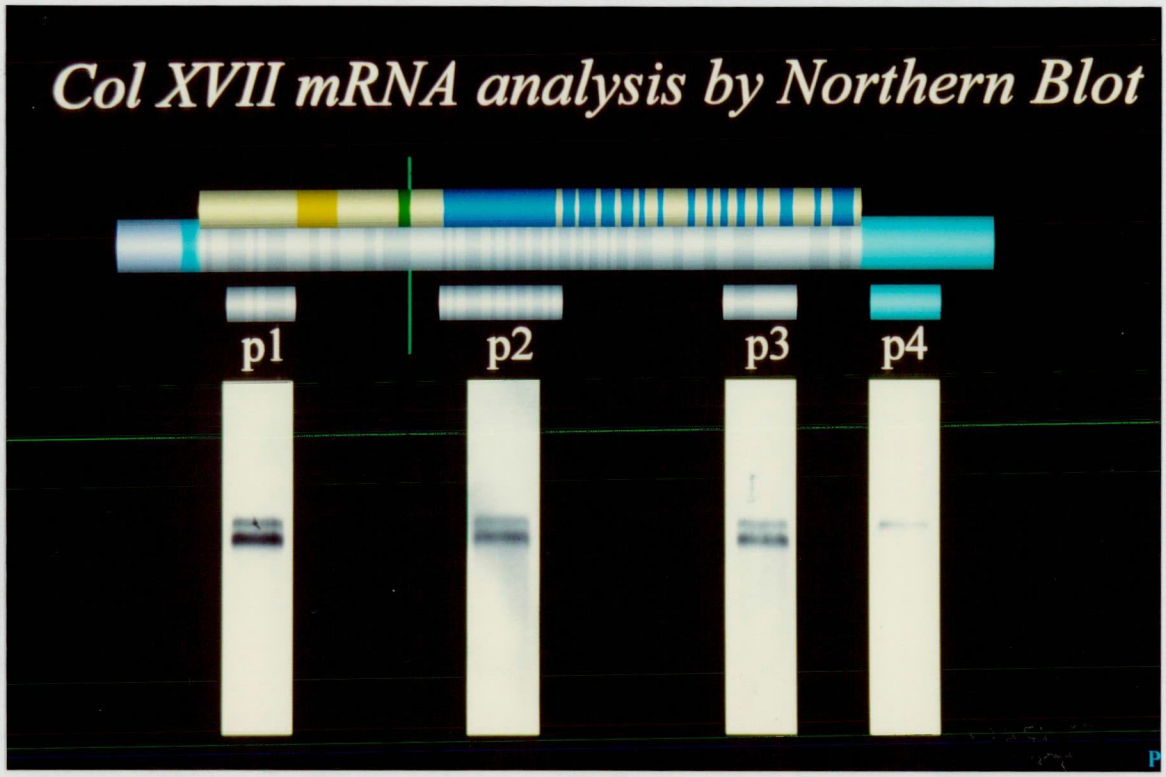

Figure 12.: Northern blot analysis shows the smaller mRNA species to be deleted in the 3'UTR sequence. Schematic representation of the BP180 protein with the cytoplasmic part to the left, the transmembrane region in green, and the extracellular part to the right. Putative collagen sequences are indicated in dark blue. The four RNA probes used for detection of mRNA with their target sequences indicated underneath. Northern blot signals with, from left to right, probes 1-4.

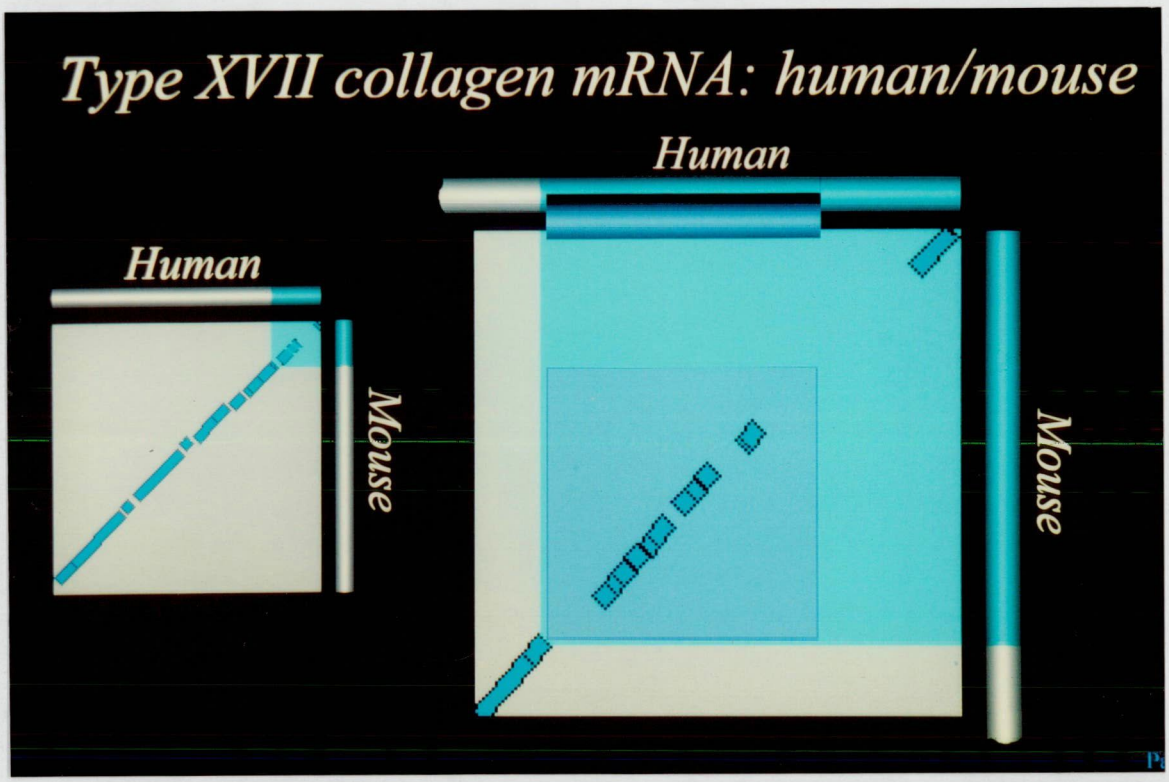

Figure 13.: The complete sequence of the 3'UTR of mouse type XVII collagen. This figure shows the homology between human and mouse mRNA. To the left of the slide the sequence of human (above) and mouse (to the right) show considerable homology for the protein coding sequences (in grey), as it has already been reported, but also in the 3'UTR more than average homology is found. This is enlarged on the right of the slide. Three stretches are found in the deleted fragment (indicate area with deleted fragment above) where the homology is 78-87% which is significantly higher than the mean 3'UTR mouse/human homology value of 69% and close to the 85% homology for protein coding regions.

II/4/C The importance of autoantibody detection in clinical work: two cases (Juvenile pemphigus foliaceus and epidermolysis bullosa acquisita)

Juvenile pemphigus foliaceus

PF is extremely rare in children, its clinical findings involve gradually spreading eczematised scaly plaques with the possible development of bullae and superficial crusts (58). Early diagnosis and prompt treatment are very important and they are based on the clinical, histological and immunopathological changes. A 7-year–old girl presented with a 3-month history of generalised, painful, small blisters and superinfected erosions covered with thick, yellowish crusts. A perilesional biopsy for histology demonstrated a subcorneal blister with acantholytic cells without any inflammatory cells in the cleft. Direct immunofluorescence studies of frozen skin tissue showed positive intercellular staining for IgG, IgA and C3 within the epidermis. The circulating antidesmoglein 1 (anti-Dsg1) and anti-Dsg3 antibodies were examined by means of ELISA using synthetic, antigenic peptides (50). The patients serum gave positive reactions against one epitope of Dsg 3 and the epitope of Dsg1. The patient's circulating anti-epidermal antibodies were also examined by Western blot technique to detect anti-Dsg1 and anti-Dsg3, but there was no positive reactions (59). The treatment was commenced with 50 mg/day prednisolone and later combined with 12.5 mg of dapsone twice a day resulting in satisfactory fast resolution of the blisters (60). She has had no further lesions since that time.

Epidermolysis bullosa acquisita

Epidermolysis bullosa acquisita (EBA) is a chronic subepidermal mechanically-induced blistering disease of the skin. Clinically, pemphigoid may mimic EBA, and vice versa, EBA may mimic pemphigoid, although the simultaneous presence of EBA and pemphigoid in one person would be extremely uncommon (61). Not only the therapy but the diagnosis is also sometimes difficult (62). A 40 year-old Hungarian male presented with complaints of blisters, erosions and excoriation on scalp, trunk, hands and feet for more than a year (63). The diagnosis of EBA was established on the basis of anamnestic data, clinical picture, histology, immunohistology, immunoblot and fluorescent overlaying antigen mapping analysis (64). Other techniques could be used as well as electronmicroscopy, immunelectromicroscopy (65). DIF revealed linear depositions of IgG and C3 along the epidermal basement membrane zone. The serum of the patient was shown to be positive by the ELISA technique using a substrate

of synthetic oligopeptide of the 230 kDa bullous pemphigoid antigen (66). To solve the differential diagnostical problem (EBA vs. pemphigoid) frozen skin samples lacking the EBA antigen/type VII collagen ("knockout skin") from a person with severe, mutilating dystrophic epidermolysis bullosa was used (67). It was the substrate for indirect IF to test for the presence of circulating antibodies against non-EBA antigens in the patient. Indirect IF with patient's serum on skin lacking EBA antigen, showed no binding of IgG or IgA. Western blot with patient's serum using dermal extract showed a band of 290 kDa, corresponding to the EBA-antigen (68). This method, called "knockout skin" can be applied to differentiate between autoimmune subepidermal bullous disorders. The treatment of the patient with high doses of prednisolone and azathioprine was partially successful. Following a 6-month period without any drugs, new mechanically-induced blisters were developing only on the extremities and much less frequently than earlier.

5. CONCLUSIONS

A/ Earlier, the diagnosis of bullous disease was based only on the DIF and IIF methods. We tried to compare different systems (IIF, Western blot, ELISA) and developed an ELISA using synthetic antigenic epitopes and fused recombinant antigens to detect disease-specific antibodies at patients with pemphigus and pemphigoid.

We compared the sensitivity of immunoblot and IIF technique at sera of patients with BP and PV. In 60 % of the 20 BP patients and similarly in the 15 PV patients with negative IIF results, positive reaction could be detected. We demonstrated that the immunoblot technique is an excellent method to identify circulating anti-epidermal antibodies in patients with autoimmune bullous dermatoses and its sensitivity is higher than that of the immunofluorescence.

To make the diagnosis simplier and quicker we used synthetic peptides in ELISA system. Epitopes to the Dsg1 and Dsg3 antigens were structured, synthesised and used in ELISA at 32 patients with PV. The sensitivity of these epitopes was 34-40%. When we used the mixture of these recombinant epitopes in the same way, positivity of the reaction reached the 65%. Thus, with this relatively simple tool we could detect PV antibodies in similar frequency to the immunoblotting. These recombinant epitopes were not developed further to reach higher sensitivity.

We investigated the antibody-reactivity against BP230 and BP180 antigens with several methods. When we compared the IIF, immunoblot and ELISA techniques, we found that 22% sera of 34 patients with BP gave positive reaction with IIF, 61% sera by means of immunoblotting and 67,6% sera by means of ELISA using two-two synthetic epitopes.

We further developed these antigens and prepared ELISA system using recombinant antigens containing only short antigenic epitopes of the two BP antigens fused to a GST partner. When the best combination of the recombinant fusion proteins were applied, 90% of the 43 BP sera gave positive reaction. We conclude that this kind of investigation offers new possibilities to determine the circulating antibodies in BP patients and that this system could form the basis of a rapid and simple system for the diagnosis of BP.

B/ We have analysed BP180 mRNA expression in normal human keratinocytes. We used RNA probes directed to sequences encoding intracellular and extracellular fragments of BP180 on Northern blot and for the first time found two COL17A1 transcripts in normal keratinocytes. By BLAST homology search alignments we extended the 3'UTR of the known BP180 mRNA sequence and identified 3 cDNAs contained 610 bp deletion. The deleted part was verified by Northern blot analysis, reverse transcription-PCR and sequencing. We compared the 3'UTR BP180 human sequences with that of mice and and found substantially higher (78-87%) sequence identity of four stretches than the mean one (69%). This may suggest that these sequences are important for proper functioning of the mRNA and indicate that the smaller mRNA may be post-transcriptionally regulated. Because the transcripts differ in their 3'UTR sequence, future research will be aimed at revealing a possible function for this sequence, that may be connected to mRNA regulation, stability, translational initiation, stabilisation, or a yet unrecognised function.

C/ PF was diagnosed in a child using immunofluorescence and ELISA technique. DIF showed positive intercellular staining within the epidermis, while the IIF demonstrated no circulating antibodies. Western blot technique can not show circulating antibodies, but using ELISA system with synthetic, antigenic peptides (anti-Dsg1 and anti-Dsg3), the serum gave positive reaction against both epitopes.

A patient with symptoms characteristic to EBA, but with positive reaction in ELISA system to the BPAG1 was presented. The differential diagnostical problem (EBA vs. pemphigoid) was solved using IIF method on "knockout skin": the patient's serum was incubated with skin

samples lacking the EBA antigen/type VII collagen. This IIF examination showed no binding of IgG or IgA, which supported the EBA diagnosis. In the abow cases the novel methods (ELISA with antigenic epitopes, "konckout skin") could help and facilitate the diagnosis and differential diagnosis among bullous diseases.

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