The Significance of Immunological Methods and Techniques of Molecular Biology in Research and Clinical Practice of Immunodermatological Disorders

(PhD dissertation)

Kiss Mária

1996

Albert Szent-Györgyi Medical University
Department of Dermatology

The Significance of Immunological Methods and Techniques of Molecular Biology in Research and Clinical Practice of Immunodermatological Disorders

(PhD dissertation)

Kiss Mária

SZEGED

1996

Content

Publication list for the dissertation

I. **Kiss M**, Husz S.
Sejtmag antigének (ENA) izolálása borjútimuszból és ENA ellenes antitestek vizsgálata rutin bőrgyógyászati beteganyagban.
Bőrgyógy. Vener. Szle 63, 245-250 (1987)

II. Morvay M, Hunyadi J, Kószó F, **Kiss M**, Dobozy A, Simon N.
Über das Vorkommen von Porphyria cutanea tarda und Lupus erythematodes.
Derm. Mschr. 175, 20-27 (1989)

III. **Kiss M**, Husz S, Soós J, Szalay F.
Antimitochondriális antitestek kimutatása immundiffúziós, ELISA és immunfluoreszcenciás módszerekkel.
Orv. Hetil. 131, 67-73 (1990)

IV. **Kiss M**, Husz S, Dobozy A.
Antinukleáris antitestek, valamint anti-SSA/Ro és anti-SSB/La antitestek vizsgálata polimorf fényexanthemában.
Bőrgyógy. Vener. Szle. 66, 255-259 (1990)

V. **Kiss M**, Husz S, Dobozy A.
The occurrence of antinuclear, anti-SSA/Ro and anti-SSB/La antibodies in patients with polymorphous light eruption.
Acta Dermato-Venereol.(Stockh.) 71, 341-343 (1991)

VI. Pokorny Gy, Németh J, Marczinovits I, **Kiss M**, Hudák J, Husz S.
Primary Sjögren's syndrome from the viewpoint of an internal physician.
Int. Ophtalmol. 15, 401-406 (1991)

VII. **Kiss M**, Husz S, Dobozy A.
Keringő autoantitestek vizsgálata autoimmun betegségben szenvedő betegek szérumában és immunkomplex precipitátumaiban.
Orv. Hetil. 133, 1103-1110 (1992)

VIII. Jánossy T, Vízler Cs, Krizsa J, Hunyadi J, **Kiss M**, Nyirati I, Dobozy A, Végh P.
Long-term survival and use of human skin xenografts in immunosuppressed mice.
Transplantation proceedings 26, 1268-1270 (1994)

IX. **Kiss M**, Husz S, Dobozy A.
Az immunoblot technika eredményessége a hólyagos bőrgyógyászati kórképek diagnosztikájában.
Orv. Hetil. 135, 2197-2199 (1994)

X. Beetz A, **Kiss M**, Michel G, Husz S, Kemény L, Ruzicka T.
Neuropeptides substance P and calcitonin gene related peptide upregulate interleukin-8 receptor mRNA in human keratinocytes.
J. Invest. Dermatol. 103, 430(A) (1994)

XI. **Mária Kiss**, M Wlaschek, P Brenneisen, G Michel, C Hommel, TS Lange, D Peus, Lajos Kemény, Attila Dobozy, K Scharffetter-Kochanek, T Ruzicka
α-Melanocyte stimulating hormone induces collagenase/matrix metalloproteinase-1 in human dermal fibroblasts.
Biological Chemistry Hoppe-Seyler 376, 425-430. (1995)

XII. **Kiss Mária**
A PCR technika és alkalmazása a klinikai diagnosztikában.
Lege Artis Medicinae, Aktuális kérdések 5, 1108-1113 (1995)

XIII. **Mária Kiss**, Lajos Kemény, Sándor Husz, Günter Michel, Thomas Ruzicka, Attila Dobozy
α-Melanocyte stimulating hormone induces the interleukin-8 production in human dermal fibroblasts but not in keratinocytes.
J. Invest. Dermatol. 105, 489 (Abstract) (1995).

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

XV. Sándor Husz, **Mária Kiss**, Ilona Marczinovits, Gábor Tóth, Katalin Molnár, János Molnár, Attila Dobozy
Detection of bullous pemphigoid antibodies by means of synthetic peptides as antigenic epitopes.
Acta Microbiol. et Immunol. Hung. (közlésre elfogadva)

XVI. Molnár Katalin, **Kiss Mária**, Husz Sándor, Dobozy Attila
Autoantitest vizsgálatok lupus erythematodesben.
Bőrgyógy. Vener. Szle. 72, 49-53 (1996)

INTRODUCTION

In the last few decades of biological sciences Immunology has represented one of the fastest growing disciplines. Immunological research has always been strongly motivated by medical problems. In the pathomechanisms of most diseases the immune system plays either a primary, causative or a secondary role. The various immunological techniques that have been developed and the knowledge gained through basic research in immunology has had an exspecially great impact in Clinical Dermatology. Many dermatological diseases are known now to be directly related to disorders of the immune system.

The understanding of the autoimmune pathomechanism and the application of immunological methods in the everyday medical practice have greatly improved patient care. It has been a very rewarding experience for me, a biologist, to set up a laboratory for special immunological investigations at the Department of Dermatology of Szeged, which has helped clinical research as well as everyday clinical practice. The first part of this dissertation briefly describes results related to Immunodermatology.

It has long been suspected based on clinical experiences that the nervous system may also play a crucial role in the pathomechanism of certain skin diseases. At present there is very little that we know about possible neurological regulatory mechanisms of skin biology. Different neuropeptides, that are released at the sensory neuronal endings and are kown to participate in the development of neurogenic inflammation and exert growth-factor-like effects, are likely candidates as regulatory molecules. The second part of the dissertation contains my recent work regarding the investigation of the effects of different neuropeptides on the biology of the important cellular components of the skin, keratinocytes and fibroblasts.

I. Investigations of autoantibodies in patients with systemic autoimmune diseases and in autoimmune bullosus dermatoses

A. Antibody investigations in systemic autoimmune diseases

Background

In a number of autoimmune diseases, the presence of the so-called antinuclear antibodies (ANA) is a dominant feature. These diseases include systemic lupus erythematosus (SLE), mixed connective tissue disease (MCTD), polymyositis, dermatomyositis, scleroderma and Sjögren's syndrome. The autoantigens that are targets of such antibodies are often large cellular complexes containing protein and nucleic acid components (1,2).

Autoantibodies reacting with various common cellular constituents are detected in sera from many patients with systemic autoimmune disorders. In 1948 Hargraves et al. (3) described the LE cell test as a first serological method for the diagnosis of systemic lupus erythematosus (SLE), which is the prototype of such diseases. The LE cell test covers only one of many autoantibodies collectively termed ANAs. Up till now ANA, anti-ENA (Extractable Nuclear Antigen) and other autoantibodies have become an important diagnostic tool for the diagnosis of autoimmune disorders and for defining certain subgroups of these diseases. Remarkable associations between specificities of ANAs and clinical expression of the disease have already been described (4).

In recent years a panel of techniques has been developed which allowing one to distinguish autoimmune diseases by their characteristic serological profile.

The types of autoantibodies, that occur most frequently, are demonstrated in Table I.

Most of them are directed to DNA-protein complexes or RNA-protein complexes, ribonucleoproteins (RNPs). Anti-Sm antibodies seemed to be highly specific for SLE, while anti-RNP antibodies have been associated with MCTD, but have also been found in SLE and less frequently in other diseases. Both antigens are RNA-protein complexes and play an essential role in mRNA splicing processes.

SSA/Ro and less frequently SSB/La antibodies are considered to be important in relation to annular erythema and photosensitivity. SSA/Ro antibodies are highly characteristic for a special clinical type of SLE (subacute cutaneous lupus erythematosus; SCLE) and in newborn infants with neonatal LE. Anti-cardiolipin and anti-mitochondrial antibodies may also have some diagnostic and prognostic importance.

During the last ten years we adopted and modified some methods for the identification of autoantibodies in our patients with different autoimmune diseases and gradually established these methods in our laboratory diagnostics. Meanwhile we tried to evaluate the clinical importance of these investigations.

Table 1

Autoantibodies against autoantigens:	**Disease specificity**	**References**
ENA complex	LE, MCTD	5
DNA (double-stranded)	SLE	6
Sm	SLE	7
RNP	MCTD, LE	8
SSA/Ro	SLE, SCLE, NLE	9,10
SSB/La	SLE, SCLE	9
M2 (mitochondrial inner membrane)	PBC	11
M4, M8 (mitochondrial outer membrane)	PBC	11
Phospholipids (cardiolipin)	SLE, vasculitis	12

Aims

— to establish new screening and typing methods for identifying different autoantibody types and subtypes;

— to correlate the occurrence of different antibodies with disease specificity and the clinical status;

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 and normal human 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.

Isolation of extractable nuclear antigens (ENA)

Acetone-precipitated protein extracts of newborn calf thymus nuclei (extractable nuclear antigen; ENA) served as antigen substrate, prepared to Sönnichsen et al (13). It contains many different nuclear antigens.

Detection of anti-ENA antibodies by counter-immunoelectrophoresis

This is another important screening test for the detection of ANAs. 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 (14).

This method was especially important when purified autoantigens were not available.

Isolation and investigations of circulating immune complexes (CIC)

CIC were isolated from patients' sera by means of polyethylene-glycol (PEG) precipitation. The precipitation was performed using 2% PEG 6000 solution at 4 °C for 24 h. After washing and centrifugation, the pellet was used for further measurements. The total protein content and levels of main immunoglobulin classes (IgG, IgA, IgM) were determined by radial immunodiffusion.

Isolation of mitochondrial autoantigens

Intact mitochondria were isolated from rat liver. For the preparations of mitochondrial subfractions, isolated mitochondria were treated by digitonin (15) to remove the outer mem-

brane and then mitoplasts (consisted of mitochondrial inner membrane and matrix) were separated from the outer membrane fraction by differential centrifugation.

Detection of different autoantibodies by using enzyme linked immunosorbent assay (ELISA)

Nowadays, a great variety of different purified autoantigens are available for ELISA investigations and for rapid screening tests.

At the beginning SSA/Ro and SSB/La antigens were isolated from calf thymus and human spleen (16), later highly purified autoantigens were used. Antigens were solved in PBS pH 7.4 in 0.1-1 μg/100 μl concentration. 100 μl-s were brought into wells of microplate (Biohit; high binding capacity) and allowed to coat overnight at 4 ^{o}C. Plates were washed two times with distilled water and remaining binding sites were blocked by 1% bovine serum albumin (BSA) in PBS for 1.5 h at 37 ^{o}C. Plates were washed again three times with PBS-Tween (PBS, contained 0.05% Tween-20) and 100 μl of 200x diluted serum samples were added per well, incubated for 1 h at 37 ^{o}C and washed extensively with PBS-Tween. 100 μl of peroxydase-conjugated antihuman IgG, 1:5000 diluted in PBS-Tween, were then added to each well and incubated for 1 h at 37 ^{o}C. Subsequently the plates were washed again with PBS-Tween and then 100 μl substrate solution (17 mg ortho-phenylene diamine, OPD, solved in 50 ml phosphate-citric acid buffer pH 5, 20 μl H_2O_2) was added into each well and the color allowed to develop for 10 minutes at room temperature in the dark. The reaction was stopped by adding 50 μl 4N H_2SO_4 to each well. Plates were read on an ELISA reader at 492 nm. Serum samples were tested in triplicate and the results averaged. Ten healthy control sera were always involved in each microplate. Autoantibody positivity was accepted when the special ELISA optical density (OD) was higher than the mean value (X) increased by two standard deviations (SD).

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

Immunoblotting studies were performed according to Hashimoto et al. (17) with slight modifications.

Normal human skin was obtained from patients undergoing plastic surgery. The skin pieces were incubated in a mixture of 1M NaCl, 1 mM EDTA and 10μM phenylmethylsulfonyl-fluoride (PMSF) at 4 ^{o}C for 72 hours. The epidermis was then easily separated from the dermis and homogenized 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 ^{o}C, the homogenizate was centrifuged at 15 000g and the supernatants were stored at -70 ^{o}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 (18) and then transferred to nitrocellulose (19) 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 visualized with diamino-benzidine.

Results and discussion

A/1. The occurrence of circulating anti-ENA antibodies among dermatological patients

First we have elaborated, based on the relevant data from the literature, a simple and reliable method for the demonstration of circulating anti-ENA antibodies. Antigen was isolated from newborn calf thymus. To measure antibodies from the serum counter-immunoelectrophoresis was used. Out of 250 sera of dermatological patients anti-ENA antibodies could be shown in 15 cases (Table 2).

The majority of positive sera belongs to the group of autoimmune disorders (3 SLE, 3 DLE, 2 Raynaud's syndrome, 1 PSS, 2 vasculitis, 1 idiopathic thrombocytopenic purpura, 1 sterile eosinophil pustulosis). In one case of each, lichenoid dermatitis and polymorphous light eruption, anti-ENA antibodies in the serum could also be identified. According to the biochemical feature of Sm autoantigen (its immunogenicity is RNase resistant) we could make a distinction between anti-Sm and anti-RNP antibodies (20). We identified anti-Sm antibodies in one SLE and one DLE patients. It was remarkable, that in our anti-Sm positive DLE patient the disease later become systemic.

We concluded that the occurrence of anti-ENA antibodies may reflect a severe autoimmune disease in most cases. Especially patients with DLE who have circulating anti-ENA antibodies appear to be at an increased risk for systematization of their diseases.

Table II

The occurrence of anti-ENA antibodies in different dermatological diseases

Diagnosis	Examined cases (n)	Positive cases (n)
Chronic urticaria	42	-
Porphyria cutanea tarda	37	-
Bullous pemphigoid	10	-
Vasculitis	14	2
Pemphigus	15	-
SLE	5	3
DLE	8	3
Erythema nodosum	7	-
Erythema exsudativum multiforme	2	-
Erythema gyratum repens	2	-
Erythema elevatum	2	-
PSS	6	1
Eczema	11	-
Polymorphous light eruption	6	1
Alopecia	6	-
Dermatitis	8	-
Raynaud' syndrome	2	2
Lichen	4	-
Necrobiosis lipoidica	4	-
Scleroderma	4	-
Idiopathic thrombocytopenic purpura	1	1
Sterile eosinophilic pustulosis	1	1
Quincke oedema	2	-
Allergodermia	3	-
Parapsoriasis	2	-
Drug allergy	3	-
Lichenoid dermatitis	2	1
Other	41	-
Total	250	15

A/2. The demonstration of anti-mitochondrial antibodies and the distinction of their subtypes; the diagnostic and prognostic importance

Primary biliary cirrhosis (PBC) is a chronic autoimmune disorder characterized by progressive destruction of the intrahepatic bile ducts, leading to cholestasis, cirrhosis and hepatic failure.

First Walker et al. described the occurrence of circulating antimitochondrial antibodies (AMA) in the patients with PBC (21). Later it became clear that 90-95% of patients with PBC have AMA and the detection of AMA is one of the most important diagnostic factor for this disease. Moreover, AMA positivity was demonstrated in some cases of scleroderma (22).

Four major mitochondrial antigens related to PBC have been described. M2 antigen on the inner mitochondrial membrane; M4, M8 and M9 antigens on the outer mitochondrial membranes. Various combinations of antibodies to these antigens have been found in PBC patients and utilized to categorize the patients according to their specific profiles with potential clinical and prognostic relevance (11).

We have studied the occurrence of AMA in 20 patients with PBC, in 30 with autoimmune diseases and in 28 patients with porphyria cutanea tarda, accompanied by a secondary anti-hepatomitochondrial autoaggressive process caused by toxic accumulation of porphyrins in the liver.

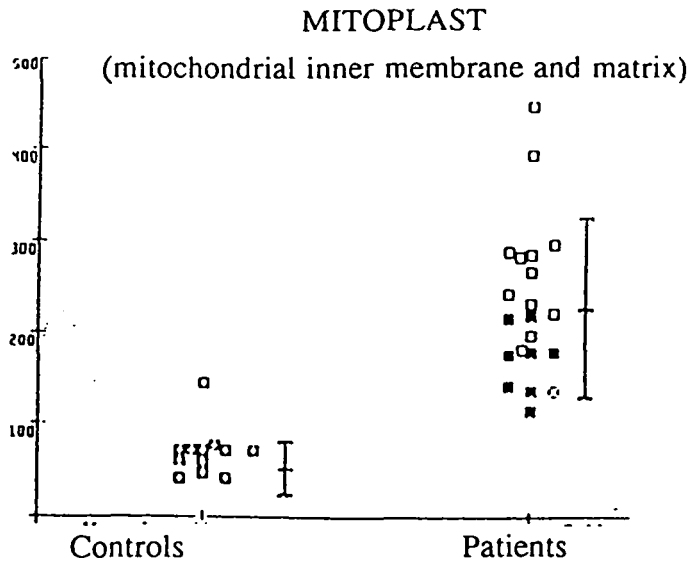
Sonicated rat liver mitochondria fractions were used as antigens. Ouchterlony immunodiffusion and ELISA investigations were performed with different mitochondrial subfractions. It was demonstrated that in all 20 patients with PBC, where immunofluorescence also revealed AMA, there was an increase (Fig 1) in the quantity of antibodies against mitochondrial inner membrane fractions (it contains the disease specific M2 autoantigen) and in 7 cases even against the outer membrane fractions (Fig 2). These 7 were the most serious cases clinically. Two of them died within half a year because of liver insufficiency. The others also had an extreme high levels of serum bilirubin (more than ten times the normal value).

AMA could not been found in the other groups of patients, except for one case of SLE.

These investigations allowed the differentiation of AMA subtypes and promoted the establishment of the diagnosis and prognosis of PBC.

Fig 1

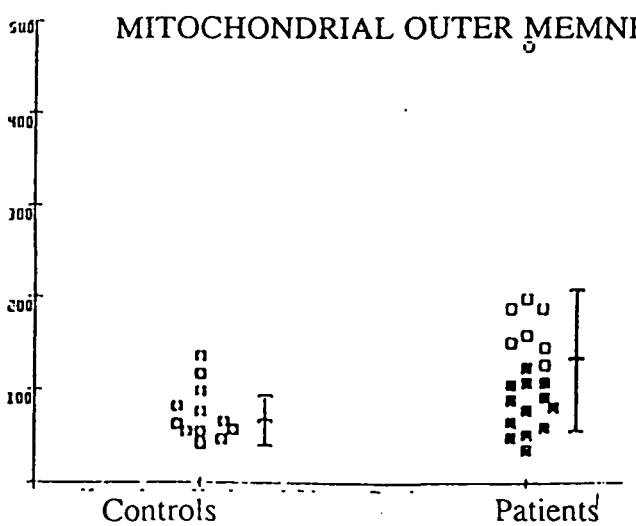
ELISA OD x 1000



Investigations of mitochondrial antibodies in patients with PBC I.
Controls: x = 69.58 SD = 28.18 n = 12
Patients: x = 231.77 SD = 83.89 n = 21
($p < 0.001$)

Fig 2

ELISA OD x 1000



Investigations of mitochondrial antibodies in patients with PBC II.
Controls: x = 71.38 SD = 27.89 n = 12
Patients: x = 128.57 SD = 93.46 n = 21
($p < 0.05$)

A/3. Circulating autoantibodies in patients with polymorphous light eruption

Continuing the investigations of the occurrence of anti-ENA antibodies in different disease conditions, we experienced that ENA positivity in patients with polymorphous light eruption is not so rare.

Polymorphous light eruption (PMLE) is an idiopathic, acquired syndrome characterized by a delayed abnormal response to light and a varied morphology of recurrent erythema, papules, vesicles or plaques on light exposed areas of skin. Because lupus erythematosus (LE) is often exacerbated by sun exposure and because the cutaneous lesions of LE may show some clinical similarities to those of PMLE, the possible relationship of those two diseases has often been discussed (23,24).

Our study demonstrated the occurrence of antinuclear, anti-SSA/Ro and anti-SSB/La antibodies in a certain proportion of 22 patients with PMLE. Twenty-two patients (9 males and 13 females, age range 20-62 years, mean 36 years) participated in this study. To exclude other diseases with light sensitivity such as LE and porphyrias, immunoflourescence investigations and appropriate laboratory tests were performed. PMLE diagnosis was supported by phototesting and histological examinations. Twenty healthy blood donors served as controls, none complaining of light sensitivity.

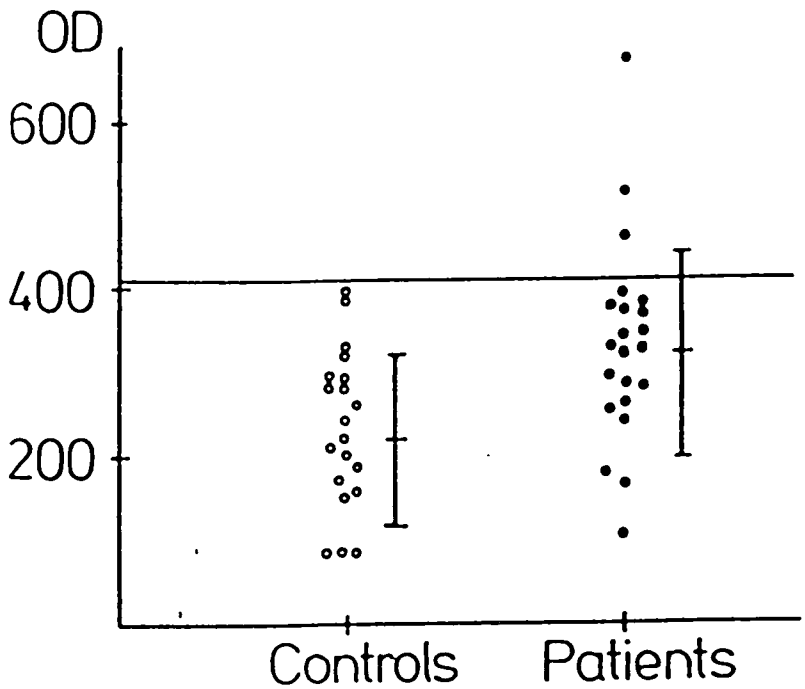
Antinuclear antibodies were not demonstrated at all by indirect immunofluorescence in our patients with PMLE. 4 sera from 22 patients were found to have precipitating antibodies to calf thymus extract (ENA). The enzyme digestion studies with trypsin and ribonuclease, revealed that in each anti-ENA positive case the antigen(s) proved to be trypsin and ribonuclease-sensitive. We think that these antibodies must be of anti-RNP type.

The serum levels of anti-SSA/Ro antibodies in the patients were mildly but significantly elevated as compared to the healthy controls (Fig. 3). In 3 cases, the titres of anti-SSA/Ro antibodies exceeded our control limit ($x+2SD$).

We could find no significant difference between the patients and controls in the serum levels of anti-SSB/La antibodies (Fig. 4). Two patients proved to be anti-SSB/La positive.

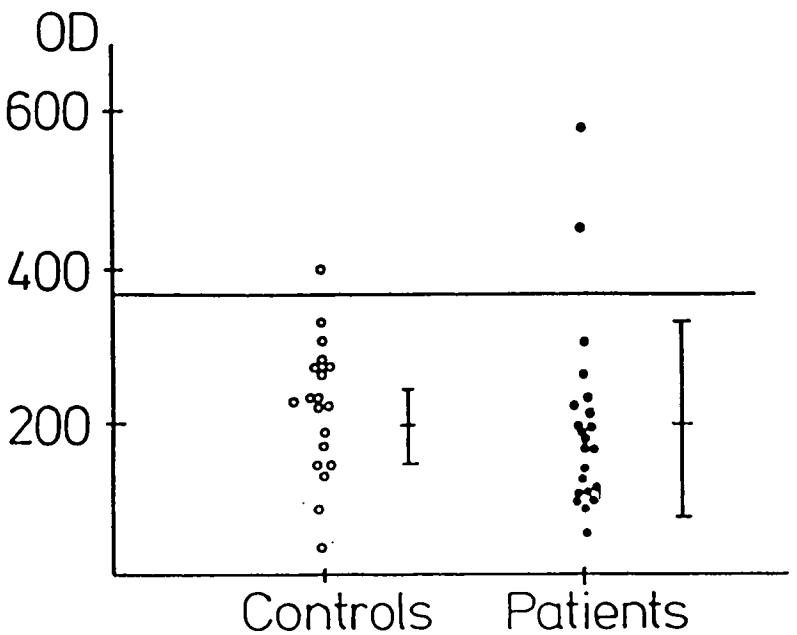
All serological findings are summarized in Table III.

Fig. 3



Anti-SSA/Ro antibody titres are expressed as ELISA optical density units (OD).
Controls: $\bar{x}$ = 208 SD = 101 n = 20
Patients: $\bar{x}$ = 323 SD = 134 n = 22 $p < 0.001$

Fig. 4



Anti-SSB/La antibody titres are expressed as ELISA optical density units (OD).
Controls: $\bar{x}$ = 197 SD = 81 n = 20
Patients: $\bar{x}$ = 191 SD = 108 n = 22 Not significant

Table III

Serological findings in patients with polymorphous light eruption (n=22)

Patients	ANA	anti-ENA	anti-SSA/Ro	anti-SSB/La
P.G.	-	+	-	-
Sz.A.	-	+	+	-
R.Z.	-	-	+	+
K.F.	-	+	+	+
J.J.	-	+	-	-
Others	-	-	-	-

Antibodies against RNA protein molecules SSA/Ro, SSB/La and nuclear RNP occur frequently in patients with systemic lupus erythematosus (SLE) and in patients with other autoimmune disorders (25). In spite of the fact that none of our patients with PMLE showed any sign of LE by laboratory, clinical and immunohistological investigations, we demonstrated serological abnormalaties in some cases with PMLE similar to autoimmune disorders.

In a 7-year follow-up study, Jansén and Karvonen (26) concluded that PMLE is not a predisposing condition for the development of SLE or other collagen diseases. This view coincides with that of several previous investigators (27,28). Our long-term clinical practice also supports this opinion.

However, there must be an explanation for the common presence of special antibodies in both conditions. Photosensitivity is a common precipitating factor and some clinical and experimental data confirmed the relationship between the photosensitivity and the presence of circulating anti-SSA/Ro antibodies.

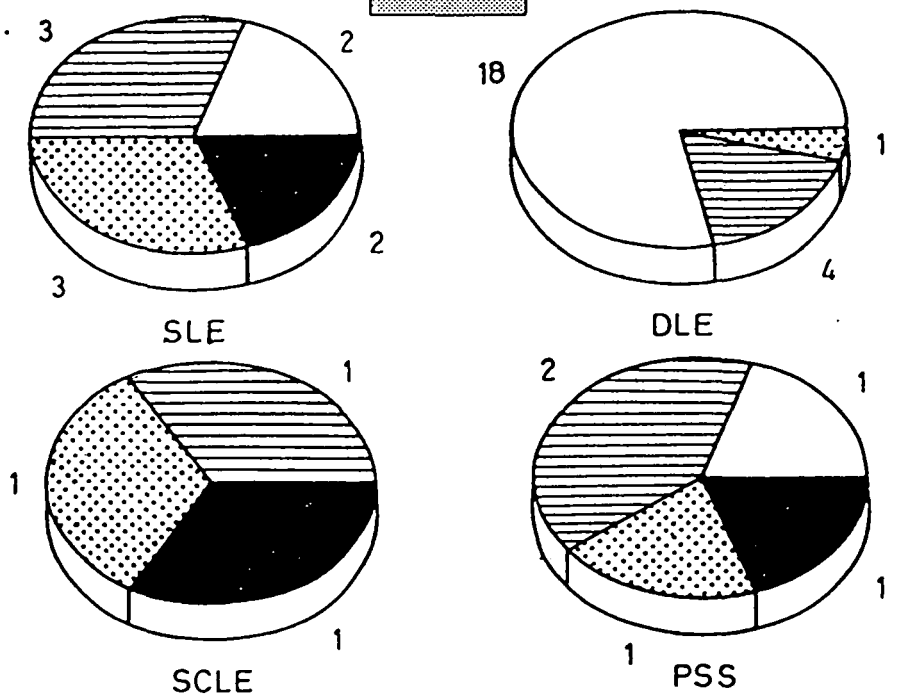
A humoral autoimmune response to the SSA/Ro antigen is seen in several disease states that involve the skin. Circulating anti-SSA/Ro antibodies can be found in the majority of patients with different autoimmune disorders (25) and many of them were accompanied by photosensitivity. It has also been demonstrated that medium and short-wave ultraviolet light exposure produces an increased expression of SSA/Ro antigen in the cytoplasm and plasma membrane of human epidermal keratinocytes in vitro (29).

Our finding, that the serum levels of anti-SSA/Ro antibodies were mildly but significantly elevated in the patients with PMLE is a new evidence for the relationship of photosensitivity and anti-SSA/Ro antibodies.
It must be considered that a late autoimmune course is not excluded, especially in those patients who have both anti-ENA and anti-SSA/Ro antibodies.

A/4. The occurrence of circulating autoantibodies in the immune complex precipitates of patients with autoimmune diseases

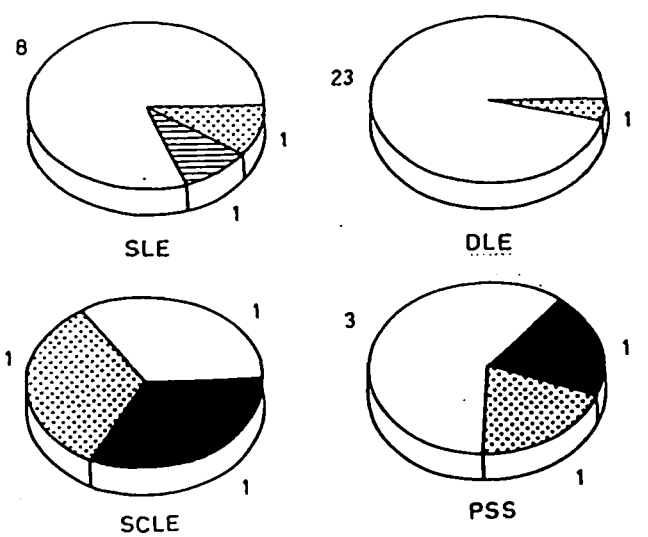
The natural history of systemic autoimmune diseases is one of relapses and remissions that occur at irregular intervals. These characteristics have prompted the search for serological factors which would serve as markers of clinical activity.
It is well-known that the titers of circulating immune complexes (CIC) are generally high in patients with different autoimmune diseases and the CIC may be involved in the elimination processes. The direct pathogenetic role of the immune complex deposits has already been proved in certain disorders.
Therefore, we investigated the possible connection between the occurrence of different autoantibodies in the serum and the immune complex precipitates of the patients. We were also interested if the autoantibody content of CIC has prognostic importance.
Antibodies against ENA, SSA/Ro and SSB/La were investigated in parallel in the serum and the immune complex precipitates of 41 patients with autoimmune disorders (10 SLE, 23 DLE, 3 SCLE, 5 PSS). In the majority of patients the protein content of isolated immune complexes was increased and the increase attributed solely to IgG.
In the majority of patients the antibodies could also be detected in the immune complex precipitates (Fig. 5 and Fig. 6). However, it was demonstrated that in some cases the autoantibody spectrum of the serum didn't coincide with that of the immune complex precipitate. Anti-ENA antibodies were demonstrated in the sera of 18 patients (9 SLE, 5 DLE, 2 SCLE and 2 PSS) and could be detected in 5 patients with SLE, 1 with DLE and 2 with PSS in the immune complex precipitate as well. In some patients antibodies appeared not only in the serum but also in immune complexes, even in 4 patients (2 SLE, 1 SCLE, 1 PSS) antibodies were detected only in the immune complex precipitate. These patients were symptom-free at the time of the antibody measurements. During the careful clinical controlling it was revealed that the disease soon exacerbated in three of them.

Fig. 5



The occurrence of SSA/Ro antibodies in sera and immune complex precipitates in patients with different autoimmune disorders

Fig. 6



The occurrence of SSB/La antibodies in sera and immune complex precipitates in patients with different autoimmune disorders

	serum negative	immune complex negative
	serum positive	immune complex positive
	serum positive	immune complex negative
	serum negative	immune complex positive

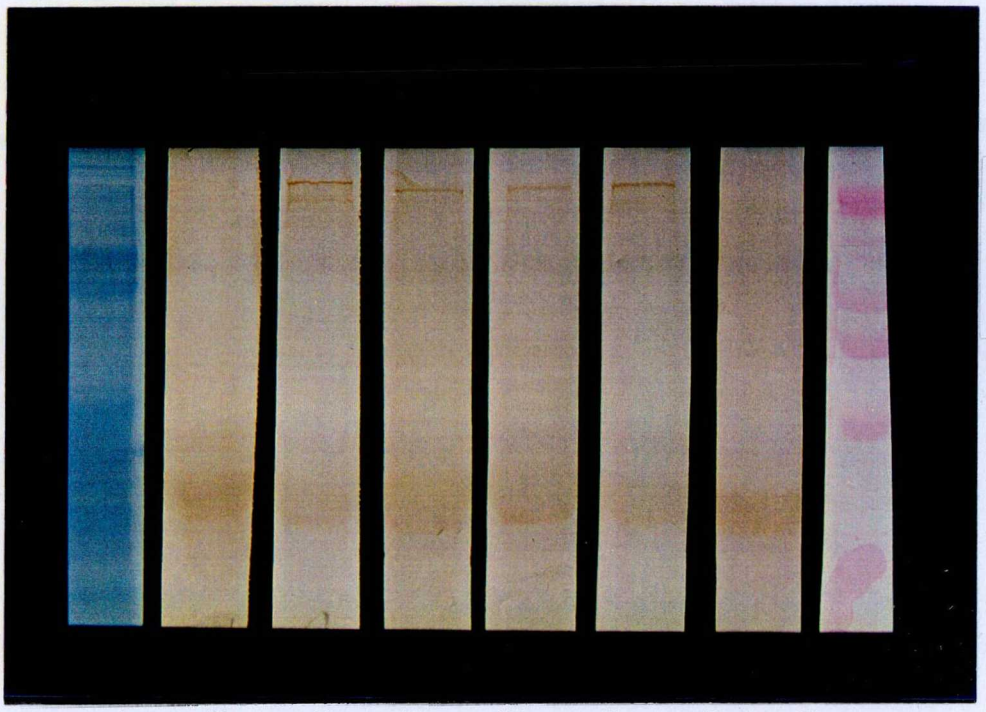
B. Investigations of autoantibodies in patients with autoimmune bullous dermatoses — pemphigus vulgaris and bullous pemphigoid

The diseases of pemphigus group and bullous pemphigoid are characterized by autoantibodies to antigens expressed by keratinocytes or constituents of the dermal-epidermal junction, which can be demonstrated by DIF. Bullous pemphigoid (BP) is a subepidermal blistering disease characterized by circulating autoantibodies which react with antigens in the lamina lucida of the basement membrane zone (BMZ). The attachment of the basal keratinocytes to the underlying basement membrane is stabilized by hemidesmosomal complexes. The two BP antigens (the 230 kD and 180 kD proteins, or BPAG1 and BPAG2, respectively) are major constituents of these hemidesmosomes (30,31). Recent molecular cloning of complementary and genomic DNA sequences of BPAG1 and BPAG2 has demonstrated that these two proteins are distinct gene products (32,33). Specifically, the 230 kD BP antigen is a noncollagenous intracellular protein, in contrast the 180 kD BP antigen is a collagenous transmembrane protein. Some 50-70% of BP sera recognize the BPAG1 antigen, but a minority of BP sera recognize other lower molecular weight bands, most commonly BPAG2. Pemphigus is an intraepidermal blistering disease group characterized by autoantibodies reactive with antigens located in the intercellular spaces or on the surface of epidermal cells. In patients with pemphigus vulgaris (PV) autoantibodies are directed against a 130 kD antigen (a member of cadherin protein family) that forms a complex with a protein of desmosomal and adherent junctions, called plakoglobin (34,35). In pemphigus foliaceus (PF), the autoantibodies are specific for a 160 kD antigen, called desmoglein I.

B/1. Immunoblot studies for the detection of anti-epidermal antibodies

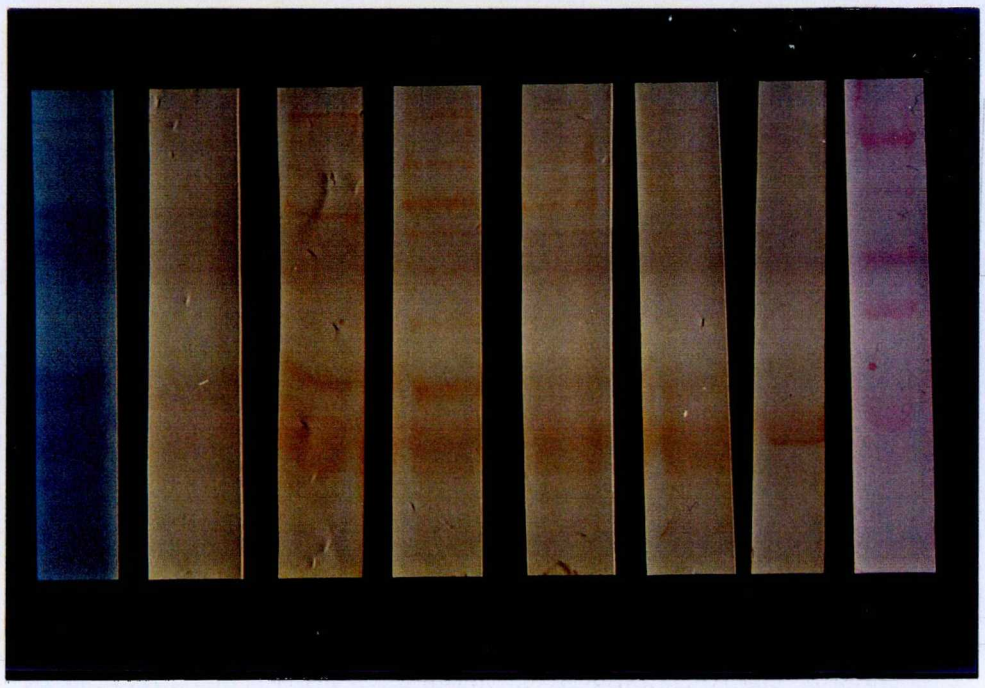
Our study included the determination of target antigen specificity of the sera of 34 patients with BP and 15 patients with PV by immunoblotting methods, and the results were correlated with immunofluorescence pictures. In addition, the Western blot patterns of the patients with BP were correlated with certain clinical data. Western immunoblotting with its great sensitivity provided a new possibility for the detection of different antiepidermal antibodies in patients with bullous dermatoses. In 60% of our BP patients and 60% of our PV patients with negative IIF results, molecular weight specific antigen-antibody reactions were demonstrated by Western blotting on salt-split epidermis extracts. The Western blot patterns of a group of BP patients are demonstrated in Fig. 7. All sera including healthy controls yielded a diffuse reactivity in the range of 30-60 kD. The strong specific bands with peroxidase-labelled antihuman IgG show the reaction between the 230 kD BP antigen and the antibody of patients' sera. In this group of patients specific reactivity can be demonstrated only against the "major", BPAG1 antigen. In another group of BP patients, multiple specific bands were seen on Western blotting (Fig. 8).

Fig. 7



Blots of BP sera showing one dominant band at 230 kD with peroxidase-labelled antihuman IgG. Lane A: Blot of epidermal extract stained with Coomassie brilliant blue, Lane B: normal serum, Lane C-F: positive BP sera, Lane G: negative BP serum, Lane H: migration of standard molecular weight markers.

Fig. 8



Blots of BP sera showing multiple bands with peroxidase-labelled antihuman IgG. Lane A: Blot of epidermal extract stained with Coomassie brilliant blue, Lane B: normal serum, Lane C-F: positive BP sera, Lane G: negative BP serum, Lane H: migration of standard molecular weight markers.

Specific reactions were demonstrated against the BPAG2 antigen (180 kD) and some other epidermal components.

The Western blotting patterns of the patients with BP were compared with certain clinical findings (Table IV), but no specific correlation was found.

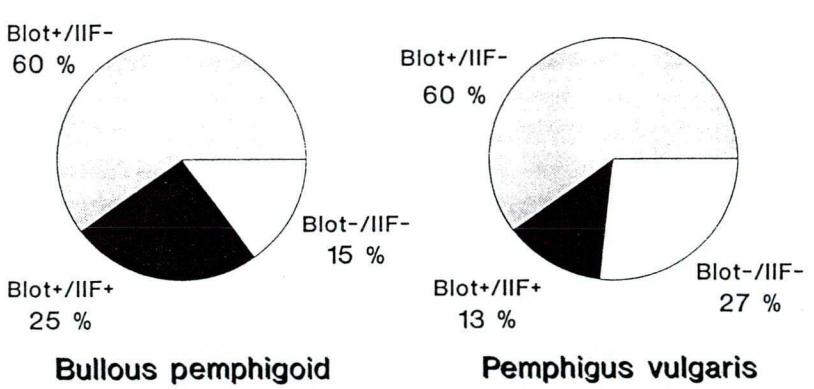
In 4 patients with BP, DIF revealed IgA deposition besides C3 along the BMZ. IgA antibodies against the BPAG1 antigen were demonstrated in 2 of them by means of Western blotting (Fig. 10).

For PV patients, the results revealed that 11 sera yielded a specific 130 kD protein band against the PV antigen (Fig. 11). Most of the sera gave additional specific bands at 210 and 80 kD, with lower intensity. The sera of 4 patients did not give any specific bands, and it is noteworthy that 3 of them were in clinical remission. This finding, together with experimental and clinical evidences supports the fact that pemphigus antibodies play a direct role in the pathogenesis of the disease.

There was considerable diversity in the patients' immunoblotting patterns, especially in BP cases. Although the number of our patients is limited, it seems that each patient yields an individual Western blot pattern besides the common reaction against the 230 kD BPAG1. A similar opinion has been reported by Shapiro et al. (36) who demonstrated that healthy individuals and patients with autoimmune diseases have antibodies, some tissue-specific and some not, which are present in a pattern that is unique to each individual.

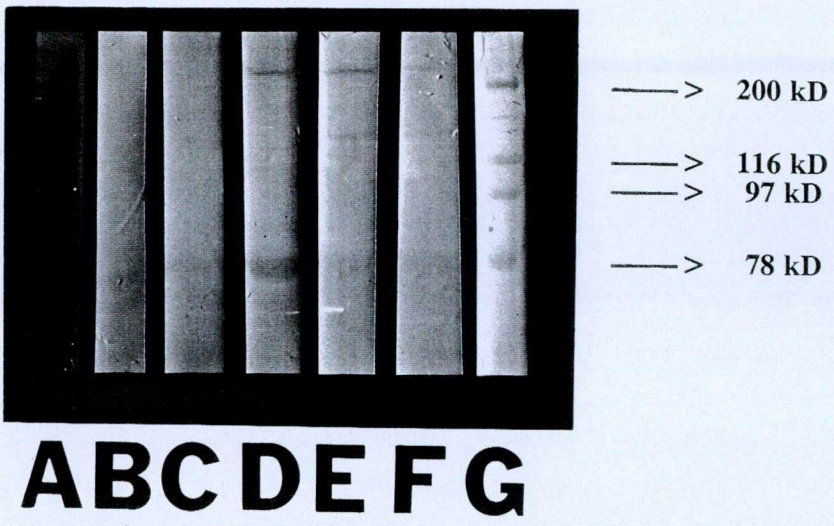
Western blot is an excellent technique to identify circulating autoantibodies in different autoimmune bullous dermatoses. In our study, Western immunoblotting proved to be a more sensitive assay than IIF for the identification of different antibodies (Fig. 9). Moreover, it provides a new possibility for the distinction of linear IgA dermatosis (LAD) and BP. LAD is characterized by circulating IgA antibodies against a 285 kD antigen of dermal extracts but patients with LAD lack the reactivity against the 230 kD BPAG1 epidermal antigen.

Fig. 9



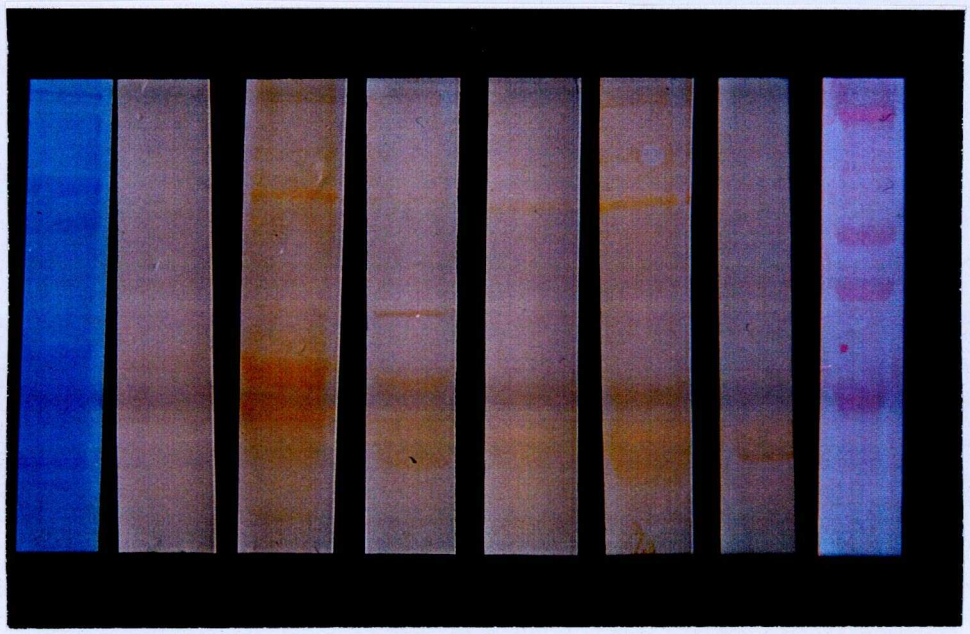
Correlation between Western immunoblotting and IIF results

Fig. 10



Blots of BP sera showing one dominant band at 230 kD with peroxidase-labelled antihuman IgA. Lane A: Blot of epidermal extract stained with Coomassie brilliant blue, Lane B: normal serum, Lane D-E: positive BP sera, Lane C, F: negative BP sera, Lane G: migration of standard molecular weight markers.

Fig. 11



Blots of PV sera showing dominant bands at 130 kD and 85 kD with peroxidase-labelled antihuman IgG. Lane A: Blot of epidermal extract stained with Coomassie brilliant blue, Lane B: normal serum, Lane C-F: positive PV sera, Lane G: negative PV serum, Lane H: migration of standard molecular weight markers.

Table IV

Correlation between Western blotting patterns and clinical picture of patients with BP

Clinical findings	Single 230 kD (n = 8)	Multiple bands (n = 9)	Blot negative (n = 3)
Age at onset (years)	60-82	48-82	60-72
Male/Female	5/3	4/5	2/1
Lesion morphology	3 typical 3 atypical	5 typical 4 atypical	1 typical 1 atypical
Severity	2 mild 2 moderate 2 severe	6 moderate 3 severe	2 moderate
Mucosal lesions	2	2	1

No clinical data available: 3 patients

B/2. Development of an ELISA system for the rapid diagnosis of BP

As the BP autoantigen structures (BPAG1, BPAG2) and their amino acid sequences are well known, we have addressed the question of whether the demonstration of circulating antibodies against major (BPAG1; 230 kD) and minor (BPAG2; 180 kD) antigens is possible by means of an ELISA technique with synthetic antigenic peptides. With the help of PeptideStructure software, two-two matched antigenic epitopes were chosen and synthetized. The sera of 33 patients with BP were investigated in parallel by the Western blot method and by the ELISA technique with the synthetic peptides.

Summarized results of the correlation between ELISA and immunoblotting investigations are demonstrated in Table V.

Among the patients with BP, 24 sera proved positive for at least one synthetic peptide. Positive reactions with the major BP antigen were found in 21 patients with the immunoblot technique and in 20 patients by ELISA with the synthetic peptides, and positive reactions were found in 9 patients by the immunoblot technique and 18 patients by the ELISA using the synthetic antigenic epitopes of the minor BPAG2 antigen (Table VI). All control sera were negative for both antigens in ELISA investigations. In 3 patients with PV, characteristic bands against pemphigus vulgaris antigen were identified by means of the immunoblot technique, but all other cases were negative. In our investigations with synthetic antigenic epitopes the ELISA technique revealed serum positivity in 68% of the patients with BP. Our results suggest that the epitope of the BPAG2 antigen gaves much higher positivity than that of the 230 kD BPAG1 protein by ELISA respective to immunoblotting method. There-

fore, to increase the usefulness of the 230 kD antigen as a diagnostic tool, other epitopes within the protein should be learned.

Table V

Correlation between ELISA and immunoblotting investigations for the detection of characteristic autoantibodies in patients with BP

	ELISA positive	ELISA negative
Blot positive	15	6
Blot negative	8	4
DIF positive	23	10

Table VI

Correlation of the results of ELISA and immunoblotting according to different antigens

Bullous pemphigoid (n=34)	230 kD BP antigen	180 kD BP antigen
ELISA positive	20	18
Blot positive	21	9

We consider that this kind of investigation offers new possibilities to determine the circulating antibodies in patients with BP. By using a mixture of these synthetic antigenic epitopes, it is possible to diagnose patients with BP in a relatively simple way by means of an ELISA system, which is much cheaper, and doesn't require a highly equipped laboratory.

Conclusions

Autoantibodies have become an important diagnostic tool for the diagnosis of systemic autoimmune disorders and autoimmune bullous dermatoses and for defining certain subgroups of these diseases.
Therefore, we adopted and modified some methods for the identification of autoantibodies in our patients with different autoimmune diseases and gradually established those methods in our laboratory diagnostics. Meanwhile we tried to correlate the occurrence of different antibodies with disease specificity and the clinical status.

Main consequencies of our work were as follows;

1) A simple and reliable method for the demonstration of circulating antibodies against extractable nuclear antigens (ENA) is very useful in everyday clinical practice of autoimmune disorders. Based on the investigation of the 250 sera of dermatological patients we concluded that the occurrence of anti-ENA antibodies may reflect a severe autoimmune disease in most cases. Especially in patients with DLE who have circulating anti-ENA antibodies appear to be at an increased risk for systematization of their diseases.

2) The detection of anti-mitochondrial antibodies (AMA) is one of the most important diagnostic factor for primary biliary cirrhosis (PBC), a chronic autoimmune liver disorder. Moreover, AMA positivity was demonstrated in some cases of scleroderma. We have studied the occurrence of AMA in 20 patients with PBC, in 30 with autoimmune diseases and in 28 patients with porphyria cutanea tarda, accompanied by a secondary anti-hepatomitochondrial autoaggressive process. AMA were demonstrated in all 20 patients with PBC but could not been found in the other groups of patients. In all patients with PBC antibodies were found against mitochondrial inner membrane fractions (it contains the disease specific M2 autoantigen) and in 7 cases even against the outer membrane fractions. These 7 were the most serious cases clinically. These investigations allowed the differentiation of AMA subtypes and promoted the establishment of the diagnosis and prognosis of PBC.

3) We demonstrated the occurrence of antinuclear, anti-SSA/Ro and anti-SSB/La antibodies in a certain proportion of 22 patients with polymorphous light eruption (PMLE). This is an idiopathic, acquired syndrome characterized by a delayed abnormal response to light and the cutaneous lesions of PMLE show some similarities to

those of lupus erythematosus. Our finding, that the serum levels of anti-SSA/Ro antibodies were mildly but significantly elevated in the patients with PMLE is a new evidence for the relationship of photosensitivity and anti-SSA/Ro antibodies.

4) It is well-known that the titers of circulating immune complexes (CIC) are generally high in patients with different autoimmune diseases and the CIC may be involved in the elimination processes. Therefore, we investigated the possible connection between the occurrence of different autoantibodies in the serum and the immune complex precipitates of 41 patients with autoimmune disorders. In the majority of patients the protein content of isolated immune complexes was increased and the increase attributed solely to IgG. In some patients antibodies appeared not only in the serum but also in immune complexes, even in 4 patients antibodies were detected only in the immune complex precipitate. Convincing clinical correlation couldn't be demonstrated because of the limited number of patients.

5) We demonstrated that the immunoblot technique is an excellent method to identify circulating anti-epidermal antibodies in patients with autoimmune bullous dermatoses (pemphigus vulgaris or bullous pemphigoid) and its sensitivity is better than that of the indirect immunofluorescence. Immunoblotting studies with salt-split human epidermis were performed on sera from 15 patients with pemphigus vulgaris (PV) and 20 patients bullous pemphigoid (BP). As concerns the 15 PV patients, 11 sera yielded a 130 kD specific protein band against the PV antigen. The sera of 4 patients did not yield specific bands; 3 of them were in clinical remission. 17 sera of the 20 BP patients yielded a 230 kD protein band against the major BP antigen and 4 of them gave another specific band at 180 kD against the minor BP antigen. The immunoblot pattenrs correlated only weakly with the clinical findings in BP patients.

6) As the major (230 kD) and the minor (180 kD) BP autoantigen structures and their amino acid sequences are known, with the help of PeptideStructure software, two-two antigenic epitopes were chosen and synthetized. The sera of 33 patients with BP were investigated parallel by the immunoblot method and by the ELISA technique with the synthetic peptides. Among the patients with BP, 24 sera proved positive for at least one synthetic peptide. We consider that this kind of investigation offers new possibilities to determine the circulating antibodies in BP patients. By using a mixture of synthetic antigenic epitopes, it is possible to diagnose patients with BP in a relatively simple way.

II. The role of neuropeptides in the skin

Background

There is increasing evidence that the nervous system can modulate various immunological responses, including certain inflammatory events in the skin, through the secretion of neural cytokines termed neurokines or neuropeptides (37,38). The discovery over the past two decades of more than 50 biologically active polypeptides in the central and peripheral nervous system has transformed our knowledge of neurotransmission and its regulation. In addition to neurotransmitter and neuroendocrin roles, neuropeptides have mitogenic actions in tissues and can modulate responses of the immune system and can also modulate the functions of different cell types of the skin.

A number of neuropeptides have been demonstrated in cutaneous nerves: substance P (SP), neurokinin A, neuropeptide K, calcitonin gene related peptide (CGRP), somatostatin, vasoactive intestinal polypeptide (VIP) (39,40,41,42,43,44). Proopiomelanocortin related peptides (ACTH, α-, β-melanocyte stimulating hormone) were also demonstrated histochemically in human skin (45). The human keratinocytes in vitro can express mRNA for the proopiomelanocortin (46). This suggests that the skin can produce opioid peptides regulating emotion and the sensation of pain or discomfort.

Neuropeptides mediate their effects on cells by interacting with specific cell surface receptors. In accordance with this, SP receptors have been demonstrated on mast cells, polymorphonuclear leukocytes and macrophages (47,48,49). Furthermore, the specific binding of SP has been detected on mouse keratinocytes and recently on human keratinocytes as well (50,51). VIP receptors were also demonstrated on the keratinocye surface (52).

The exact mode of actions of neuropeptides in skin is not yet known. The vasodilatator effect may be due to to a direct effect of cutaneous vasculature (53) or to action on mast cells, since these peptides are known to be potent releasers of histamine from mast cells.

Substance P

Of all neuropeptides, the tachykinins, especially the ubiquitous 11-amino-acid SP is perhaps the best characterized and considered an important neurotransmitter in primary sensory neurons. The innervation of SP in the skin has been investigated by a number of studies using immunocytochemistry (54,55). Innervation with SP-fibers is generally scanty in human skin, with the densest innervation being on palms, soles and axillary skin. SP is a potent immunomodulator as well. It has been shown to stimulate the proliferation of human T

lymphocytes (56) and the phagocytosis and chemotaxis of neutrophils (57). It was recently found that SP is able to stimulate the proliferation of the PAM 212 mouse transformed keratinocyte cell line (58), to stimulate murine keratinocytes to secrete IL-1 and GM-CSF (59) and to induce cytokine secretion from macrophages (60). Capsaicin, which induces the release of SP from nerve endings (61), inhibits immunoglobulin secretion in the rat (62), and also inhibits the stimulation of human B lymphocyte differentiation by SP and neurokinin A (63).

SP has been presumed to play a role in a variety of inflammatory skin diseases, such as psoriaisis — Neukarinnen et al. (64) demonstrated a significantly increased length of thin epidermal SP fibres in psoriatic lesional skin — urticaria (65), atopic dermatitis (66), and increased levels of SP were found in tissue extracts from psoriatic lesional skin, urticaria and atopic dermatitis.

Calcitonin gene related peptide (CGRP)

CGRP is another important neuropeptide, structurally resembling to calcitonin and it encoded by the calcitonin/CGRP multigene complex, has been implicated in neuromodulation and in the physiological regulation of blood flow. It is a potent vasodilalator, presents in peripheral neurons and is released at the local sites of inflammation, and can act as an immunomodulator. CGRP has been shown to colocalize with SP in human skin peripheral nerve endings. CGRP inhibits both mitogen-stimulated T lumphocyte proliferation and Langerhans cell antigen presentation (67,68).

There is some indirect data, that CGRP may be involved in the pathogenesis of some skin diseases, such as psoriasis, atopic dermatitis and Raynaud's syndrome (69).

α-Melanocyte stimulating hormone (α-MSH)

The melanocyte stimulating hormones, also referred to as melanocortins or melanotropins were first identified by their ability to stimulate vertebrate pigmentation (70). The melanocortin peptide family consists of α-MSH, β-MSH, gamma-MSH and ACTH, all characterized by a common heptapeptide core. These peptides and some endorphins, lipotropins are generated from a common precursor, the proopiomelanocortin (POMC). POMC is transcribed predominantly in pituitary gland, but its expression has also been detected in the central nervous system (CNS) and in a variety of peripheral tissues, in cells of the immune system and has recently been found in skin (45,71).

Melanocortins appear to be involved as regulators in an ever growing number of physiological processes in cells and tissues of diverse functions. Depending on the site of their production and target tissue, they can either act as hormones, neurotransmitters, growth factors

or biological response modifiers. The skin is a recognized target tissue for α-MSH and other POMC derived peptides (72,73). Patients with pathologically increased plasma concentrations of ACTH reveal hyperpigmentation and skin atrophy. Elevated concentrations of α-MSH are also associated with skin hyperpigmentation (74).
Recently POMC peptides (predominantly gamma-MSH and ACTH) were demonstrated in paraffin-embedded histological skin samples from different pathological conditions (45) and human cultured keratinocytes were shown to be able to produce α-MSH and ACTH. Ultraviolet B (UVB) irradiation highly stimulated α-MSH synthesis (75). Cell surface receptors for α-MSH have been detected and characterized in normal human melanocytes (76) and α-MSH binding sites were also demonstrated in an immortalized keratinocyte cell line (77). However, melanocortin receptors have not yet been demonstrated on human dermal fibroblasts.

There is increasing evidence that keratinocytes and fibroblasts may participate in inflammatory processes and immune responses of the skin by the production of biological mediators or cytokines. Keratinocytes can be induced to express various immunocompetent cell surface molecules (78). In addition, they are capable of phagocytosis (79) and motility response to certain chemotactic stimuli (80) and secrete a number of cytokines with potent immunological inflammatory and proliferative processes (81).
Thus neuropeptides are present in human skin, they can modulate the functions of immune cells, keratinocytes and fibroblasts in the skin. Accordingly, since only limited data is available on the interaction of neuropeptides with these cell types, it appeared important to study the effects of neuropeptides on human keratinocytes and dermal fibroblasts.
Interleukin-8 (IL-8) is a chemotactic cytokine with proinflammatory and growth-promoting activities. Recently it has been shown to influence several functions of keratinocytes including HLA-DR expression, chemotaxis and proliferation. We could detect mRNA homologous to neutrophil IL-8 receptor (IL-8R) in a human epidermal cell line as well as specific IL-8 binding to the cells (82). Elevated IL-8 and IL-8R mRNA levels have been demonstrated in lesional psoriatic skin (83).

Aims

- Because of the well-known role of α-MSH in UV-mediated skin alteration, we addressed the question whether α-MSH was able to regulate interstitial collagenase/ matrix metalloproteinase-1 (MMP-1) in mRNA and protein levels in human dermal fibroblasts. This enzyme and the other MMPs are responsible for the breakdown of dermal interstitial collagen and can play a role in the cutaneous photoaging.
- to study the effects of the α-MSH on the expression of IL-8 and IL-8 receptor in human dermal fibroblasts.
- to study the effects of neuropeptides SP, CGRP and α-MSH on the expression of IL-8 and IL-8 receptor in human keratinocytes.

Methods

Cell culture

Fibroblast cultures were established by outgrowths from foreskin biopsies of healthy human donors, with an average age of 5 years. The cells were maintained in DMEM supplemented with sodium ascorbate (50 μg/ml), glutamine (300 μg/ml), penicillin (400 U/ml), streptomycin (50 μg/ml), 10% FCS and grown on plastic petri dishes in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C (84).
A spontaneously immortalized, nontumorigenic human skin keratinocyte cell line "HaCat" was used for the experiments. Cells were maintained in RPMI 1640, with glutamine (300 μg/ml), penicillin (400 U/ml), streptomycin (50 μg/ml), 10% FCS in the same conditions as fibroblasts.

Neuropeptide treatment

Confluent keratinocyte and fibroblast monolayer cultures were used for the experiments. α-MSH was dissolved in distilled water and added to the medium to a final concentration of 10^{-5} M, 10^{-8} M or 10^{-11} M α-MSH for 30 min. SP and CGRP was added to the medium to the final concentration of 10^{-8} M. Total RNA was isolated immediately and at 1, 3, 6, 12,

24 h after the neuropeptide treatment. Total RNA from mock controls were isolated at 0 and 24 h. There was no effect of different neuropeptide concentrations on cell morphology and viability as determined by phase contrast microscopy and trypan blue exclusion assay.

RNA extraction and Northern blot analysis

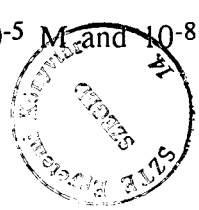
Total RNA was isolated from cells following established procedures (85). For Northern blot analysis, 4 μg of total RNA were separated by electrophoresis on 1% agarose gels under denaturating conditions and subsequently blotted onto nitrocellulose. Hybridizations with specific cDNA probes were carried out using deoxy-cytosine-5-^{32}P-triphosphate oligolabelled cDNA probes for interstitial collagenase, tissue inhibitor of matrix metalloproteinases (TIMP-1) and β-actin as published elsewere (86,87). Following hybridization, filters were washed and then exposed with intensifying screens to X-ray film (Kodak X-Omat AR) at -80 °C. After development, the collagenase/β-actin and collagenase/TIMP-1 mRNA ratios were densitometrically determined and plotted against the post-incubation period.

Reverse transcription (RT) - Polymerase chain reaction (PCR) analysis

Samples (9 μl; 1 μg) of total RNA were heated to 65 °C for 10 min and cooled on ice. Reverse transcription of RNA was performed for 1 h at 37 °C in 20 μl with 10 U M-MLV reverse transcriptase (Gibco BRL) and 0.2 μg/μl oligo-dT primer per sample in 1 x RT buffer and 1 mM of each of dATP, dTTP, dGTP and dCTP. After reverse transcription the mixture was heated to 94 °C for 10 min to inactivate the enzyme, then diluted 10x with sterile distilled water.
PCR amplifications were carried out in 30 μl volumes overlayed by mineral oil. Each 30 μl reaction mixture contained 0.25 μg of each primer (specific for IL-8 or IL-8 receptors respectively) 0.5 U Taq polymerase, 0.125 mM of each of dATP, dTTP, dGTP and dCTP in 1 x PCR buffer, 10 μl of diluted cDNA sample and 12 μl sterile distilled water. Amplification was performed in an automatic thermocycler (Hybaid). The amplification products were electrophoresed in 2% agarose gels, and the bands were visualized with ethydium bromide.
The results were evaluated densitometrically.

Immunofluorescence studies for the detection of collagenase in fibroblasts

Human fibroblasts were seeded on slides at a density of 10^4 cells/cm^2 in 100 μl DMEM with 10% FCS. After 24 h cells were incubated with 10^{-5} M and 10^{-8} M α-MSH for 30



min at 37 °C in a humidified atmosphere of 5% CO_2. Fibroblasts were washed twice with PBS and fed with fresh DMEM. After 48 h the cells were fixed. Slides were washed twice with PBS and the cells were incubated with the monoclonal antibody against MMP-1 at a concentration of 2 μg/μl for 30 min at 4 °C in a humidified chamber. An antibody (mouse IgG_1) directed against MMP-1 was utilized. Subsequently, cells were extensively rinsed with PBS and then incubated with a dichlorotriacinyl aminofluorescein (DTAF)-conjugated secondary antibody (rabbit anti-mouse IgG; Dianova) at a dilution of 1:100 for 20 min at 4 °C. The slides were analyzed by means of fluorescence photomicroscope (Zeiss, Axioplan, Germany) at a magnification of 50x and 250x, respectively.

Determination of the collagenolytic activity in the cell culture supernatants

Fibroblasts were cultured and treated with α-MSH as described. At 24, 48, and 72 h after α-MSH treatment cell culture supernatants were collected.
The collagenolytic activity of fibroblast supernatants were measured by an established procedure (88). Briefly, microplate (Dynatech) wells were covered with collagen type I (Seromed, Berlin, Germany) at a concentration of 10 μg/well. In order to determine the collagenolytic activity, cell culture supernatants were serially diluted, subsequently 100 μl of each sample was pipetted into 96 well microplates coated with collagen type I and incubated at 37 °C for 20 h. After incubation microplates were stained with Coomassie Brilliant Blue for 1 h at room temperature. Following extensive washing with distilled water, microplates were dried and extinctions were measured by an ELISA reader at 630 nm. Statistical analysis was done with the Student t test.

Measurement of IL-8 secretion

IL-8 concentrations of cell culture supernatants were assessed by a specific enzyme-linked immunosorbent assay (R&D system).

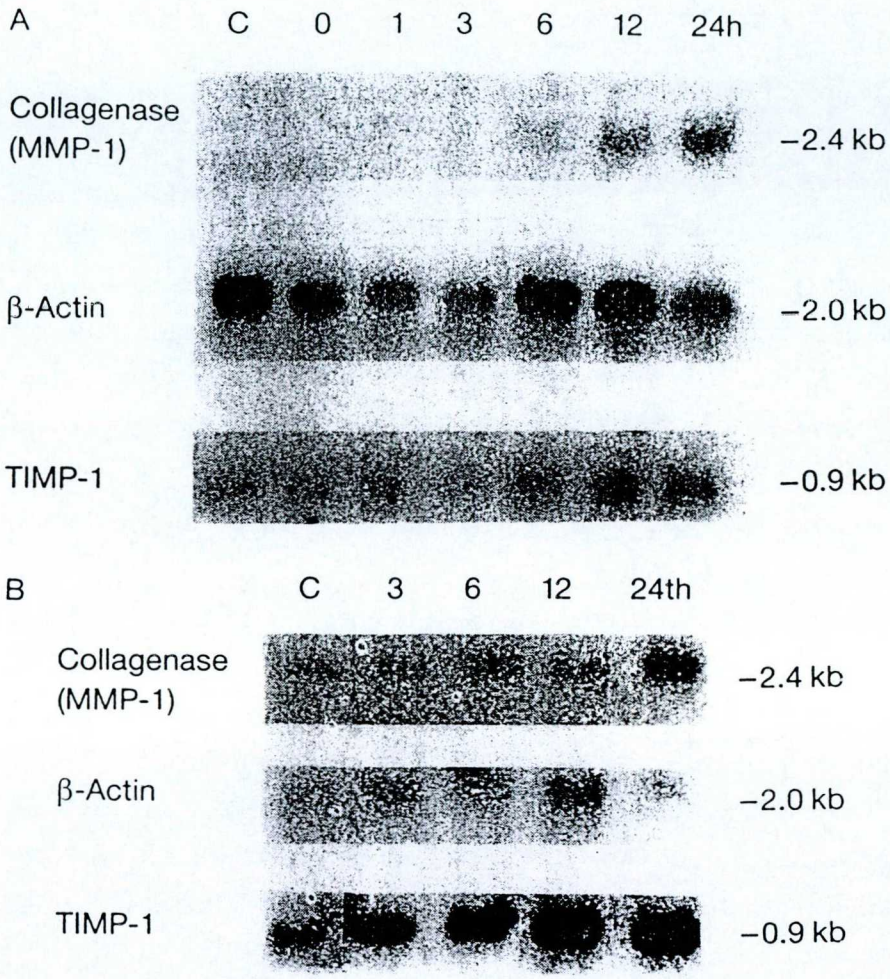
Results and discussions

A. The effect of α-MSH on the collagenase/MMP-1 expression in human dermal fibroblasts

The chronic exposure to UV light is accompanied by skin pigmentation and guantitative and qualitative alterations of dermal extracellular matrix proteins, among them collagen type I as the major structural component of the dermis. The interstitial fibroblast-derived collagenase (MMP-1) (EC 3.4.24.7.) belongs to a family of matrix degrading metalloproteinases and is characterized by its distinctive ability to cleave α-chains of interstitial collagens. Collagenase/MMP-1 activity is inhibited by its tissue inhibitor of metalloproteinases (TIMP-1). Thus, the net enzyme activity is at least partly regulated by the balanced synthesis of these two components.
We provided the first evidence that α-MSH was able to increase the expression of steady state mRNA levels of collagenase/MMP-1 in cultured human dermal fibroblasts.

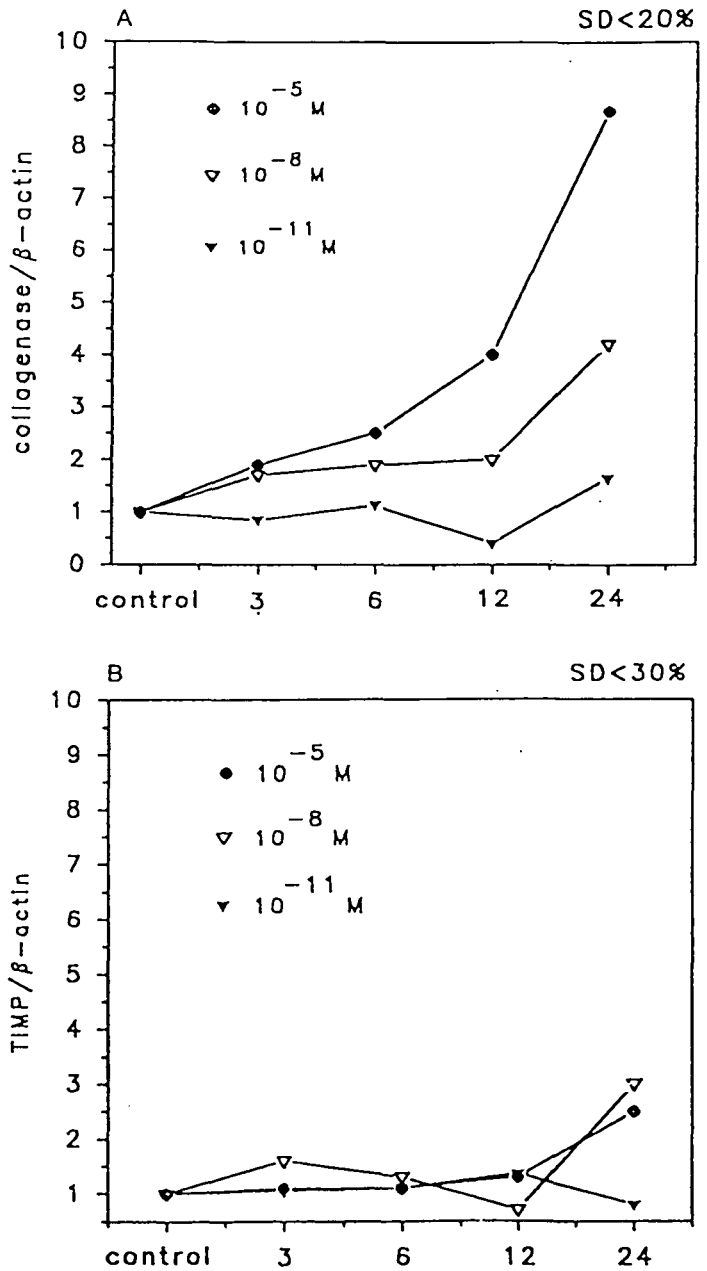
In order to study the effect of α-MSH on the steady state levels of collagenase/MMP-1 and TIMP mRNAs time course experiments were performed. Total RNA was isolated at 0, 1, 3, 6, 12, and 24 h following α-MSH treatment and subjected to Northern blot analysis. Treatments of fibroblasts at a concentration of 10^{-5} M of α-MSH resulted in a time dependent induction of steady state levels of collagenase/MMP-1 mRNA (Fig. 12/A). This induction occurred already at 6 h and was 9-fold at 24 h compared to the mock treated controls. At an α-MSH concentration of 10^{-8} M, a 4-fold induction was detected at 24 h post-treatment (Fig. 12/B), suggesting that α-MSH induction of collagenase mRNA levels was concentration-dependent (Fig. 13/A). An α-MSH concentration of 10^{-11} M did not effect specific collagenase mRNA levels. TIMP mRNA levels were only slightly induced at an α-MSH concentration of 10^{-5} and 10^{-8} M, but the induction was less than that of collagenase/MMP-1 mRNA when normalized to the β-actin (Fig. 13/B).

Fig. 12



Determination of Specific mRNAs from Cultured Human Dermal Fibroblasts by Northern Blot Hybridization.
After treatment with 10^{-5} M α-MSH (A) and with 10^{-8} M α-MSH (B). Total RNA from fibroblast cultures was isolated at 0, 1, 3, 6, 12, and 24 h after treatment with α-MSH (10^{-5} M) for 30 min and also at 3, 6, 12, 24 h after treatment with α-MSH (10^{-8} M) for 30 min. 'C' represents the untreated control. The RNA was fractionated by gelelectrophoresis under denaturating conditions and blotted onto nitrocellulose. After sequential hybridization with cDNA probes for human collagenase/MMP-1, β-actin and TIMP-1, the filter was processed for autoradiography. The Northern blots show representative experiments.

Fig. 13

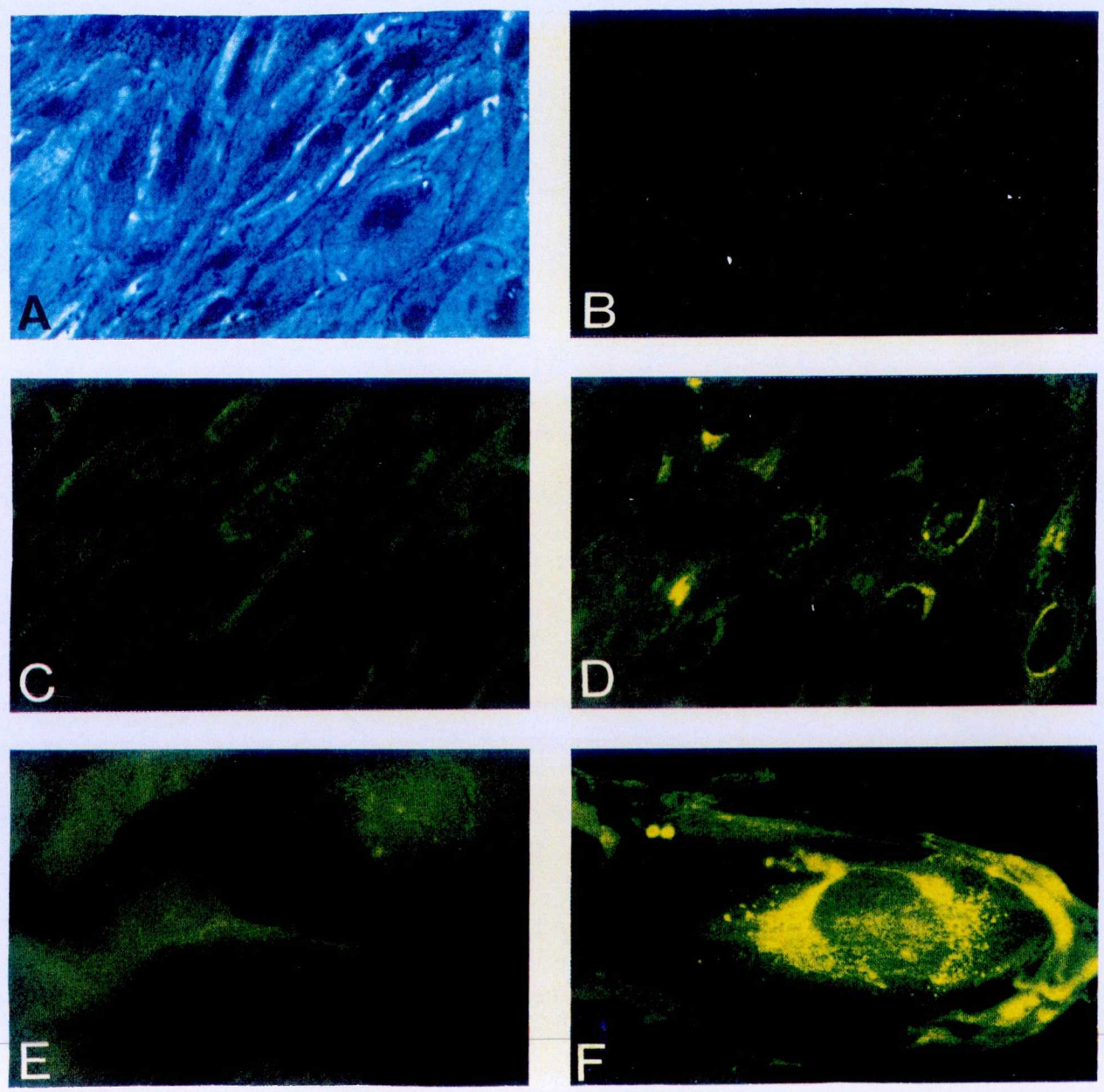


Densitometric Evaluation of the Steady State Specific mRNA Levels in α-MSH Treated Fibroblasts.

The analysis of Figure 2 makes use of the data of independent experiments including those of Figure 1. Collagenase/β-actin mRNA ratios (A) and TIMP/β-actin mRNA ratios (B) in α-MSH treated human dermal fibroblasts. The relative amounts of specific mRNAs coding for interstitial collagenase and TIMP-1 were determined densitometrically and plotted against the time (h) of the post-incubation period. The standard deviations did not exceed 20% or 30% respectively.

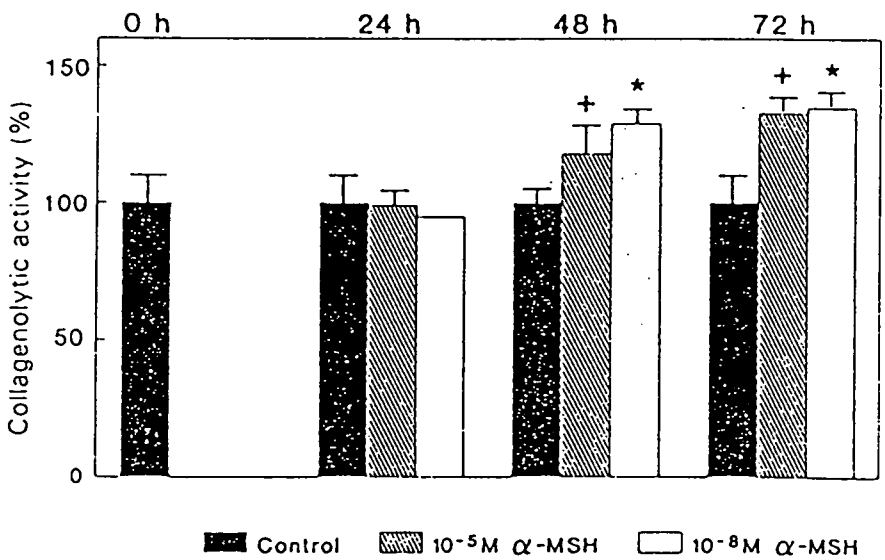
Collagenase protein expression and localization were studied in α-MSH treated cells using a monoclonal antibody against collagenase (Fig. 14). In untreated cells there was only a weak diffuse staining in the cytoplasms of fibroblasts (Fig. 14/C,E). Following treatment with α-MSH staining was more intense, and in contrast to the diffuse staining pattern of the control, revealed a pronounced perinuclear staining pattern (Fig. 14/D,F).

Fig.14



In order to study whether the increased collagenase mRNA was translated into the corresponding protein and to analyze the secretion and activation, the overall collagenolytic activity in fibroblast supernatants was determined and expressed as a percent of the mock-treated controls. At 24 h post-treatment, there was no difference in the collagenolytic activity of the fibroblast supernatants from α-MSH treated cells compared to the untreated controls. However, at 48 h post treatment a significant increase in the collagenolytic activity was found in the supernatants of α-MSH treated cells compared to the untreated controls. The differences between the controls and treated cells became more pronounced after 72 h (Fig. 15).

Fig. 15



Collagenolytic Activities of Cell Culture Supernatants from α-MSH Treated Human Dermal Fibroblasts.
After α-MSH treatment fibroblasts were cultured for 24,48 and 72 h in DMEM with 0.2% lactalbumin hydrolisatum without FCS. The collagenolytic activity of supernatants was determined as described in Materials and Methods and were expressed as % of time-matched controls. Significant differences were found in the collagenolytic activity of supernatants between controls and α-MSH treated fibroblasts in both 10^{-5}M and 10^{-8}M concentrations at 48 h and 72 h post incubation, respectively ($p < 0.001$).

In this study we have demonstrated that α-MSH is able to induce collagenase/MMP-1 mRNA steady state levels in human dermal fibroblasts, followed by an increase in translation of collagenase mRNA and secretion of the collagenolytic activity in fibroblast culture supernatants. Collagenase/MMP-1 mRNA up-regulation by α-MSH was detected as early as 24 h post treatment, an increase in collagenolytic activity observed at 48 h and to a higher extent at 72 h. Gronovitz et al. (89) have found the same kinetics of collagenase/MMP-1 expression and activation in tumor necrosis factor-α-treated fibroblasts.

When human skin is exposed to UV irradiation, a highly complex cascade of events occurs and there is growing evidence that melanocortins, among them the α-MSH, play a central role in these UV-mediated skin processes. Accordingly, α-MSH can act on tyrosinase activity — a rate limiting enzyme of the melanin pathway — via a cAMP dependent pathway in both normal melanocytes and melanoma cells (90). Clinical investigations have also suggested a synergism between MSH or/and ACTH and UV exposure in increasing melanin content (91). In this report we added a new aspect of α-MSH effects in that α-MSH modulates connective tissue metabolism by the induction of interstitial collegane/MMP-1.

Even though the actual concentration of α-MSH in the dermis after UV irradiation as well as melanocortin receptors have not yet been identified on human dermal fibroblasts, we would like to suggest that α-MSH — by the induction of fibroblast-derived collagese — contributes to the loss of interstitial collagen — the major pathological hallmark of photoaging.

B. The effect of α-MSH on the IL-8 and IL-8R expression in human dermal fibroblasts

Recently we have shown that the α-MSH is able to induce fibroblast-derived collagenase mRNA and protein production in human dermal fibroblasts.

Moreover, recent reports have suggested that α-MSH plays an important role in ultraviolet (UV) irradiation mediated skin changes, including pigmentation, connective tissue damage and inflammation. α-MSH is known to be able to regulate production of cytokines in different tissues.

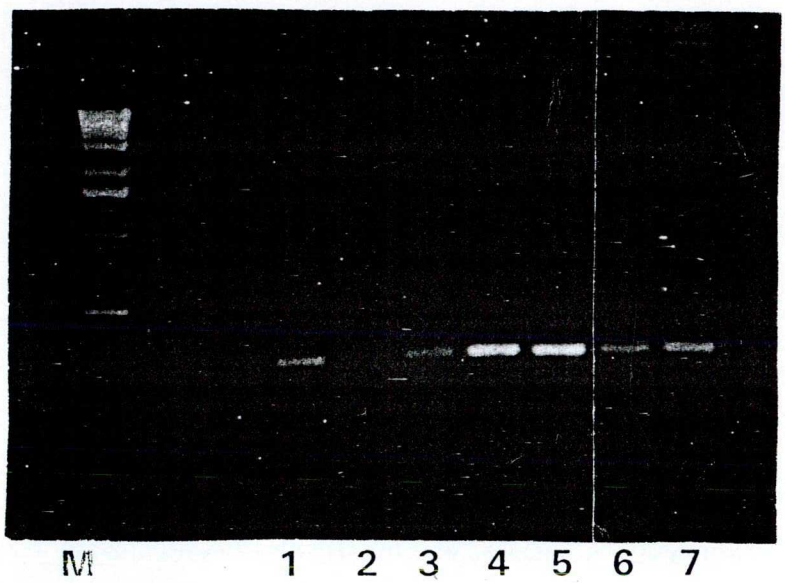
Human fibroblasts can produce the chemotactic cytokine IL-8 and it has been shown that fibroblasts produce much higher amounts of IL-8 than keratinocytes to different stimuli (92). It was also demonstrate that IL-8 was able to influence the collagen turnover in the dermis by the down-regulation of collagen biosynthesis (93).

Thus, we have investigated whether α-MSH could regulate the IL-8 production in human fibroblasts. The cells were treated with 10^{-8} M α-MSH for 30 min then cells were fed by

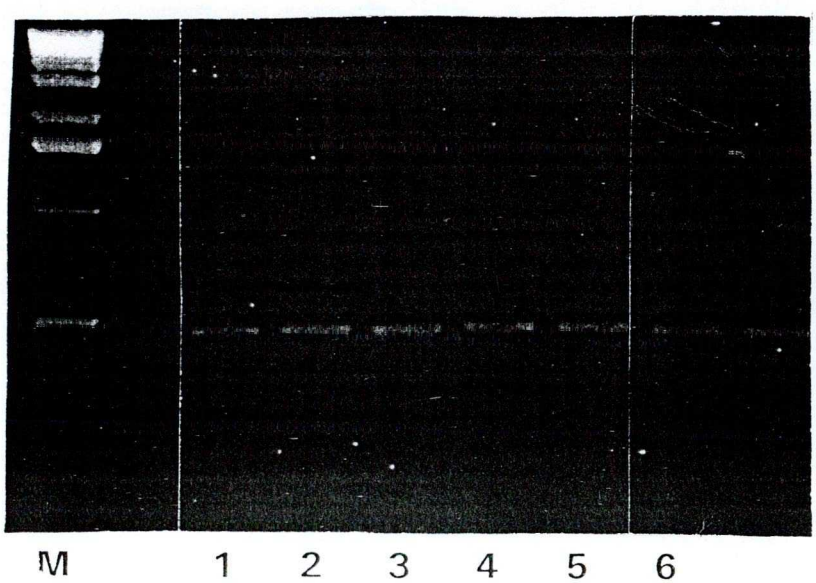
fresh medium, their total RNA was isolated in different time intervals. After reverse transcription, PCR reactions were performed using IL-8 and IL-8R specific oligonucleotid primers with β-actin as an internal control (Fig. 16). α-MSH caused a considerable induction in IL-8 mRNA expression in fibroblasts 3-6 h post treatment (Fig. 17).

Fig.16

PCR of IL-8 mRNA from 10^{-8} M α-MSH treated fibroblasts



PCR of β-actin mRNA from 10^{-8} M α-MSH treated fibroblasts

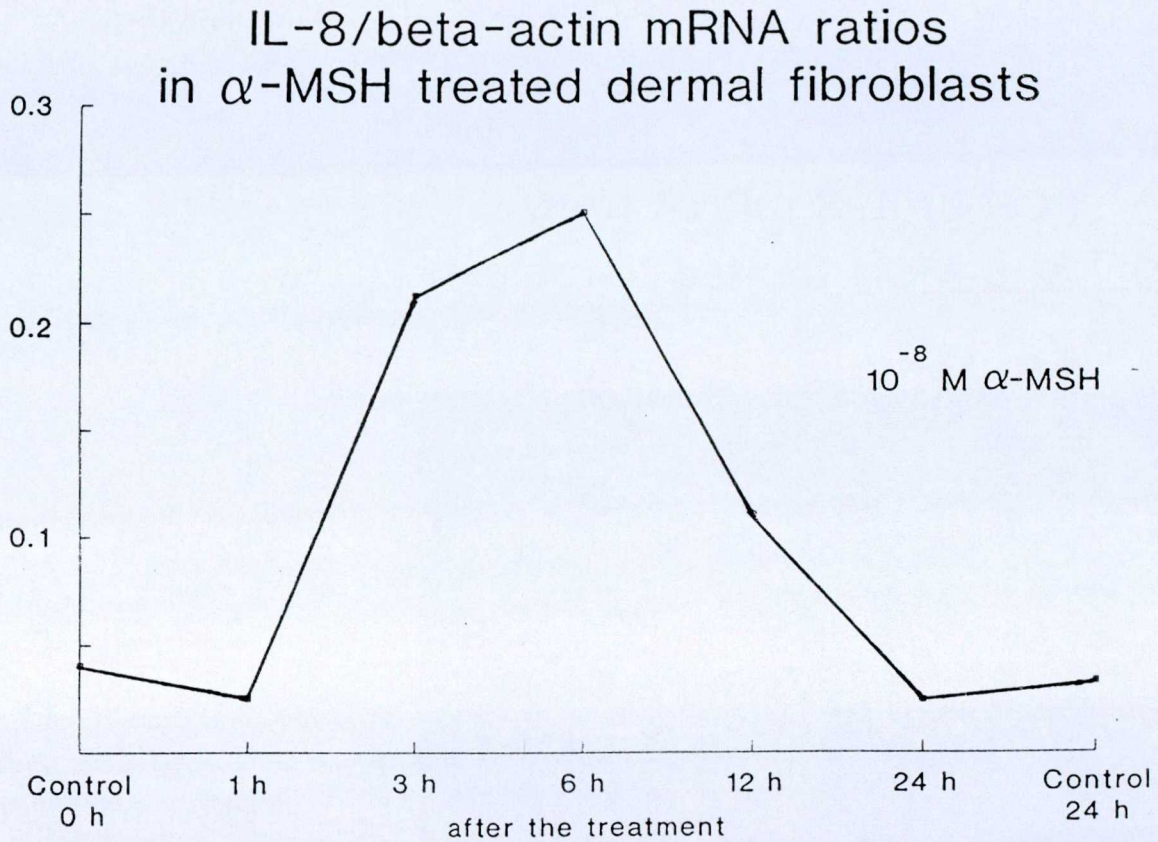


M = Molecule weight marker

1 = Untreated **control cells (0 h)**

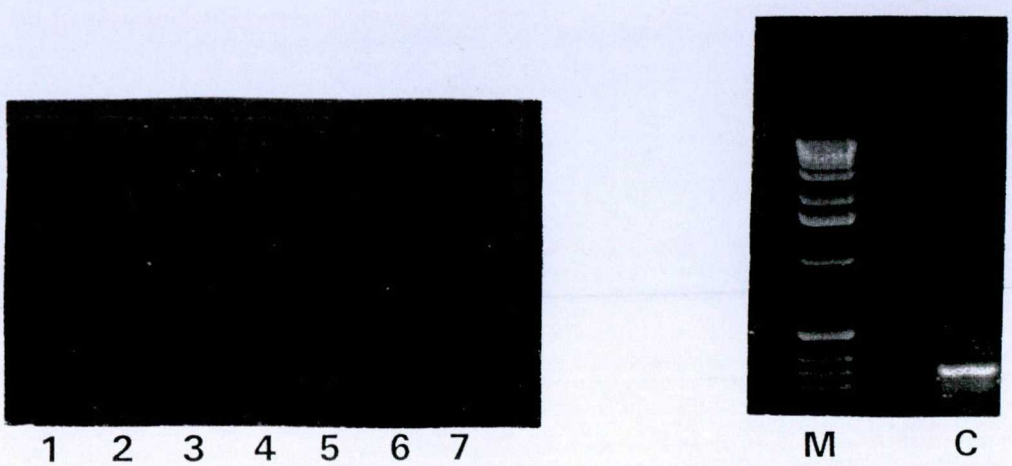
2 = α-MSH treated fibroblasts **(1 h)**
3 = α-MSH treated fibroblasts **(3 h)**
4 = α-MSH treated fibroblasts **(6 h)**
5 = α-MSH treated fibroblasts **(12 h)**
6 = α-MSH treated fibroblasts **(24 h)** post treatment
7 = Untreated **control cells (24 h)**

Fig.17



IL-8R mRNA was not detected in control fibroblasts at all, but IL-8R specific bands also appeared 3-6 h α-MSH post treatment (Fig. 18).

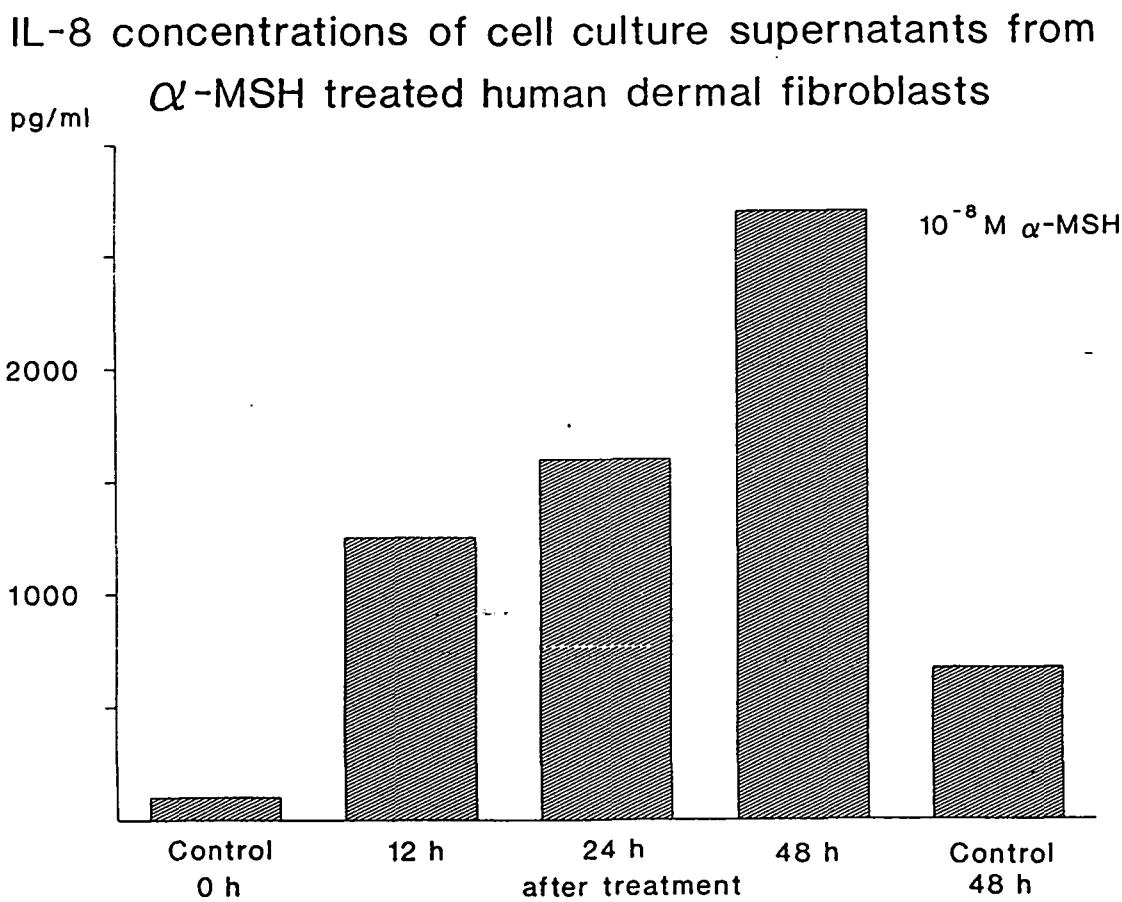
Fig.18



Legends for the figure see Fig.17

The IL-8 content of the cell culture supernatants was increased dramatically with a maximum at 48 h after the treatment (Fig 19).

Fig.19



A variety of dermatoses is characterized by the infiltration of neutrophils into skin, induction of IL-8 production in fibroblasts may play an important role in inflammatory skin diseases. Since IL-8 can down-regulate the collagen production in fibroblasts and α-MSH is able to induce their interstitial collagenase production and activity, it seems that both α-MSH and IL-8 may be important in the regulation of collagen turnover as well.

C. The effects of SP, CGRP and α-MSH on the IL-8 and IL-8R expression of human keratinocytes

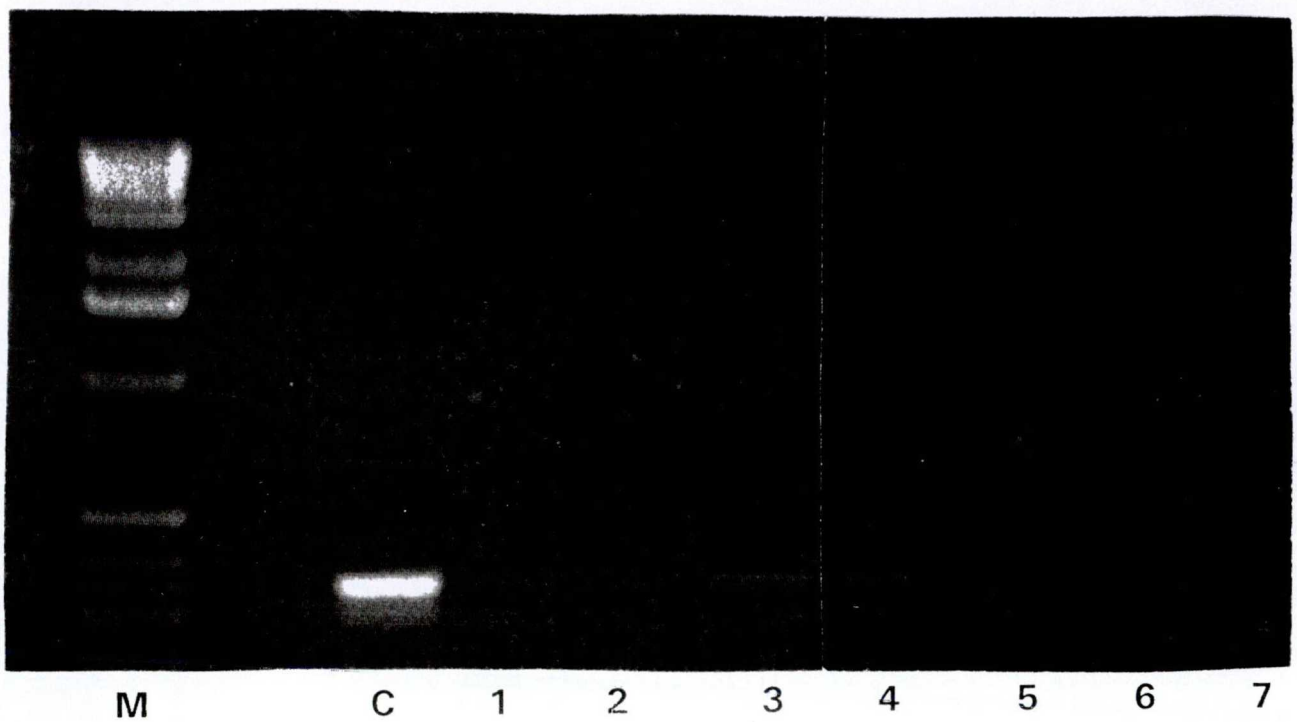
Neurogenic mechanisms are implicated in inflammatory skin diseases, particularly in psoriasis. Neuropeptides can be found in the skin of healthy subjects and have been presumed to play a role in a variety of inflammatory skin diseases. SP and CGRP coexist in human skin and the SP binding capacity of human keratinocytes was also demonstrated (51).

We studied the effects of neuropeptides SP and CGRP on the mRNA expression of IL-8 and IL-8R in a human transformed keratinocyte cell line HaCat.

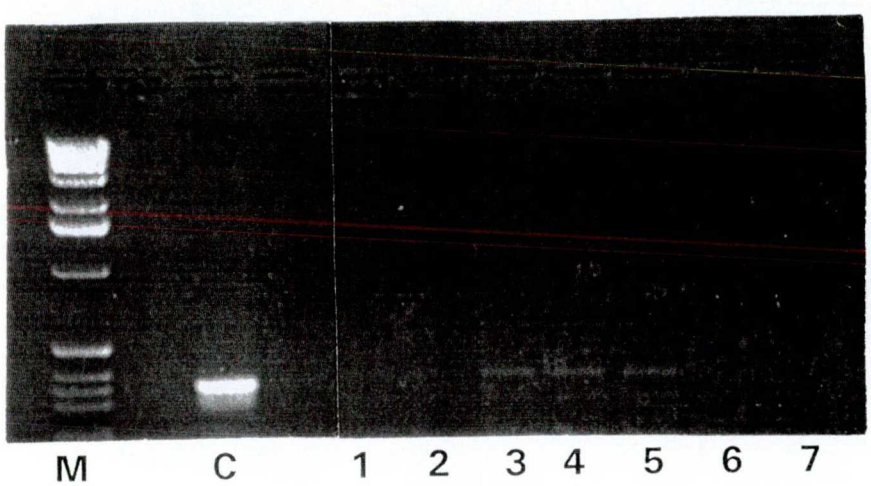
SP and CGRP at 10^{-8} M concentration caused a considerable induction of the IL-8R mRNA expression in HaCat cells (Fig. 20). In untreated controls the IL-8R mRNA expression was under the detection limit. The induction started 1-3 hours after the treatment with neuropeptides and lasted approximately 12 hours. In contrast, a constitutive IL-8 expression was found in all samples and was uneffected by neuropeptide treatment of cells (Fig. 21). No effect of α-MSH was found on the IL-8 and IL-8R mRNA expression in keratinocytes.

Fig.20

PCR of IL-8R mRNA from 10^{-8} M CGRP treated HaCat cells



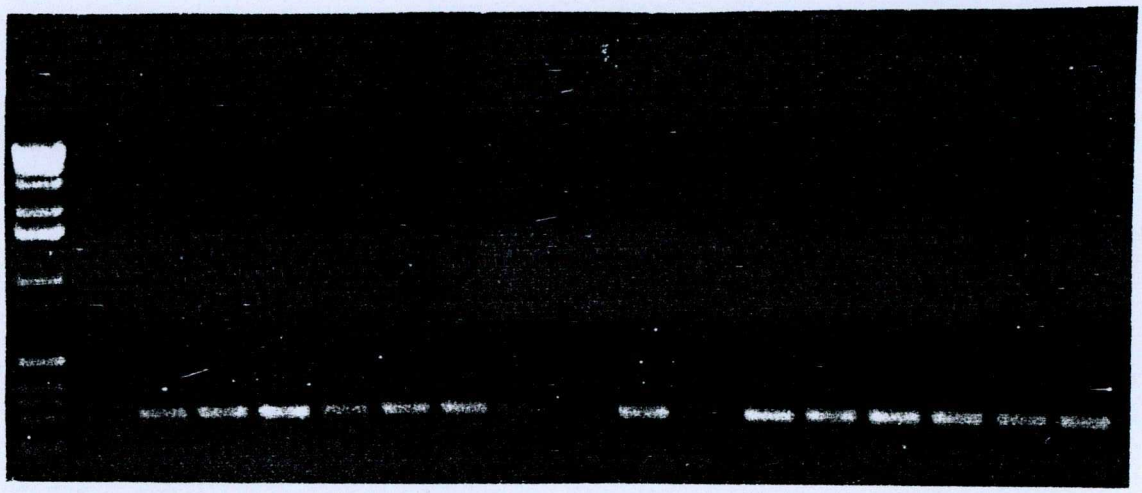
PCR of IL-8R mRNA from 10^{-8} M SP treated HaCat cells



M = Molecule weight marker
C = Positive IL-8R control (clone)
1 = Untreated **control cells (0 h)**
2 = Neuropeptide treated HaCat cells **(1 h)**
3 = - " - **(3 h)**
4 = - " - **(6 h)**
5 = - " - **(12 h)**
6 = - " - **(24 h)**
post treatment
7 = Untreated **control cells (24 h)**

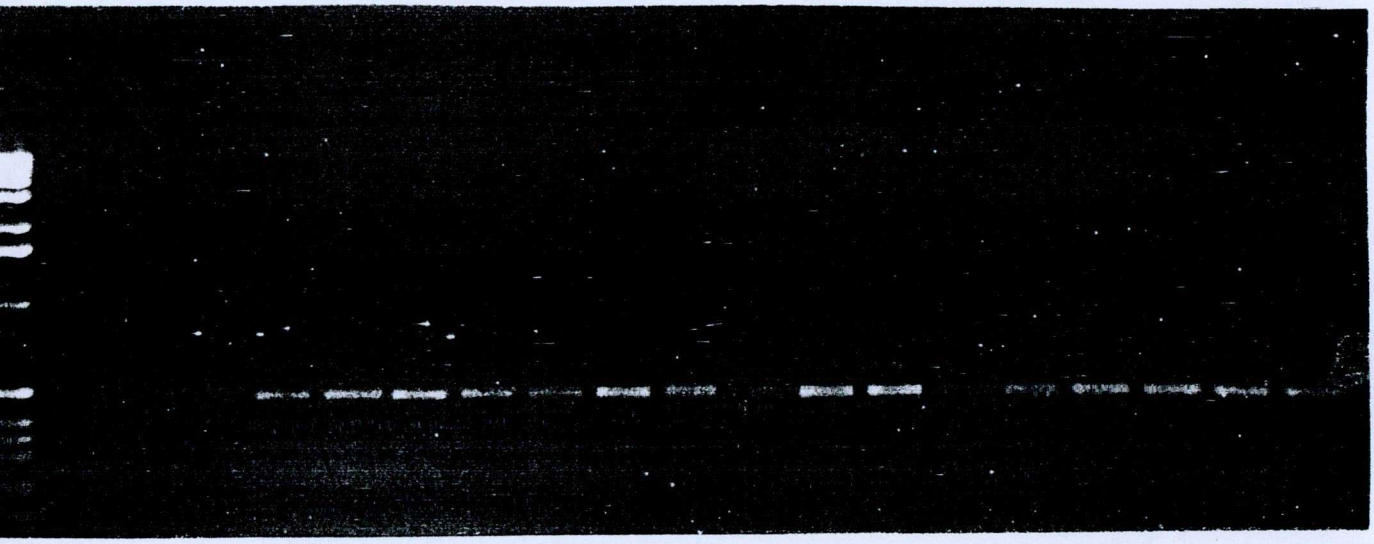
Fig.21

PCR of IL-8 mRNA from 10^{-8} M CGRP and SP treated HaCat cells



M 1 2 3 4 5 6 7 8 9 10 11 12 13 14

PCR of beta-actin mRNA from 10^{-8} M CGRP and 10^{-8} M SP treated HaCat cells



M 1 2 3 4 5 6 7 8 9 10 11 12 13 14

1 = Untreated **control cells (0 h)**
2, 9 = Neuropeptide treated HaCat cells **(0 h)**
3,10 = - " - **(1 h)**
4,11 = - " - **(3 h)**
5,12 = - " - **(6 h)**
6,13 = - " - **(12 h)**
7,14 = - " - **(24 h)**
post treatment

8 = Untreated **control cells (24 h)**

Conclusions

In this study we have demonstrated that α-MSH is able to induce collagenase/MMP-1 mRNA steady-state levels in human dermal fibroblasts, followed by an increase in translation of collagenase mRNA and secretion of collagenolytic activity in fibroblast culture supernatants. Collagenase/MMP-1 mRNA up-regulation by α-MSH was detected as early as 24 h post treatment, an increase in collagenolytic activity observed at 48 h and to a higher extent at 72 h.

Even though in our *in vitro* experiments the proteolytic enzyme activity of collagenase was induced only by 35% of the control, extrapolation to the in vivo situation with repeated α-MSH release following UV irradiation may result in profound connective tissue damage of the skin. This possibility is supported by the clinical experience that photoaging results from chronic UV exposure of the skin over years and decades.

Interestingly, UVB can stimulate the production and secretion of α-MSH and ACTH from human keratinocytes (75). Thus, paracrine effects of α-MSH released by keratinocytes following UV irradiation might play a crucial role in UV induced alterations of collagen metabolism in fibroblasts. The induction of collagenase synthesis and activation is a novel α-MSH effect, and so far has not been reported for other proopiomelanocortin peptides, such as ACTH. Hence, besides its immunomodulatory capacities α-MSH play an important role in the UV mediated regulation of connective tissue metabolism of the skin.

Even though the actual concentration of α-MSH in the dermis after UV irradiation as well as melanocortin receptors have not yet been identified on human dermal fibroblasts, we would like to suggest that α-MSH — by the induction of fibroblast-derived collagenase — contributes to the loss of interstitial collagen — the major pathological hallmark of photoaging.

We provided the first evidence that α-MSH was able to upregulate the IL-8 mRNA production in human dermal fibroblasts. Moreover, the IL-8 content of the cell culture supernatants was increased dramatically with a maximum at 48 h after the α-MSH treatment. IL-8R mRNA was not detected in control fibroblasts at all, but IL-8R mRNA also appeared α-MSH post treatment.

α-MSH is known to be able to regulate production of cytokines in different tissues. Human fibroblasts can produce the chemotactic cytokine IL-8 and it has been shown that IL-8 can influence the collagen turnover in the dermis.

A variety of dermatoses is characterized by the infiltration of neutrophils into skin, induction of IL-8 production in fibroblasts may play an important role in inflammatory skin diseases. Since IL-8 can down-regulate the collagen production in fibroblasts and α-MSH is able to induce their interstitial collagenase production and activity, it seems that both α-MSH and IL-8 may be important in the regulation of collagen turnover as well.

IL-8 is a potent chemotactic and proinflammatory cytokine, produced in the skin by a variety of cells in response to inflammatory stimuli. The effects of IL-8 are mediated by binding specific high-affinity receptors. The effects of IL-8 may be modulated in receptor level. Recently, increased expression of epidermal IL-8R has been observed in psoriasis, an inflammatory and hyperproliferative skin disease.

We demonstrated that both SP and CGRP could upregulate the IL-8R mRNA expression in a keratinocyte cell line HaCat, but no effect on IL-8 production was found. In contrast, α-MSH influenced neither IL-8 nor IL-8R expression in HaCat cells. These findings shed new light on the interaction of the nervous system and inflamatory cells contributing to neurogenic inflammation in skin.

REFERENCES

1. Tan EM, Chan EKL, Sullivan K, Rubin RI. Antinuclear Antibodies (ANAs): diagnostically specific immune markers and clues toward the understanding of systemic autoimmunity. Clin. Immunol. Immunopathol. 1988; 47: 121-141.
2. Meurer M, Franz S, Braun-Falco O. Diagnostic significance of autoantibodies in connective tissue diseases. J. Eur. Acad. Derm. Vener. 1991; Pilot issue 5-21.
3. Hargraves MM, Richmond H, Morton R. Presentation of two bone marrow elements: the "tart" cell and the "L.E." cell. Mayo Clin. Proc. 1948; 23: 25-28.
4. Van Venrooij WI, Van de Putte LB. The clinical significance of anti-nuclear antibodies in connective tissue diseases. Semin. Clin. Immunol. 1991; 3: 27-32.
5. Holman HR. Partial purification and characterization of an extractible nuclear antigen which reacts with SLE sera. Ann. N. Y. Acad. Sci. 1965; 124: 800-806.
6. Koffler D, Miller TE, Faiferman I. Antipolynucleotide antibodies: The rheumatic connection. Human Pathol. 1983; 14: 406-418.
7. Tan EM, Kunkel HG. Characteristics of a soluble nuclear antigen precipitating with sera of patients with systemic lupus erythematosus. J. Immunol. 1966; 96: 464-471.
8. Nakamura RM, Tan EM. Autoantibodies to nonhistone nuclear antigens and their clinical significance. Human Pathol. 1983; 14: 392-400.
9. Martinez-Lavin M, Vaughan JH, Tan EM. Autoantibodies and the spectrum of Sjögren's syndrome. Ann. Intern. Med. 1979; 91: 185-190.
10. Sontheimer RD. Immunobiological significance of SSA/Ro antigen-antibody system. (Editorials) Arch. Dermatol. 1985; 121: 327-330.
11. Smith ND, Boyer JL. Are antimitochondrial antibodies of prognostic value in primary biliary cirrhosis? Hepatology 1986; 6: 739-741.
12. McNeil HP et al. Immunology and clinical importance of antiphospholipid antibodies. Adv. Immunol. 1991; 49: 193.
13. Sönnichsen N, Ziegler H, Cebecauer L, Barthelmes H, Albrechtnebe H. Preparation nuklearer Antigene (ENA) aus Kalbsthymi, Nachweis von anti-ENA-antikörpern bei Patienten mit Autoimmunkrankheiten. Dt. Gesundh.Wesen. 1983; 31: 1211-1214.
14. Kurata N, Tan EM. Identification of antibodies to nuclear acidic antigens by counter-immunoelectrophoresis. Arthritis Rheum. 1976; 19: 574-580.
15 Schnaitman C, Greenwalt JW. Enzymatic properties of the inner and outer membranes of rat liver mitochondria. J. Cell. Biol. 1968; 38: 158.
16. Lieu TS, Jiang M, Steigerwald JC, Tan EM. Identification of the SSA/Ro cellular antigen with autoimmune sera. J. Immunol. Methods 1985; 71: 217-228.
17. Hashimoto T, Ogawa MM, Konohana A, Nishikawa T. Detection of pemphigus vulgaris and pemphigus foliaceus antigens by immunoblot analysis using different antigen sources. J. Invest. Derm. 1990; 94: 327-331.
18. Laemmli UK. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 1970; 277: 680-685.
19. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 1979; 76: 4350-4354.
20. Gibbons JJ, Augustynek D, Tsai CC, Roodman ST. Characterization of RNP and Sm ribonucleoprotein nuclear antigens. Mol. Immunol. 1982; 19: 765-777.
21. Walker JG, Doniach D, Roitt IM, Sherlock S. Serological tests on the diagnosis of primary biliary cirrhosis. Lancet I, 1965; 827-831.

22. Berg PA, Klein R. Clinical and prognostic relevance of different mitochondrial antibody profiles in primary biliary cirrhosis. Molec. Aspects Med. 1985; 8: 235-247.

23. Cahn MM, Levy EJ, Shaffer B. Polymorphous light eruption. Arch. Dermatol. 1963; 88: 756-758.

24. Wojnarowska F. Simultaneous occurrence in identical twins of discoid lupus erythematosus and polymorphic light eruption. J. Royal Soc. Med. 1983; 76: 791-792.

25. Wilson MR. Antinuclear antibodies and anticytoplasmic antibodies in lupus erythematosus. Lupus erythematosus. Third edition. In Wallace DJ, Dubois EL ed. Philadelphia, Lea & Febiger; 1987.

26. Jansén CT, Karvonen J. Polymorphous light eruption. A seven-year follow-up evaluation of 114 patients. Arch. Dermatol. 1984; 120: 862-865.

27. Fisher DA, Epstein JH, Kay DN, Tuffanelli DL. Polymorphous light eruption and lupus erythematosus. Arch. Dermatol. 1970; 101: 458-461.

28. Panet-Raymond G, Johnson WC. Lupus erythematosus and polymorphous light eruption. Arch. Dermatol. 1973; 108: 785-787.

29. LeFeber WP, Norris DA, Ryan SR, Huff JC, Lee LA, Kubo M, Boyce ST, Kotzin BL, Weston WL. Ultraviolet light induces binding of antibodies to selected nuclear antigens on cultured human keratinocytes. J. Clin. Invest. 1984; 74: 1545-1551.

30. Labib RS, Anhalt GJ, Patel HP, Mutasim DF, Diaz LA. Molecular heterogeneity of the bullous pemphigoid antigens as detected by immunoblotting. J. Immunol. 1986; 136: 1231-1235.

31. Robledo MA, Kim S.-C, Korman NJ, Stanley JR, Labib RS, Futamura S, Anhalt GJ. Studies of the relationship of the 230-kD and 180-kD bullous pemphigoid antigens. J. Invest. Derm. 1990; 94: 793-797.

32. Sawamura D, Li K, Chu M-L, Uitto J. Human bullous pemphigoid antigen (BPAG1). J. Biol. Chem. 1991; 266: 17784-17790.

33. Giudice GJ, Emery DJ, Diaz LA. Cloning and primary structural analysis of the bullous pemphigoid autoantigen, BP180. J. Invest. Dermatol. 1992; 99: 243-250.

34. Korman NJ, Eyre RW, Klaus-Kovtun V, Stanley JR. Demonstration of an adhering-junction molecule (plakoglobin) in the autoantigens of pemphigus foliaceus and pemphigus vulgaris. New Engl. J. Med. 1989; 321: 631-635.

35. Amagai M, Klaus-Kovtun V, Stanley JR. Autoantibodies against a novel epithelial cadherin in pemphigus vulgaris, in a disease of cell adhesion. Cell 1991; 67: 869-877.

36. Shapiro PE, McAllister G, Lerner EA, Lerner MR. Healthy individuals and patients with systemic lupus erythematosus have unique, person-specific spectra of antibodies detectable on immunoblots. Clin. Immunol. Immunopathol. 1991; 59: 129-138.

37. Lynch DR, Snyder SH.: Neuropeptides: multiple molecular forms, metabolic pathways and receptors. Annu. Rev. Biochem. 1986; 55: 773-799.

38. Hokfelt T, Bean A, Ceccatelli S. Overview of peptide systems with special reference to coexistence with classical transmitters. In: Racagni G, Brunello N, Fukuda T, eds. Biological Psychiatry. Proceedings of the 5th World Congress of Biological Psychiatry. Florence, June 9-14 1991. Amsterdam, Elsevier 1991: 534-536.

39. Johansson O. A detailed account of NPY immunoreactive nerves and cells of human skin. Comparison with VIP, substance P and PHI containing structures. Acta Physiol Scand 1986; 128: 147-153.

40. Wallergren J, Ekman R, Sundler F.: Occurrence and distribution of neuropeptides in the human skin. Acta Dermato-Venereol. (Stockh.) 1987; 67: 185-192.

41. Foreman JC.: The skin as an organ for the study of pharmacology of neuropeptides. Skin. Pharmacol. 1988; 1: 77-92.

42. Pincelli C, Fantini F, Gianetti A.: Neuropeptides and skin inflammation. Dermatology 1993; 187: 153-158.

43. Eedy DJ.: Neuropeptides in skin. Br. J. Dermatol. 1993; 128: 597-605.

44. Lotti T, Hautmann G, Panconesi E. Neuropeptides in skin. Clinical review. J. Am. Acad. Derm. 1995; 33: 482-496.

45. Slominski A, Wortsman J, Mazurkiewicz JE, Matsuoka L, Dietrich J, Lawrence K, Gorbani A, Paus R. Detection of proopiomelanocortin-derived antigens in normal and pathologic human skin. J. Lab. Clin. Med. 1993; 122, 658-666.

46. Schauer E, Trautinger F, Köck A, Schwarz A, Bhardwaj-A, Simon M, Ansel JC, Schwarz T, Luger TA. Proopiomelanocortin-derived peptides are synthetized and released by human keratinocytes. J. Clin. Invest. 1994; 93: 2258-2262.

47. Piotrowski W, Devoy MAB, Jordan CC et al. The substance P receptor on rat mast cells and in human skin. Agents Actions 1984; 14: 420-424.

48. Watson SP. Are the proposed substance P receptor subtypes, substance P receptors? Life Sci 1984; 35: 797-808.

49. Pascual DW, Blalock JE, Bost KL. Antipeptide antibodies that recognize a lymphocyte substance P receptor. J. Immunol. 1989; 143: 3697-3702.

50. Pincelli C, Fantini F, Giardino L, Zanni M, Calza L, Sevignani C, Gianetti A.: Autoradiographic detection of substance P receptors in normal and psoriatic skin. J. Invest. Dermatol. 1993; 101: 301-304.

51. Kemény, L., Restorff, B., Michel, G., Ruzicka, T.: Specific binding and lack of growth-promoting activity of substance P in cultured keratinocytes. J. Invest. Dermatol. 1994; 103: 605-606.

52. Halgerstrand A, Jonzon B, Dalsgaard CJ, Nilsson J.: Vasoactive intestinal polypeptide stimulates cell proliferation and adenylate cyclase activity of cultured human keratinocytes. Proc. Natl. Acad. Sci. USA 1989; 86: 5993-5996.

53. Kowalski ML, Kaliner MA. Neurogenic inflammation, vascular permeability, and mast cells. J. Immunol. 1988; 140: 3905-3911.

54. Eedy DJ, Johnston CF, Shaw C, Buchanan KD.: Neuropeptides in psoriasis: An immunohistochemical and radioimmunoassay study.J. Invest. Dermatol. 1991; 96: 434-438.

55. Gianetti A, Fantini F, Cimitan A, Pincelli C.: Vasoactive intestinal polypeptide and substance P in the pathogenesis of atopic dermatitis. Acta Dermato-Venereol. (Stockh.) Suppl. 1992; 176: 90-92.

56. Payan DG, Brewster DR, Goetzl EJ. Specific stimulation of human T lymphocytes by substance P. J Immunol 1983; 131:1631-1645.

57. Payan DG, McGillis JP, Renold FK, Mitsuhashi M, Goetzl EJ.: Neuropeptide modulation of leukocyte function. Ann. NY Acad. Sci. 1987; 496: 182-188.

58. Tanaka T, Danno K, Ikai K, Inamura S. Effects of substance P and substance K on the growth of cultured keratinocytes. J Invest Dermatol 1988; 90:399-401.

59. Brown J, Perry P, Hefeneider S, Ansel J. Effect of substance P on murine keratinocyte cytokine production. In: Oppenheim JJ, Powanda MC, Kluger MJ, Dinarello CA eds. Molecular and Cellular Biology of Cytokines. Alan Liss, New York (in press)

60. Lotz M, Vaughan JH, Carson DA.: Effect of neuropeptides on production of inflammatory cytokines by human monocytes. Science 1988; 241: 1218-1221.

61. Jancsó G, Király E, Jancsó-Gábor A. Pharmacologically induced selective degeneration of chemosensitive primary sensory neurones. Nature 1977; 270: 741-743.

62. Eglezos A, Andrews PV, Boyd RL, Helme RD. Effects of capsaicin treatment on immunoglobulin secretion in the rat -further evidence for involvement of tachykinin-containing afferent nerves. J Neuroimmun 1990; 26: 131-138.

63. Laurenzi MA, Persson MAA, Dalsgaard CJ, Ringden O. Stimulation of human B lymphocyte differentiation by the neuropeptides substance P and neurokinin A. Scan J Immunol 1989; 30: 695-701.

64. Naukkarinen A, Harvima I, Paukkonen K, Aalto M-L, Horsmanheimo M. Immunohistochemical analysis of sensory nerves and neuropeptides, and their contacts with mast cells in developing and mature psoriatic lesions. Arch. Derm. Res. 1993; 285: 341-346.

65. Tóth-Kása I, Jancsó G, Obál F Jr, Husz S, Simon M.: Involvement of sensory nerve endings in cold and heat urticaria.J. Invest. Dermatol. 1983; 80: 34-36.

66. Coulson IH, Holden CA.: Cutaneous reactions to substance P and histamine in atopic dermatitis. Br. J. Dermatol. 1990; 122: 343-349.

67. Umeda Y, Takamiya M, Yoshizaki H. Inhibition of mitogen-stimulated T lymphocyte proliferation by calcitonin gene-related peptide. Biochem. Biophys. Res. Commun. 1988; 154: 227-235.

68. Hosoi J, Murphy GF, Egan CL, Lerner EA, Grabbe S, Asahina A, Granstein RD. Regulation of Langerhans cell function by nerves containing calcitonin gene-related peptide. Nature 1993; 363: 159-163.

69. Bunker CB, Reavley C, O'Shaughnessy DJ, Dowd PM. Calcitonin gene-related peptide in treatment of severe peripheral vascular insufficiency in Raynaud's phenomenon. Lancet 1993; 342: 80-83.

70. Allen BM. The results of exstirpation of the anterior lobe of the hypophysis and of the thyroid of Rana pipiens larvae. Science 1916; 44: 755-758.

71. Farooqui JZ, Medrano EE, Abdel-Malek Z, Nordlund J. The expression of proopiomelanocortin and various POMC-derived peptides in mouse and human skin. Ann. N. Y. Acad. Sci. 1993; 680: 508-510.

72. Slominski A, Paus R, Wortsman J. On the potential role of proopiomelanocortin in skin physiology and pathology. Mol. Cell. Endocrinol. 1993; 93: C1-C6.

73. Pawelek JM. New possibilities for regulation of skin physiology (Editorials) J. Lab. Clin. Med. 1993; 122: 627-628.

74. Pears JS, Jung RT, Bartlett W, Browning MCK, Kenicer K, Thody AJ. A case of skin hyperpigmentation due to α-MSH hypersecretion. Brit. J. Dermatol. 1992; 126: 286-289.

75. Köck A, Schauer E, Schwarz T, Luger TA. MSH and ACTH production by human keratinocytes. A link between the neuronal and the immune system. J. Invest. Dermatol. 1990; 94: 543A.

76. Thody AJ, Hunt G, Donatien PD, Todd C. Human melanocytes express functional melanocyte-stimulating hormone receptors. Ann. N. Y. Acad. Sci. 1993; 680: 381-390.

77. Chakraborty A, Pawelek J MSH receptors in immortalized human epidermal keratinocytes: A potential mechanism for coordinate regulation of the epidermal-melanin unit. J. Cell. Physiol. 1993; 157: 344-350.

78. Hunyadi J, Simon M Jr., Dobozy A. Immune-associated surface markers of human keratinocytes. Immunol. Lett. 1992; 31: 209-216.

79. Csató M, Bozóky B, Hunyadi J, Dobozy A. Candida albicans phagocytosis by separated human epidermal cell. Arch. Dermatol. Res. 1986; 279: 136-139.

80. Michel G, Kemény L, Ried C, Arenberger P, Kenderessy SzA, Peter RU, Ruzicka T. Interleukin-8-receptor mediated chemotaxis of human epidermal cells. FEBS Lett. 1992; 305: 241-243.

81. Barker JNWN, Mitra RS, Griffiths CEM, Dixit VM, Nickoloff, BJ. Keratinocytes as initiators of inflammation. Lancet 1991; 337: 211-214.

82. Kemény L, Kenderessy SzA, Michel G, Ruzicka T, Dobozy A. Identification of specific receptors for interleukin-8 on human epidermal cells. Exp. Dermatol. 1992; 1: 98(A).

83. Schulz BS, Michel G, Wagner S, Süss R, Beetz A, Peter RU, Kemeny L, Ruzicka T. Increased expression of epidermal IL-8 receptor in psoriasis. Down-regulation by FK-506 in vitro. J. Immunol. 1993; 151: 4399-4406.

84. Fleischmajer R, Perlish JS, Krieg T, Timpl R. Variability in collagen and fibronectin synthesis by scleroderma fibroblasts in primary culture. J. Invest. Dermatol. 1981; 76: 400-403.

85. Sambrook J, Frisch EF. & Maniatis T. Molecular cloning: a laboratory manual. 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y. 1989.

86. Herrmann G, Wlaschek M, Lange TS, Prenzel K, Goerz G, Scharffetter-Kochanek K. UVA irradiation stimulates the synthesis of various matrix-metalloproteinases (MMPs) in cultured human fibroblasts. Exp. Dermatol. 1993; 2, 92-97.

87. Wlaschek M, Bolsen K, Herrmann G, Schwarz A, Wilmroth F. Heinrich PC, Goerz G, Scharffetter-Kochanek K. UVA-induced autocrin stimulation of fibroblast-derived collagenase by IL-6: A possible mechanism in dermal photodamage?. J. Invest. Dermatol. 1993; 101, 164-168.

88. Nethery A, Lyons JG, O'Grady RL. A spectrophotometric collagenase assay. Annal. Biochem 1986; 159: 390-395.

89. Gronowicz G, Hadjimichael J, Richards D, Cerami A, Rossomando EF. Correlation between tumor necrosis factor-alpha (TNF-alpha)-induced cytoskeletal changes and human collagenase gene induction. J. Periodont. Res. 1992: 27: 562-568.

90. Jimenez M, Kameyama K, Maloy WL, Tomita Y, Hearing VJ. Mammalian tyrosinase biosynthesis, processing and modulation by melanocyte-stimulating hormone. Proc. Natl. Acad. Sci. USA 1988; 85: 3830-3834.

91. Fitzpatrick TB, Breathnach AS. Das Epidermale Melanin-Einheit System. Dermatologische Wochenschrift 1963; 147: 481-489.

92. Kristensen MS, Paludan K, Larsen CG, Zachariae CO, Deleuran BW, Jensen PK, Jorgensen P, Thestrup-Pedersen K. Quantitative determination of IL-1α-induced IL-8 mRNA levels in cultured human keratinocytes, dermal fibroblasts, endothelial cells and monocytes. J. Invest. Derm. 1991; 97, 506-510.

93. Unemori EN, Amento EP, Bauer EA, Horuk R.: Melanoma growth-stimulatory activity/GRO decreases collagen expression by human fibroblasts. Regulation by C-X-C but not C-C cytokines. J. Biol. Chem. 1993; 268(2), 1338-1342.

ACKNOWLEDGMENTS

I thank *Prof. Dr. Attila Dobozy* for guiding me with help and professional advice in my work and providing me the opportunity to work at his Department.

Dr. Sándor Husz has given me invaluable advice and help throughout my professional career. I am grateful for his constant support through the years.

I have been fortunate to work closely with *Drs. Lajos Kemény, Günter Michel, Meinhard Wlaschek and Prof. Thomas Ruzicka*, without them this work would not have been possible.

This work was supported by grants from OTKA 2716, OTKA T5192, OMFB 5268 and Deutsche Forschungsgemeinschaft (Ru 292/4-1 and Scha 411/2-2).