basal cells from the quiescent to the cycling state (11), the analogue of the specific Ki67-negative G_1 phase between G_0 and S phase, as has been suggested for leukocytes (3).

It should be noted that the cytoplasmic and the nuclear binding sites are simultaneously present in the few cells in unstimulated epidermis that are cycling (Fig. 1 *a*). This excludes the possibility of one binding site moving after stimulation, from the cytoplasm to the nucleus. The nature of the nuclear binding site is not known, but is very probably a protein, since its expression in lymphocytes can be inhibited by cycloheximide (3); the nature of the cytoplasmic binding site, however, remains obscure. Further research is required to clarify these points.

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Use of Sodium-chloride Separated Human Skin in Detection of Circulating Anti-basement Membrane Zone Antibodies

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The sensitivity of the indirect immunofluorescence (IIF) technique for detection of circulating basement membrane zone (BMZ) antibodies was evaluated, employing NaCl-separated human skin and intact skin as substrate. Consecutive serum samples from 12 patients with clinically, histologically and immunohistologically verified bullous pemphigoid (BP) were investigated in parallel on both substrates, in dilutions ranging from 1:10 to 1:1,280. All BP sera showed linear deposits of IgG at the BMZ on intact skin, with titres ranging from 10 to 160. On NaCl-separated skin, all BP sera produced a linear epidermal fluorescent band for IgG, with titres ranging from 80 to 1,280. None of the sera showed deposits of IgM anti-BMZ antibodies. Sera from 5 healthy donors (dilutions 1:10) produced no fluorescence, either on intact or on NaCl-separated skin. The serum-titres of circulating anti-BMZ IgG antibodies in 2 patients with corticosteroid-resistant BP were significantly reduced (from 160 to <10) during treatment with plasmapheresis, when using NaCl-separated skin as substrate for IIF, whereas the serum-titres showed insignificant reduction (from 20 to <10), when using intact skin as substrate. We conclude that the IIF method is more sensitive for detection of circulating anti-BMZ antibodies, when NaCl-separated skin as compared with intact human skin is employed as substrate. *Key words: Immunofluorescence; NaCl-separated skin; Bullous pemphigoid.*

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The direct (DIF) and indirect immunofluorescence (IIF) techniques are widely used in the diagnosis of bullous diseases of the skin. By DIF on skin from

patients with bullous pemphigoid (BP) linear deposits of IgG at the basement membrane zone (BMZ) can be demonstrated in 50–90% and linear deposits of complement C3 in 80–100% of the patients. By IIF circulating anti-BMZ IgG antibodies can be detected in sera from approximately 70% of BP patients (1).

However, conventional IIF does not yield any information as to the more precise localization of the antigen in the BMZ. By employing human skin, which had been separated in the BMZ by treatment with hypertonic NaCl, as substrate in IIF assay, sera from patients with BP were found to react with an antigen located at the epidermal side of the split skin, whereas sera from patients with epidermolysis bullosa acquisita (EBA) reacted with an antigen, located at the dermal side of the split skin (2). A recent study demonstrated that the use of human split skin as substrate increased the diagnostic sensitivity of the IIF technique, compared with the sensitivity, when using intact skin, mucous membranes, or monkey esophagus as substrate (3).

In this study we have evaluated the sensitivity of the IIF assay for determining the titres of anti-BMZ antibodies in a series of BP patients, employing NaCl-separated skin as substrate, compared with intact skin as substrate.

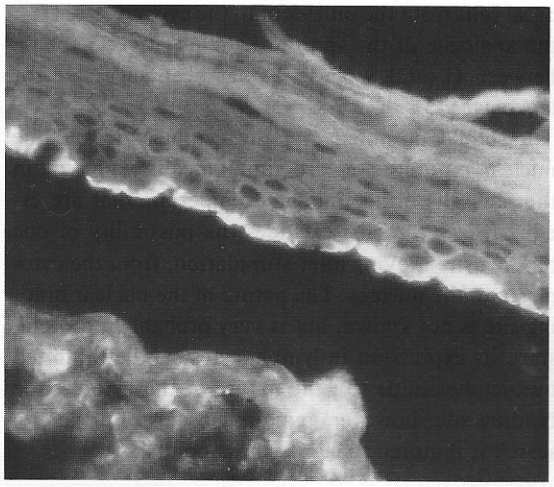

Fig. 1. Indirect immunofluorescence on NaCl-separated skin with serum from a patient with bullous pemphigoid. Linear staining for IgG on the epidermal side of the split is seen (×400).

MATERIALS AND METHODS

Immunoreagents

Fluorescein-conjugated F(ab')$_2$-fragments of rabbit anti-human IgG (γ-chain specific) and IgM (μ-chain specific), and fluorescein-conjugated swine anti-rabbit immunoglobulins (Ig) were obtained from DakoPatts (Copenhagen, Denmark). Rabbit antiserum against human laminin was kindly donated by Dr R. Albrechtsen, University Institute of Pathological Anatomy, Copenhagen, Denmark.

Patients and controls

Serum samples were collected from 12 patients, consecutively admitted to the Department of Dermatology, Odense University Hospital, with clinically, histologically and immunohistologically verified BP. In addition, serial samples from 2 patients with severe BP were examined. In both cases, treatment with high-dose prednisolone and azathioprine failed to control disease activity and plasmapheresis was instituted. Plasmapheresis was performed every second day for 10 days, with exchange of 2 litres of plasma at each plasmapheresis. The plasma was substituted with human albumin and isotonic saline. Clinical improvement was seen after two plasmaphereses; after four plasmaphereses, no disease activity was recorded in either case. Samples were obtained immediately before and 24 h after each plasma exchange. Sera from 5 healthy donors served as controls. All sera were heat-inactivated (at 56°C for 30 min.) and stored at −70°C until assayed.

Skin samples

Keratomed skin (0.5 mm thick, 3.0 cm wide and 4.0 cm long) from the flexor surface of the forearm was obtained from a human cadaver (8 h) without skin disease. The skin was rinsed in phosphate-buffered saline (PBS) (pH 7.2). One cm^2 was mounted in Tissue Tek, OCT compound (Miles Scientific), quick frozen in CO_2 and stored at −20°C. This was used as intact skin. For preparation of split-skin the previously described technique (4, 5, 6) with some modifications was used. The keratomed skin was rinsed in PBS and immersed in cold (4°C) 1.0 M NaCl and slowly agitated for 24 h at 4°C. The epidermis was gently separated from the dermis, but left in place. Specimens of 0.5 cm^2 were cut with razor blades, mounted and quick-frozen as above. Some specimens were fixed in 2.5% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.3), and prepared for electron microscopy using conventional methods.

Indirect immunofluorescence

IIF was performed in parallel on 5 µm thick cryostat sections of intact and NaCl-separated normal skin. The cryostat sections were incubated with serial two-fold dilutions of BP or control sera (diluted 1:10 to 1:2,560 in PBS containing 5% normal swine serum (PBS/NSS) (DakoPatts)) for 30 min at room temperature. Following two washes in PBS, the sections were incubated for 30 min at room temperature with FITC-conjugated anti-human IgG or anti-human IgM (diluted 1:20 in PBS/NSS). The fluorescence titre was determined as the lowest dilution of serum giving visible fluorescence. Rabbit anti-laminin antiserum was applied diluted 1:100 and developed with FITC-conjugated swine anti-rabbit Ig.

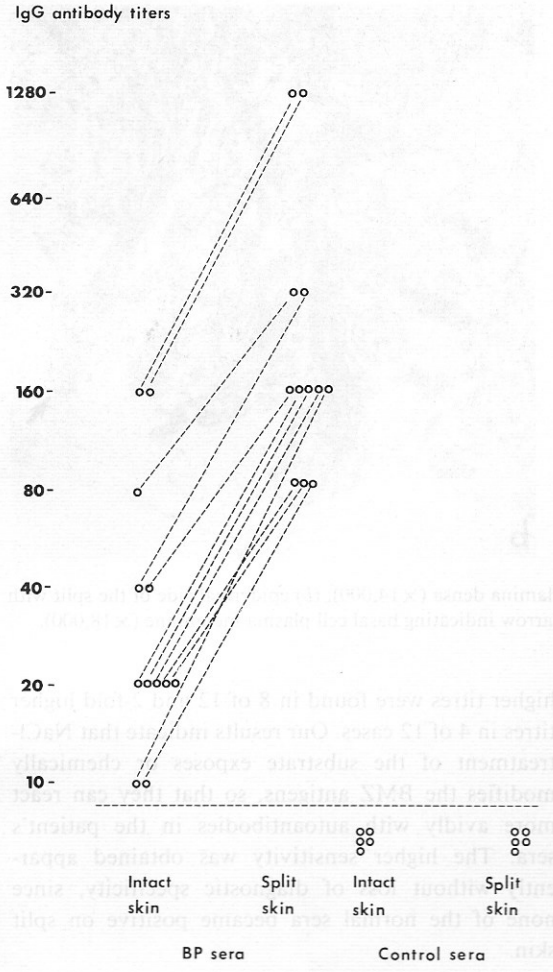

Fig. 2. Serum titres of anti-BMZ IgG antibodies in 12 patients with bullous pemphigoid (BP) and 5 control sera, determined by indirect immunofluorescence on intact or NaCl-separated human skin.

RESULTS

Comparison of anti-BMZ antibody titres on intact and split skin

On intact skin all BP sera produced a linear fluorescent band at the BMZ for IgG, with fluorescence titres ranging from 10 to 160. On NaCl-separated skin all BP sera produced a linear fluorescent band for IgG at the epidermal side of the split, with no staining on the dermal side (Fig. 1). The fluorescence titres ranged from 80 to 1,280 and in all sera the titres were 2–3-fold higher on split skin than on intact skin (Fig. 2). None of the BP sera produced deposits of IgM anti-BMZ antibodies, either on intact or on split skin.

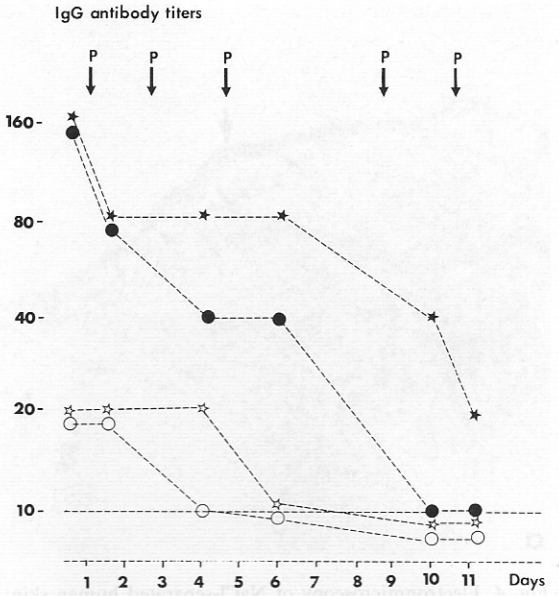

Fig. 3. Serial measurements of anti-BMZ IgG antibody titres in 2 patients with bullous pemphigoid, treated with plasmapheresis, employing intact skin (case 1: ○–○, case 2: ☆–☆) or NaCl-separated skin (case 1: ●–●, case 2: ★–★) as substrate in indirect immunofluorescence investigations. Arrows indicate time of plasmaphereses.

None of the control sera produced fluorescence (in dilutions 1 : 10), either on intact or on split skin.

In the 2 patients with BP, treated with plasmapheresis, the anti-BMZ antibody titres were reduced from 160 to <10, when using split skin as substrate (Fig. 3), whereas the titre reduction on intact skin was much less pronounced (from 20 to <10).

Determination of level of cleavage

Electron microscopy demonstrated that the NaCl-treated skin was separated through the lamina lucida, leaving the electron dense lamina densa on the dermal side of the split (Fig. 4 *a*) and the basal cell plasma membrane on the epidermal side of the split (Fig. 4 *b*).

On intact rabbit skin, anti-laminin antiserum produced intense linear fluorescence at the BMZ, whereas on NaCl-separated skin a dermal fluorescent band was seen, without staining on the epidermal side of the split.

DISCUSSION

In this study we have evaluated the assay sensitivity of the split skin IIF technique for detecting circulating

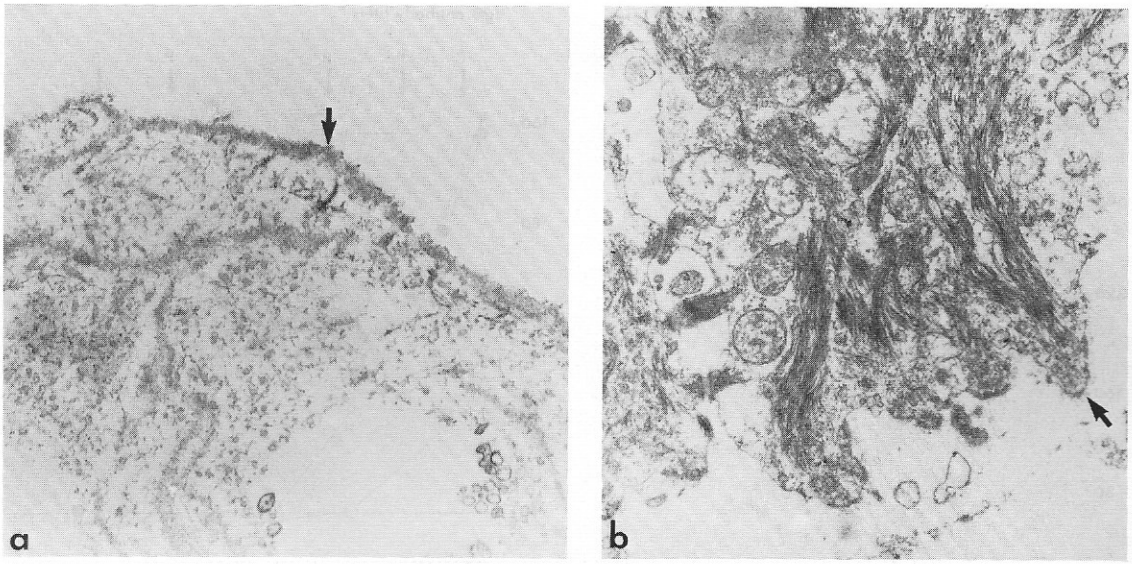

Fig. 4. Electronmicroscopy of NaCl-separated human skin: (*a*) the dermal side of the split with arrow indicating the lamina densa (×14,000); (*b*) epidermal side of the split with arrow indicating basal cell plasma membrane (×18,000).

anti-BMZ IgG antibodies in a small series of patients with BP.

Staining of the split skin with anti-laminin antibodies indicated that the cleavage occurred through the lamina lucida, leaving laminin at the dermal side of the split. This was confirmed by electron microscopy, showing that the cleavage occurred through the lamina lucida.

Variation in BP antibody reactivity with various substrates has previously been described (3, 7, 8). The highest diagnostic sensitivity of the IIF technique was observed when using monkey and guinea pig esophagus as substrate (7). When human skin is employed as substrate, the results are influenced by the site from which the skin is obtained, due to regional and individual variations in the expression of the BP antigen. Flexor surfaces, as used in our study, have been shown to have a high amount of BP antigens (8). When employing split skin as substrate, a higher diagnostic sensitivity was demonstrated, than when using intact skin: on split skin, anti-BMZ IgG antibodies were detected in 17 of 20 (85%) sera from patients with clinically and histiologically verified BP, compared with only 10 of 20 (50%) sera, when intact skin was used (3).

In our study the difference in sensitivity between the two substrates was quantified by means of serum titrations. In all patients examined, the split skin technique yielded higher antibody titres: three-fold higher titres were found in 8 of 12 and 2-fold higher titres in 4 of 12 cases. Our results indicate that NaCl-treatment of the substrate exposes or chemically modifies the BMZ antigens, so that they can react more avidly with autoantibodies in the patient's sera. The higher sensitivity was obtained apparently without loss of diagnostic specificity, since none of the normal sera became positive on split skin.

A lack of correlation between the pemphigoid antibody titres and clinical activity has previously been described (9, 10). In contrast to this, we observed a significant reduction in the IgG anti-BMZ antibody titres in the 2 patients with BP, undergoing plasmapheresis, when using split skin as substrate. Furthermore, the reduction in antibody titres correlated with remission of the disease activity. By contrast, IIF on intact skin was inefficient in monitoring the treatment by measurement of circulating anti-BMZ antibodies.

Based on these preliminary observations, we conclude that the use of split skin as substrate in the IIF technique may be useful in the diagnosis of bullous diseases. Apart from giving diagnostic information regarding the localization of the antigens in the BMZ, the method has a higher diagnostic sensitivity. However, a precise estimation of the diagnostic specificity and sensitivity requires larger study poulations and studies of other patient groups as well.

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Clonidine-induced Immune Complex Disease

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We report upon a 46-year-old woman, who developed immune complex disease after treatment with clonidine for one year. The diagnosis was verified with histological demonstration of IgG and IgM complexes as well as complement C1q, C3c and C4 between muscle fibres and at the dermo-epidermal junctions. The patient's symptoms abated and the abnormal results of blood tests reverted to normal following cessation of clonidine therapy.

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Local deposition of circulating immune complexes or complex formation "*in situ*" may lead to inflammatory injury to the tissue, i.e. immune complex disease (ICD) (1). Drug-induced ICD is rare, but it has been reported following treatment with hydralazine, procainamide, α-methyldopa or chlorthalidone (2, 3). Clonidine is an α_2-receptor stimulating agent used for treatment of hypertension, prophylaxis of hemicrania, and treatment of menopausal flushes. The most common adverse effects are drowsiness and dryness of the mouth (4). In this report we present a case of clonidine-induced ICD.

CASE REPORT

The patient is a 46-year-old woman, who was formerly completely healthy. Due to menopausal flushes she had been treated with tablets of clonidine 25 μg twice a day for one year. During the last 9 months prior to admission, she had noticed an increasing depigmentation of the skin of her right upper extremity, including diffuse swelling of the forearm and tingling in the first, second and third fingers. Similar, though less distinct symptoms appeared at the same time on the left arm.

The physical examination of the right arm showed a depigmented skin area beginning on the upper arm and ending in the middle of the forearm on the ulnar side. The edema and the solid induration included the hand. The symptoms of the