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A New Marker of Epidermal Differentiation Associated with the Membrane Coating Granules: Characterization and Applications to Pathology

J. BAILLY, [1]M. DARMON, [1]L. JUHLIN[2] and M. DÉMARCHEZ[1]

[1]CIRD GALDERMA, Route des Lucioles, Sophia Antipolis, Valbonne, FRANCE and [2]Department of Dermatology, University Hospital, Uppsala, Sweden

A murine monoclonal antibody, BC12, was obtained after immunization against suprabasal human keratinocytes. In the epidermis of normal human skin, the antigen recognized by BC12 (BC12 antigen) is located at the apex of keratinocytes in the upper stratum spinosum and stratum granulosum but is absent in other layers. The BC12 antigen is also present in hair follicles. Immunoblotting performed on keratinocyte subpopulations confirmed the presence of the BC12 antigen in differentiated keratinocytes only. Two-dimensional immunoblotting showed that the BC12 antigen corresponds to a set of polypeptides with an apparent molecular weight of approximately 33kD. In keratinocyte cultures, the antigen is present only in stratified areas. The distribution of the BC12 antigen, as studied by indirect immunofluorescence and immunoelectron microscopy, and its presence in certain subcellular fractions of epidermal cells suggest that it is a component of membrane coating granules (MCGs) or that it is associated with these structures. Strikingly, in psoriasis, eczema and many other diseases, the BC12 antibody does not label the epidermis, but vessels in dermal papillae. The BC12 antibody may thus be a useful tool in the study of keratinocyte differentiation and MCG physiology, and, also, in pathology. ***Key words:*** **Monoclonal antibody; keratinocyte.**

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M. Démarchez, CIRD Galderma, 635, Route des Lucioles, BP 87, 06902 Sophia Antipolis Cedex, Valbonne, France.

The architecture of the epidermis reflects the time course of keratinocyte differentiation since the successive layers of this stratified epithelium (basal, spinous, granular and cornified) are formed by cells which progress along the various stages of differentiation. At the same time as they migrate outwardly, four main changes, characterized both by morphological and biochemical criteria, occur sequentially: 1) Specific additional keratins, K1 and K10, appear as soon as keratinocytes detach from the basement membrane (1–3); 2) Membrane coating granules (MCGs) appear in the upper stratum spinosum (4, 5); 3) Keratohyaline granules containing filaggrin appear in the stratum granulosum (6, 7); 4) The formation of cornified envelopes from previously synthesized precursors takes place in the upper stratum granulosum (8, 9).

Membrane coating granules (also named lamellar granules, Odland bodies, cementosomes or keratinosomes) are cytoplasmic rounded organelles present in the cells forming the upper layers of epidermis. They are assembled within the keratinocytes of the upper stratum spinosum and stratum granulosum and are then translocated to the apex and periphery of the cells. Eventually, at the interface between the stratum granulosum and the stratum corneum, they fuse with the plasma membrane and excrete their contents into the intercellular spaces. This excretion is thought to play an important role in the barrier function of the epidermis and in desquamation (4, 10–12).

One approach to study epidermal differentiation is to produce monoclonal or polyclonal antibodies against stage-specific markers. Such antibodies have been produced for keratins (13–16), keratohyaline granules and filaggrin (17), cornified envelopes and their precursors, involucrin and loricrin (18, 19), and recently against MCGs (20).

In the present study, a monoclonal antibody, BC12, raised against whole suprabasal keratinocytes, is described. In normal human epidermis, this antibody recognizes an antigen located in the cells of the stratum spinosum and stratum granulosum which seems to be associated with the membrane of the MCGs, and which is different from the previously described MCG-associated antigen (20) recognized by the AE17 antibody. In pathological states, BC12 was found to be a very useful and discriminative reagent.

MATERIALS AND METHODS

I) *Keratinocyte cultures*

Fresh human skin was obtained from discarded skin of healthy human subjects undergoing plastic surgery. Culture of normal human keratinocytes (NHK) was performed as previously described (21). HESV (22) and SV-K14 (23) cell lines were grown in DMEM containing 10% fetal calf serum.

II) *Immunization and production of monoclonal antibodies*

A suspension of keratinocytes prepared from human skin was incubated in culture medium for 1 h at 37°C in a Petri dish coated with type I collagen (24). The non-adherent cells, mainly of suprabasal origin, were used as immunogen either immediately or after being kept frozen in liquid nitrogen. BALB/c mice were immunized intraperitoneally (2.5×10^6 cells) and subcutaneously (2.5×10^6 cells). They were boosted five times at 3-week intervals. Fusions were performed 5 days after the last immunization. Splenocytes (6×10^7 cells) were fused with SP2O myeloma cells (2×10^7 cells) according to the general method of Kohler & Milstein (25). Ten days after the fusion, the supernatants of the wells containing hybridoma colonies were screened by indirect immunofluorescence on frozen skin sections. Twenty hybridoma colonies that exhibited interesting reactivity were selected and cloned. BC12 was isolated from 1 of these hybridoma colonies.

III) *Immunotyping and purification of immunoglobulin*

The isotype of immunoglobulins was determined by the Ouchterlony double immunodiffusion technique (Serotec, England). BC12 belongs to the IgG1 class. Immunoglobulins were purified from ascites fluid with protein A sepharose CL 4B (Pharmacia) (26).

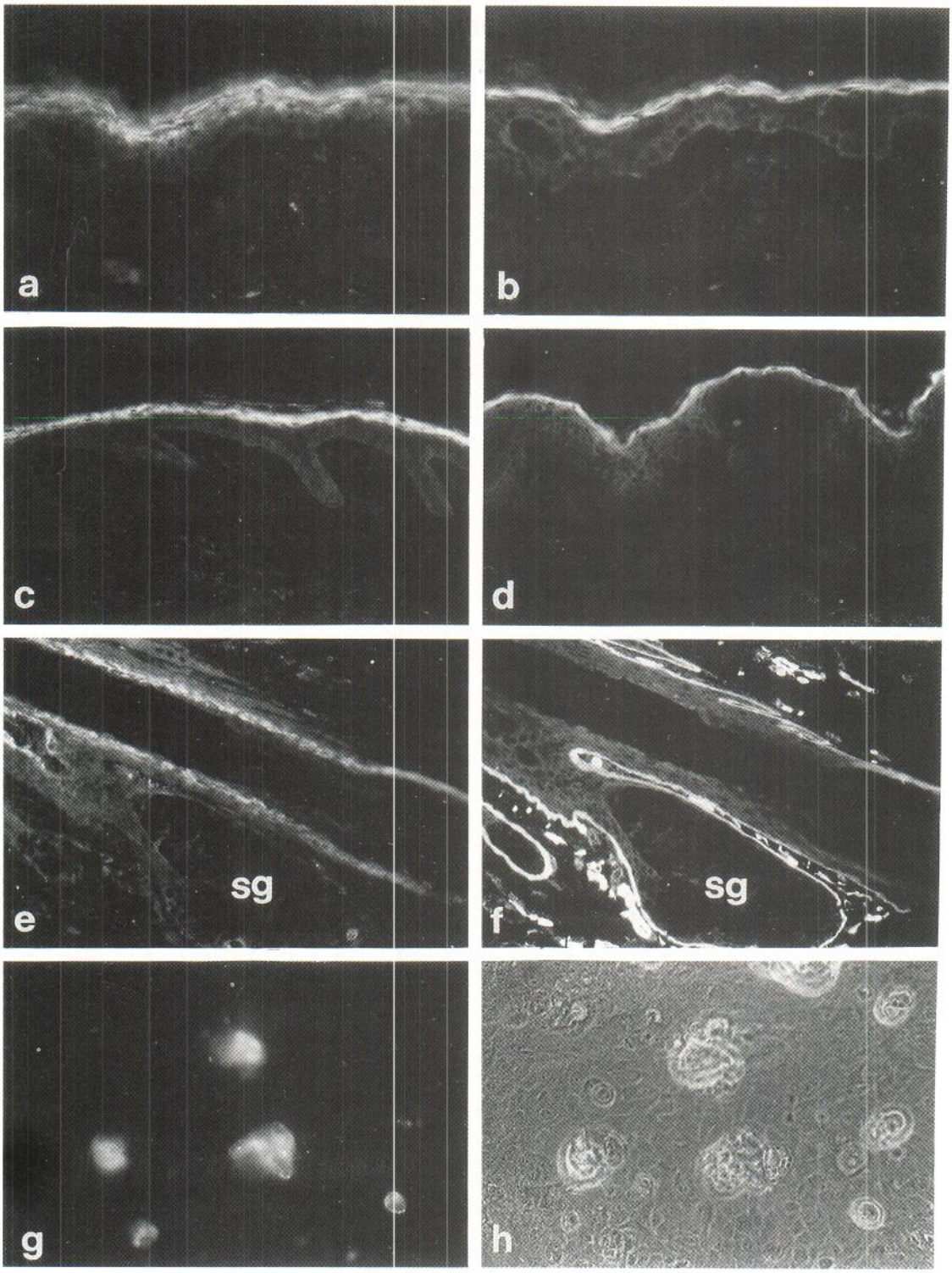

Fig. 1. Frozen sections of normal human skin stained for BC12 antigen (*1a* and *e*), involucrin (*1b*), AE17 antigen (*1c*), filaggrin (*1d*), laminin (*1f*) (× 60). Keratinocyte culture stained for BC12 antigen (*1g*); Phase contrast microphotography of the same zone as in Fig. *1g*. sg: sebaceous gland.

IV) *Indirect immunolabelling*

Human skin tissue specimens, frozen in liquid nitrogen immediately after excision, were embedded in Tissue-Tek (Miles Inc., USA). 6 µm-thick cryostat sections were cut at −20°C, air-dried and immunolabelled at room temperature by the indirect method. Sections were fixed in acetone at 4°C for 5 min, washed in PBS (phosphate buffered saline), incubated for 30 min with BC12 immunoglobulins diluted in PBS (dilution 1:100), washed with PBS and incubated with a FITC-labelled rabbit anti-mouse IgG antibody (Dakopatts A/S. Denmark; dilution 1:300). After washing in PBS, sections were mounted in 90% glycerol in PBS, containing p-phenylenediamine at a final concentration of 5mM and observed with a Zeiss photomicroscope III equipped with a III R S fluorescence vertical illuminator. For the study of skin from various dermatoses, acetone-fixed 6 µm-thick frozen sections were investigated for binding with the BC12 antibody (supernatant at

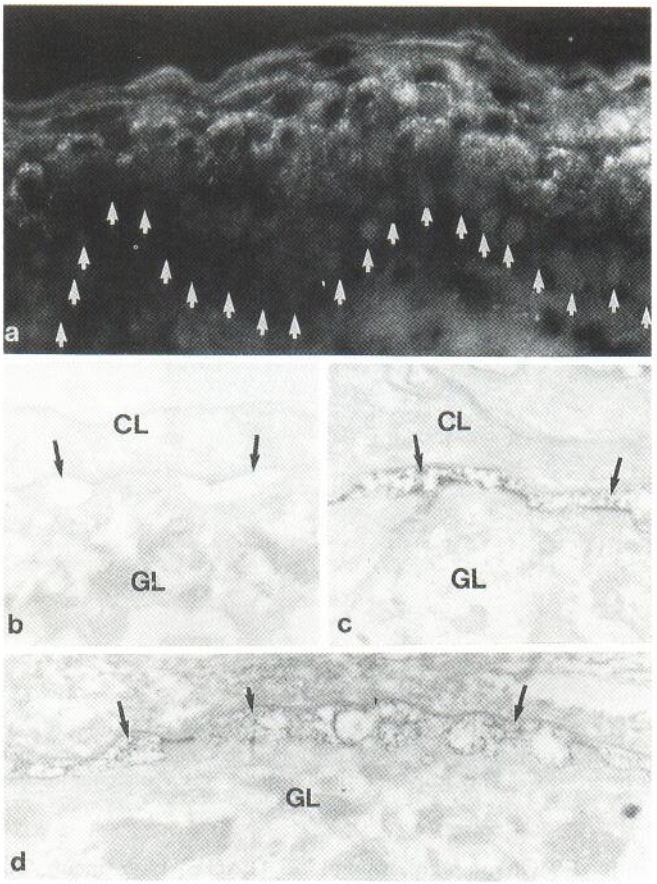

Fig. 2. Frozen section (*2a*) (×150) and ultrathin section (*2b, c* and *d*) (X 30000) of normal human skin stained with BC12 antibody (*2a, c* and *d*) or SP20 supernatant (*2b*). CL: cornified layer; GL: granular layer.

a dilution of 1/8) by using the peroxidase antiperoxidase (PAP) technique (27). Cells in culture were studied after fixation with paraformaldehyde (PFA) 3% for 5 min and staining by indirect immunofluorescence as described previously.

V) *Electron microscopy*

15 µm-thick frozen sections of human skin were fixed with 8% paraformaldehyde in PBS for 6 min, incubated with BC12 (dilution 1:100) or SP2O cell supernatant at room temperature for 1 h and were then washed in PBS. The immunoreaction was revealed using the Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, Ca., USA). Before revelation of peroxidase activity, the sections were fixed with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH4) for 30 min at room temperature. Peroxidase activity was revealed by incubation of the sections with 1% 3–3′-diaminobenzidine and 0.01% H_2O_2. After washing, the sections were postfixed in 1% osmium tetroxide for 30 min and then prepared for electron microscopy examination. Ultrathin sections were observed without counterstaining with a Temscan Jeol 1200 EX electron microscope.

VI) *Electrophoresis and Western blots*

One- and two-dimensional PAGE electrophoreses were performed as described by Laemmli (28) and O'Farrell et al. (29) respectively. Western immunoblotting was processed according to Towbin et al. (30), with modifications described by Dunn (31) as an attempt to renature the proteins before transfer onto nitrocellulose. Two-dimensional immunoblots were counterstained with India ink (32).

VII) *Isolation of keratinocyte subpopulations*

Three subpopulations were isolated for immunochemical studies: basal cells, spinous cells and a mixture of granular and horny cells (24). To this end, a total suspension of NHK was prepared from freshly obtained surgical skin samples (33). The pool of granular and cornified layers, not dissociated by trypsin, were isolated by filtration on gauze during the preparation of the cell suspension of normal human keratinocytes (24). The basal cells were obtained by panning of the dissociated suspension on type I collagen (24). Cells which do not attach after panning are mainly spinous cells (24).

VIII) *Partial purification of BC12 antigen*

In an attempt to purify the antigen recognized by BC12, purification of MCGs was performed according to Freinkel & Traczyk (10). Briefly, fresh human skin samples were frozen in liquid nitrogen, and then incubated in a water bath at +56°C for 75 s. Thereafter, the epidermis was separated from the dermis with forceps, homogenized in a Potter homogenizer at +4°C in 0.25 M sucrose supplemented with protease inhibitors. The homogenate was centrifuged at 700 g for 15 min. Subcellular fractions were prepared by sequential differential centrifugation at 3,000 g, 17,000g and 100,000 g for 20, 30 and 60 min. The MCGs were concentrated in the 17,000 g pellet. Each pellet and the 100,000 g supernatant were extracted in Laemmli sample buffer (28) at 95°C for 2 min and analyzed by Western blotting as previously described.

RESULTS

I) *Indirect immunofluorescence*

The BC12 antibody was found to label human skin (Fig. 1), but not mouse, rabbit, rat, pig or chicken skin (data not shown). For comparison, the localization of the BC12 antigen (Fig. 1a), involucrin (Fig. 1b), AE17 antigen also associated to MCGs (Fig. 1c), and filaggrin (Fig. 1d) is shown in normal human skin. In the epidermis, BC12 stains cytoplasmic granules in the spinous and granular cells (Fig. 1a). In spinous cells, the labelled granules seem to be located above the nucleus in the apical part of the cytoplasm. The resulting image has a characteristic bridge shape (Fig 2a). In the granular cells, the staining is also cytoplasmic and apical but matches the elongated shape of the cells. Moreover, in some areas, the

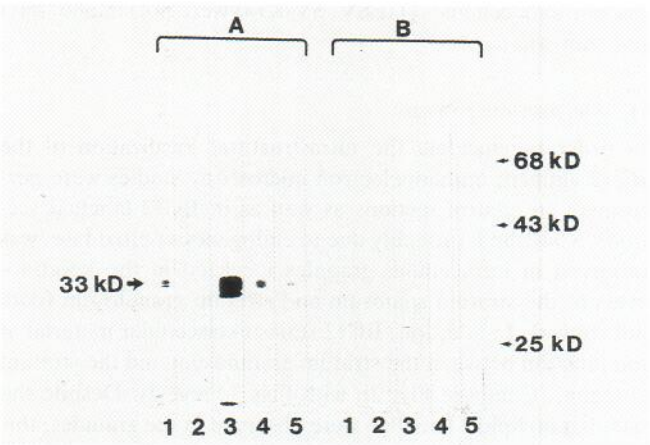

Fig. 3. Immunoblotting of protein extracts obtained from keratinocyte subpopulations.
a) protein extracts incubated with BC12 antibody; lane 1: whole normal human skin; lane 2: basal keratinocytes; lane 3: spinous keratinocytes; lane 4: pool of granular and horny layers; lane 5: cultured normal human keratinocytes.
b) same protein extracts incubated with SP_2O supernatant.

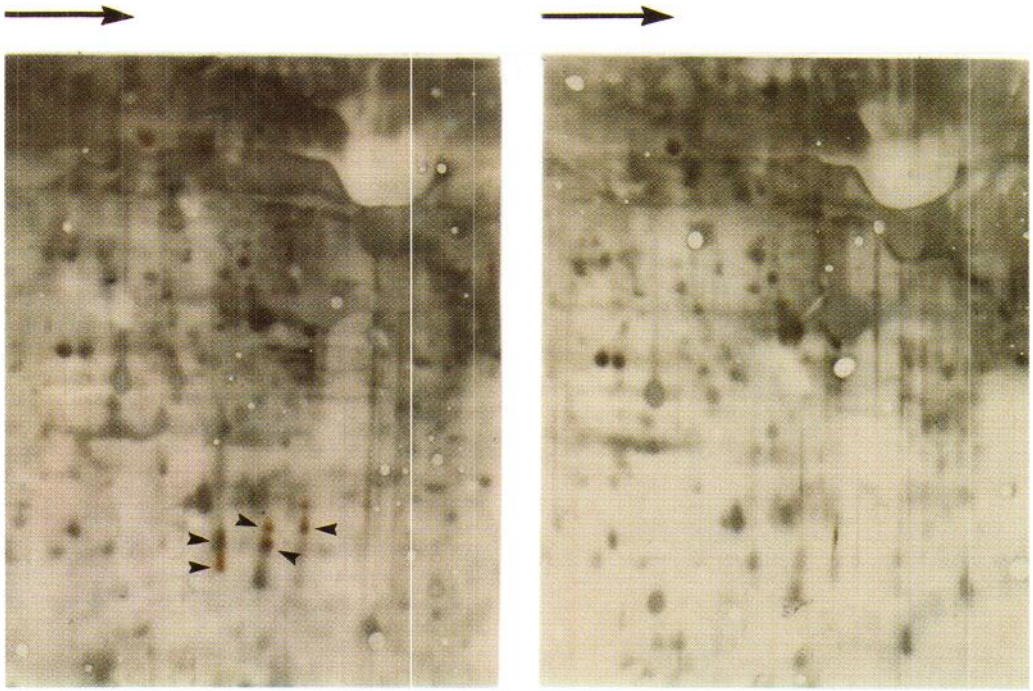

Fig. 4. Two-dimensional immunoblotting of total epidermis protein extract incubated with: *left*: BC12 antibody; *right*: SP_2O supernatant. Black coloration corresponds to the protein pattern (India ink staining). Brown coloration corresponds to the specific immunostaining. The individual spots marked by arrowheads are in the 33kD MW range with pi ranging from 6 to 7. The arrows on the top indicate the direction of the isoelectrofocusing pH gradient.

staining seems to be reinforced at the level of plasma membranes, especially in the granular layers. The staining disappears at the junction between the stratum granulosum and the stratum corneum. It must be noted that a very strong staining of the corresponding zone of the hair follicle is also observed (Figs. 1e and f). Basal cells of the sebaceous glands are also labelled (Fig. 1e).

In normal human keratinocyte cultures, staining is only observed in foci of cells corresponding to stratified areas of the cultures (Figs. 1g and h). Poorly differentiated transformed keratinocyte cell lines (HESV, SV-K14) were not stained (data not shown).

II) *Electron microscopy*

In order to elucidate the ultrastructural localization of the BC12 antigen, immunoelectron microscopy studies were performed. In control sections as well as in BC12 labelled sections, a labelling, probably due to endogenous peroxidase, was observed in intracellular granules localized in the keratinocytes of the stratum spinosum and stratum granulosum (data not shown). In addition, BC12 stains extracellular material at the junction between the stratum granulosum and the stratum corneum (Compare Fig. 2b with Figs 2c and d). Despite the fact that no lipidic lamellae were observed in the granules, this distribution of BC12 antigen strongly suggests that it is associated with MCGs.

III) *Immunoblotting*

A partial renaturation (31) of the epidermal proteins was necessary for detecting any immunoreactivity of the BC12 antibody. On one-dimensional gel (PAGE) blots incubated with BC12, several bands (one major and one or two minor) of an apparent molecular weight (MW) of approximately 33 kD were labelled. This reaction was obtained both with total epidermal protein preparations (Fig. 3a, lane 1) and with proteins extracted from cultured NHK (Fig. 3a, lane 5). In order to confirm the specific distribution of the BC12 antigen in the spinous and granular layers, immunoblotting was per-

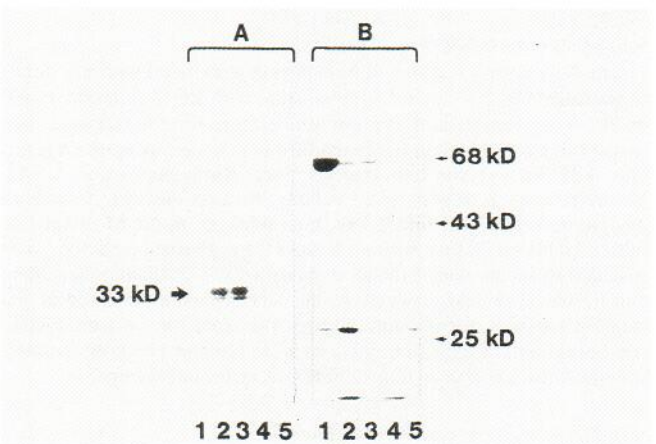

Fig. 5. Immunoblotting of protein fractions obtained by differential centrifugation (partial purification of MCGs).
a) protein extract fractions incubated with BC12 antibody; lane 1: 100,000 g supernatant; lane 2: 100,000 g pellet; lane 3: 17,000 g fraction; lane 4: 3,000 g fraction; lane 5: 700 g fraction.
b) same fractions incubated with BC10, a monoclonal antibody of the same isotype (our unpublished results) which recognizes a 68kD antigen and which was used as a control.

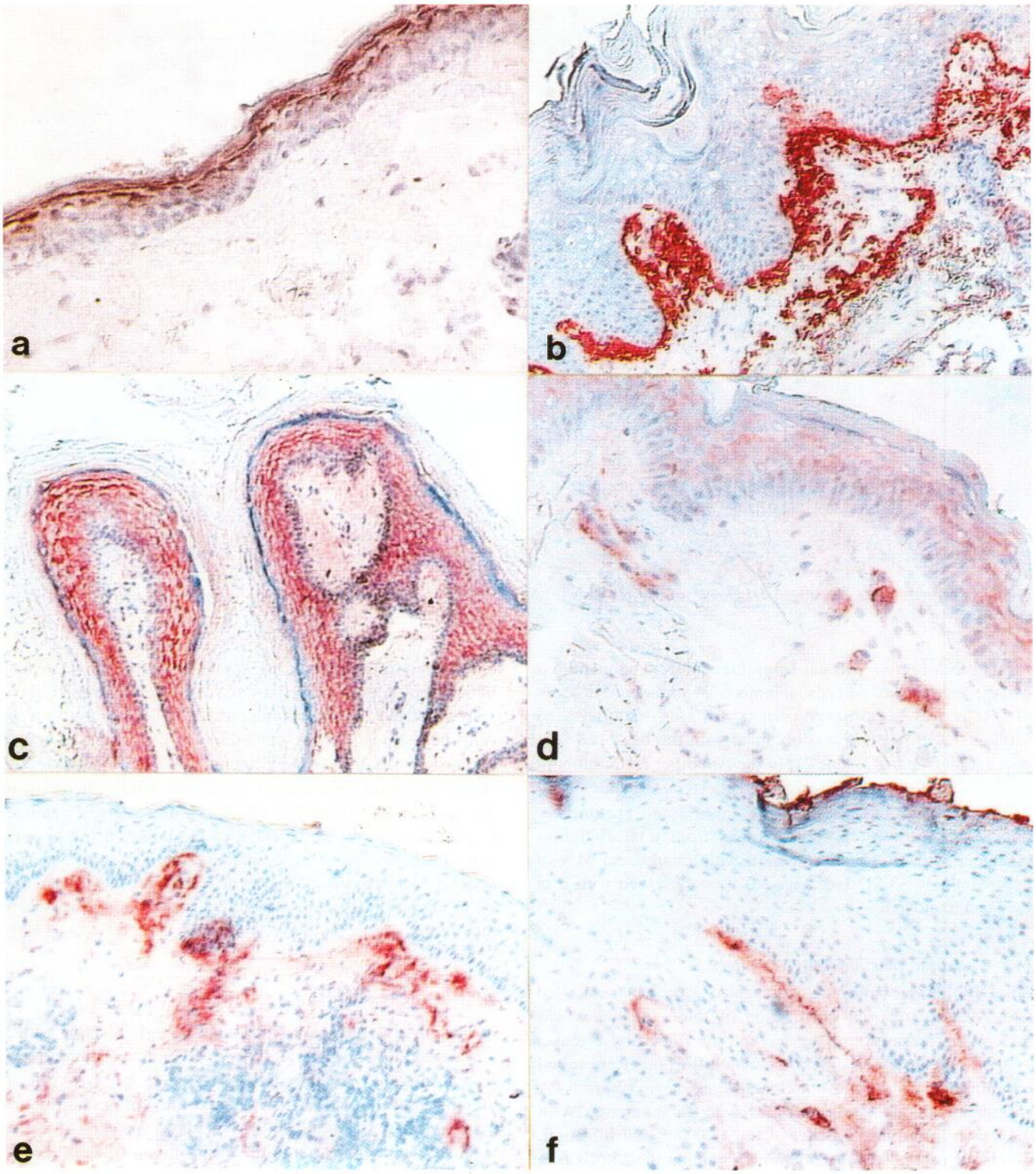

Fig. 6. Expression of BC12 antigen in dermatological diseases.
a) normal skin (× 62): upper spinous cells are labelled; *b*) actinic keratosis (× 31): epidermis is not labelled, but the vessels in dermal papillae stain intensely;
c) seborrheic keratosis (× 31): epidermis is strongly stained, but not dermis;
d); urticaria (× 62): epidermis and the vessels in dermis are moderately stained.
e) lichen planus (× 31): epidermis is not stained, but the vessels in dermal papillae are stained; *f*) prurigo nodular (× 31): epidermis is not stained but the vessels in dermal papillae are stained intensely.

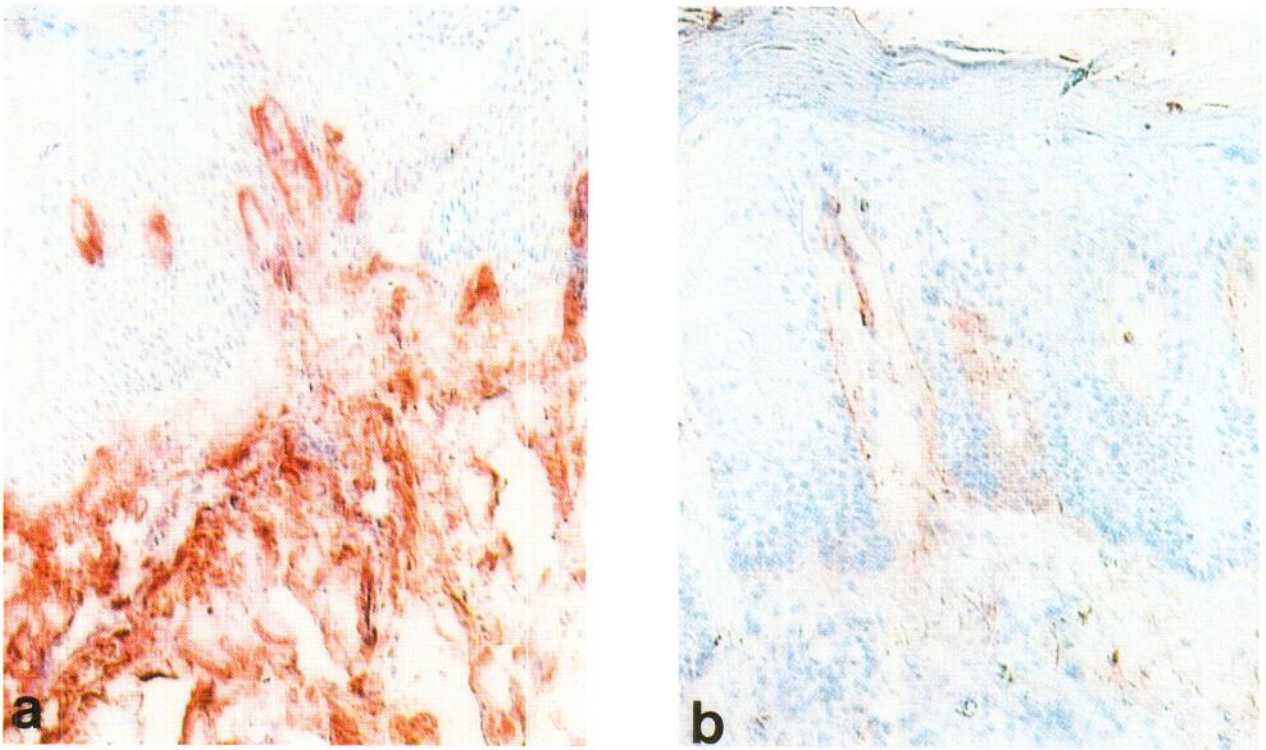

Fig. 7. Expression of BC12 antigen in eczema (*a*) and psoriasis (*b*) (×31). Epidermis is not labelled but the vessels in the dermal papillae are stained.

formed with protein extracts from three NHK subpopulations corresponding to basal cells, spinous cells and a pool of granular cells and horny layer corneocytes (see Materials and Methods). No reaction was obtained with the basal cell subpopulation (see Fig 3, lane 2). In the two other NHK subpopulations, BC12 antibody recognized the typical bands of apparent MW of 33 kD (see Fig. 3a, lanes 3 and 4). On two-dimensional blots of protein extracts from total epidermis incubated with BC12, several spots with an apparent MW of approximately 33 kD (see Fig. 4a) were observed with a pi between 6 and 7.

IV) *Partial purification of BC12 antigen*

Since BC12 was believed to recognize a protein associated with MCGs, a partial purification of these organelles was undertaken according to the method developed by Freinkel and Traczyk (10). The different isolated fractions were tested by immunoblotting after one-dimensional electrophoresis. A strong reaction with several bands having an apparent MW of 33kD was found in the pellet of the 17,000 g centrifugation, which corresponds to the fraction containing the MCGs (see Fig. 5a, lane 3). A similar reaction was observed in the 100,000 g pellet (see Fig. 5a, lane 2), which contains membrane material. No reaction was seen in the other fractions (Fig. 5a, lanes 1, 4 and 5).

V. *Reactivity of BC12 antibody in dermatological diseases*

As shown in Figs. 6, 7 and Table I, the reactivity of BC12 antibody in pathological states is very interesting and discriminative. In normal subjects, BC12 labels the epidermis and hair follicles but does not label any structure in the dermis.

In seborrheic keratosis, epidermal labelling is more precocious and stronger in the epidermis. In the lesions of actinic keratosis, psoriasis, various eczemas, and Darier's disease, the BC12 antibody did not label the epidermis but labelled instead the vessels and surrounding structures in the dermal papillae. In psoriatic lesions, no labelling of the hair follicles was evident. In the lesions of lichen planus, pityriasis rosea, prurigo nodularis, porphyria, purpura, urticaria and melanoma, the BC12 antibody labelled both the epidermis and the vessels in the dermis.

DISCUSSION

In the present study, a monoclonal antibody reacting with a new epidermal antigen is described. By indirect immunofluorescence, it stains granules located at the periphery of cells in the upper layers of the stratum spinosum and granulosum of human epidermis. Immunoelectron microscopy confirmed this reactivity and demonstrated the localization of the antigen in cytoplasmic and membrane-associated vesicles with a distribution very similar to that of MCGs in human epidermis. Besides their characteristic cellular and tissue distribution, another criterion allowing the identification of MCGs might be the observation, by electron microscopy, of intact lipidic lamellae in the granules. However, despite several modifications of the processing used for preparing the samples, preservation of MCG lipidic lamellae was not obtained in our immunoelectron microscopy studies.

Table I. *Staining with BC12 antibody in normal and diseased skin*

Diagnosis	Number of subjects	Epidermis	Vessels in dermal papillae	Hair follicles
Normal skin	10	+	–	+
Actinic keratosis	2	–	++	ns
Alopecia areata	3	+	–	+
Alopecia (androgen)	1	+	–	+
Atopic dermatitis	3	–	+	+
Basal cell carcinoma	4	+	–	+
Cystic fibrosis	1	+	–	ns
Darier's disease	2	–	++	+
Eczema	4	–	+	+ (one)
Erythema annulare	2	+	–	–
Erythema multiforme	1	+	–	seb.gland pos.
Exanthema (penicillin)	1	+	+	+
Lichen planus	5	–	++	ns
Mastocytoma	1	+	–	ns
Melanoma	2	(+)	+	ns
Mycosis fungoides	7	+	–	+
Naevus (benign)	3	+	–	ns
Naevus (dysplastic)	2	+	–	+
Keratodermia	1	+	–	ns
Ichthyosis vulgaris	1	+	–	+
Pityriasis rosea	1	+	+	ns
Porphyria cutanea tarda	1	+	+	ns
Prurigo nodularis	2	(+)	+++	+
Purpura	1	+	+	ns
Psoriasis (plaque)	8	–	+(+)	+ (one)
Seborrheic keratosis	4	++	–	ns
Squamous cell carcinoma	2	+	–	+
Urticaria (acute)	2	+	+	+
Urticaria (chronic)	4	+	+	+
Urticaria (cold)	2	+	+	+
Urticaria (pressure)	2	+	+	+
Verruca vulgaris	2	+	–	ns

ns: no follicles seen

Recently O'Guin et al. (20) described two monoclonal antibodies, AE17 and AE18, which recognize a basic 25kD protein associated with MCGs displaying, as studied by IIF and electron microscopy, a localization very similar to that of BC12 antigen. However, our immunoblotting data revealed that the BC12 antigen (apparent MW 33kD and pi 6–7) is different from the basic 25kD protein reacting with AE17 and AE18.

A partial purification of MCGs was performed according to the method of Freinkel & Traczyk (10). Immunoblotting of the different fractions obtained by this technique showed that the BC12 antigen is present in two fractions, the 17,000 g pellet expected to contain MCGs, and the 100,000 g pellet corresponding to the membrane fraction. This result suggests that the BC12 antigen is localized in the MCG membrane. Interestingly, it was observed that treatment of frozen sections of human skin with a mixture of methanol/chloroform (2:1) increased the staining obtained with BC12 by indirect immunofluorescence (data not shown). Thus, the BC12 antigen might be associated with lipid structures partially masking the antigen.

The study of a large panel of dermatological diseases suggests that BC12 might be a very interesting reagent for pathology. The staining in upper epidermal layers is a landmark of terminal differentiation and is not present in several diseases in which upper layers are altered. The staining in hair follicles is absent in a larger number of pathological conditions. The most striking observation, though, is the appearance of staining with BC12 in (or around) vessels of dermal papillae in several diseases with an inflammatory component. We do not know whether the antigen recognized in the dermis is similar to the BC12 epidermal antigen, but we could speculate that endothelial or circulating cells could release antigens similar to BC12 in pathological conditions.

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