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Expression of Gastrin-releasing Peptide Receptor in Human Skin

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Bombesin-related peptides are expressed in the skin of batrachians and mammals. As gastrin-releasing peptide belongs to this family, we searched for the presence and distribution of gastrin-releasing peptide receptors (GRPr) in the skin of healthy human adults by immunohistochemistry, flow cytometry and electron microscopy.

The results indicated that GRPr are expressed on nerves and vessels in the dermis, on eccrine sweat glands, sebaceous glands and erector pili muscle. Within epidermis, staining was localized only on basal and suprabasal layer cells, or in the whole epidermis, according to the samples studied. Interestingly, suprabasal epidermal dendritic cells occasionally showed a strong labelling. Some of these epidermal dendritic cells were identified as Langerhans' cells by immunoelectron microscopy studies. Flow cytometry analysis of crude epidermal cell suspensions resulted in the expression of GRPr on about 43% of the cells.

Therefore, we investigated whether human GRPr could modulate Langerhans' cells antigen-presenting functions. For this purpose, we added increasing concentrations of GRP (10^{-12} to 10^{-5} M) to mixed epidermal cell lymphocyte reactions. Allogeneic T-cell proliferation was not significantly modified when added to GRP-pretreated epidermal cells.

In conclusion, we demonstrated the presence of GRPr in human skin, suggesting that GRP may modulate epidermal cell functions but does not modify antigenic presentation. ***Key words: neuropeptides; allogeneic T-cell response; Langerhans' cells.***

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Gastrin-releasing peptide (GRP) was first isolated from porcine gastrointestinal tract. GRP has since been characterized and cloned from rat, canine, guinea pig and human species (1). GRP is considered as the mammalian equivalent of the amphibian bombesin (BOM), because it reproduces all the biological effects of BOM, first harvested from the skin of *Bombina bombina*, an orange-coloured frog (2). Indeed, GRP shares with BOM a common amino acid sequence in the C-terminal that is necessary for its biological activity (3). Radioimmunoassays and immunohistochemistry studies localized BOM-like immunoreactivity in mammalian brain and peripheral tissues such as gastrointestinal tract and lung. In the skin, very few observations have been reported in the literature about peptides of the BOM family. Studies analysing the concentration of BOM in pig skin showed that this neuropeptide was present in low concentrations (0.6 to 1.5 pmol/g of tissue), variable with the anatomical localization (4). By contrast, immunohistochemistry techniques failed to reveal BOM expression in normal human skin (5).

In the present study, we searched for the presence of GRP receptors (GRPr) in human skin. For this purpose, normal human skin biopsies were studied by immunohistochemistry and epidermal cell suspensions were analysed by flow cytometry and electron microscopy. We found that GRPr were present in the dermis, the epidermis and especially on Langerhans' cells (LC). We examined the ability of GRP to modulate the LC allostimulating function by using mixed epidermal cell-lymphocyte reaction.

MATERIAL AND METHODS

Generation of GRPr antiserum

A peptide was synthesized (M A P N N C S H L N L D L D V D) by Néosystem (Strasbourg, France) corresponding to the N-terminal of the mouse GRPr. The peptide was conjugated to bovine serum albumin through ethyl-carbodiimide condensation for the production of polyclonal antisera from rabbit.

Skin biopsies

Eleven punch biopsies from human skin (arm, abdomen and breast) were obtained from healthy men and women (22 to 57 years old). Samples were immediately fixed in Zamboni solution (10% formalin solution, produced from 4% paraformaldehyde, containing 14% saturated picric acid) for 3 h at 4°C. Skin pieces were washed in phosphate buffer solution containing 0.3 M sucrose and 0.15 mM sodium azide for 18 h at 4°C. The skin pieces were frozen in liquid nitrogen, sectioned perpendicularly at 4 μm or 10 μm on a cryostat and processed for indirect immunohistochemistry.

Immunohistochemistry

Skin sections were incubated for 20 min with normal goat serum, then incubated overnight at 4°C in humid conditions with primary antibody (Ab) (1/100). After two washes in phosphate buffer solution, the sections were incubated for 30 min at room temperature with fluorescein isothiocyanate (FITC) – conjugated goat anti-rabbit IgG (1/50) (Zymed Labo, San Francisco, CA, USA). Sections were rinsed, mounted with Fluoprep (bioMérieux, France) and observed with a Zeiss fluorescence microscope.

Immunoenzymological staining was performed with streptavidin/biotin coupled with peroxidase (LSAB kit DAKO). Sections were counterstained with Mayer hematoxylin, mounted in Glycergel (Dako).

For negative controls, the first antiserum was replaced by antisera preabsorbed with 10 nM of the GRPr peptide (not conjugated to bovine serum albumin).

Epidermal cell suspensions

Human epidermal cell suspensions were obtained from normal skin and removed during plastic surgery. Skin samples were freed of fatty tissue and split-cut with a keratome set. The resulting skin slices were incubated for 18 h at 4°C with 0.05% trypsin (Difco Laboratories, Detroit, MI) and epidermis was separated from dermis with fine forceps. In some experiments, dispase II (2 mg/ml, Boehringer Mannheim GmbH, Mannheim, Germany) was used instead of trypsin and, in that case, epidermal sheets were cut into small pieces and floated for an additional hour at 37°C in deoxyribonuclease (DNase, 16 U/ml, Sigma Chemicals, St Louis, Mo). In both cases, epidermal fragments were pooled in HBSS (Gibco) supplemented with 10% fetal

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calf serum. Single epidermal cell suspensions were obtained by repeated pipetting of the epidermal sheets and filtration through sterile gauze.

Enrichment and purification of LC from epidermal cell suspensions was achieved by three consecutive gradient centrifugations on Lymphoprep (Flobio SA, Courbevoie) for 20 min at 400 X g as previously described (6). After each centrifugation, the cells at the interface were washed and counted. Routinely, they contained 30–50% LC after the second centrifugation step and more than 75% LC after the third one.

Flow cytometry

All staining procedures were carried out on ice. Phosphate buffer containing 1% bovine serum albumin and 0.1% sodium azide was used for Ab dilution and washing.

Five suspensions of crude epidermal cells from different donors were studied. The cells were pelleted by centrifugation (400 g for 10 min), incubated with normal goat serum, washed and incubated with primary Ab for 1 h at 4°C. After two washes, cells were incubated for 30 min with FITC goat anti-rabbit IgG (1/50) (Zymed Labo, San Francisco, CA, USA).

As a negative control, normal rabbit serum was used in place of anti-GRPr. Cells were subsequently washed and fixed in 1% paraformaldehyde.

Suspensions were analysed using a FACSTAR (Type IV, Becton Dickinson). Data acquisition was triggered by cell size (forward versus 90° light scatter) in order to eliminate cell debris. For each assay, the value of the fluorescence intensity corresponded to the mean fluorescence intensity. The percentage of positive cells was determined by comparison with the negative control. The statistics were calculated using over 10^4 cells.

Immunoelectron microscopy

Enriched LC suspensions were incubated with anti-GRPr Ab diluted at 1/100 for 1 h at 4°C and then stained with 10 nm-gold labelled goat anti-rabbit IgG (H + L) for 1 h on ice. Controls were performed by replacing the first antibody by normal rabbit serum. Cell suspensions were fixed with 2% glutaraldehyde and processed for electron microscopy analysis. LC were identified by the presence of cytoplasmic Birbeck granules, and the number of gold granules along the cell membrane was counted. Results are expressed as the number of particles per 100 μm of cell membrane.

Isolation of T-cells

Mononuclear cells were obtained from peripheral blood of healthy donors by centrifugation on Ficoll-Hypaque (Pharmacia). Cells were layered on plastic Petri dishes for 2 h at 37°C in the culture medium. Nonadherent cells were harvested and T-cells were obtained after rosetting with 2-amino-ethylisothiouronium bromide hydroxybromide-treated sheep red blood cells. Residual accessory cells were eliminated by a further adherence to plastic for 1 h at 37°C. Preliminary experiments indicated that contamination by monocytes in the resulting T-cell population was below 1% and that the T-cells were unable to proliferate in response to ConA (5 μg/ml, Sigma).

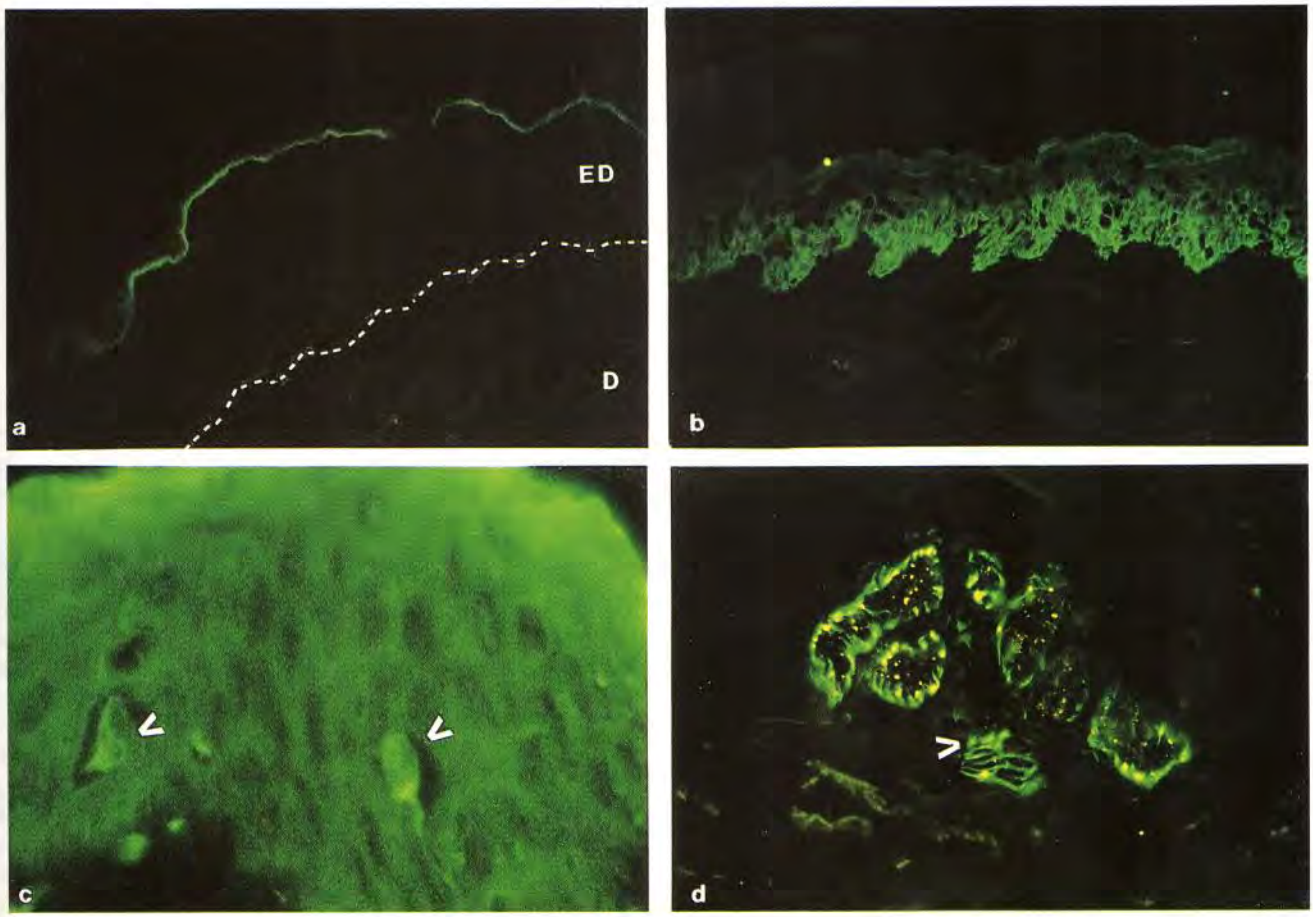

Fig. 1. Sections were processed for immunofluorescence detection of gastrin-releasing peptide receptors (GRPr). (*a*) No positive immunoreactivity was found in any of the control sections obtained with preabsorbed antiserum (×25). (*b*) Moderate to strong GRPr immunoreactivity in the basal and suprabasal epidermal layers (×25). (*c*) A diffuse GRPr immunoreactivity was observed in all the layers of the epidermis of some patients (*c*), as well as in some dendritic suprabasal cells (*c*; *arrow-head*) (×150). In the dermis, the secretory loops of sweat glands and a nerve fibre display a strong GRPr immunoreactivity (*d*; *arrow-head*) (×40). (*a*, *b*, *c*: 4 μm and *d*: 10 μm thick section). *ED*: epidermis; *D*: dermis.

T-cell proliferative assays

Mixed epidermal cell-lymphocyte reaction was carried out in U-bottom microtitre plates. The culture medium was RPMI-1640 (Sigma), supplemented with 10% human Ab serum and antibiotics. Purified LC (10^3 cells/well) were preincubated for 3 h at 37°C in the presence, or not, of varying concentrations of GRP (Sigma) before adding the allogeneic T-cells (10^5 cells/well). In three independent experiments, LC suspensions were cultured for 2 days in culture medium supplemented, or not, with GM-CSF (200 U/ml), then washed, enumerated and used in the proliferative assays.

After 5 days at 37°C, the cultures were pulsed with 1 μCi of [^{3}H] methylthymidine (1 Ci/mmol, Amersham, France). The cells were harvested 18 h later onto fiberglass filters and processed for liquid scintillation counting. Results are expressed as the mean cpm ± SD of triplicate cultures.

RESULTS

Immunohistochemistry

Results showed expression of GRPr in normal human skin. In the epidermis, immunoreactivity to GRPr was observed only on basal and suprabasal keratinocytes (Fig. 1b) (7 samples) or in the whole epidermis (4 samples) with a strong intensity. These two kinds of stainings could not be related to age, sex or to the region from which the biopsies were obtained. Occasionally, we could detect a very strong immunoreactivity on suprabasal cells, sometimes showing a dendritic morphology (Fig. 1c).

In upper and lower dermis, moderate to strong GRPr staining was found on isolated nerve fibres and nerve trunks, sometimes running beneath the epidermis. The blood vessels, independent of their size and situation in the dermis, showed a strong GRPr reactivity. The papillary capillary loops of the superficial vascular plexus were positive. Arterioles showed GRPr labelling on both endothelial cells and muscular layer.

Eccrine sweat glands were strongly stained with anti-GRPr (Fig. 1d). We observed that the secretory coils of sweat glands were strongly stained. Sebaceous glands and hair follicles were moderately stained with GRPr Ab. The erector pili muscles were also labelled.

It should be noted that none of the structures described above were stained with antiserum previously absorbed on the immunogenic peptide or only with the second antiserum (Fig. 1a).

Flow cytometry

Labelling with anti-GRPr antibodies was carried out on five different crude epidermal cell suspensions obtained after trypsin treatment. Fig. 2 illustrates a representative experiment, showing significant staining with anti-GRPr antibodies on epidermal cell suspensions obtained after trypsin treatment of skin samples. Results showed that 42.93% ± 9.1% of epidermal cells expressed GRPr. By contrast, epidermal cell suspensions were not stained by GRPr Ab, when obtained by the use of dispase/DNase. We conclude therefore that GRPr are expressed on keratinocytes. It should be noted that the staining levels were variable from one experiment to another.

Immunoelectron microscopy

Immunogold electron microscopy studies were performed using enriched LC suspensions. The labelling appeared as

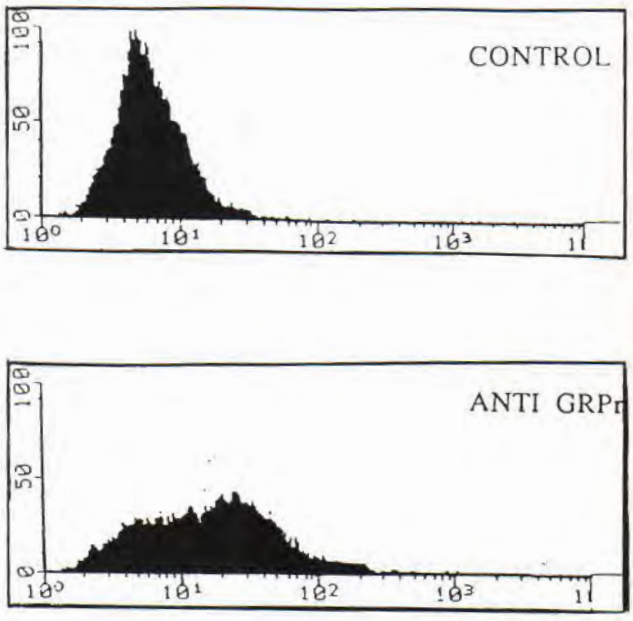

Fig. 2. Expression of gastrin-releasing peptide receptors (GRPr) on fresh epidermal cells obtained by trypsin treatment and analysed by flow cytometry. Representative histograms show fluorescence intensities of cells stained with normal rabbit serum (CONTROL) or with GRPr Ab (ANTI GRPr).

single particles in direct apposition with the plasma membrane. LC were identified by the presence of intracytoplasmic Birbeck granules. When enriched LC were obtained by trypsinization, many LC expressed GRPr. In two independent experiments, we found that 34% of LC (25 cells observed) were stained and had 10.4 to 42.9 gold particles per 100 μm of membrane, while 26% of the control cells (25 cells observed) had 2.0 to 10.0 gold particles per 100 μm of membrane (Fig. 3). Staining to GRPr was negative on enriched LC suspensions obtained by dispase/DNase treatment.

Mixed epidermal cell-lymphocyte reaction

We determined the effects of human GRP on the primary LC-induced allogeneic T-cell proliferation. To this end, purified or partly enriched LC suspensions were treated for 3 h at 37°C with GRP at different concentrations before adding the responding allogeneic T-cells. Seven independent assays carried out with different donors showed that GRP at 10^{-12} to 10^{-5} M did not significantly affect T-cell proliferation. The same results were observed using LC cultured for 2 days with GM-CSF.

DISCUSSION

In this report, we demonstrated the presence of GRPr in normal human skin. GRPr were observed on different structures in the dermis, including sweat glands, sebaceous glands, erector pili muscle, hair follicles, blood vessels and nerves. Although GRPr on human sweat glands were not reported in previous studies, GRP has already been shown in exocrine glands (the Harderian gland) of the frog *Rana esculenta* (7). The role of GRP in sweat gland function remains to be defined. However, it could be hypothesized that GRP might

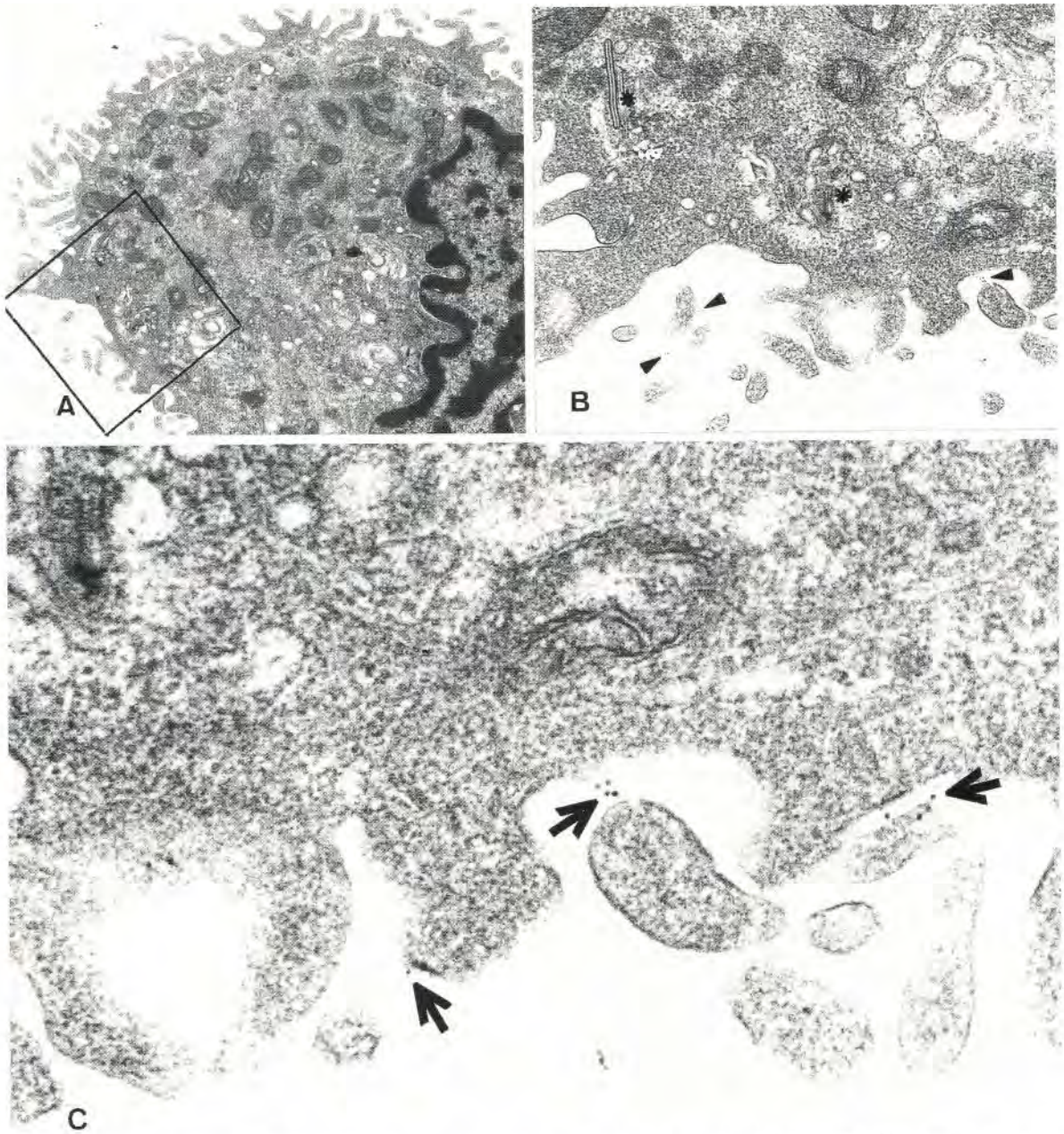

Fig. 3. Immunoelectron micrograph of gastrin-releasing peptide receptors positive Langerhans' cells (LC) (A). B and C are successive enlargements of the framed area in A. The labelling appears as single particles in direct apposition with the plasma membrane (*arrows*). LC were identified by the presence of intracytoplasmic Birbeck granules (denoted in B by an *asterisk*). Scale bars: A: 1 μm; B and C: 0.1 μm.

control sweat secretion and be involved in cutaneous thermoregulation, since different authors reported that some BOM-related peptides have potent effects on thermoregulation (8). We showed that the receptor for GRP is expressed on erector pili muscle and blood vessels. As it is known that GRP or BOM acts on the contraction of smooth muscles (3), GRP may modulate pili erection and blood flow by modulating vascular motility.

We found a specific expression of GRPr in the epidermis on both keratinocytes and LC. In all cases, the receptors were localized on the cell surface. We determined by cytofluorometry that only keratinocyte suspensions obtained by trypsinization expressed GRPr. By contrast, GRPr expression was never found when dispase/DNase was used to prepare the epidermal cell suspensions. Thus, GRPr seem to be dispase/DNase-sensitive. Although GRPr staining was observed in keratinocyte suspensions isolated by the means of trypsin, it is possible that cleavage of GRPr may still occur with longer incubation times.

The functional significance of the presence of GRPr on keratinocytes must be clarified, especially by investigating its effects on keratinocyte proliferation. Indeed, GRP/BOM have been implicated in the proliferation and DNA synthesis of various types of cells, including Swiss 3T3 cells (9) and tumour cells (10). Since most GRPr are localized in the proliferative basal and suprabasal layers of epidermis, they may be involved in the growth phase of keratinocytes. Experiments are in progress to analyse the effects of GRP on in vitro keratinocyte

proliferation. The presence of neurotransmitter receptors on epidermal cells has been reported only recently. Receptors for acetyl choline (11), vasoactive intestinal polypeptide (12) and substance P (13) have been identified on human keratinocytes. These signal substances can be released from nerve fibres entering the epidermis and contacting keratinocytes by membrane-membrane apposition (14). However, neurotransmitters could also be secreted from the keratinocytes themselves, as demonstrated for proopiomelanocortin (15) or acetylcholine (11). Whether GRP is released in human skin requires further investigation.

An interesting finding in this study is the presence of GRPr on human epidermal LC. Several observations have suggested interactions between the immune system and the nervous system. Indeed, it has been demonstrated that calcitonin gene-related peptide can inhibit murine LC antigen-presenting function (16).

It has been demonstrated that GRP has receptors and effects on Ag-presenting cells such as macrophages or monocytes (17, 18). GRP was shown to increase the phagocytic function of murine monocytes and macrophages (17, 18) and to decrease their capacity to support concanavalin A-mediated T-cell proliferation (19). All of these results suggest that GRP may regulate the Ag-presenting capacity of LC. We failed here, however, to demonstrate significant effects of GRP on human LC allostimulatory function. The use of trypsin can impair the functional part of GRPr without modifying the immunoreactive part. We therefore assumed that LC might recover GRPr on the cell surface after a 2-day culture with GM-CSF. We found, however, that allogeneic T-cell proliferation induced by cultured LC was not altered by addition of GRP at 10^{-12} M to 10^{-5} M.

In conclusion, this study demonstrates the presence of GRPr in normal human skin, in both keratinocytes and LC. Although GRP does not modify antigenic presentation functions of LC, it may be involved in other epidermal cell functions, including keratinocyte proliferation. The development of certain cutaneous diseases such as psoriasis (hyperproliferative keratinocytes) seems to be related to the nervous system and has been associated with abnormal neuropeptide regulation (16). Further studies are needed, however, to assess the role of this neuropeptide in normal as well as pathological conditions.

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