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STUDIES ON THE PLASMA MEMBRANE OF NORMAL AND PSORIATIC KERATINOCYTES

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
IN DE WISKUNDE EN NATUURWETENSCHAPPEN
AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF.DR.J.H.G.I.GIESBERS
VOLGENS BESLUIT VAN HET
COLLEGE VAN DEKANEN IN HET
OPENBAAR TE VERDEDIGEN OP
DONDERDAG 28 JUNI 1984
DES NAMIDDAGS OM 4 UUR

DOOR

JOAN MARIA GOMMANS
GEBOREN TE NIJMEGEN

kmps repro meppel

1984

De geschiedenis van een reis
een onderzoeker en zijn onderzoek
ondernemen deze.
Kijk met aandacht naar het gedrag
van deze twee.
Vindt het bevreemdend, ook al
is het niet vreemd.

(Vrij naar: de uitzondering en de regel, leerstuk van Bertolt Brecht, 1930).

Aan allen, die op enigerleiwijze hebben bijgedragen aan het tot stand komen van dit proefschrift, mijn oprechte dank.

The present investigations have been carried out in the Department of Dermatology, Catholic University, Nijmegen, The Netherlands with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O., grant 95-90).

CONTENT

Studies on the plasma membrane of normal and psoriatic keratinocytes

INTRODUCTION

The skin is an organ with an unusual shape which encloses and supports all other organs of the body. The outer part of the skin is the epidermis which protects the body against the environment. The physical barrier itself is formed by the horny layer, which is the end product of the epidermis. Apart from the keratinocytes, whose task is the production of the horny layer, some other cell populations are also present in the epidermis, there are 2-5% Langerhans cells which have an immunological function and probably, in addition, play a regulatory role. In the basal layer of the epidermis are 2-5% melanocytes which protect against radiation by injecting keratinocytes with melanin granules. At the same position as the melanocytes are about 1% Merkel cells, which are specialised nerve cells and designed for communication through the epidermis and some indeterminate cells, which are probably pre-Langerhans cells and/or pre-melanocytes.

But most of the epidermis (90-95%) is composed of keratinocytes which can be divided into 3 compartments. First the basal layer or germinative layer where cells divide and proliferate, second the spinal or mid-epidermal layer where cells differentiate and become incapable of dividing and finally, the granular layer, the top layer where all cell components are removed and the keratinocyte becomes a flake of keratin which is, in its turn, a part of the horny layer; this is the final product of the keratinization.

A changed and therefore often inefficient horny layer is seen in many skin diseases, for example in psoriasis where it is much thicker but less effective than in normal skin. In psoriasis the keratinization is incomplete; cell fragments such as the nucleus are found in the horny cells. Although the epidermis in psoriasis is much thicker, elongations of the dermis beneath the epidermis reach at some places nearly to the horny layer. The thickened badly keratinized horny layer is easily broken and these cracks reach the dermis resulting in an ineffective barrier function in the psoriatic lesion.

Because the horny layer is formed by the keratinocytes it is reasonable to assume that the abnormal keratinization (which occurs in the psoriatic lesion) is a reflection of a fault in the proliferation and/or differentiation of the psoriatic keratinocytes. Differentiation and proliferation are modulated by intrinsic and external factors. Intrinsic factors include the DNA structure of the species and the transcription regulation mechanisms which are determined during embryological development of the tissue. In addition, the behaviour of the cell can be modulated by a variety of external factors, the latter depend on communication via a receptor in or on the surface of the keratinocyte. Because most regulatory factors are of protein origin, the receptors have to be on the surface, the plasma membrane of the keratinocyte. In consequence, differences in the plasma membrane receptors can be reflected by differences in proliferation and differentiation.

That is why this investigation on "studies on the plasma

membrane of normal and psoriatic keratinocytes" was undertaken. The plasma membrane encircles the cell. It has, just as the skin envelops the body, to enclose all cell organelles and to protect the cell against its environment. Communication, however, must be provided for. The first suggestions about the construction of the plasma membrane were made by Danielli and Davson (1935). They postulated a bilayer of lipid, with the fatty acid chains pointing to the inner part of the bilayer and the polar heads forming the two outer leaflets of the unit-membrane in contact with the external aqueous phase. This construction is strong enough to enwrap the cell organelles but the communication through the membrane depends upon specific receptors situated in the lipid bilayer.

The receptors are mostly glycoproteins. The globular integral protein part is dispersed in a matrix of fluid lipid molecules (Singer and Nicolson, 1972). Portions of the protein molecules exposed to the extracellular space contain most of the polar amino-acid residues and also the carbohydrate moieties of glycoproteins. The portions embedded in the lipid bilayer are rich in non-polar amino-acids. The extent to which each protein integrates into the lipid interior varies and the proteins may span the width of the membrane and can thus be associated, for example, with enzymes which are situated in the cytoplasm. The "face" of a receptor is mostly determined by the sugar composition of the carbohydrate moieties of the glycoprotein. More than 85% of the glycoproteins in the plasma membrane of

the keratinocyte have an N-glycosidic linkage between the polysaccharide chain and the peptide, that is a covalent binding between the anomeric carbon of N-acetylglucosamine and the nitrogen of the amide group of asparagine. The amino acid sequence adjacent to the glycosylated asparagine is always asparaginyl-X-serine or asparaginyl-X-threonine, where X can be any amino acid. While the peptide is formed concomitantly with the formation of the polysomes, passage of the peptide begins through the membrane bilayer of the endoplasmic reticulum into the intravesicular space.

In the dolichol phosphate cycle sugars are transferred from sugar nucleotides to a lipid-linked oligosaccharide. This consists of two residues of N-acetylglucosamine, nine residues of mannose and three residues of glucose. The lipid dolichol appears to transport the hydrophilic sugars into or through the hydrophobic membrane environment of the intravesicular space of the endoplasmic reticulum. Glucose addition serves as a signal for completion and therefore transfer (Lennarz, 1983). Transfer of the lipid-linked oligosaccharide from the lipid intermediate to asparagine residues of the growing polypeptide chains probably occurs soon after these residues emerge on the inner side of the endoplasmic reticulum.

After completion of the peptide chain, the primary glycoprotein is transferred to the Golgi complex where it is trimmed by α-glucosidases and α-mannosidases to the pentamannosyl-di-N-acetylglucosamine linked to protein. Elongation of the saccharide chain occurs via specific sugar transferases and an α-mannosidase

will further trim off some mannoside residues till there remain of the primary glycoprotein at least the trisaccharide mannosyl-di-N-acetylglucosamine linked to protein. Then the saccharide chain can be elongated by sugar transferases. In human epidermis the sugars N-acetylglucosamine, L-fucose, mannose, galactose, N-acetylgalactosamine and sialic acid are found as components of the glycoproteins of the plasma membrane of the keratinocyte. The subsequent addition of galactose and N-acetylneuraminic acid form the completed complex units. The mature glycoprotein finally migrates towards the plasma membrane within a transport vesicle. Situated in the plasma membrane, the carbohydrates serve as important recognition markers on the glycoprotein.

A changed composition of the sugar moities of the glycoprotein in the plasma membrane will thus imply a modified receptor and therefore an altered interaction of the receptor with its messenger.

In this study of the plasma membrane of normal and psoriatic keratinocytes, attention is focussed onto the oligosaccharide chain of the glycoproteins. Differences in sugar composition and structure in the plasma membrane of normal, psoriatic uninvolved and psoriatic lesional keratinocytes have been investigated.

Because skin is a solid tissue, the receptors on the plasma membrane can quantitatively and qualitatively only be studied when the keratinocytes are free in suspension. In Ch.2 a method is therefore developed for making cell suspensions with minimal

damage to the plasma membrane. Also the morphological characterization of normal and psoriatic keratinocytes is reported. In Ch.3 it is shown that the plasma membrane receptors are unaffected by the preparation of the cell suspension. Ch.4 is concerned with the uptake of radioactive sugars and their incorporation into glycoconjugates; the characterization of these glycoconjugates is described in Ch.5. After developing a method for lectin binding on keratinocytes (Ch.6), this technique is used in Ch.7 to study differences in receptor structure and binding affinity of the plasma membrane-bound glycoconjugates of normal and psoriatic keratinocytes. The last research project (Ch.8) deals with the terminal sugar on the oligosaccharide chain, namely sialic acid, which is responsible for the electric charge on the receptor. In the discussion an attempt has been made to integrate these data, and by comparing the values of normal and psoriatic keratinocytes to get a better understanding of the pathogenesis of psoriasis.

REFERENCES

Danielli, J.F. and Davson,H. (1935) A contribution to the theory of permeability of thin films. Journal of cellular physiology, 5, 495-508.

Singer, S.J. and Nicolson, G.L. (1972) The fluid mosaic model of the structure of cell membranes. Science, New York, 175, 720-731

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Studies on the plasma membrane of normal and psoriatic keratinocytes

1. PREPARATION OF MATERIAL AND MORPHOLOGICAL CHARACTERIZATION

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SUMMARY

A method is described for the preparation of isolated keratinocytes suitable for subsequent biochemical studies Scanning electron microscopy showed that the maturation process is accompanied by an increase in cell size and a shortening and eventual loss of microvilli. Psoriatic keratinocytes are distinguishable by exhibiting longer microvilli at all levels of maturation

It is now beyond doubt that the regulation of cell division and differentiation is significantly altered in the psoriatic keratinocyte. Observations at the ultrastructural level (Orfanos *et al*, 1973; Orfanos, Mahrle & Runne, 1976) have suggested that the fault which is responsible for these abnormalities may be located in the plasma membrane, in particular involving the 'glycocalyx'

Such a concept is wholly compatible with our knowledge of membrane function in general, for example·

(1) Many of the substances known to control cell cycle parameters are mediated via specific membrane receptors (Emmelot, 1977).

(2) Malignant transformation of cells is accompanied by changes in the cell surface (Nicolson, 1976, Bramwell & Harris, 1978)

(3) Experimental modulation of the cell surface can lead to changes in mitotic rate, cell mobility, cell–substratum and cell–cell interactions (Pardee, 1975; Edelman, 1976, Pastan & Willingham, 1978)

Turning to the molecular level of organization, certain alterations have been reported in the psoriatic membrane which are compatible with a specific defect in glycoconjugate (glycoprotein or glycolipid) metabolism These include a reduced response of the adenyl cyclase receptor for catecholamines (Yoshikawa *et al*, 1975; Iizuka *et al*, 1978), a reduced activity of membrane-bound ATPase activity (Mahrle & Orfanos, 1974) and certain immunological changes (reviewed by Guilhou, Meynadier & Clot, 1978)

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We are therefore studying various aspects of the metabolism of membrane-bound glycoconjugates in the psoriatic lesion The present paper deals with the preparation and morphological characterization of isolated, viable keratinocytes suitable for biochemical investigation

MATERIALS AND METHODS

Chemicals

Trypsin (grade III) and ribonuclease (from bovine pancreas, 5 × recrystallized) were obtained from the Sigma Chemical Co , Missouri. TC 199 medium was supplied by Difco (Detroit), penicillin and streptomycin sulphate by Mycofarm, Delft, and the calf serum by the Animal Laboratory of this University. All other chemicals were of Analytic Reagent quality and were supplied by Merck (Darmstadt, Germany).

Skin samples

Control specimens were obtained from healthy, paid volunteers (aged 18–46). Samples of lesion and uninvolved skin were taken from patients with chronic, stable plaques (usually of the back or upper arm) and who had used no local or systemic therapy for at least 2 weeks prior to biopsy

After cooling the skin surface with an ethyl chloride spray, specimens were cut using a Castroviejo keratotome in conjunction with a steel guard limiting the area of biopsy to about 1 cm^2. Keratotome shims were selected to give an average thickness of 0 2 mm for normal and psoriatic uninvolved skin and 0 3 mm for psoriatic lesion.

Preparation of cell suspension

The biopsy was floated, dermal face downwards, in a plastic Petri dish containing a solution of trypsin (0 25 mg/ml) and dithioerythritol (3 mg/ml) in 10 ml of phosphate-buffered saline (PBS. 137 mM NaCl, 2 68 mM KCl; 8 1 mM $Na_2H\ PO_4$ and 1 47 mM $KH_2\ PO_4$ in distilled, pyrogen-free water). After 30 min at 37°C, the intact specimen was transferred to a second Petri dish containing 2 ml of TC 199 supplemented with 20% calf serum, 200 units/ml penicillin and 100 μg/ml streptomycin sulphate. The dermis was peeled from the epidermis and discarded. The remaining contents of the dish (medium, epidermis and some detached cells) were transferred to a tube and agitated gently ('Vortex' mixer) for 1 min.

After removing the transparent horny layer, 50 μl of a solution of ribonuclease (2 5 mg/ml in phosphate-buffered saline) was added, and the tube incubated for a further 30 min at 37°C. The contents of the tube were finally filtered through a Phywé Korbfilter (pore size 50 μm; Phywe, Gottingen, Germany) and the residue washed with 2 ml medium. The cells were pelleted by centrifugation (100 *g* for 10 min), resuspended in an appropriate volume of medium (usually 0·5 ml) and an aliquot of the suspension counted in a Burker counting chamber

All operations were, as far as possible, conducted under sterile conditions

Preparation of material for scanning E M.

The cells were washed once with PBS and resuspended at a concentration of $1–4 \times 10^6$ cells per ml. Fifty microlitres of this suspension was spread on a clean object glass (7 × 7 mm) mounted in a covered Petri dish, and the cells allowed to settle on to the surface of the glass (37°C, 30 min) The attached cells were fixed with 2% glutaraldehyde in PBS (room temperature for 2 h), washed overnight at 4°C, and post-fixed with osmium tetroxide (room temperature for 1 h). After dehydration through graded aqueous acetones, the 100% acetone was replaced by liquid carbon dioxide, the preparation dried using the critical point method and coated with a thin layer of gold. The photographs were made on a Philips 500 Scanning Electron Microscope at an acceleration voltage of 12 kV.

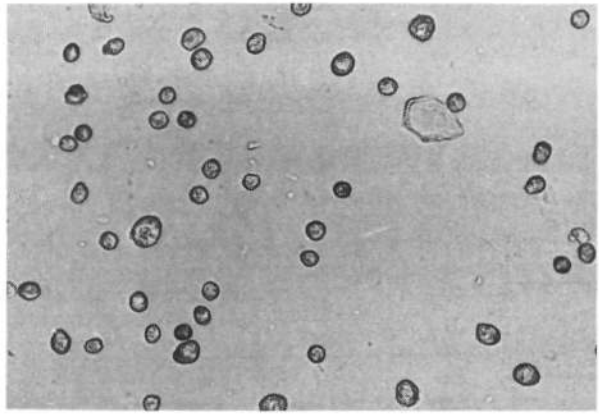

FIGURE 1. A typical preparation of isolated keratinocytes viewed by light microscopy (unstained; × 350).

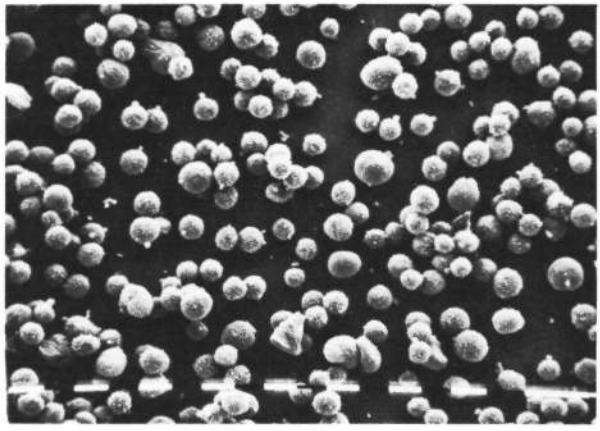

FIGURE 2. A typical preparation viewed by SEM. (Marker bars are each 10 μm.)

RESULTS

In general the preparative method described here yielded $2\text{–}4 \times 10^6$ cells. The suspension usually contained a large preponderance of single cells, with occasional ($< 10\%$) clusters of two or more cells (Fig. 1). The viability, assessed by the ability to exclude Trypan blue, was always better than 98%.

Figure 2 illustrates the appearance of a typical preparation from a healthy control as viewed by SEM; note the high degree of morphological heterogeneity. Cells originating from different regions of

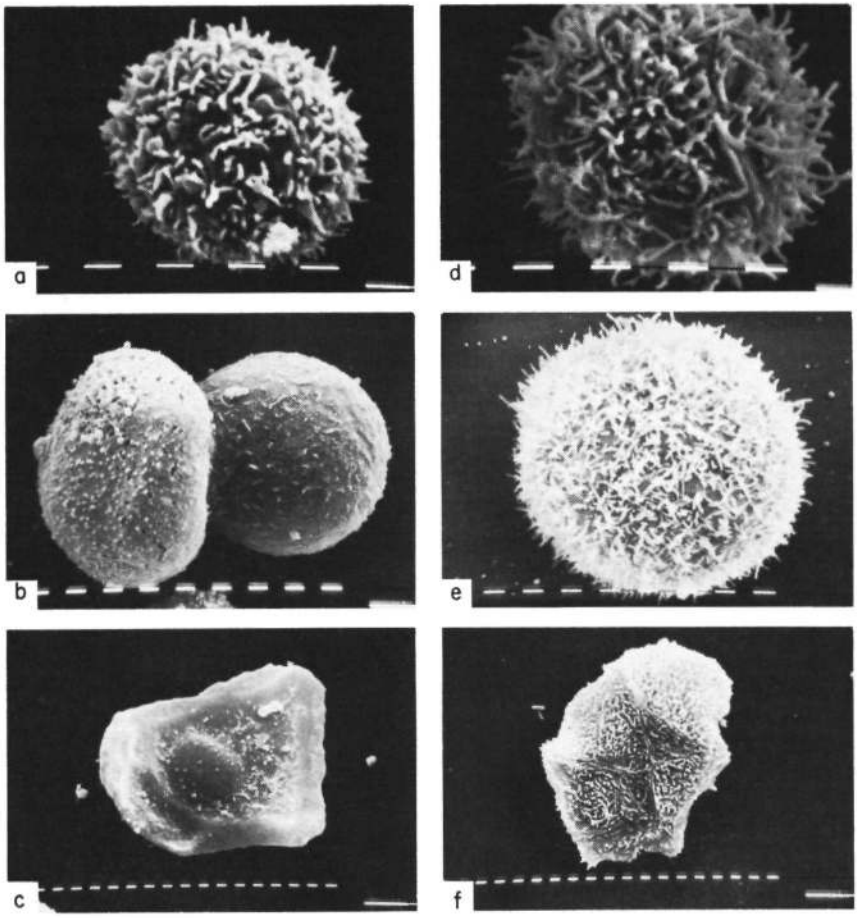

FIGURE 3. Individual cells from normal epidermis (a–c) and psoriatic lesion (d–f). (a) and (d) basal cells; (b) and (e) upper spinous cells; (c) and (f) corneocytes. (Marker bars are each 1 μm; note the differences in magnification between the photographs.)

the epidermis were identified by comparison with similar preparations of trypsinized but intact epidermis.

Figure 3a–c shows the characteristic appearances of basal cells, upper squamous cells, and corneocytes. It is clear that the maturation process is accompanied by a considerable increase in size, a shortening and eventual loss of microvilli, and ultimately by the transition from a spherical to a more or less polygonal shape.

The most clear cut change in keratinocytes from the psoriatic lesion was that cells at all levels exhibited much longer microvilli (Fig. 3d–f). Preparations from the uninvolved skin of psoriatic patients frequently showed similar changes, although to a lesser extent.

DISCUSSION

The use of isolated cells is mandatory for many investigations into membrane structure or function. The practical difficulty when studying solid tissues (especially mechanically tough tissues such as human epidermis) is that any procedure which is sufficiently drastic to separate the cells is liable to cause extensive changes in the plasma membrane. The technique described here has been developed in an attempt to minimize such damage. In particular, the use of dithioerythritol to cleave the desmosomes (Hentzer & Kobayasi, 1976) and the addition of calf serum as trypsin inhibitor before mechanical disruption both represent significant improvements over simple trypsinization. Evidence will be presented in subsequent papers to show that loss of glycoconjugates (in terms of surface carbohydrate or functional receptor groups) does not exceed 20%.

The most significant finding from the SEM studies is that there is a clear and consistent difference between normal and psoriatic keratinocytes at all levels of maturation. Our observations offer strong support for the 'microvillous transformation' described by Orfanos *et al.* (1973) and confirm that our preparative procedure does preserve these abnormalities for further investigation at the molecular level.

ACKNOWLEDGMENTS

The authors wish to thank the many volunteers (with and without psoriasis) who provided us with specimens of skin for use in this investigation.

REFERENCES

Bramwell, M.E. & Harris, H. (1978) An abnormal membrane glycoprotein associated with malignancy in a wide range of different tumours. *Proceedings of the Royal Society of London*, **201**, 87.

Edelman, G.M. (1976) Surface modulation in cell recognition and cell growth. *Science*, **192**, 218.

Emmelot, P. (1977) The organization of the plasma membrane of mammalian cells structure in relation to function. In *Mammalian Cell Membranes* (Ed. by G A. Jamieson and D.M. Robinson), 1st edn, Vol. 2, p. 40. Butterworths, London

Guilhou, J J , Meynadier, J & Clot, J. (1978) New concepts in the pathogenesis of psoriasis. *British Journal of Dermatology*, **98**, 585.

Hentzer, B. & Kobayasi, T. (1976) Dissociation of human adult epidermal cells by disulphide-reducing agents and subsequent trypsinization. *Acta dermato-venereologica*, **56**, 19.

Iizuka, H., Adachi, K , Halprin, K.M. & Levine, V. (1978) Cyclic AMP accumulation in psoriatic skin: differential responses to histamine, AMP and epinephrine by the uninvolved and involved epidermis. *Journal of Investigative Dermatology*, **70**, 250.

Mahrle, G. & Orfanos, C.E. (1974) Decreased membrane-bound ATP-hydrolytic activity in psoriatic epidermis. *British Journal of Dermatology*, **91**, 529.

Nicolson, G.L. (1976) Trans-membrane control of the receptors on normal and tumour cells II. Surface changes associated with transformation and malignancy. *Biochimica et biophysica acta*, **458**, 1.

Orfanos, C.E., Schaumburg-Lever, G., Mahrle, G. & Lever, W.F. (1973) Alterations of cell surfaces as a pathogenetic factor in psoriasis. Possible loss of contact inhibition of growth. *Archives of Dermatology*, **107**, 38.

Orfanos, C.E., Mahrle, G. & Runne, U. (1976) Verteilungsstorung oberflachlicher Glycoconjugate der psoriatischen Zellmembran. *Archives of Dermatological Research*, **256**, 39.

PARDEE, A.B. (1975) The cell surface and fibroblast proliferation. Some current research trends. *Biochimica et biophysica acta*, **417**, 153.

PASTAN, J. & WILLINGHAM, M. (1978) Cellular transformation and the 'morphologic phenotype' of transformed cells. *Nature*, **274**, 645.

YOSHIKAWA, K., ADACHI, K., HALPRIN, K.M. & LEVINE, V. (1975) On the lack of response to catecholamine stimulation by the adenyl cyclase system in psoriatic lesions. *British Journal of Dermatology*, **92**, 619.

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Studies on the plasma membrane of normal and psoriatic keratinocytes

2 CYCLIC AMP AND ITS RESPONSE TO HORMONAL STIMULATION

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SUMMARY

Cyclic AMP levels have been determined for the first time in isolated keratinocytes Values were more reproducible than those reported using epidermal slices Evidence is presented to show that damage to hormone receptors is minimal

Other observations include the following

(1) Keratinocytes from psoriatic lesions showed reduced 'resting' levels of cyclic AMP as well as a diminished response to adrenaline

(2) Cyclic AMP levels were maximal in the basal cells, falling dramatically in fully differentiated keratinocytes

(3) The topical application of a corticosteroid (fluocinolone acetonide) did not modulate the response of adenyl cyclase to hormonal stimulation

Voorhees & Duell (1971) formulated the concept that certain aberrations of psoriatic epidermis (in particular the alterations of cell cycle parameters and the accumulation of glycogen) might result from a defect in the adenyl cyclase-cyclic AMP cascade Since this time, ample confirmation has been provided that the ability of adenyl cyclase to respond to catecholamines is indeed reduced in the psoriatic lesion Nevertheless, the original reports that the cyclic AMP content of the lesion is in fact low have since been denied by several workers, notably Yoshikawa *et al* (1975a)

All previous investigators have employed either intact epidermis or broken cell preparations, despite the obvious limitations of these materials for receptor studies The present experiments, using the isolated keratinocyte preparation described previously (Gommans *et al*, 1979), were undertaken with two main objectives first, to evaluate this preparation in biochemical terms, and second, to obtain further evidence regarding the role of cyclic AMP in the pathogenesis of psoriasis

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MATERIALS AND METHODS

Materials

Histamine dihydrochloride and theophylline were purchased from the Sigma Chemical Co. Adrenaline bitartrate was supplied as a sterile solution (1 mg/ml) in physiological saline (O.P.G., Utrecht, NL). Reagents for the measurement of cyclic AMP were obtained from the Radiochemical Centre, Amersham (assay kit TRK 432). All other chemicals were as described previously (Gommans *et al.*, 1979).

The selection of healthy volunteers and psoriatic patients, the method of biopsy and the preparation of isolated keratinocytes have already been reported (Gommans *et al.*, 1979).

General techniques

Approximately 10^6 keratinocytes, suspended in medium (Gommans *et al.*, 1979), were washed twice with phosphate-buffered saline (PBS), resuspended in 200 μl PBS containing 7·5 mmol/l theophylline, and pre-incubated for 20 min at 37 C. The exact cell density of the final suspension was determined.

Following the pre-incubation, the cell suspension was mixed briefly (Vortex mixer) and three samples, each of 50 μl, were withdrawn. One sample was added to a tube, pre-heated to 37°C, containing 20 μl adrenaline (150 μmol/l in PBS); a second was added to a similar tube containing 20 μl histamine (4·5 mmol/l in PBS), and the third sample was injected rapidly into a preheated tube containing 150 μl 0·1 mol/l HCl at 100°C. This last (used to determine the 'resting' level of cyclic AMP) was kept at 100°C for 2 min before cooling to room temperature. The tubes containing adrenaline and histamine were incubated at 37°C for 5 min; aliquots of 50 μl were then injected into 150 μl of 0·1 mol/l HCl at 100°C as before.

All three heat-inactivated samples were sonicated (MSE Ltd, Crawley, Sussex) for 20 s to complete the disruption of the cells, and re-heated for a further 2 min at 100°C. After cooling and centrifuging duplicate 50 μl aliquots of the supernatant were withdrawn from each tube. These were dried *in vacuo* over KOH at 0°C. The dry samples were re-dissolved in 20 μl PBS and (together with appropriate blanks and standards) the cyclic AMP levels were determined using the Gilman procedure (Gilman, 1970). The assay technique described by the Radiochemical Centre was modified by reducing sample and reagent volumes; in our hands the reproducibility was about $\pm$0·05 pmol.

Time curves for hormone stimulation

Preliminary experiments were carried out in which the period of incubation with adrenaline or histamine was varied over the range 0–6 min. In all other respects the technique was as described above.

Receptor regeneration studies

In certain experiments the cells were incubated in the original medium (supplemented with antibiotics and 20% calf serum) at 37°C for periods of up to 12 h after trypsinization. The suspension was then washed twice with PBS and the sensitivity to hormone stimulation determined as before.

Study of individual epidermal layers

Cow snout was used for this investigation because of its much greater thickness (about 0·5 mm) compared with human epithelium. After gross dissection of the skin, successive slices were cut with a keratotome (nominal setting 0·1 mm). Each slice was allocated to one of three pools (superficial, mid-spinous or basal) according to its macro- and microscopical appearance.

The pooled slices were trypsinized and all subsequent procedures carried out as described above.

Pre-treatment with topical corticosteroid

Approximately 80 mg fluocinolone acetonide, 0·2% (Synalar forte, Sarva) was spread over an area of about 2 × 2 cm on one shoulder of healthy volunteers. Occlusive dressing was applied both over the treated area and over a control site on the opposite shoulder. After 24 h the dressings were removed, the sites cleaned with alcohol and biopsies taken.

Trypsinization and subsequent procedures were again as previously described.

Protein determination

Aliquots of keratinocytes from healthy controls were washed twice in PBS and disrupted in an all-glass homogenizer (Microwet grinder, Townson and Mercer, Croydon, Surrey). The protein content of the homogenate was determined by the method of Lowry *et al* (1951) using bovine serum albumin as standard.

RESULTS

Time curves for hormonal stimulation

The time courses for the stimulation of normal keratinocytes by adrenaline and histamine are shown in Fig. 1, the results of two independent experiments are illustrated. It is seen that maximum cyclic AMP levels are reached within about 4 min of hormone addition.

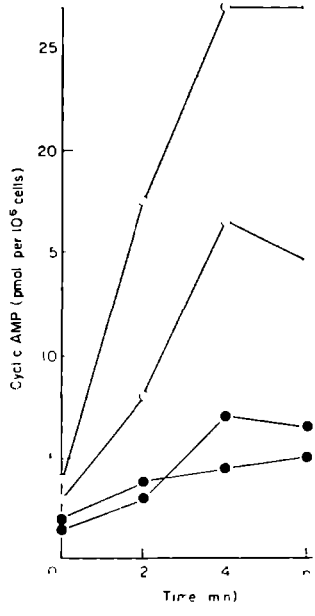

FIGURE 1. Time courses for the stimulation of isolated keratinocytes by adrenaline ( ) and histamine (●). Two different preparations were studied in each case.

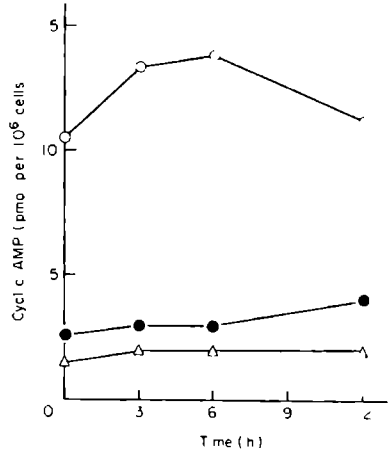

FIGURE 2. Effects of allowing a recovery-period after trypsinization and before determination of hormone sensitivity (○) resting levels, (△) plus adrenaline, (●) plus histamine.

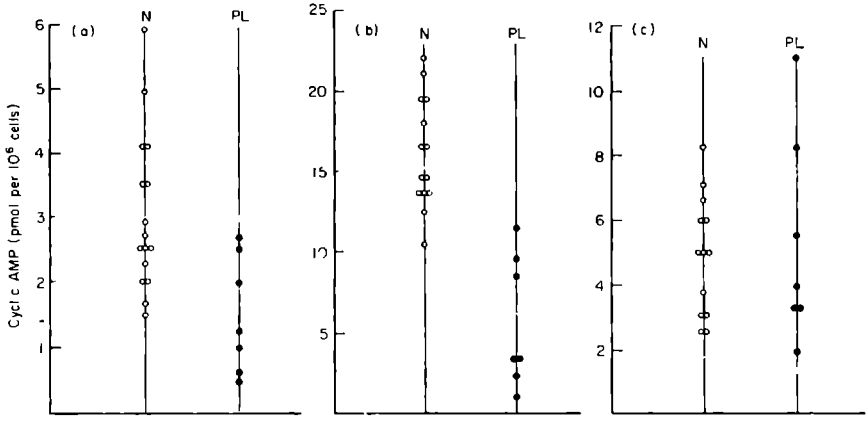

FIGURE 3 Absolute values for cyclic AMP levels in normal ( ) and psoriatic (●) keratinocytes. (a) resting levels, (b) following adrenaline stimulation, (c) following histamine stimulation.

Receptor regeneration studies

The effect of allowing the keratinocytes to 'recover' for various periods after trypsinization is illustrated in Fig 2. It is seen that there is an increase of about 25% in the sensitivity to adrenaline after 3–6 h incubation

Changes in the psoriatic lesion

Absolute values for cyclic AMP levels ('resting' and following hormonal stimulation) in normal and psoriatic keratinocytes are shown in Fig 3a–c Both the resting levels of cyclic AMP and the levels

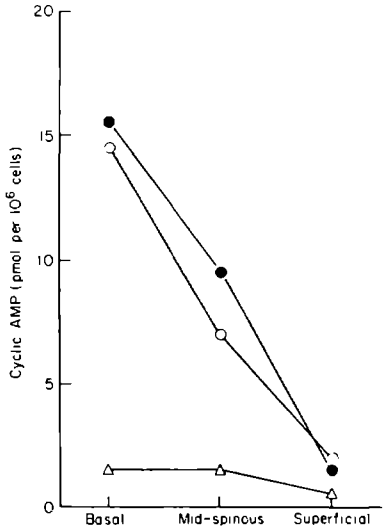

FIGURE 4. Cyclic AMP levels in keratinocytes prepared from different regions of cow snout epidermis. Symbols are as in Fig. 2.

TABLE 1. Effects of pre-treatment with fluocinolone acetonide on 'resting' cyclic AMP levels and sensitivity to hormones

	Cyclic AMP (pmol per 10^6 cells)	
	Treated	Control
Unstimulated	2 8	2 6
Adrenaline stimulated	15 9	17 9
Histamine stimulated	4 7	5 0

following adrenaline stimulation are significantly reduced in cells from the psoriatic lesion (Wilcoxon test, $P<0.05$).

Variation with depth

The cyclic AMP levels of basal, mid-spinous and superficial keratinocytes from cow snout are shown in Fig. 4. It is seen that both the 'resting' level and the response to hormonal stimulation are maximal in the basal layer, falling to very low values in the fully differentiated tissue.

Effect of corticosteroid

The values for control and steroid-treated sites are shown in Table 1 (mean of three experiments). The differences are not statistically significant ($P>0.05$).

TABLE 2 Hormone response of isolated keratinocytes in comparison to intact epidermis Note that in this table our results have been recalculated as pmol per mg protein

	Cyclic AMP (pmol per mg protein)	
	Isolated keratinocytes	Intact epidermis (Iizuka *et al*, 1978)
Adrenaline stimulated	44 1	58 0
Histamine stimulated	12 1	11 7

Protein content

The mean protein content of washed keratinocyte preparations from ten healthy controls was 411 ± 58 μg protein/10^6 cells

DISCUSSION

The advantages offered by an isolated cell suspension may be illustrated by comparing the present results with those of Iizuka *et al* (1978), who carried out similar experiments using intact epidermis The response time of isolated keratinocytes to hormonal stimulation in the presence of theophylline, for example, was less than 4 min compared with 10–20 min for whole epidermis More important, the wide variations in hormonal responsiveness between individual patients reported by Iizuka *et al* (1978) were not found in the present investigation The reason for the improved reproducibility is not clear, but possibilities include the elimination of diffusion limitations, the absence of dermal components, and the removal of endogenous mediators which may be present in the interstitial material

The most serious reservation regarding the use of isolated keratinocytes is, of course, the possibility of membrane damage resulting from trypsinization However, the following evidence suggests that such damage is minimal in our preparations

(a) Our absolute figures for cyclic AMP levels in normal keratinocytes (recalculated to a protein basis, Table 2) are in good agreement with values obtained using whole epidermis

(b) Experiments in which cells were allowed to 'recover' following trypsinization (Fig 2) show little increase in hormonal stimulation*

(c) The reduced response of psoriatic keratinocytes to adrenaline stimulation (about 30% of normal, see Fig 1b) is consistent with figures obtained using intact epidermis (Voorhees *et al*, 1974, Yoshikawa *et al*, 1975b, Iizuka *et al*, 1978)

Despite considerable dispute, it is now accepted that the cyclic AMP level of psoriatic epidermis *in vivo* is about normal if expressed in terms of DNA, i e per cell (Yoshikawa *et al*, 1975a) Moreover, after pre-incubation with theophylline but in the absence of added hormones the cyclic AMP level of whole epidermis from the lesion seems actually to be elevated (Iizuka *et al*, 1978) It is therefore of interest that the cyclic AMP content of washed, isolated psoriatic keratinocytes after pre-incubation with theophylline (but again in the absence of added hormones) was lower than normal (Fig 1a) An hypothesis consistent with these data is that the level of endogenous 'first messengers' in the psoriatic plaque is abnormally high Alternatively, the difference may be related to oxygen

* Evidence will be presented in a subsequent communication to show that turnover of cell surface glycoprotein continues linearly during this period

tension; in epidermal slices, the higher oxygen uptake of the lesion will result in hypoxia, and hence higher cyclic AMP levels (Yoshikawa *et al.*, 1975c), whereas in isolated keratinocytes such an effect is unlikely.

The values obtained from individual layers of cow snout epithelium are consistent with histochemical observations that adenyl cyclase activity is maximal in the basal layer (Mahrle & Orfanos, 1975). The data clearly support the concept that cyclic AMP is concerned with the control and coordination of proliferation rather than with the events occurring during the process of keratinization. A second point emerging from this work is that cow epithelium resembles pig in showing roughly equal sensitivities to adrenaline and histamine (Iizuka *et al.*, 1976, 1977); human epidermis seems unusual in showing a much lower response to histamine.

The final experiments, in which the responses of keratinocytes to hormonal stimulation were determined following the application of fluocinolone acetonide, were prompted by reports that in some tissues steroids may act by modulating the number of catecholamine and other receptors on the cell membrane (Rajerison *et al*, 1974; Wolfe, Harden & Molinoff, 1976; Roberts *et al.*, 1977). Such a concept would provide a theoretical basis for the efficacy of corticosteroids in proliferative skin disease. Unfortunately our results (Table 1) do not support the idea of a mechanism of this kind in epidermis, at least for the particular steroid investigated.

REFERENCES

GILMAN, A.G. (1970) A protein binding assay for adenosine 3′,5′-cyclic monophosphate. *Proceedings of the National Academy of Sciences*, **67**, 305.

GOMMANS, J.M., BERGERS, M., VAN ERP, P.E.J., VAN DEN HURK, J.J M.A., MIER, P.D. & ROELFZEMA, H. Studies on the plasma membrane of normal and psoriatic keratinocytes. 1. Preparation of material and morphological characterization. *British Journal of Dermatology*, **101**, 407.

IIZUKA, H., ADACHI, K., HALPRIN, K.M. & LEVINE, V. (1976) Histamine (H_2) receptor-adenylate cyclase system in pig skin (epidermis). *Biochimica et biophysica acta*, **437**, 150.

IIZUKA, H., ADACHI, K., HALPRIN, K.M. & LEVINE, V. (1977) Epidermal adenylate cyclase stimulation of the histamine (H_2) receptor by tolazoline. *Journal of Investigative Dermatology*, **69**, 442.

IIZUKA, H., ADACHI, K., HALPRIN, K.M. & LEVINE, V. (1978) Cyclic AMP accumulation in psoriatic skin differential responses to histamine, AMP and epinephrine by the uninvolved and involved epidermis. *Journal of Investigative Dermatology*, **70**, 250.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951) Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265.

MAHRLE, G. & ORFANOS, C.E. (1975) β-Adrenerge Stimulation membrangebundener Adenylcyklase in normaler Epidermis und mangelnde Stimulierbarkeit bei Psoriasis. *Archives for Dermatological Research*, **253**, 195.

RAJERISON, R, MARCHETTI, J., ROY, C., BOCKAERT, J. & JARD, S. (1974) The vasopressin-sensitive adenylate cyclase of the rat kidney. *Journal of Biological Chemistry*, **249**, 6390.

ROBERTS, J.M., INSEL, P.A, GOLDFIEN, R D. & GOLDFIEN, A. (1977) α-Adrenoreceptors but not β-adrenoreceptors increase in rabbit uterus with oestrogen. *Nature*, **270**, 624.

VOORHEES, J.J. & DUELL, E.A. (1971) Psoriasis as a possible defect of the adenyl cyclase-cyclic AMP cascade. *Archives of Dermatology*, **104**, 352.

VOORHEES, J.J., DUELL, E.A., STAWISKI, M. & HARRELL, E.R. (1974) Cyclic nucleotide metabolism in normal and proliferating epidermis. *Advances in Cyclic Nucleotide Research*, **4**, 117.

WOLFE, B.B., HARDEN, T K. & MOLINOFF, P.B. (1976) β-Adrenergic receptors in rat liver effects of adrenalectomy. *Proceedings of the National Academy of Sciences of the United States of America*, **73**, 1343.

YOSHIKAWA, K., ADACHI, K., HALPRIN, K.M. & LEVINE, V. (1975a) Is the cyclic AMP in psoriatic epidermis low? *British Journal of Dermatology*, **93**, 253.

YOSHIKAWA, K., ADACHI, K, HALPRIN, K.M. & LEVINE, V. (1975b) On the lack of response to catecholamine stimulation by the adenyl cyclase system in psoriatic lesions. *British Journal of Dermatology*, **92**, 619.

YOSHIKAWA, K., ADACHI, K., HALPRIN, K.M. & LEVINE, V. (1975c) Cyclic AMP in skin effects of acute ischaemia. *British Journal of Dermatology*, **92**, 249.

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Studies on the plasma membrane of normal and psoriatic keratinocytes. 3. Uptake of labelled sugars and their incorporation into glycoconjugates

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SUMMARY

We report the uptake of four labelled sugars by keratinocytes isolated from normal epidermis, psoriatic 'uninvolved' skin and psoriatic lesions Our findings include the following

(1) The rate of uptake of all sugars by the psoriatic lesion is increased

(2) This abnormally high uptake diminishes dramatically during 22 h incubation *in vitro*

(3) There is a striking abnormality in the metabolism of fucose by psoriatic keratinocytes, our data suggest an increased rate of incorporation of fucose into glycoconjugates

We have already described morphological changes (Gommans *et al*, 1979a) and functional disturbances (Gommans *et al*, 1979b) of the plasma membrane in psoriatic keratinocytes The remaining papers in this series will deal with the glycoconjugates (glycoprotein, glycolipid and proteoglycans) which are essential components of the membrane and its receptors

In this communication we report the uptake of labelled sugars by isolated keratinocytes and their incorporation into glycoconjugates In particular, we have asked two questions First, to what extent can the known membrane abnormalities in psoriasis (Mahrle & Orfanos, 1977) be characterized in terms of a specific biochemical defect? Second, if such a defect exists, is this also manifest in the clinically uninvolved skin of the psoriatic patient?

MATERIALS AND METHODS

Materials

L-(1-^{14}C)fucose (61 Ci/mol), N-acetyl-D-(1-^{14}C) glucosamine (59 Ci/mol), D-(1-^{14}C) galactose (60 7 Ci/mol), D-(1-^{14}C) mannose (59 Ci/mol), L-(6-^{3}H)fucose (17 1 Ci/mmol) and D-(6-^{3}H)glucosamine hydrochloride (38 Ci/mmol) were obtained from the Radiochemical Centre, Amersham All other chemicals were as described previously (Gommans *et al*, 1979a)

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The selection of healthy volunteers and psoriatic patients, the method of biopsy and the preparation of isolated keratinocytes have already been reported (Gommans *et al.*, 1979a).

Labelling procedures

Keratinocytes (usually $1-2 \times 10^6$) from healthy controls, psoriatic lesions or psoriatic 'uninvolved' skin were suspended in 0·5 ml TC 199 medium supplemented with 20% calf serum and containing 200 units/ml penicillin and 100 μg/ml streptomycin sulphate. The appropriate radioactive sugar was added (1 μCi/ml for ^{14}C-labelled sugars or 10 μCi/ml for ^{3}H-labelled sugars) and the suspension was incubated at 37° C. At the end of the appropriate incubation period, the cells were pelleted by centrifugation (400 *g*, 5 min) and washed three times with 0·5 ml portions of TC 199 medium without serum, the washings being discarded. The washed cells were finally extracted three times with 0·5 ml portions of ice-cold trichloroacetic acid (5% w/v), with centrifugation at 2300 *g* for 10 min. Radioactivity was measured in the pellet after solubilization in 1% NH_3 ('TCA-insoluble sugar') and in the pooled extracts ('TCA-soluble sugar').

Three time-schemes were employed:

(1) A single labelling period of 3 or 4 h (t = 3 or 4).

(2) A single labelling period of 22 h (t = 22).

(3) Pre-incubation in TC 199 plus serum for 22 h followed by a labelling period of 3 or 4 h (t = 22/3 or 22/4).

Scintillation counting

All samples were counted in an Isocap 300 (Searle Ned. B.V., Uithoorn, NL) after addition of 10 ml 'aquasol' (NEN, Inc., Boston, Mass.). External standard ratios were used for quench correction.

RESULTS AND DISCUSSION

Uptake and distribution of radioactivity

Our results are summarized in Fig. 1. In each group of experiments we have indicated the mean total radioactivity of the keratinocytes, together with the distribution into TCA-soluble material (free sugar, sugar phosphates, UDP-sugars, etc.) and TCA-insoluble material (glycoprotein, glycolipid, proteoglycans).

The effects of different concentrations of 'cold' sugar in the incubation medium are seen by comparing the results using ^{3}H-labelled fucose and N-acetylglucosamine with the corresponding experiments in which ^{14}C-labelled sugars were employed. It is clear that the use of the very high specific activity ^{3}H-label (i.e. the addition of less 'cold' sugar to the medium) results not only in a higher absolute level of radioactivity taken up, but also in a rather higher percentage of the total activity appearing in the TCA-insoluble fraction. This latter observation implies that the synthesis of glycoconjugates in the Golgi apparatus may be the rate-limiting step in the overall pathway.

Assumptions and theoretical model

The interpretation of data such as these necessitates a theoretical model which must be based on certain simplifying assumptions. Here we may reasonably assume that the label, after entry into the cell, passes through two consecutive metabolic pools, namely the unconjugated sugars (TCA-soluble pool) and the conjugated sugars (TCA-insoluble pool), as shown in Fig. 2. If the cell is in metabolic equilibrium, the pool sizes are constant and $V_1 = V_2 = V_3$ ('turnover rate').

These two parameters (pool size and turnover rate) may in principle be evaluated by the appropriate choice of labelling period.

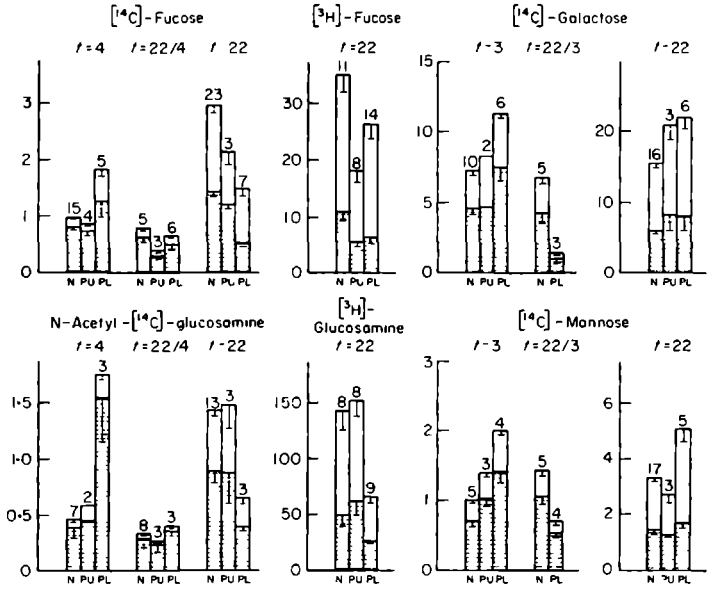

FIGURE 1. Radioactivity (c.p.m. per 10^3 cells) in the TCA-soluble fractions (shaded bars) and the TCA-insoluble fractions (open bars). The figure above each bar indicates the number of specimens measured, and the vertical lines represent the s.e.mean Time-schedules are described in the text ('labelling procedures'). Abbreviations are: N = normal epidermis, PU = psoriatic uninvolved, PL = psoriatic lesion.

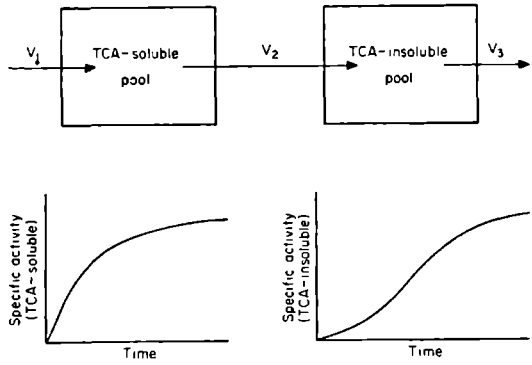

FIGURE 2. Idealized model for transit of the labelled sugar through the cell. The implicit assumptions are described in the text. Note that V_1, V_2 and V_3 represent bulk transport rates and not movement of radioactivity.

Turnover rate For very short labelling periods, the label leaving a pool is small compared with that entering, the initial rate at which radioactivity accumulates is therefore governed only by the turnover rate and is independent of pool size For the TCA-soluble pool, the calculation is simplified by the fact that the precursor (i e the extracellular sugar) remains at constant specific activity The initial rate of increase will therefore be linear, and the slope will be proportional to V_1 In the case of the TCA-insoluble pool, however, the situation is more complex, here we must also take into account the 'average' specific activity of the material entering the pool in order to compare bulk-flow rates (V_2) between different experiments This has been done for 'short' labelling periods by expressing the results as the ratio of the activity of the TCA-insoluble pool to that of the TCA-soluble pool (Fig 3)

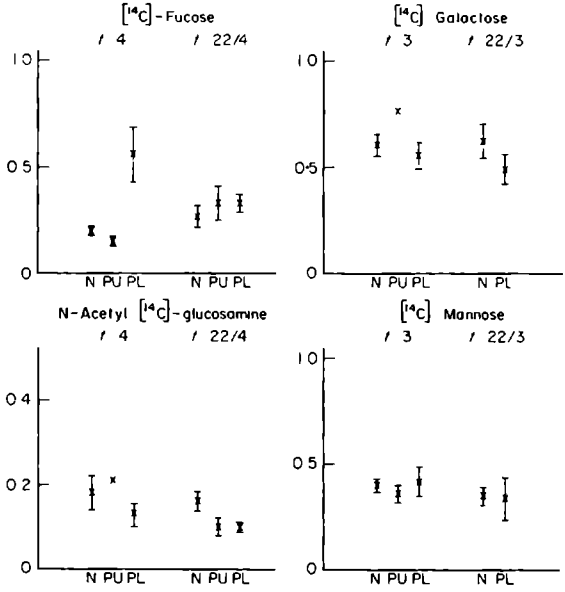

FIGURE 3 Ratios of TCA-insoluble to TCA soluble activity Abbreviations are N = normal epidermis, PU = psoriatic uninvolved, PL = psoriatic lesion

Pool sizes After a sufficiently long labelling period, a dynamic equilibrium is reached in which the specific activities of the pools are equal and constant At this point the total activity of each pool is a direct measure of its size

The practical measurement of flow-rates and pool sizes thus depends on selecting optimum values for the labelling periods The lower limit for the 'short' period is set by the minimum cpm which can be accurately determined, this was 3–4 h for routine measurements Preliminary experiments, using normal human keratinocytes, confirmed that the net uptake of radioactivity was reasonably linear during this time The choice of the 'equilibration' period is more difficult because protracted periods of maintenance *in vitro* may well lead to gross metabolic changes during the experiment Preliminary investigations indicated that the total radioactivity taken up by normal keratinocytes increases little

after 22 h, the possibility of metabolic alteration during this time is excluded by the observation that pre-incubation for 22 h followed by a 'short' labelling period (Fig 1, $t = 22/3$ or $22/4$) yield results similar to those from fresh cells ($t = 3$ or 4) This was not true, however, for psoriatic keratinocytes (see below)

The TCA-soluble pool (unconjugated sugars)

Application of this model to the data presented in Fig 1 yields two clear-cut conclusions regarding the entry-rates of sugars into the TCA-soluble pool First, using freshly-trypsinized preparations of keratinocytes, it is seen that the rates of entry of all four sugars into cells derived from psoriatic lesions are very much higher than into normal cells ($t = 3$ or 4) Secondly, the rates of entry of sugars into psoriatic keratinocytes, in contrast to normal cells, decline dramatically after 22 h pre-incubation *in vitro* ($t = 22$ 3 or $22/4$) This latter phenomenon is apparent both in keratinocytes derived from lesions and from uninvolved skin, but is particularly striking in the case of the lesions

Surprisingly, the radioactivity present in the TCA-soluble pool after a 'long' labelling period ($t = 22$) in cells derived from psoriatic lesions is not increased over the level found after a 'short' period, indeed, in the cases of fucose and N-acetylglucosamine it is significantly less This confirms our previous conclusion that the metabolic parameters of psoriatic keratinocytes change rapidly *in vitro*, and prohibits quantitative statements regarding pool sizes

The TCA-insoluble pool (glycoconjugates)

The rate of entry of a sugar into the TCA-insoluble pool (i e the rate of synthesis of glycoconjugates) is indicated by the ratio of activity (TCA-insoluble) activity (TCA-soluble) These ratios have been calculated from the data of Fig 1 and are presented in Fig 3 A striking and apparently specific abnormality is seen in the experiments using fucose Comparing freshly trypsinized keratinocytes from psoriatic lesions with those from healthy controls, it is evident that the rate of incorporation of fucose into glycoconjugates is about three times greater than normal This high value, however, returns to a figure approaching normal after 22 h pre-incubation *in vitro* ($t = 22/4$) Curiously, keratinocytes derived from psoriatic uninvolved skin behave in precisely the opposite manner, fresh cells have a somewhat low rate of incorporation of fucose, which rises significantly after pre-incubation

Again the evaluation of pool sizes is hindered by our observation that the metabolism of psoriatic keratinocytes is changing during the 'long' labelling period It is evident that the pattern of sugar incorporation into glycoconjugates (Fig 1, $t = 22$) is grossly abnormal in the psoriatic cells, but our data give no indication of whether this conclusion may be extrapolated to the situation *in vitro*

General comments

Three conclusions have been drawn from the data in Fig 1 These are firstly, that keratinocytes from psoriatic lesions take up all sugars more rapidly from the medium, secondly, that this abnormally high rate of uptake falls dramatically during incubation *in vitro*, and finally, that there seems to be a specific abnormality in the incorporation of fucose into glycoconjugates in psoriatic cells

Such conclusions are, of course, dependent on the validity of the assumptions inherent in the model used to interpret the data We have already pointed out that comparisons of pool sizes might be misleading because the rate of uptake of sugars by the psoriatic cells does not remain constant during the experiment Other potential inaccuracies in our assumptions include

(1) *De novo* synthesis of sugar, leading to dilution of the labelled material entering the cell

(2) Recycling of sugar via intracellular breakdown of glycoconjugates

(3) Metabolic utilization of the labelled sugar via alternative pathways, for example conversion to other sugars

We obviously cannot exclude any of these possibilities on the basis of the data presented. Indeed, attempts to calculate absolute pool sizes from the radioactivity after 22 h and the specific activity of the added sugar yield results at least an order of magnitude less than values generally reported for chemical assays (Yurchenko, Ceccarini & Atkinson, 1978), implying considerable levels of *de novo* synthesis or recycling However, these limitations do not necessarily invalidate qualitative statements regarding flow-rates At the worst, alternative explanations are introduced, in particular, the apparently increased incorporation of fucose into glycoconjugates of psoriatic lesions could instead reflect a diminished *de novo* synthesis of fucose (and hence a 'hotter' TCA-soluble pool) in psoriatic cells.

The striking metabolic changes observed in psoriatic keratinocytes after 22 h incubation *in vitro* are perhaps not surprising. The situation seems to parallel that occurring *in vivo*, where a transit time of only 3 days (Porter & Shuster, 1968) suggests an enormous acceleration of the various processes which are collectively termed 'keratinization' These processes include progressive losses of enzymatic activities and of membrane functions in addition to the oxidation of prekeratin to mature keratin Our present results imply that in cells from the psoriatic lesion the transport function diminishes very rapidly indeed, the relatively small changes in the percentage incorporation of three sugars into glycoconjugates after 22 h pre-incubation indicate that the Golgi apparatus and the endoplasmic reticulum maintain their integrity during this period, i e that 'cell death' has not occurred.

The findings regarding fucose metabolism are of greater interest in that they are compatible with a specific membrane defect. We are currently engaged in a more detailed study of the composition of the TCA precipitate (glycoprotein, glycolipid and proteoglycans) and also in the investigation of cell-surface material removed by trypsin in order to elucidate this abnormality more clearly.

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REFERENCES

GOMMANS, J M., BERGERS, M , VAN ERP, P E J , VAN DEN HURK, J M.A , MIER, P D & ROELFZEMA, H (1979a) Studies on the plasma membrane of normal and psoriatic keratinocytes. 1. Preparation of material and morphological characterization. *British Journal of Dermatology*, **101**, 407.

GOMMANS, J M , BERGERS, M , VAN ERP, P E J , VAN DEN HURK, J M.A., VAN DE KERKHOF, P , MIER, P D. & ROELFZEMA, H (1979b) Studies on the plasma membrane of normal and psoriatic keratinocytes 2. Cyclic AMP and its response to hormonal stimulation. *British Journal of Dermatology*, **101**, 413.

MAHRLE, G & ORFANOS, C E (1977) The plasma unit membrane. Membrane mediated growth control and its failure in psoriasis. *British Journal of Dermatology*, **96**, 215

PORTER, D. & SHUSTER, S. (1968) Epidermal renewal and amino acids in psoriasis and pityriasis rubra pilaris. *Archives of Dermatology*, **98**, 339.

YURCHENKO, P D , CECCARINI, C & ATKINSON, P H (1978) Labeling complex carbohydrates of animal cells with monosaccharides. In *Methods of Enzymology* (Ed. by V.Ginsburg), 1st ed., vol L, p. 175. Academic Press. New York.

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Studies on the plasma membrane of normal and psoriatic keratinocytes. 4. Characterization of glycoconjugates

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SUMMARY

A substantial proportion (20–50%) of radioactive sugar incorporated into glycoconjugates by normal human keratinocytes was soluble in chloroform-methanol, using (^{14}C)-galactose as precursor about half of this fraction was neutral lipid The incorporation of labelled sugars into the lipid fraction was consistently increased in keratinocytes derived from psoriatic lesions

The abnormality in fucose metabolism which we reported previously has been confirmed In particular we have shown that the molecular weight of fucosylated glycopeptides appears to be abnormally high in the untreated psoriatic lesion, possibly reflecting an increased degree of branching In psoriatic uninvolved epidermis and in treated psoriatic lesions the situation is reversed, the molecular weight being significantly lower than normal

The uptake of labelled sugars by keratinocytes and their subsequent incorporation into glycoconjugates (glycoprotein, glycolipid and proteoglycan) have already been described (Roelfzema *et al*, 1981) Certain differences between normal and 'psoriatic' keratinocytes were observed, in particular in the metabolism of fucose

We have now studied the labelled glycoconjugate pool in greater detail in order to define this abnormality more sharply Here we report its separation into lipid and non-lipid fractions and the partial characterization of these components

MATERIALS AND METHODS

Materials

Sephadex G50 was obtained from Pharmacia (Uppsala, Sweden), silica gel from Merck (Darmstadt, Germany) and papain (type III, 2 × cryst), fetuin, transferrin and thyroglobulin

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from the Sigma Chemical Co , Ltd (St Louis, Miss , U S A) Authentic samples of lipids were provided by Applied Science Laboratories, Inc , Philadelphia, U S A All other chemicals were as described previously (Roelfzema *et al* , 1981) The selection of healthy volunteers and psoriatic patients, method of biopsy and preparation of keratinocyte suspensions were as reported by Gommans *et al* (1979) For certain experiments additional biopsies were obtained from psoriatic lesions which had been treated with coal tar and dithranol (Ingram, 1954) for 1–3 days prior to excision

Labelling and isolation procedures

Incubation of cells with radioactive sugars and the subsequent isolation of a 'TCA-insoluble sugar' pellet was as before (Roelfzema *et al* , 1981) using a 3 h or 22 h labelling period

The general protocol for fractionation of the TCA-insoluble material is summarized in Fig 1 The pellet was extracted with 2 × 1 ml portions of chloroform-methanol (2 1 v/v) according to Folch, Lees & Sloan-Stanley (1957) After drying at 60°C, the residue was resuspended in 0 5 ml of 0 2 M acetate buffer (pH 6 5) containing 2 mg/ml cysteine Papain (3 mg) was added, and the mixture incubated for 24 h at 60°C The hydrolysate was cooled to 4°C and centrifuged The supernatant, which contained labelled glycopeptides and the sulphated glycosaminoglycans, was further fractionated by gel filtration on Sephadex G50

In order to avoid the possibility that labelled dolichol derivatives were present in large amounts, the residue obtained after chloroform-methanol extraction was extracted again with chloroform-methanol-water (10 10 3 by volume) Using keratinocytes which had been labelled for 22 h, this extract contained only 3–5% of the total TCA-insoluble radioactivity regardless of whether the cells were derived from healthy skin or psoriatic lesions This procedure was therefore omitted in the following experiments

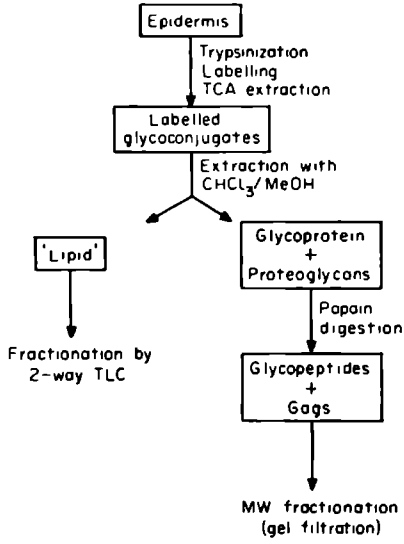

FIGURE 1 General protocol for fractionation of labelled glycoconjugates

Gel filtration

In general samples of 0 5 ml were applied to a Sephadex G50 column which had been equilibrated with 0 2 M pyridine-acetate buffer (pH 5 0) and were eluted with the same buffer The column dimensions were 2 5 × 32 cm, the flow-rate was 35 ml/h, and 2 25 ml fractions were collected In all experiments UV monitoring was used to establish the position of added marker proteins In addition, 'standard' glycopeptide mixtures were prepared by papain digestion of fetuin, desialyzed fetuin, transferrin and thyroglobulin, and the elution profiles recorded

Thin-layer chromatography (TLC)

Two-dimensional TLC was performed according to Broekhuyse (1968) Silica gel HR (containing 3% alkaline magnesium silicate) was used as adsorbant The chloroform-methanol extracts were evaporated to dryness, taken up in benzene-methanol (4 1 v/v) and applied to the plates After development with chloroform-methanol-7 M ammonia (90 54 11 by volume) the chromatograms were dried *in vacuo* for 1 h over H_2SO_4 and developed in the second direction with chloroform-methanol-acetic acid-water (90 40 12 2 by volume) Both solvent systems contained 50 mg/l of butylated hydroxytoluene Lipids were detected with iodine vapour Standards included phosphatidylcholine, phosphatidylethanolamine and cholesterol

Measurement of radioactivity

In general this was as described previously (Roelfzema *et al*, 1981) Samples of silica gel were scraped from thin-layer plates, suspended in 1 ml water and mixed with 10 ml 'Aquasol' for scintillation counting Counting efficiency was about 80% for ^{14}C and 45% for ^{3}H

RESULTS

Fractionation into lipid and non-lipid components

Preliminary experiments, using normal human keratinocytes and (^{14}C)-galactose, showed that the percentage of the total TCA-insoluble radioactivity which extracts into chloroform-methanol (i e lipid) rises from 22 ± 2% (n = 5) after a 3 h labelling period to 40 ± 2% (n = 12) after 22 h labelling The latter period was selected for subsequent experiments, four different labelled sugars were investigated and the behaviour of 'psoriatic' keratinocytes compared with controls It is seen from Fig 2 that, regardless of the sugar employed, the percentage of radioactivity in the lipid fraction was the same for 'uninvolved' psoriatic epidermis and controls, but was markedly increased using keratinocytes derived from psoriatic lesions

In all keratinocyte preparations some large, polygonal cells could be observed which still retained nuclei (Gommans *et al*, 1979) Preparations derived from healthy epidermis contained relatively few of these cells (usually about 1% of the total population), but in preparations from psoriatic lesions these accounted for up to 50% of the total Remarkably, we observed that when using (^{14}C)-galactose the percentage of radioactivity in the lipid fraction increased as a function of the percentage of the 'large' cells in the keratinocyte preparation (Fig 3) No such correlation was apparent in the cases of the other three sugars

TLC of the lipid fraction

Two samples each of the lipid extracts derived from normal, psoriatic lesion and psoriatic uninvolved epidermis (all labelled with ^{14}C-galactose) were combined in order to obtain sufficient material for visualization of the individual components Examination of chromato-

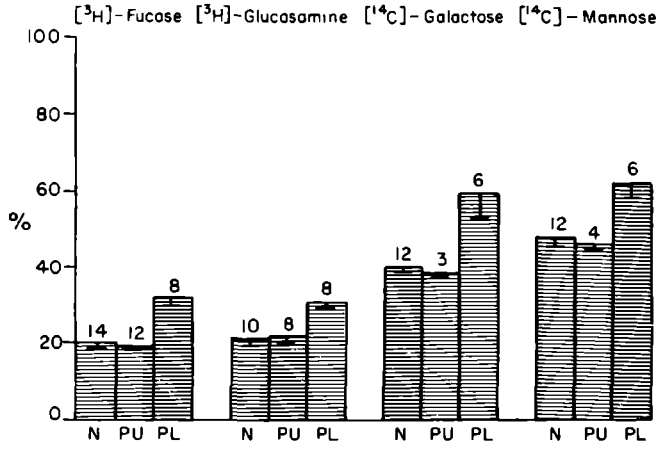

FIGURE 2 Percentage of radioactivity extracted from labelled glycoconjugates by chloroform-methanol Symbols are as follows: N = normal epidermis; PU = uninvolved epidermis from psoriatic patients; PL = psoriatic lesion.

grams treated with iodine vapour revealed a substantial proportion of material remaining at the origin, a single major component and five to six minor components.

The major component co-ran with cholesterol (i.e. near the solvent fronts) and was designated as neutral lipid. The R_F values of the various minor components indicated that these were probably phospholipids, but positive identification of the individual compounds was not made. An area corresponding to each spot was scraped off and the radioactivity measured. The remaining plate was divided into a grid of sixteen squares and the activity of each was determined; in no case did this exceed 0·5% of the total activity.

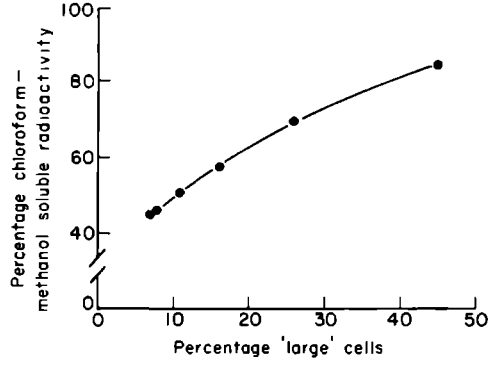

FIGURE 3 Percentage of radioactivity extracted from labelled glycoconjugates by chloroform-methanol as a function of the percentage of 'large' cells in the preparation All specimens were from psoriatic lesions and were labelled with (^{14}C)-galactose

TABLE 1 TI C of lipid fraction Keratinocytes were labelled for 22 h with (^{14}C)-galactose

Specimen	Percentage radioactivity		
	Origin	Neutral lipid	Minor components
Normal epidermis	21	51	28
Psoriatic 'uninvolved' epidermis	19	46	35
Psoriatic lesion	22	50	28

Both the percentage of neutral lipid (Table 1) and the 'patterns' of activity of the minor components were very similar for all three specimens

Gel filtration of glycopeptides and glycosaminoglycans

The molecular weight distributions of the glycopeptides solubilized by papain, using normal human keratinocytes as starting material, are illustrated in Fig 4 In general four peaks can be distinguished, although their relative contributions are very dependent on the particular sugar used for labelling Calibration with standards of known molecular weight and comparison with

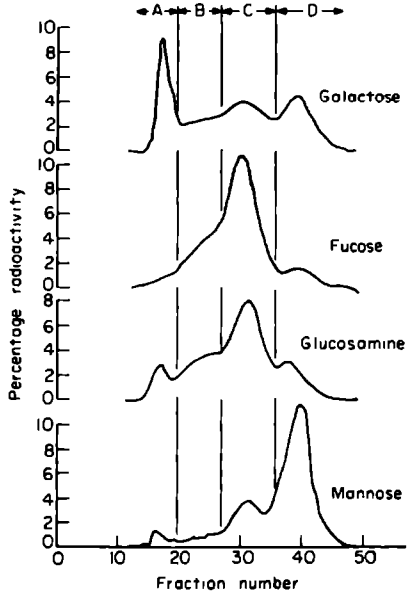

FIGURE 4 Molecular weight distribution of glycopeptides solubilized by papain, using normal human keratinocytes as starting material Curves were prepared by averaging the data for all normal specimens using a TI 59 microcomputer

TABLE 2 Percentage distribution of radioactivity into fractions A–D Symbols are as follows N = normal epidermis, PU = psoriatic uninvolved epidermis, PL = psoriatic lesion, TPL = treated psoriatic lesion Numbers of specimens are in parenthesis Values are ± s e mean

Sugar	Specimen	Fraction A	B	C	D	Ratio C/B
Galactose	N (5)	24 8 ± 2 4	17 3 ± 0 4	30 1 ± 0 9	27 0 ± 1 9	1 8 ± 0 1
	PU (3)	21 4 ± 4 6	19 1 ± 1 3	30 8 ± 1 4	28 1 ± 2 6	1 6 ± 0 1
	PL (4)	31 0 ± 11 4	15 2 ± 2 0	27 2 ± 4 0	26 2 ± 5 7	1 8 ± 0 1
Fucose	N (7)	3 9 ± 0 6	23 8 + 0 8	61 3 ± 1 8	10 1 ± 2 3	2 6 ± 0 1
	PU (9)	2 3 ± 0 3	16 9 ± 1 1	69 4 ± 1 4	10 8 ± 1 0	4 3 ± 0 4
	PL (5)	9 9 ± 2 4	30 1 ± 3 1	43 9 ± 4 1	13 9 ± 4 6	1 6 ± 0 2
	TPL (5)	2 8 ± 0 4	19 8 ± 1 7	66 8 ± 1 2	10 1 ± 1 4	3 5 ± 0 4
Glucosamine	N (7)	10 3 ± 1 6	21 7 ± 0 6	51 5 ± 1 2	16 2 ± 0 8	2 4 ± 0 1
	PU (5)	9 8 ± 1 6	16 8 ± 1 7	52 1 ± 1 0	20 7 ± 2 0	3 2 ± 0 3
	TPL (5)	5 7 ± 0 3	16 9 ± 1 4	48 9 ± 1 9	27 2 ± 2 2	3 0 ± 0 2
Mannose	N (4)	4 2 ± 1 6	5 8 ± 0 2	26 3 ± 2 8	62 6 ± 2 3	4 5 ± 0 4
	PU (3)	3 8 ± 1 8	5 7 ± 0 2	23 2 ± 1 5	65 1 ± 0 5	4 1 ± 0 2
	PL (2)	2 8	4 8	22 7	67 0	5 0

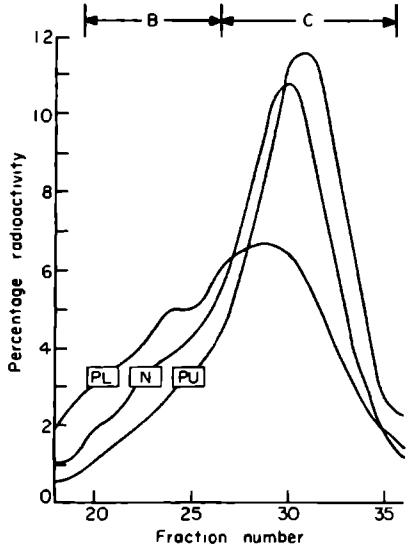

FIGURE 5 Elution profiles of glycopeptides, following labelling with (^{3}H)-fucose, showing the shift to higher mol wt in the psoriatic lesion and to lower mol wt in the psoriatic uninvolved skin Curves were prepared by averaging the data from all (^{3}H)-fucose labelled specimens Symbols are as in Fig 2

published data (Warren, Buck & Tuszynski, 1978, Krusius *et al*, 1974, Kobata, 1979) indicate that the composition of these peaks is as follows

Fraction A glycosaminoglycans and/or O-glycosidic glycopeptides (mucin-type)
Fraction B N-glycosidic glycopeptides complex type with four or more chains
Fraction C N-glycosidic glycopeptides complex type with two chains and high mannose-type chains
Fraction D oligosaccharide fragments

N-glycosidic glycopeptides, of the complex type carrying three chains (obtained by papain digestion of fetuin), were distributed equally between fractions B and C In addition it was established that removal of the terminal sialic acid group from glycopeptides resulted in a shift to a fraction of lower mol wt

To facilitate quantitative comparison of the various specimens, the areas under each peak were evaluated, these data are summarized in Table 2 It is seen that the most striking changes in the psoriatic keratinocytes occur in the distribution of fucose In particular, the ratio C/B is significantly reduced in untreated psoriatic lesions ($P < 0.01$), whereas in both 'uninvolved' psoriatic epidermis and in lesions after 1–3 days therapy this ratio is increased ($P < 0.01$ and $P < 0.05$, respectively) The actual elution profiles of (^{3}H)-fucose from the various specimens are illustrated in Fig 5, the shift to higher mol wt for untreated lesions and to lower mol wt for the 'uninvolved' psoriatic epidermis is evident

No correlation was observed between the mol wt distributions and the percentage of 'large' cells in the individual specimens

DISCUSSION

Our results make it clear that normal human keratinocytes incorporate a substantial proportion of labelled sugar into the lipid fraction This finding is in line with the report of Speak & White (1978), who studied the fate of mannose in mammary gland These workers showed that up to 20% of the radioactivity taken up by the cells was soluble in chloroform-methanol, and reported that 80–90% of this fraction was neutral lipid The consistently higher percentage of radioactivity found in the chloroform-methanol fraction using keratinocytes from psoriatic lesions (Fig 2) may well indicate a real increase in the synthesis of neutral or phospholipids in this disease, an interpretation which would support the data of Summerly, Ilderton & Gray (1978) However, the possibility of an increased incorporation into retinol derivatives, which are soluble in chloroform-methanol and which serve as intermediates in glycoprotein synthesis (De Luca, 1977) cannot be ruled out

The surprising correlation between the incorporation of galactose into glycolipids and the percentage of 'large' cells in the keratinocyte preparation (Fig 3) yields some insight into the site of galactose utilization in the epidermis These cells, which possess a rigid keratinized structure but which still retain nuclei, are clearly derived from the upper layers of the stratum spinosum Thus it would seem either that incorporation of galactose into lipid occurs chiefly in this region or that the incorporation of this particular sugar into glycoprotein and/or proteoglycans is limited to the basal and lower spinous layers

By far the most interesting observation arising from the present work is the abnormality in the molecular weight distribution of fucose-containing glycopeptides, particularly since this confirms our previous report of a specific disturbance in the metabolism of fucose by psoriatic keratinocytes (Roelfzema *et al*, 1981) The reduced C/B ratio in the untreated lesion indicates either that the fucosylated glycopeptides are more highly branched than normal or that there is

an increased sialylation of glycopeptides with a normal degree of branching. A similar shift to higher molecular weight glycopeptides has been reported in cultured BHK cells following viral transformation (Takasaki, Ikehira & Kobata 1980); in this case it was established that the degree of branching was abnormally high.

During the past few years a number of changes have been reported to occur in clinically uninvolved psoriatic epidermis. In general, however, these alterations are in the same direction as those found in the lesion but are smaller in magnitude; thus it is difficult to exclude the possibility that such observations result from the presence of 'sub-clinical' lesions in the apparently healthy epidermis. It is therefore noteworthy that the abnormality which we have demonstrated in the uninvolved psoriatic epidermis is in the opposite sense to that found in the lesion, i.e. the molecular weight of fucosylated glycopeptides is reduced. Further work in this direction is in progress.

REFERENCES

Broekhuyse, R M (1968) Phospholipids in tissues of the eye 1 Isolation, characterization and quantitative analysis by two-dimensional thin-layer chromatography of diacyl and vinyl-ether phospholipids *Biochimica et Biophysica Acta*, **152**, 307.

De Luca, L M (1977) Epithelial membranes and vitamin A In *Mammalian Cell Membranes* (Ed by G A Jamieson and D M. Robinson), 1st ed., vol 3, p 231 Butterworths, London

Folch, J , Lees, M. & Sloan-Stanley, G H (1957) A simple method for the isolation and purification of total lipids from animal tissues. *Journal of Biological Chemistry*, **226**, 494

Gommans, J M , Bergers, M , van Erp, P E J , van den Hurk, J J.M A., Mier, P D & Roelfzema, H (1979) Studies on the plasma membrane of normal and psoriatic keratinocytes. 1 Preparation of material and morphological characterization *British Journal of Dermatology*, **101**, 407.

Ingram, G T. (1954) The significance and management of psoriasis *British Medical Journal*, **ii**, 823.

Kobata, A (1979) Structures and functions of the sugar chains of cell surface glycoproteins. *Cell Structure and Function*, **4**, 169

Krusius, T., Finne, J , Kärkkainen, J & Jarnefelt, J. (1974) Neutral and acidic glycopeptides in adult and developing rat brain. *Biochimica et Biophysica Acta*, **365**, 80.

Roelfzema, H., Bergers, M van Erp, P.E.J., Gommans, J M & Mier, P D (1981) Studies on the plasma membrane of normal and psoriatic keratinocytes. 3. Uptake of labelled sugars and their incorporation into glycoconjugates. *British Journal of Dermatology*, **104**, 635.

Spear, B.K & White, D A (1978) The formation of lipid-linked sugars as intermediates in glycoprotein synthesis in rabbit mammary gland *Biochemical Journal*, **170**, 273

Summerly, R , Ilderton, E & Gray, G M (1978) Possible defects in triacylglycerol and phosphatidylcholine metabolism in psoriatic epidermis *British Journal of Dermatology*, **99**, 279

Takasaki, S., Ikehira, H & Kobata, A. (1980) Increase of asparagine-linked oligosaccharides with branched outer chains caused by cell transformation *Biochemical and Biophysical Research Communications*, **92**, 735

Warren L , Buck, C A. & Tuszynski, G.P. (1978) Glycopeptide changes and malignant transformation A possible role for carbohydrate in malignant behaviour. *Biochimica et Biophysica Acta*, **516**, 97.

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Lectin binding studies on suspensions of isolated epidermal keratinocytes

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SUMMARY

The sugar composition of the surface of isolated human keratinocytes has been investigated using lectins A method is described in which the FITC-labelled lectin is displaced with competing sugar prior to measurement This avoids errors due to aspecific binding and utilizes the minimum biological sample Evidence is presented which indicates that little cell surface glycoconjugate is lost during the preparation of the keratinocyte suspension from human epidermal slices using the technique described here Values for keratinocytes are similar to those reported for fibroblast lines, being much higher than those found using erythrocytes or lymphocytes

The epithelium of human skin is a complex structure, consisting of several distinct layers of cells. Proliferation occurs exclusively in the innermost (basal) layer Certain cells move outward and lose their mitotic ability, this stage being characterized by extensive synthesis of pre-keratin (spinous layer) The uppermost spinous cells subsequently undergo extensive morphological and biochemical changes (keratinization) to leave what is essentially a flake of mature keratin (the corneocyte) It is this last layer which provides the chemical barrier between the body and the environment, and which may therefore be regarded as the *raison d'être* of the epidermis

Clearly the ability of such a complex system to function in the face of environmental fluctuations requires active coordination of cell division, differentiation and keratinization Surprisingly, however, little is known about the homeostatic processes involved, beyond recognition that certain pharmacological mediators influence the equilibria *in vivo* or in culture Most or all of these mediators act via cell-surface receptors which depend for their function on the presence of specific sugar moieties

Lectins, which are proteins or glycoproteins originally isolated from plant seeds, are characterized by their ability to bind sugar residues Their value for the investigation of membrane-bound carbohydrate is now well documented (Hughes, 1976) It seems likely, therefore, that any changes in the distribution of surface receptors which accompany the normal processes of differentiation or pathological situations such as psoriasis may be evaluated in terms of alterations in the lectin-binding properties of the keratinocyte In this report we describe the binding of five different lectins to normal human keratinocytes

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MATERIALS AND METHODS

Chemicals

α-Methyl mannosidase, N-acetyl-D-galactosamine, D-galactose, L-fucose, N-acetyl-D-glucosamine, trypsin (grade III), ribonuclease (bovine pancreas, 5 × recrystallized) and sialidase (type VI, *Clostridium perfringes*) were obtained from the Sigma Chemical Co, Missouri Medium (TC 199) was supplied by Difco (Detroit), penicillin and streptomycin sulphate by Mycofarm, Delft, and calf serum by the Animal Laboratory of this University

The various FITC-labelled lectins (Table 1) were purchased from Pharmindustry, Clichy, France The protein content of each batch of lectin was determined by measurement of optical densities at 278 and 492 nm as described by Monsigny & Obrenovitch (1979) All other chemicals were of Analytic Reagent quality and were obtained from Merck (Darmstadt, Germany)

Skin samples and preparation of cells

This has been described in detail in a previous communication (Gommans *et al*, 1979a) In brief, samples of split-thickness skin (about 1 cm^2, 0 1 mm thick) were removed from paid volunteers using a Castroviejo keratotome After incubation with trypsin and dithioerythritol (30 min, 37 C) the epidermis was peeled from the dermis and the keratinocytes dispersed by agitation in TC 199 medium containing 20% calf serum After treatment with ribonuclease and filtration (50 μm pore size) the cells were pelleted, resuspended in an appropriate volume of medium and counted. Typically $3–6 \times 10^6$ keratinocytes could be obtained from each specimen

Determination of binding sites and association constants

All manipulations using ConA or UeA were carried out in 15 mM tris-buffered isotonic saline containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 1 g/l bovine serum albumin (TBSMA) The experiments using HPA, PNA or WGA employed phosphate-buffered saline containing 1 g/l bovine serum albumin (PBSA).

Freshly-prepared keratinocytes were washed twice in TBSMA or PBSA with centrifuging at 150 *g* and distributed into six 'Eppendorf' polypropylene tubes. Each aliquot contained $1–3 \times 10^5$ cells, a quantity which gave saturation within the range of lectin concentration used. FITC-lectin, dissolved in TBSMA or PBSA, was added to each tube to yield a total volume of 200 μl and a final lectin concentration within the range indicated in Table 1 Blanks (cells omitted) were set up corresponding to the highest and lowest lectin concentrations

After 1 h at 20°C the cells were pelleted, washed twice, and extracted with 500 μl of the appropriate eluting agent (Table 1) for 30 min. The cells were again pelleted, and the fluorescence of the extract determined for specific bindings ('Fluorispec', Baird Atomic; $\lambda_{EX} = 488$, $\lambda_{IM} = 522$ nm). 500 μl of buffer was added to the remaining pellet, and after ultrasonic treatment (MK2, MSE Scientific Instruments Ltd, England) the fluorescence of the contents was determined for aspecific binding A standard of 1–5 μg FITC-lectin in the appropriate eluting agent was employed for calibration. Cell-bound lectin was calculated by subtracting the appropriate blank, and free lectin calculated by subtraction of total bound lectin from the orginal quantity of lectin employed.

After incubation some batches were checked for possible internalization of the lectin with a fluorescence microscope (Leitz Ortholux)

A Scatchard plot (Fig. 1) was used to determine the number of specific binding sites per cell and the association constant according to the equation:

TABLE 1. A summary of the properties of the FITC labelled lectins and the experimental conditions employed. The measurement of FITC/protein ratios is described in the text

Lectin	Abbreviation in text	mol wt.	Average FITC/protein ratio	Source	Sugar specificity	Range of concentration (mg/l) employed	Eluting agent
Concanavalin A	ConA	110,000	0 25	Jack bean *Canavalia ensiformis*	Glucose Mannose	25–375	0 15 M Methyl mannoside
Helix Pomatia agglutinin	HPA	79,000	0 20	Snail *Helix pomatia*	N-acetyl-D-galactosamine	25–275	0 06 M N-Acetyl-D-galactosamine
Peanut agglutinin	PNA	108,000	0 85	*Arachis hypogea*	β-D-galactose in a non-reducing terminal position	5–75	0 15 M D-Galactose
Ulex europeus agglutinin	UeA	46,000	0 50	Gorse seeds (glycoprotein fraction I)	L-Fucose	25–375	1 M Fucose
Wheat germ agglutinin	WGA	36,000	0 25	Wheat germ *Triticum vulgare*	N-acetyl-glucosamine (sialic acid)	20–300	0 3 M N-Acetyl glucosamine

$$\frac{B}{F} = (B_{max} \times K_{ass}) - (B \times K_{ass})$$

where

B = bound lectin (M);
F = free lectin (M);
B_{max} = maximal binding (M) which is equal to the binding site concentration;
K_{ass} = association constant (M^{-1})

Regression lines were calculated using a Texas Instrument SR-51-II and experiments yielding a correlation coefficient of $< |0\,90|$ were discarded.

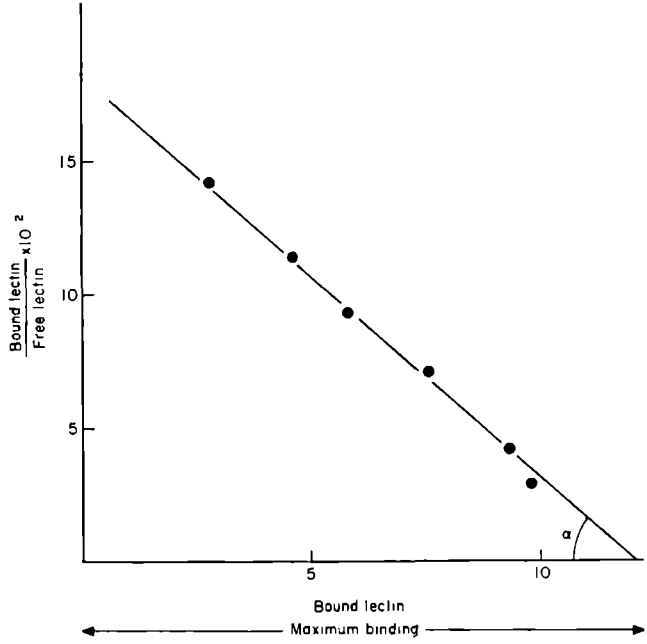

FIGURE 1. A typical Scatchard plot for ConA binding to keratinocytes Bound and free lectin are expressed as μg/ml. In this experiment the number of cells employed was 2 03 × 10⁵ per tube.

Maintenance of keratinocytes in vitro

In certain investigations keratinocytes were maintained *in vitro* for various periods prior to lectin binding studies. Two alternative techniques were employed. In some experiments the cells, suspended in TC 199 medium supplemented with 20% calf serum, were allowed to attach to the surface of glass vessels during the incubation period; they were subsequently dislodged by gentle friction with a rubber policeman. In other experiments aliquots of the cell suspension were placed in Eppendorf polypropylene tubes and agitated on a reciprocal mixer adjusted to the minimum amplitude necessary to prevent settling. In all cases the cells were afterwards washed twice in the appropriate buffer and the lectin binding measured in the usual way.

Incubation of isolated keratinocytes with trypsin or sialidase

The effect of trypsin was investigated by incubating $1-2 \times 10^6$ cells with 0 025% w/v trypsin and 0 3% w/v dithioerythritol in an Eppendorf polypropylene tube (total volume 1 ml) agitated on a reciprocal shaker at 37 C In certain experiments (Fig 2) the basal medium was TC 199 and the incubation period was 30 min, viability after incubation, as measured by the exclusion of trypan blue, was 80–90% For other experiments (Table 4), phosphate-buffered saline (pH 7 3) was employed and the incubation period was 10 min, this resulted in a viability of only about 60%

Incubation with sialidase employed 0 5 units/ml of the enzyme The basal medium was phosphate-buffered saline at a pH of 6 5 and the incubation period 10 min Conditions were in other respects as for trypsin Viability was again about 60%

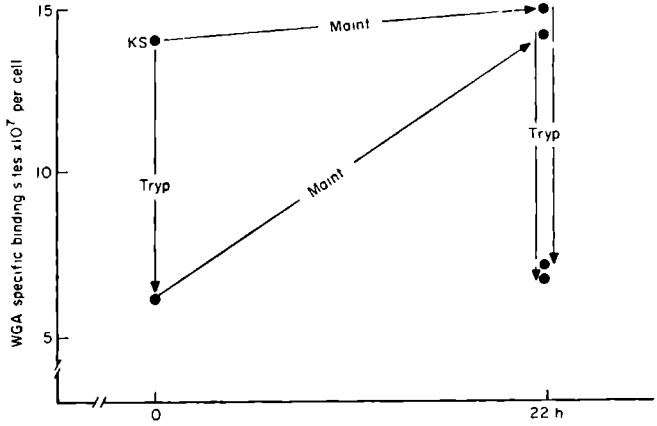

FIGURE 2 Results of various protocols illustrating the reversibility of receptor cleavage by trypsin and recovery during maintenance *in vitro* KS = freshly prepared keratinocytes, tryp = exposure to trypsin (no serum, 37 C, 30 min), maint = incubation under suspension conditions (TC 199 + 20% serum, 37 C, 22 h)

RESULTS

Specific binding of all five lectins to normal human keratinocytes was observed No fluorescence of the nuclear membrane, a uniform fluorescence on the cell surface and a fluorescent halo were observed, indicating that internalization of the lectins had not occurred In general the Scatchard plots were reasonably linear (Fig 1), values for the calculated number of binding sites and for the association constants are listed in Table 2 With the exception of HPA the proportion of aspecifically-bound lectin was rather low (less than 20% of the total bound lectin) In the case of UeA the aspecific binding was proportional to the concentration of lectin over the observed range, thus no values for aspecific binding sites could be calculated

The effect of maintenance of the isolated keratinocytes *in vitro* prior to binding measurements is shown in Table 3 It is clear that incubation under conditions such that attachment to the substratum is prohibited has relatively little effect However, attachment to the surface of a glass culture vessel results in a striking increase in the number of specific binding sites, in the case of WGA this was nearly a twenty-fold increase over the freshly-prepared cells Measurements after longer periods

TABLE 2. Binding sites (±s.d.) per cell and association constants (±s.d.) for the individual lectins. All cell suspensions were derived from whole epidermis; morphology indicated these preparations to contain an average of about 20% basal and 3.4% 'keratinized' cells, the remainder being designated 'squamous'

Lectin	Nature of binding	Number of specimens	Binding sites × 10^7	Association constant × 10^6
ConA	Specific	53	6 6 ± 2 5	1 71 + 0 40
	Aspecific	43	1 13 ± 0 35	3 7 ± 1 6
HPA	Specific	11	0 89 ± 0 19	1 81 ± 0 59
	Aspecific	11	0 82 ± 0 39	0 51 ± 0 20
PNA	Specific	11	1 00 + 0 33	7 1 ± 2 1
	Aspecific	10	0 19 ± 0 08	5 9 ± 2 2
UeA	Specific	8	13 8 ± 3 4	0 13 ± 0 03
	Aspecific			
WGA	Specific	27	14 0 ± 4 6	0 78 ± 0 23
	Aspecific	26	1 87 ± 0 87	0 63 ± 0 21

TABLE 3. Effect of 22 h maintenance *in vitro* on the lectin binding of keratinocytes. Figures are numbers of binding sites per cell ± s.d. Numbers of experiments are in parenthesis

Lectin	Fresh keratinocytes	22 h Maintenance in suspension	22 h Maintenance with attachment
ConA	6.6 ± 2 5 (53)	10 4 ± 6 7 (4)	51 1 ± 24 4 (10)
WGA	14 0 ± 4 6 (27)	15 0 ± 4 6 (14)	264 ± 91 (4)

TABLE 4. Effect of incubating keratinocyte suspensions with trypsin or sialidase. Conditions are described in the text. The control value is taken as 100%

Lectin	Trypsin (%)	Sialidase (%)
ConA	38	115
HPA	58	124
PNA	15	145
UeA	75	166
WGA	36	56

of attachment yielded a mean value of $109 \pm 32 \times 10^7$ binding sites per cell (five experiments) after 46 h and a single value of 424×10^7 binding sites per cell after 6 days *in vitro* for ConA.

The results of incubating keratinocyte suspensions with trypsin or sialidase are summarized in Table 4. The effect of trypsin, as might be expected, is a marked reduction in the binding of all lectins. Following incubation with sialidase, the binding of ConA, HPA, PNA and UeA increase significantly; this is in accord with the concept that removal of sialic acid will reduce steric hindrance and render non-terminal sugars more 'accessible'. Due to possible protease activity in the sialidase preparations this increase may not be optimal. The reduction of binding sites for WGA confirms

the report of Boldt *et al* (1977) that this lectin binds specifically to sialic acid as well as to N-acetyl glucosamine In certain experiments, incubation with trypsin was followed by 22 h maintenance in suspension The results of various protocols are illustrated in Fig 2 It is seen that the loss of surface glycoconjugates is entirely reversible, recovery being complete within 22 h

DISCUSSION

The techniques described here provide a sensitive, convenient and reproducible method of determining lectin binding sites on epidermal keratinocytes The advantages of fluorescent labelling over radioactive labelling have already been discussed (Monsigny & Obrenovitch, 1979) In addition the displacement of specifically bound lectin by competing sugar prior to measurement obviates interference from non-specific binding, the alternative technique commonly employed of using a separate 'blank' composed of lectin plus cells in the presence of the competing sugar obviously doubles the number of cells required, a serious problem when dealing with human biopsy material A theoretical disadvantage of our method is that some specifically bound lectin is lost in the washes, and could result in a low value for apparent binding sites However, preliminary measurements showed that for no lectin does this loss exceed 10% of the bound material

Enzyme kinetics and receptor binding kinetics show many common features The Scatchard plot is a modified Michaelis-Menton equation and therefore three assumptions are implicit, (1) the reaction is at equilibrium and reversible, (2) the lectin concentration is markedly greater than the binding concentration and (3) no cooperative effects between the binding sites occur In the present study these requirements seem to be fulfilled The range of lectin concentrations employed (selected after preliminary experiments) reached about seven times the $1/K_{ass}$ values, and no more than about 10% of the total lectin was bound to the cells Linearity of the plots indicated an absence of cooperative effects, and also confirmed that the specifically bound lectin was attached to the sugar moiety in a relatively uniform configuration This does not, of course, imply that the individual sugars were present in similar oligosaccharide sequences

A more serious possibility is the loss of surface glycoprotein during the trypsinization procedure used to isolate keratinocytes It should be emphasized, however, that in our technique (Gommans *et al*, 1979a), and in contrast to methods described elsewhere, the separated epidermis is removed from the trypsin solution into medium containing serum *before* mechanical disruption, thus the surface of the isolated cells is never exposed directly to the enzyme Deliberate subsequent exposure of the cells to trypsin (Fig 2) does indeed cause serious loss The data illustrated in Fig 2 suggest strongly that the surface glycoprotein levels in the fresh keratinocyte suspension (KS) are very similar to those after 22 h 'recovery', and thus presumably to those of the cell surface *in vivo* This conclusion is similar to that reached using adrenaline stimulation of adenyl cyclase activity as a 'functional' test of membrane receptor integrity (Gommans *et al*, 1979b) These data also confirm the metabolic viability of our keratinocyte preparations

Comparison of our absolute values with those reported previously (see, for example, review by Sharon & Lis, 1975) is of particular interest since the present data are apparently the first quantitative measurements pertaining to cells of epithelial origin It is clear that our figures, at least for ConA and WGA, are an order of magnitude higher than those found for erythrocytes or lymphocytes, corresponding more closely to those reported for cultured fibroblast lines This may, of course, reflect the unusually large surface area of keratinocytes rather than any specific difference in surface composition Investigations are currently in progress to establish whether changes in the lectin-binding profile accompany the normal processes of keratinocyte differentiation or occur in pathological situations such as psoriasis

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REFERENCES

BOLDT, D.H., SPECKART, S.F., RICHARDS, R.L. & ALVING, C.R. (1977) Interactions of plant lectins with glycolipids in liposomes. *Biochemical and Biophysical Research Communications*, **74**, 208.

GOMMANS, J.M., BERGERS, M., VAN ERP, P.E.J., VAN DEN HURK, J.J.M.A., MIER, P.D. & ROELFZEMA, H. (1979a) Studies on the plasma membrane of normal and psoriatic keratinocytes. 1. Preparation of material and morphological characterization. *British Journal of Dermatology*, **101**, 407.

GOMMANS, J.M., BERGERS, M., VAN ERP, P.E.J., VAN DEN HURK, J.J.M.A., VAN DE KERKHOF, P., MIER, P.D. & ROELFZEMA, H. (1979b) Studies on the plasma membrane of normal and psoriatic keratinocytes. 2. Cyclic AMP and its response to hormonal stimulation *British Journal of Dermatology*, **101**, 413.

HUGHES, R.C. (1976) Lectins. In. *Membrane Glycoproteins*, p. 135. Butterworths, London, Boston.

MONSIGNY, C.S. & OBRENOVITCH, A. (1979) Quantitative fluorimetric determination of cell-surface glycoconjugates with fluorescein-substituted lectins. *European Journal of Biochemistry*, **96**, 295.

SHARON, N. & LIS, H. (1975) Use of lectins for the study of membranes. In *Methods in Membrane Biology* (Ed. by E.D.Korn), vol. 3, p. 147. Plenum Press, New York.

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Studies on the plasma membrane of normal and psoriatic keratinocytes. 5. Lectin binding

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SUMMARY

The glycocalyx of epidermal keratinocytes from psoriatic patients has been investigated by means of lectins Striking changes were found in the levels of glucose and/or mannose (concanavalin A) and of N-acetylglucosamine and/or sialic acid (wheat germ agglutinin) on the surface of cells from the psoriatic lesion Smaller but significant changes were seen in the clinically uninvolved epidermis of the patient A marked increase in the affinity of the cell surface for *Ulex europus* agglutinin (fucose-specific) confirms our previous reports of structural alterations in fucose-containing oligosaccharides in psoriasis

In previous papers in this series we have reported profound abnormalities in the metabolism of certain sugars by psoriatic keratinocytes Disturbances include an increased rate of entry of labelled sugars into the cell and of their incorporation into glycoconjugates (Roelfzema *et al*, 1981a) and changes in the structure of the oligosaccharide fragments remaining after proteolytic digestion of cellular glycoprotein (Roelfzema *et al*, 1981b) Such findings are compatible with the altered cell cycle kinetics of the psoriatic lesion, since the surface receptors which couple the external milieu to the intracellular regulators are mostly glycoprotein in nature (Kahn, 1976)

A direct analysis of the carbohydrate content of glycoconjugates located on the cell surface would therefore be very useful in extending these findings Unfortunately the difficulties in isolating pure plasma membrane fractions coupled with the very limited amount of tissue available from human biopsy makes this approach impractical However, lectins (specific sugar-binding proteins) can be used as sensitive and reliable tools for the study of cell-surface sugars (Sharon & Lis, 1975), and the application of this technique to normal human epidermis has recently been described (Gommans & van den Hurk, 1981) In the present paper we report the extension of this work to keratinocytes derived from psoriatic lesions and the uninvolved epidermis of psoriatic patients

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MATERIALS AND METHODS

Materials

The sources of the chemicals used in this investigation have been detailed in a previous publication (Gommans & van den Hurk, 1981) The protein content of each batch of FITC-labelled lectin was determined by measurement of optical densities at 278 nm and 492 nm Sellotape employed for the 'stripping' experiments was Tesafilm (Beiersdorf B V , Almere, NL)

Patients with psoriasis vulgaris were selected who had chronic, stable plaques on the trunk or upper limbs and who had received no topical or systemic therapy for at least 2 weeks prior to investigation Control subjects were healthy volunteers with no personal or family history of psoriasis Skin samples of about 1 cm^2 were obtained using a Castroviejo keratotome, set to 0 3 mm depth for psoriatic lesions or 0 2 mm for healthy or psoriatic uninvolved skin The preparation of isolated keratinocyte suspensions was as described by Gommans *et al* (1979), briefly, the skin slices were exposed to trypsin for 30 min in the presence of dithioerythritol, the epidermis was disrupted mechanically after neutralizing the trypsin with calf serum, and the keratinocytes were washed by centrifugation and counted

Lectin-binding assays

The lectins studied were concanavalin A (Con A) which binds mannose or glucose, *Helix pomatia* agglutinin (HPA) binding N-acetyl-D-galactosamine, peanut agglutinin (PNA) binding D-galactose in a non-reducing terminal position, *Ulex europus* agglutinin (UEA) binding L-fucose and wheat germ agglutinin (WGA) which binds N-acetyl-D-glucosamine and sialic acid All lectins were used as fluorescein isothiocyanate (FITC) derivatives to permit fluorometric quantification

The techniques employed have been described fully by Gommans & van den Hurk (1981) In brief, six aliquots of suspended keratinocytes were incubated with different concentrations of each FITC-labelled lectin After pelleting the cells and washing to remove excess lectin, the bound material was displaced with the appropriate sugar and determined Non-specific binding was estimated by direct fluorometry of the resuspended residue after sonication The number of binding sites (i e 'available' sugar molecules) on the surface of the cell and the association constant (Ka) between the lectin and the sugar moiety was calculated by means of Scatchard plots .

Basal-cell and upper spinous cell enriched preparations

In certain experiments the standard procedure for the preparation of keratinocytes was modified by reducing the time of incubation with trypsin-dithioerythritol to 15–20 min After mechanical separation of epidermis from dermis, an appreciable fraction of the epidermal keratinocytes remained adhering to the dermis, from which they could be freed by vigorous agitation in medium containing 20% calf serum Morphological evidence confirmed that this sub-population consisted mainly of basal or lower spinous cells

Preparations enriched in upper spinous cells were obtained by a second trypsinization of the stratum corneum remaining from the standard procedure, followed by repeated agitation In general up to $0\ 3 \times 10^6$ upper spinous cells could be obtained from each biopsy, the preparations containing 60–100% of nucleated keratinocytes which remained polygonal (i e did not 'round up') in suspension The majority of these cells did not exclude trypan blue The yield of both sub-populations was sufficient for the determination of Con A binding only

Epidermal regeneration studies

Experiments were carried out in which the stratum corneum was 'stripped' by repeated application of sellotape to the skin of healthy volunteers (Pinkus, 1951) and the lectin binding characteristics of keratinocytes determined during the subsequent regenerative response.

Three areas of skin were stripped on the back of each subject. After marking each area (about 4 cm^2), Tesafilm® was applied firmly and peeled away to remove a layer of adherent corneocytes. Following about fifteen consecutive applications, the underlying epidermis became red and moist, and no further corneocytes were seen adhering to the tape. Biopsies of the stripped areas were obtained at 24, 48 and 72 h. Keratinocytes were prepared from each sample and lectin binding measurements (Con A and WGA) made in the usual way.

RESULTS

The numbers of binding sites per cell for the various lectins are summarized in Table 1. These results are quite clear: the number of 'available' glucose and/or mannose molecules (Con A binding) is roughly tripled and the number of N-acetylglucosamine and/or sialic acid molecules (WGA binding) is nearly doubled on the surface of keratinocytes from the psoriatic lesion. More surprisingly, there is a significant increase (about double) in the quantity of glucose/mannose on the surface of cells from the apparently healthy skin of the patient. The distribution of the remaining sugars appears to be normal.

The data regarding association constants are summarized in Table 2. These are more difficult to interpret, since the avidity with which a lectin binds to a particular sugar depends not only on the nature of the sugar, but also on the configuration of the oligosaccharide moiety in which it occurs (Narasimhan *et al.*, 1979). Nevertheless, it is clear from these data that the carbohydrate composition of the psoriatic cell surface is grossly abnormal.

Binding of Con A to upper spinous cell and basal cell enriched preparations is shown in Table 3, together with figures for the percentage of upper spinous cells (i.e. cells which do not 'round up' after trypsinization) in standard preparations from healthy skin and psoriatic lesions. Several facts emerge from these figures. First, it is evident that 'available' glucose and/or mannose increases dramatically during maturation of the keratinocyte. Secondly, it is clear that there are real differences between the corresponding normal and psoriatic sub-populations; in

TABLE 1 Number of binding sites (+standard deviation) per cell Data for normal keratinocytes are taken from Gommans & van den Hurk (1981)

		Normal		Psoriatic uninvolved			Psoriatic lesion		
Lectin	Nature of binding	Number of specimens	Binding sites × 10^7	Number of specimens	Binding sites × 10^7	*P*	Number of specimens	Binding sites × 10^7	*P*
Con A	Specific	53	6 6 ± 2 5	24	11 2 ± 4 7	0 025	35	18 1 ± 0 1	0 005
	Non-specific	43	1 13 ± 0 35	23	1 46 ± 0 59	0 025	32	2 75 ± 1 41	0 005
HPA	Specific	11	0 89 ± 0 19	11	1 00 ± 0 36	n s	11	0 83 ± 0 27	n s
	Non-specific	11	0 82 ± 0 39	11	0 71 ± 0 28	n.s	11	0 73 ± 0 18	n s
PNA	Specific	11	1 00 ± 0 33	9	1 21 ± 0 49	n s	7	1 44 ± 0 75	n s
	Non-specific	10	0 19 ± 0 08	7	0 20 ± 0 09	n s	5	0 24 ± 0 03	n.s.
UEA	Specific	8	13 8 ± 3 4	6	13·6 + 6 7	n s	8	12 0 ± 6 2	n s.
WGA	Specific	27	14 0 ± 4 6	11	14 8 ± 5 9	n s	15	25 0 ± 15 6	0 005
	Non-specific	26	1 87 ± 0 87	9	1 84 ± 0 96	n.s.	15	3 59 ± 2 76	0 010

TABLE 2 Association constants (± standard deviation) between the various lectins and the cell Data for normal keratinocytes are taken from Gommans & van den Hurk (1981)

		Normal		Psoriatic uninvolved			Psoriatic lesion		
Lectin	Nature of binding	Number of specimens	Association constant × 10^6	Number of specimens	Association constant × 10^6	*P*	Number of specimens	Association constant × 10^6	*P*
Con A	Specific	53	1 71 ± 0 40	24	1 46 ± 0 39	0 005	35	1 13 ± 0 30	0 005
	Non-specific	43	3 71 ± 1 56	23	3 30 ± 1 65	n s	32	3 28 ± 1 34	n s
HPA	Specific	11	1 81 ± 0 59	11	1 76 ± 0 82	n s	11	2 00 ± 0 65	n s
	Non-specific	11	0 51 ± 0 20	11	0 57 + 0 26	n s	11	0 73 + 0 18	0 050
PNA	Specific	11	7 1 + 2 1	9	5 4 ± 2 7	n s	7	6 6 ± 2 5	n s
	Non-specific	10	5 9 ± 2 2	7	4 8 ± 2 9	n s	5	3 3 ± 0 7	0 005
UEA	Specific	8	0 13 ± 0 03	6	0 17 ± 0 04	n s	8	0 31 ± 0 15	0 005
WGA	Specific	27	0 78 ± 0 23	11	0 70 + 0 21	n s	15	0 39 ± 0 14	0 025
	Non-specific	26	0 63 ± 0 21	9	0 55 + 0 11	n s	15	0 47 ± 0 20	0 025

TABLE 3 Number of Con A binding sites per cell for basal cell and upper spinous cell preparations The percentage of upper spinous ('polygonal') cells in standard preparations from healthy epidermis and psoriatic lesions is shown in column 3 Numbers of experiments are in parentheses

	Binding sites (± s d) × 10^7		Percentage of upper spinous cells in
Specimen	Basal cells	Upper spinous cells	standard preparations
Normal	2 6 ± 0 3 (3)	136 ± 36 (3)	3 4 ± 2 5 (17)
Psoriatic lesion	7 9 ± 2 2 (4)	30 ± 4 (4)	18 9 + 15 4 (25)

TABLE 4 Number of binding sites per cell for Con A and WGA at various times after stripping with Sellotape Numbers of experiments are in parentheses

	Binding sites (× 10^7) per cell	
Hours after stripping	Con A	WGA
0	6 6 (53)	14 0 (27)
24	7 9 (3)	11 6 (2)
48	10 3 (4)	13 7 (2)
72	8 0 (3)	12 7 (2)

particular the surface of the psoriatic basal cell is much richer in glucose/mannose than its normal counterpart.

Table 4 shows Con A and WGA binding to keratinocytes at various times after 'stripping' the stratum corneum from the skin of healthy volunteers. It is seen that the number of binding sites for Con A peaked at about 50% above normal after 48 h, whereas the level of WGA binding remained constant throughout the experiments.

DISCUSSION

Our results indicate that the cell surface of keratinocytes from psoriatic lesions has an altered carbohydrate composition. This difference cannot be explained solely by the presence of larger microvilli in the psoriatic keratinocytes (Gommans *et al.*, 1979) because the binding of the various lectins increases in varying degree and also because the association constants are markedly altered. Thus we must assume either structural changes in the oligosaccharide moieties in particular glycoproteins or an alteration in the relative amounts of the different types of glycoprotein. The marked increase in the association constant of UEA (fucose-binding) is noteworthy, and may be correlated with the changes in molecular weight distribution of total membrane glycopeptides found after labelling with fucose (Roelfzema *et al.*, 1981b).

The enormous increase in Con A binding which accompanies the maturation of normal keratinocytes (Table 3, column 1) is an obvious consequence of the increased surface area, but may in part be an artefact resulting from membrane damage and hence to access of the lectin to internal binding sites. Since the percentage of apparently mature keratinocytes is very much greater in the psoriatic lesion than in healthy epidermis, it is in any case necessary to exclude the possibility that such a change in distribution could account for the abnormalities discussed above. It is in fact clear (column 2) that this is not the case, both sub-populations of psoriatic keratinocytes being markedly different from normal.

The final question concerns the specificity of these changes for psoriasis. Since the hall-mark of the psoriatic lesion is the increased proliferative rate, it seemed of interest to evaluate a parallel experimental situation. We selected for this purpose the regenerative response following 'stripping' with Sellotape. The relatively small increase in Con A binding (presumably mannose) together with the complete absence of any change in WGA binding would seem clearly to differentiate this situation from that found in the psoriatic lesion. Although these data are of course insufficient to permit firm conclusions, it seems probable that the structural alterations in cell surface glycoprotein which occur in the psoriatic lesion are not secondary to the increased proliferation.

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REFERENCES

GOMMANS, J M , BERGERS, M , VAN ERP, P E.J. VAN DEN HURK, J M M A , MIER, P D & ROELFZEMA, H. (1979) Studies on the plasma membrane of normal and psoriatic keratinocytes 1 Preparation of material and morphological characterization *British Journal of Dermatology*, **101**, 407.

GOMMANS, J M & VAN DEN HURK, J J M A (1981) Lectin binding studies on suspensions of isolated epidermal keratinocytes *British Journal of Dermatology*, **104**, 641

KAHN, C R. (1976) Membrane receptors for hormones and neurotransmitters. *The Journal of Cell Biology*, **70**, 261.

NARASIMHAN, S , WILSON, J R , MARTIN E & SCHACHTER, H. (1979) A structural basis for four distinct elution profiles on concanavalin A-sepharose affinity chromatography of glycopeptides *Canadian Journal of Biochemistry*, **57**, 83

PINKUS, H. (1951) Examination of the epidermis by the strip method of removing horny layers *Journal of Investigative Dermatology*, **16**, 383

ROELFZEMA, H., BERGERS, M , VAN ERP, P E J., GOMMANS, J.M. & MIER, P D (1981a) Studies on the plasma membrane of normal and psoriatic keratinocytes. 3 Uptake of labelled sugars and their incorporation into glycoconjugates, *British Journal of Dermatology*, **104**, 635

ROELFZEMA, H , BERGERS, M , VAN ERP, P E J , GOMMANS, J M & MIER, P D (1981b) Studies on the plasma membrane of normal and psoriatic keratinocytes 4 Characterization of glycoconjugates *British Journal of Dermatology*, **105**, 509

SHARON, N & LIS, H (1975) Use of lectin for the study of membranes In *Methods in Membrane Biology* (Ed by E D Korn), vol 3, p 147 Plenum Press, New York

Electrophoretic mobilities of keratinocytes from psoriatic lesions and normal epidermis.

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SUMMARY

We describe the first measurement of the electrophoretic mobility of cells derived from a solid tissue. Keratinocytes, like other mammalian cells, carry a negative electric charge; the mobility of keratinocytes derived from psoriatic lesions is greater than those of normal epidermis, indicating an increased charge. This is compatible with previous suggestions of an increased level of sialic acid on the cell surface in psoriasis.

The electrophoretic mobility of a cell is determined by its 'zeta-potential' or surface charge (Coker, Perry and Plummer, 1981). This charge is negative in all mammalian cells, its magnitude depending on the composition of the cell surface carbohydrate (glycoprotein and glycolipid). The chief contributor seems to be the negatively charged carboxyl group carried by sialic acid, a sugar which occurs in the terminal position in oligosaccharide chains (Schachter et al, 1982). Other investigations, however, have suggested that the positively-charged amino groups of amino sugars play a significant, albeit minor, role (Lochner et al, 1982).

During the last few years a number of reports have indicated membrane alterations, in particular of cell surface carbohydrate, in the lesions of psoriasis (Roelfzema et al, 1981a and b; Gommans et al, 1982). We have now attempted to confirm and extend these findings by direct measurement of the electrophoretic mobility of keratinocytes from psoriatic lesions.

MATERIALS AND METHODS

Subjects and preparation of samples

Psoriatic patients (mean age 46 ± 16 years) had stable, chronic plaques which had been untreated for at least 2 weeks. Controls (24 ± 5 years) were healthy, paid volunteers with no history of psoriasis. Biopsies (approximately 5 x 0.3 mm) were cut freehand

and cell suspensions prepared as described by Bauer et al (1980) but with omission of propidium iodide and DMSO. Cells were pelleted and washed 3 times by centrifugation (150 g, 10 min) in electrophoresis buffer (15 mmol/l sodium phosphate containing 0.27 mol/l sucrose, pH 7.9). The keratinocytes (about 5×10^5) were resuspended in 200 µl of buffer prior to electrophoresis.

Preliminary experiments to check the reproducibility of the technique were carried out using monodisperse carboxylated microspheres, diameter 3.55 µm, supplied by Polysciences (Warrington, USA). An aliquot of 20 µl of this suspension, containing about 1.5×10^7 particles, was washed 3 times and resuspended in 5 ml electrophoresis buffer before use.

Electrophoresis procedure

Electrophoresis was carried out using the 'Particle Microelectrophoresis Apparatus Mark II' (Rank Brothers, Cambridge, England), depicted schematically in Fig. 1.

The sample was introduced into the capillary and the blackened platinum electrodes inserted. The current was adjusted to fall into the range 0.3-0.6 mA, and the precise values of current and voltage noted. Five different levels in the capillary were selected by adjustment of the focal plane of the microscope, spaced at exactly 0.3 mm intervals, and a total of 40 different cells observed at each level. The velocity of each cell was determined by timing its movement over a distance of 60 µm, using the calibrated eyepiece; to overcome asymmetry problems, the recorded velocity was averaged from two consecutive

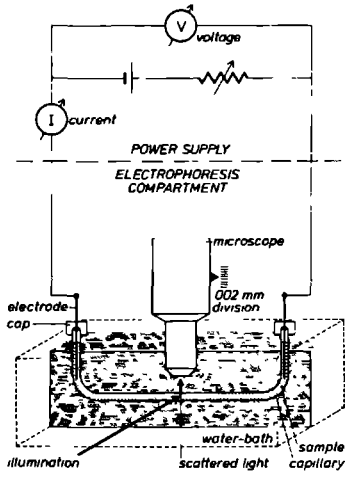


Figure 1 Schematic diagram of electrophoresis equipment. Illumination is at right angles to the plane of the paper. The water bath is thermostatically controlled at 25° C. The internal diameter of the capillary is about 2 mm and its horizontal length 4.8 cm.

measurements using opposite polarities.

Calibration and calculation of mobility

The resistance of the sample-filled capillary was calculated ($^{V}/_{I}$) and converted to specific resistance (ρ) by calibration with 0.1 mol/l KCL solution (77.58 Ω cm). The inner diameter of the capillary (2 r) was determined by focussing on the upper and lower inside surfaces. The field strength (E) at the point of viewing could then be calculated as:

$$E = \frac{I \times \rho}{\pi r^2}$$

Since the glass walls of the capillary are negatively charged in the presence of the electrophoresis buffer, this leads to electro-osmotic streaming of the oppositely charged solvent near the walls to the appropriate electrode.
A correction may be applied using the following equation (Alexander and Johnson, 1950):-

$$v = v_{obs} + v_o - 2\, v_o\, (^{l}/_{r})^2$$

where v = electrophoretic velocity of the particle, v_{obs} = observed velocity, v_o = electro-osmotic velocity and l = distance of the plane of focus from the axis of the capillary. Refraction effects at the glass-liquid interfaces, however, require an additional correction in the ratio $^{l}/_{r}$, leading to:-

$$v = v_{obs} + v_o - 2\, v_o\, (^{l}/_{r} + \text{shift})^2$$

Values of v_{obs} at each of the 5 levels were fitted to this equation to yield the best values for v, v_0 and shift.

The electrophoretic mobility (u) was finally calculated from the corrected velocity and the field strength:-

$$u = {}^{v}/_{E}\ cm^2.\ sec^{-1}.volt^{-1}$$

RESULTS

The preliminary experiments with carboxylated microspheres enabled us to optimize the technique and to establish the reproducibility of the measurements. It is seen from Table 1 (upper line) that the reproducibility is in fact extremely high, the standard deviation between the results of 10 experiments being about 2% of the mean. Even this low figure probably reflects real variations in the physical characteristics of the microspheres (variation coefficient between individual particles of 12.6%) more than intrinsic errors in the measurements.

The data for normal and psoriatic keratinocytes are also shown in Table 1. It is seen that cells from the psoriatic lesion carry a negative charge about 20% greater than those from healthy skin ($P < 0.001$, Students t-test). The variation between individual cells from any one suspension was considerably greater than the variation between microspheres.

Table 1 Electrophoretic mobilities of carboxylated microspheres, keratinocytes from healthy controls and keratinocytes from psoriatic lesions. Col. 2 shows means ± SD of 10 experiments; the variation coefficient (col. 3) indicates the 'spread' of individual values within any one experiment.

Material	Mean ± SD ($cm^2.sec^{-1}.volt^{-1} \times 10^{-4}$)	Variation coefficient
Carboxylated microspheres	3.644 ± 0.081	12.6% ± 1.3%
Normal keratinocytes	1.262 ± 0.138	23.2% ± 3.6%
Psoriatic keratinocytes	1.483 ± 0.095	21.2% ± 5.0%

No correlation was observed between electrophoretic mobility and age of the subject in either the psoriatic or the control group.

DISCUSSION

It is clear from our data that measurement of the mobility of a cell in an electric field (as might be expected from a purely physical technique) offers a reproducibility and precision greatly exceeding that of most biological or biochemical approaches. It has, in the past, been applied to various blood-derived cell types, and changes in electrophoretic mobility have been shown to accompany malignancy (Tantz et al, 1977). Our present work, however, seems to be the first application of this method to the study of any solid tissue. The major criticism here, of course, is the theoretical possibility of loss of carbohydrate during cell isolation; this problem has been discussed in our earlier publications and seems in practice to be minimal (Gommans and van den Hurk, 1981). It is pertinent to note that the actual cell surface charge (zeta potential) may be calculated directly from the mobility, and indeed some authors express their data in this way. Such calculations, however, involve a number of assumptions, and for practical purposes the use of mobilities is perhaps safer (Loeb et al, 1960).

Turning to psoriasis, it has been clear for some years that the surface of the keratinocyte in the lesion is abnormal. This was first convincingly demonstrated by Mahrle and Orfanos (1974), whose electron microscopical studies indicated a disturbance of the carbohydrate-rich outer coat of the cell ('glycocalyx'). Subsequent investigations in our own laboratories have confirmed this by means of a number of different approaches. First, we demonstrated a functional disturbance of the β-adrenergic receptor (Gommans et al, 1979); secondly we described alterations in amount and composition of cellular glycoconjugates (Roelfzema et al, 1981a and b), and finally we have shown that the affinity of various lectins (sugar-specific binding proteins) to the cell surface is abnormal in the psoriatic lesion (Gommans et al, 1982). It is therefore reassuring to link these changes by the direct determination of a single, specific physical parameter characterizing the cell surface.

Elevation of electrophoretic mobility which occurred just prior to, and during cell division (Mayhew, 1966) was interpreted by Kramer (1967) to be a change in the spatial arrangement of sialic acid in the region of the membrane which determines surface charge ("electrokinetic shear layer"). Thus a greater negative surface charge may result from either increased sialic acid levels, and/or from an altered topography of the plasma membrane (Smith and Walborg, 1977). Our present data are therefore compatible with several earlier observations. Structural analysis of fucose-

labelled glycopeptides (Roelfzema et al, 1981b) indicated an increased degree of branching in psoriasis; since sialic acid occurs only on the terminal position, this seems compatible with a shift to more negative surface charge. Further, binding of the lectin wheat germ agglutinin is doubled in the psoriatic lesion (Gommans et al, 1982) and the effects of sialidase treatment suggested that an increased level of sialidation might be responsible. Again, our present results offer direct confirmation of this concept. Several outstanding questions remain, including the specificity of our findings for psoriasis and the relationship of cell surface charge to processes such as proliferation and differentiation in normal epidermis. Investigations along these lines are currently in progress.

REFERENCES

Alexander, A.E. & Johnson, P. (1950) Colloid Science. University Press, Oxford.

Bauer, F.W., Crombag, N.H.C.M.N., De Grood, R.M. & De Jongh, G.J. (1980) Flow cytometry as a tool for the study of cell kinetics in epidermis. Investigations on normal epidermis. British Journal of Dermatology, 102, 629

Coker, E.N., Perry, M.C. & Plummer, D.T. (1981) Changes in the zeta potential of chick erythrocytes after the addition of insulin . Biochemical Society Transactions, 9, 89

Gommans, J.M., Bergers, M., Van Erp, P.E.J., Van den Hurk, J.J.M.A., Van de Kerkhof, P., Mier, P.D. & Roelfzema, H. (1979) Studies on the plasma membrane of normal and psoriatic keratinocytes. 2. Cyclic AMP and its response to hormonal stimulation. British Journal of Dermatology, 101, 413.

Gommans, J.M. & Van den Hurk, J.J.M.A. (1981) Lectin binding studies on suspensions of isolated epidermal keratinocytes. British Journal of Dermatology, 104, 641

Gommans, J.M. Van den Hurk, J.J.M.A., Bergers, M., Van Erp, P.E.J., Mier, P.D. & Roelfzema, H. (1982) Studies on the plasma membrane of normal and psoriatic keratinocytes. 5. Lectin binding. British Journal of Dermatology, 106, 317

Kramer, P.M. (1967) Configuration change of surface sialic acid during mitosis. Journal of Cell Biology, 33, 197

Lochner, J.E., Seaman, G.V.F., Blume, P. & Malley, A. (1982) Early electrochemical events in lectin lymphocyte interaction. Cell Biophysics, 4, 15

Loeb, Overbeek & Wiersma (1960). The electric double layer around a spherical colloid particle. M.I.T. press, Cambridge Mass., U.S.A.

Mahrle, G. & Orfanos, C.E. (1974) Decreased membrane-bound ATP-hydrolytic activity in psoriatic epidermis. British Journal of Dermatology, 91, 529

Mayhew, E. (1966) Cellular electrophoretic mobility and the mitotic cycle. Journal of General Physiology, 49, 717

Roelfzema, H., Bergers, M., Van Erp, P.E.J., Gommans, J.M. & Mier, P.D. (1981a) Studies on the plasma membrane of normal and psoriatic keratinocytes. 3. Uptake of labelled sugars and their incorporation into glycoconjugates. British Journal of Dermatology, 104, 635

Roelfzema, H., Bergers, M., Van Erp, P.E.J., Gommans, J.M. & Mier, P.D. (1981b) Studies on the plasma membrane of normal and psoriatic keratinocytes. 4. Characterization of glycoconjugates. British Journal of Dermatology, 105, 509

Schachter, H., Narasimhan, S., Gleeson, P., Vella, G.J. & Brockhausen, I. (1982) Oligosaccharide branching of glycoproteins: biosynthetic mechanisms and possible biological functions. Philosophical Transactions of the Royal Scociety of Londen Series B300, 145

Smith, D.F. & Walborg,E.F.Jr. (1977) The tumor cell periphery: carbohydrate components. In: Mammalian Cell Membrane (Ed. by G.A.Jamieson and D.M.Robinson), vol. 3, p.122 Butterworths, London

Tautz, C., Schneider, W., Laier, E. & Brugmann, G. (1978) Der elektrophoretische Mobilitäts(EM)Test : Untersuchungsmethode zur Unterscheidung von malignen und nicht malignen Tumoren. Klinische Wochenschrift 56, 175

DISCUSSION

General

The cellular hyperactivity in psoriasis has long been regarded as an epidermal phenomenon (Shuster 1971). But it is now reported that the cells resident in the psoriatic dermis also have abnormally high rates of proliferation when maintained in culture and those from the uninvolved skin are almost as active as those from the lesion (Priestley 1983). Even the markers of the capillary situated beneath the psoriatic lesion are grossly elevated (van de Kerkhof 1983) and, although the monocyte is not really abnormal in stable psoriasis (Geerdink 1984), the polymorphonucleocytes are changed (Kreuger 1981). All these factors, with presumably still undiscovered ones, have a role in the pathogenesis of psoriasis. All are linked together and influencing each other, so the location of the primary defect is uncertain. But attacking the disease by studying one factor of the homeostasis will throw some light on the interactions of the different components and may so elucidate the nature of the defect in psoriasis. As explained in the introduction, this study is therefore concerned with differences in the plasma membrane composition of normal and psoriatic keratinocytes.

Technical aspects

In this section the most important methods are described, with their advantages and limitations. The method described in chapter 2 very gently liberates keratinocytes from the epidermis by the proteolytic enzyme trypsin with dithioerythritol as

a reducing agent. Although changes in suspensions of human keratinocytes due to trypsin are reported (Barton 1981), these changes are mostly due to higher trypsin concentrations, longer incubation times and a direct trypsinization of the keratinocytes. In our method the enzyme trypsin has to diffuse through some dermis and the basal lamina. Before dermis and epidermis are separated, the trypsin is inhibited by serum proteins and the epidermal cell suspension is made in a medium with serum. It is proven in chapter 3 that at least the beta-adrenergic receptor on the plasma membrane of the keratinocyte is unaffected by our mild trypsinization procedure. This was confirmed by Takeda et al (1983) who found no significant loss of adrenaline-stimulated cyclic AMP synthesis by mild trypsinization (0.05-0.8%). It is shown in chapter 6 that the proteolytic enzyme trypsin removes carbohydrates of the plasma membrane by direct treatment, indicated by a decreased number of binding sites for lectins. But it is also shown in this chapter that if keratinocytes, with a plasma membrane damaged by trypsin, are incubated in culture medium for 22 hours, the plasma membrane will be repaired; after 22 hours of incubation the plasma membrane has the same number of binding sites for lectins as the keratinocyte freshly prepared from the epidermis. This means that the number of glycoconjugates on the plasma membrane after 22 hours in vitro is the same as in vivo. This is of great importance for the work done in chapter 4 where keratinocytes are incubated with radioactive sugars for 22 hours and thus will incorporate these sugars into a plasma membrane resembling that in vivo. The glycoconjugates studied in

chapter 5 are derived from total cellular membrane preparations but as is proven by Roelfzema et al (1983a) the gel chromatography profiles from glycoproteins of the plasma membrane are in agreement with those derived from the total cellular membrane fraction. The three-dimensional structure of the plasma membrane of the keratinocytes was examined with a scanning electron microscope (chapter 2). The most extensive microvilli are seen on the smallest cells (presumably of the basal layer) When the diameter of the keratinocytes is becoming bigger (probably during differentiation) the extrusions are shortening. Lavker et al (1982) report that numerous microvilli are specific for epidermal stem cells

Abnormalities in psoriasis

Before attempting to provide a theoretical framework to interpret these data, the differences between normal (N), psoriatic uninvolved (PU) and psoriatic lesional skin (PL) will be summarized. In chapter 2 we found with the scanning electron microscope a clear and consistent difference between the three dimensional structures of the plasma membrane of the keratinocytes of N and PL at all levels of maturation. This means that although the diameter of the keratinocytes is the same for N and PL at each level of maturation, the microvillous extrusions of the psoriatic cells seem to be responsible for a larger surface and different architecture of the plasma membrane of the PL keratinocytes. When the beta-adrenergic receptors on the plasma membrane of suspended keratinocytes from N and PL skin are compared (chapter 3), lower

resting levels of cyclic AMP and a reduced response to adrenaline stimulation for PL are found. Although higher levels of cyclic AMP have been reported in PL skin slices (Iizuka 1978) it is possible that these higher values are not really 'resting' levels, as discussed in chapter 3. The reduced response of the PL keratinocytes to adrenaline can be due to changed receptors on the plasma membrane of the PL keratinocytes.

Using lectins which share the ability to bind stereo-specifically and reversibly to carbohydrates (in particular to the sugar moieties of glycoproteins and glycolipids) the oligo-saccharide portion of receptors in the plasma membrane of keratino-cytes was examined in chapter 8. Differences in binding affinities and the number of the lectin molecules which can be bound to the plasma membranes of N, PU and PL keratinocytes are found for some lectins. Thus two and three times more ConA molecules specific for mannose can be bound to PU and PL keratinocytes respectively, each with reduced association constants, compared with the binding to N keratinocytes. Also the lectin WGA (specific for N-acetyl glucosamine) has two times more binding sites on PL keratinocytes with a lower association constant, compared with N keratinocytes. Only the lectin UEA (specific for fucose) has the same number of binding sites, but a significantly higher association constant for PL keratinocytes. Keratinocytes derived from different layers of the epidermis have different numbers of binding sites, at least for the lectin ConA, as is also reported for studies on epidermal cross-sections (Reano 1982). N basal cells possess one third of the binding sites compared with PL basal keratinocytes. The differentiated N upper spinal cells have four times more

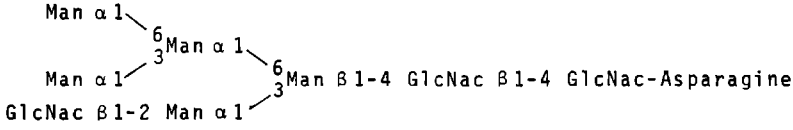
binding sites for ConA than the differentiated PL keratinocytes, which only show a threefold increase during differentiation. It can be calculated from the presented data that the increase in binding sites for ConA to N keratinocytes should be sharply limited to the upper spinal layer, whereas for PL keratinocytes there is a uniform transition to more binding sites during differentiation.

There is no lectin available which binds exclusively to the terminal sugar sialic acid. Although the lectin WGA binds to N-acetyl glucosamine and sialic acid as is shown in chapter 6. Because sialic acid has a negative charge, it forms the main contribution to the negative charge on the cell surface. The cell surface charge determines the mobility of the cell in an electric field. In chapter 8 a higher electrophoretic mobility is found for PL keratinocytes than for the N cells, indicating a higher sialic acid content on the plasma membrane of PL keratinocytes. Quantitative changes on the plasma membrane of PL keratinocytes are examined with the lectin binding studies. Qualitative differences between N, PU and PL keratinocytes are investigated by means of the incorporation of radioactive sugars. Freshly prepared PL keratinocytes show for all sugars an increased uptake, but only an increased incorporation of fucose into the glycoconjugates (chapter 4). All these differences diminish when the keratinocyts are preincubated in vitro for 22 hours. The sugars fucose, glucosamine, galactose and mannose are all incorporated into the lipid fraction, but no differences are found when the individual lipid components

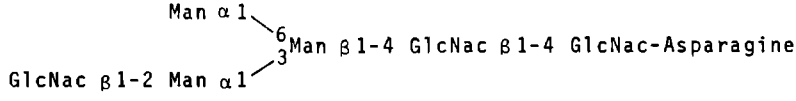
derived from N, PU and PL keratinocytes are compared (chapter 5). In the same chapter it is shown that mainly fucose and glucosamine are incorporated in N-glycosidic glycopeptides and only fucose is incorporated into glycopeptide fractions; these are different in material derived from N, PU and PL keratinocytes.

Theoretical aspects

The biosynthesis of N-glycosyl oligosaccharides starts within the rough endoplasmic reticulum by removing the 3 glucose residues followed by removal within the Golgi complex of four α-2 linked mannose (Man) residues. This yields a key intermediate to which N-acetyl glucosamine (GlcNac) is transferred, resulting in the hybrid oligosaccharide.



When GlcNac is attached to the β linked mannose, yielding a bisected complex, the oligosaccharide synthesis is frozen, and only the elongation of a single antenna by addition of a galactose and possibly a sialic residue may happen. When the hybrid oligosaccharide is not bisected, 2 mannose molecules may be trimmed off to give



At various stages of the synthesis of the oligosaccharide chains

fucose can be attached to the asparagine-linked GlcNac in α 1-6 linkage but this is prevented by bisecting and/or galactosylation of one of the antennae (Schachter 1982). In the urine of patients with the lysosomal storage disease fucosidosis glycopeptides are found in which fucose is also attached in α 1-3 linkage to GlcNac bound to α1-3 mannose (Strecker 1974). In human lactotransferrin at least two glycopeptides are found, one with only fucose α1-6 bound to the asparagine-linked GlcNac, another with an extra α1-3 fucose to an antenna. Of the second glycoprotein one needs the half-concentration, compared to the first glycoprotein, to inhibit the precipitation reaction of red blood cells by the lectin UEA (Debray 1981) indicating a stronger binding of UEA to glycoproteins when an extra fucose molecule is attached to the oligosaccharide chain. When, in the synthesis of N-glycosyl oligosaccharides, a second GlcNac molecule is attached via β1-4 to the α1-3 linked mannose, a triantennary structure results but fucose in 1-6 linkage to asparagine-linked GlcNac is mostly seen in bisected biantennary hybrids, bisected biantennary complexes and biantennary complexes. Fucose in α1-2 linkage to a galactosyl residue and in α1-3 linkage to a GlcNac residue of N-acetyllactosamine have been detected in antennae (Stanelone 1982) from glycoproteins derived from porcine submaxillary glands and human milk respectively. Assuming that free lectin binding to keratinocytes is in the same order as the precipitation reaction of lectins by isolated carbohydrate-containing macromolecules, the free lectin ConA will only be bound to the plasma membrane of the cell if the α-D-mannopyranosyl ring is available with

free hydroxyl groups at positions C-3, C-4 and C-6 (Goldstein 1976). Differences in composition of the oligosaccharide chain bound to mannose influence the minimum concentrations of these structures necessary to inhibit red cell agglutination by ConA (Debray 1981) indicating the effect of the antennae composition on the association constant of the lectin binding to the keratinocytes. If ConA is conjugated to Sepharose two interacting α mannosyl residues also unsubstituted at C-3, C-4 and C-6 are required for binding (Ogata 1975). Residues linked to these two interacting mannoses can either strengthen or weaken the binding to ConA-Sepharose. Also bisecting weakens the interaction with ConA-Sepharose.It is now known that the lectin WGA interacts with glycoconjugates with a high density of terminal non-reducing GlcNac or sialic acid residues (Monsigny 1980) but interactions between the lectin WGA and cell-surface glycoconjugates are rather complex and have to be interpreted very carefully.

Conclusions

In this section an attempt is made to correlate the results of the different experiments on N, PU and PL keratinocytes.
In PL keratinocytes we found almost double the number of binding sites for the lectin WGA on the plasma membrane and a higher cell surface charge compared to N keratinocytes, indicating more sialic acid residues and more available GlcNac on the plasma membrane. Although a larger cell surface obviously implies more membrane components the differences in the enhancements and the changed association constant of the WGA binding indicate different structures of these glycoconjugates on the plasma membrane

of PL keratinocytes. In actively growing fibroblasts glycoproteins were found with higher molecular weights due to a higher sialidation (Gamberg 1981) but we found in activated keratinocytes, due to stripping, no differences in WGA binding sites.

We have seen that fucose labelling of PL glycoproteins yields glycopeptides which have, on average, a higher molecular weight than those derived from N keratinocytes. It has recently been shown (Roelfzema 1983b) that the same high molecular weight fraction is also synthesized in N keratinocytes (basal and differentiated). This fraction is not bound to a ConA-Sepharose column and only a minor fraction of these fucose-labelled glycoproteins is built into the plasma membrane. It was also shown that the main molecular weight fraction of PU-derived fucose-labelled glycopeptides is synthesized by N differentiated keratinocytes as a ConA-Sepharose bound fraction. The main molecular weight fraction of N fucose-labelled glycopeptides is synthesized by the N basal cells as a ConA-Sepharose bound fraction. This means that the bulk of fucose-labelled glycopeptides derived from PL and PU keratinocytes are products which also occur in N keratinocytes but to a much lesser extent, although differences in sialic acid content of the two groups are reported.

When comparing the qualitative radioactive labelling experiments with the quantitative lectin binding measurements concerning the lectin ConA, it seems that we have found some contradictory results. Although PL keratinocytes have more binding sites for the free lectin ConA, their fucose-labelled higher molecular

weight fraction is unbound to ConA-Sepharose. The PU keratinocytes have also more binding sites for the free lectin ConA and their fucose-labelled lower molecular weight fraction is possibly bound to ConA-Sepharose.

Also taking into account that the number of binding sites for the fucose specific lectin UEA is unchanged, it is reasonable to assume that we are dealing with at least two different features.

I. A fucose-containing glycopeptide, which can be bound to ConA-Sepharose when synthesized by N or PU keratinocytes. This glycopeptide has at least two interacting αMan residues with unsubstituted C-3, C-4 and C-6 positions and presumably somewhat shorter antennae when derived from PU cells. When this fucose-containing glycopeptide is derived from PL cells, it lacks at least the two interacting αMan residues, possibly as a result of bisecting, substituation at C-3, C-4 and/or C-6, and/or elongation of the antennae by (for example) N-acetyl lactosamine, galactose and sialic acid. Each of these substituents weakens the binding of the glycopeptide to ConA-Sepharose and the addition of sialic acid would also explain the higher electrophoretic mobility of the cells. (Extra sialic acid and GlcNac was already indicated by the enhanced WGA binding). Binding of the free lectin ConA to this structure should still be possible but with a lower association constant. The higher association constant of the lectin UEA to PL keratinocytes indicates at least one extra fucose on the antennae of this glycopeptide. But the same number of binding sites of UEA on N, PU and PL cells indicates also an equal

or diminished number of mannose molecules responsible for free ConA binding to this glycopeptide.

II. As is also proven by Roelfzema (1983b) not all fucose labelled glycopeptides bind the lectin ConA, since in N keratinocytes the number of binding sites for UEA is double that of ConA. The enhancements of the ConA binding sites for PU and PL keratinocytes are therefore probably due to the appearance of glycoconjugates in the plasma membrane which are not fucosylated and have no free galactose in a non-reducing terminal position.

Since labelled mannose is found to be incorporated in oligosaccharide fragments, one may suspect that these kinds of glycoproteins have a small molecular weight, being possibly a high-mannose type with some GlcNac substituents, indicated by the low association constant. It should be noted that within the range of ConA concentrations used for the lectin studies second order reactions were rarely found, indicating nearly the same association constant for the fucosylated and non-fucosylated glycopeptides on the plasma membrane.

Future prospects

It is shown that during differentiation the number of binding sites per cell for ConA is not altered until the keratinocyte reaches the granular layer, where the whole cell is changing. On the plasma membrane of the PL keratinocyte, however, the number of binding sites increases continuously during differentiation, indicating an increased number of the high-mannose type glycoproteins in the plasma membrane.

It may now be concluded that in psoriatic keratinocytes at least two different mannose containing glycopeptides are expressed, the fucosylated type, which is also present in N keratinocytes, but in very small amounts and the non-fucosylated high mannose type. The latter is not present in N keratinocytes, but is increasing in number on the plasma membrane of psoriatic cells during differentiation.

Since the synthesizing cell, rather than the developing glycoprotein determines the nature of the glycan structure (Hatton 1983), it seems reasonable that the presumed high mannose type of oligosaccharide structure is synthesized in all psoriatic keratinocytes, but not in N ones; PL cells would seem to transfer about twice the amount to their plasma membrane than do the PU keratinocytes.

The determination of the glycan type (transferring sugars by their transferases) for a glycoprotein takes place in the Golgi complex, where in psoriasis presumably the fault is located in the processing of the fucosylated type oligosaccharide. It may be postulated that the fucosylated glycopeptide is responsible for the increased cell production and the high mannose type is due to the faulty differentiation process. It seems that future study of the glycoproteins is of great value. One of the most perplexing features of glycoproteins is the heterogeneity of their carbohydrate chains. In order to correlate glycoproteins with their function, the nature of their complex carbohydrate chains should be thoroughly investigated.

REFERENCES

Barton, P. and Marks, R. (1981) Changes in suspensions of human keratinocytes due to trypsin. Archives of Dermatological Research, 271, 245

Debray, H., Decout, D., Strecker, G., Spik, G. and Montreuil, J. (1981) Specificity of twelve lectins towards oligosaccharides of glycopeptides related to N-glycosylproteins. European Journal of Biochemistry, 117, 41

Gamberg, C.G. (1981) Membrane glycoproteins and glycolipids : structure, localization and function of the carbohydrate. In: Membrane structure, p.127. New Comprehensive Biochemistry, vol. 1. Elsevier/North-Holland Biomedical Press. Amsterdam-New York-Oxford.

Geerdink, J.P.M., De Mulder, P.H.M., Franzen, M.R., Gommans, J.M., Van Rennes, H., Van Erp, P.E.J., Bergers, M. and Mier, P.D. (1984) Monocyte function is normal in quiescent psoriasis. Journal of Investigative Dermatology, 82, 122

Goldstein, I.J. (1976) Carbohydrate binding specificity of concanavalin A. In: Concanavalin A as a Tool, p. 55. By H.Bittiger and H.P.Schnebli. John Wiley & Sons. London, New York, Sydney, Toronto.

Hatton, M.W.C., März, L. and Regoeczi, E. (1983) On the significance of heterogeneity of plasma glycoproteins possessing N-glycans of the complex type : a perspective. Trends in Biochemical Sciences, 8, 287

Iizuka, H., Adachi, K., Halprin, K.M. and Levine, V. (1978) Cyclic AMP accumulation in psoriatic skin: differential responses to histamine, AMP and epinephrine by the uninvolved and involved epidermis. Journal of Investigative Dermatology, 70, 250

Van de Kerkhof, P.C.M., Van Rennes, H. and Mier, P.D. (1983) Quantification of alkaline phosphatase in lesions and uninvolved skin of psoriatic patients. Acta Dermatovener (Stockholm), 63, 231

Kreuger, G.C. (1981) Psoriasis: current concepts of its aetiology and pathogenesis. In: 1981 Yearbook of Dermatology, 23. Yearbook Medical Publishers, Chicago.

Lavker, R.M. and Sun, T. (1982) Heterogeneity in epidermal basal keratinocytes: morphological and functional correlations. Science, 215, 1239

Monsigny, M., Roche, A.C., Sene, C., Maget-Dana, R. and Delmotte, F. (1980) How does wheat germ agglutinin bind sialo-glycoconjugates? European Journal of Biochemistry, 104, 147

Ogata, S., Muramatsu, T. and Kobata, A. (1975) Fractionation of glycopeptides by affinity column chromatography on Concanavalin A-Sepharose. Journal of Biochemistry, 78, 687

Priestly, G.C. and Adams, L.W. (1983) Hyperactivity of fibroblasts cultured from psoriatic skin. I. Faster proliferation and effect of serum withdrawal. British Journal of Dermatology, 109, 149

Reano, A., Faure, M., Jaques, Y. Reichert, U., Schaefer, H. and Thivolet, J. (1982) Lectins as markers of human epidermal cell differentiation. Differentiation 22, 205

Roelfzema, H. and Van Erp, P.E.J. (1983a) Studies on the plasma membrane of normal and psoriatic keratinocytes. 6. Cell surface and shed glycoproteins. Journal of Investigative Dermatology, 80, 24

Roelfzema, H. and Van Erp, P.E.J. (1983b) Glycoproteins composition of psoriatic epidermis in relation to growth control. Journal of Investigative Dermatology, 80, 20

Schachter, H., Narasimhan, S., Gleeson, P., Vella, G.J. and Brockhausen,I. (1982) Oligosaccharide branching of glycoproteins: biosynthetic mechanisms and possible biological functions. Philosophical Transactions of Royal Society London, B300, 145

Shuster, S. (1971) Research into psoriasis - the last decade. British Medical Journal, 3, 236

Strecker, G. and Montreuil, J. (1979) Glycoproteines et glycoproteinases. Biochimie, 61, 1199

Takeda, J., Adachi, K., Halprin, K.M., Nemoto, O., Levine, V. and Woodyard, C. (1983)"Leaky" epidermal cells contain a complete receptor-mediated adenylate cyclase system with an accessible GTP regulatory protein. Journal of Investigative Dermatology, 81, 241

SUMMARY

The thesis consists of 9 chapters.
In chapter 1 the aims of the investigation and the synthesis of glycoproteins are described. The investigations of glycoconjugates, situated in the plasma membrane, are presented in the following chapters.
In chapter 2 a new method is described for preparing keratinocyte suspensions from pieces of skin and the cell morphology is examined with the scanning electron microscope.
In chapter 3 it is shown that the plasma membrane bound receptor of the enzyme adenyl cyclase is unaffected by the new cell isolation procedure and that the non-activated psoriatic keratinocytes have a reduced level of cyclic AMP.
In chapter 4 the incorporation into glycoconjugates of the four sugars appearing in the plasma membrane is studied. The uptake of all sugars by the keratinocytes derived from the psoriatic lesion (PL) is enhanced, but this high uptake disappears when the cells are pre-incubated for 22 hours in vitro. The metabolism of the sugar fucose is strikingly changed in PL derived cells.
In chapter 5 the glycoconjugates are characterized: the lipid fraction by 2 dimensional thin layer chromatography, glycopeptides and glycosaminoglycans by Sephadex G-50 gel filtration. The incorporation of sugars into the lipid fraction of PL keratinocytes is enhanced and the percentage incorporation of the sugar galactose seems to be related to the number of large polygonal (differentiated) cells. It may be concluded from the results of gel filtration of glycopeptides and glycosaminoglycans that

the sugars fucose and glucosamine are found as N-glycosidic glycopeptides. Moreover, differences in elution pattern are shown in fucose-labelled material derived from keratinocytes of PL, psoriatic uninvolved skin (PU) and healthy control skin (N).

In chapter 6 a method is developed for measuring the number of binding sites and association constants of lectins (a lectin binds in a characteristic way to a certain sugar in a specific configuration) to keratinocytes and

in chapter 7 these parameters are determined using keratinocytes derived from PL, PU and N. The obtained results using the lectin characteristic for the sugar mannose indicate a significantly different number of binding sites and altered association constants for cells derived from PL and PU comparing with N and the lectin specific for the sugar glucosamine only for PL in respect to N. The lectin specific for the sugar fucose possesses only a changed association constant for cells derived from PL versus N.

In chapter 8 a method is described for measuring the mobility of keratinocytes in an electric field. It is proven that PL keratinocytes move faster, due to their higher negative surface charge.

In chapter 9 an attempt has been made to integrate, as far as possible, the above data. The pathogenesis of psoriasis is discussed in the light of these new findings.

SAMENVATTING

Het proefschrift omvat 9 hoofdstukken.

In hoofdstuk I wordt het doel van het onderzoek en de synthese van glycoproteïnen beschreven. De onderzoekingen aan glycoconjugaten, gelegen in het plasma membraan, worden in de volgende hoofdstukken gepresenteerd.
In hoofdstuk 2 wordt een nieuwe methode beschreven voor het bereiden van keratinocytsuspensies uit stukjes huid; de celmorfologie is bestudeerd met de scanning electronenmicroscoop.
In hoofdstuk 3 wordt aangetoond dat de plasma membraan gebonden receptor van het enzym adenyl cyclase niet wordt aangetast door de nieuwe celisoleringsmethode en dat de niet geactiveerde psoriatische keratinocyten een verminderd niveau hebben van cyclisch AMP.
In hoofdstuk 4 wordt de inbouw bestudeerd in de glycoconjugaten van de vier suikers welke in het plasma membraan voorkomen. De opname van alle suikers door de keratinocyten afkomstig uit de psoriatische laesie (PL) is verhoogd, maar deze hoge opname verdwijnt wanneer de cellen gedurende 22 uren worden gepreïncubeerd in vitro.
Het metabolisme van de suiker fucose is opvallend veranderd bij cellen afkomstig van de PL.
In hoofdstuk 5 worden de glycoconjugaten gekarakteriseerd: de lipid fractie d.m.v. 2 dimensionale dunne laag chromatografie, glycopeptiden en glycosaminoglycanen m.b.v. Sephadex G-50 gel filtratie. De inbouw van de suikers in de lipid fractie van PL keratinocyten is verhoogd en het inbouwpercentage van de suiker galactose lijkt gerelateerd aan het aantal grote polygonale (gedifferentieerde) cellen. Uit de resultaten van de gel filtratie van de glycopeptiden en glycosaminoglycanen mag mogelijk geconcludeerd worden dat de suikers fucose en glucosamine worden aangetroffen als N-glycosidische glycopeptiden. Bovendien worden er verschillen in elutiepatronen aangetoond in het met fucose gemerkte materiaal afkomstig van keratinocyten van PL, psoriatisch niet aangedane huid (PU) en gezonde controlehuid (N).
In hoofdstuk 6 wordt een methode ontwikkeld voor het meten van het aantal bindingsplaatsen en associatieconstanten van lectinen (een

lectine bindt op een karakteristieke manier aan een bepaald suiker in een specifieke configuratie) aan keratinocyten en in hoofdstuk 7 worden deze parameters bepaald aan keratinocyten afkomstig van PL, PU en N. De verkregen resultaten geven aan dat bij gebruik van het lectine, karakteristiek voor de suiker mannose, een significant verschillend aantal bindingsplaatsen en een veranderde associatieconstante aanwezig zijn bij cellen afkomstig van PL en PU vergeleken met N en het lectine specifiek voor de suiker glucosamine alleen voor PL in vergelijking met N. Het lectine specifiek voor de suiker fucose heeft alleen een veranderde associatieconstante voor de cellen afkomstig van PL t.o.v. N.
In hoofdstuk 8 wordt een methode beschreven voor het meten van de bewegelijkheid van de keratinocyten in een electrisch veld.
Het is bewezen dat de PL keratinocyten sneller bewegen door hun grotere negatieve oppervlakte lading.
In hoofdstuk 9 wordt een poging ondernomen om zo goed mogelijk de hierboven vermelde resultaten te integreren. De pathogenese van psoriasis wordt in het licht van deze nieuwe bevindingen bediscussieerd.

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STELLINGEN

I

Ofschoon de niet aangedane huid van een psoriasispatiënt morfologisch en histologisch niet van een gezonde huid te onderscheiden is, blijken enkele biochemische parameters significant veranderd te zijn.

(Dit proefschrift).

II

Het blijkt mogelijk om Langerhans cellen, die ongeveer 2% uitmaken van de totale epidermale celpopulatie, in een enkele zuiveringsstap te verrijken tot 80-90%.

III

Het human T6 antigeen op epidermale Langerhans cellen wordt in vitro na conjugatie met een antilichaam geïnternaliseerd.

IV

De expressie van classe I MHC antigenen op capillair endotheel cellen in muizenhuid allotransplantaten wordt beïnvloed door de T-cel immuunrespons van de ontvanger.

V

De diverse biochemische en morfologische karakteristieken van de verschillende epitheliale konijnenkeratinocyten verdwijnen nadat de cellen in cultuur zijn gebracht. Dat deze veranderingen reversibel zijn kan bewezen worden door de gekweekte cellen in het konijn te injecteren.

VI

Aan het superoxide anion is met betrekking tot weefselschade bij ontstekingen meer betekenis toegekend dan op grond van experimentele data gerechtvaardigd is.

VII

De rol van H_2O_2 als ontstekingsmediator wordt onvoldoende onderkend.

VIII

Het gebruik van PUVA bij de behandeling van andere dermatosen dan psoriasis suggereert dat het primaire aangrijpingspunt niet in de prolifererende keratinocyten gelegen is.

IX

Van een placebo mag verwacht worden dat het geen werking heeft. Indien het placebo toch een effect scoort, is de term placebo noch geneesmiddel op zijn plaats. De benaming van een "werkend placebo" is in de Geneeskunde nog niet uitgevonden.

X

Het verdient aanbeveling dat feministen zich verdiepen in het imkerwezen. De bijen laten namelijk zien dat een feministische samenleving uitstekend functioneert onder een dictatuur.

XI

De stikkers op de gangdeuren in het gebouw Tandheelkunde "Deur gesloten houden op last van de brandweer" zijn niet bevorderlijk voor de werkzin aangezien iedere reis van en naar de werkruimten gepaard gaat met het krijgen van een gevoel van onbehagen weer een overtreding te hebben begaan.

XII

Door het (ver)bouwen van een eigen huis krijgt men een goede indruk op welke manier er effectief bezuinigd kan worden, een aspect dat door menige researchwerker nog onvoldoende doorzien wordt.

XIII

Door het berijden van een antiek motorvoertuig blijft men zich beter bewust van de afstanden en de snelheden waarmee deze gemotoriseerd worden overbrugd.

Nijmegen, 28 juni 1984 Joan Gommans