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STUDIES OF IRRITANT REACTIONS ON EPIDERMIS: THE APPLICATION OF ENERGY DISPERSIVE X-RAY MICROANALYSIS.

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Abstract

Modern man is exposed to an increasing amount of chemicals in the environment. The skin is a complex tissue with regards to both structure and function. Contact with external stimuli can lead to various responses in the skin depending on the type of stimuli, the dose and time of exposure and the status of the exposed individual. The electron probe microanalytic technique can be used to obtain information on physiological alterations within the skin and especially the epidermis when the normal homeostasis is disturbed by contact with external stimuli. The present paper presents a review of some aspects of contact dermatitis and the possibility of using the electron probe microanalytic technique for analysis of epidermal events of this condition.

Key Words: skin, contact dermatitis, toxicology, irritant reactions, hyperplasia, energy dispersive X-ray microanalysis, guinea-pig, cryosectioning.

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Introduction

The skin constitutes the barrier between the organism and the environment. Thus the skin is continuously exposed to harmful chemicals and products in our external milieu. As a result, contact dermatitis has developed into one of the major problems in dermatological practice; this is especially true for occupational dermatology. Contact dermatitis (or contact reactions) can be divided into allergic and irritant types. Although by quantity the irritant reaction is the dominating problem, most research has been devoted to various aspects of allergic contact dermatitis. During the past five to ten years the interest in irritant dermatitis has increased. The epidermal response to irritant stimuli is also of interest for those investigating the pathogenesis of epidermal hyperplasia and neoplasia (1, 2, 23).

The skin is a complex tissue with both a histological and a functional stratification (13, 17, 18, 24). One of the major functions of the skin is to act as a barrier against diffusion and penetration of various substances. This barrier function is considered to be functionally located to the outermost cell layers, the stratum corneum (34). The stratum corneum can be considered the end product of epidermal differentiation and there is a continous renewal of this cell layer to maintain its function. This process involves cell replication in the basal part of epidermis, followed by the migration and simultaneous differentiation of the keratinocytes eventually to produce the corneocytes of stratum corneum. Based on research during recent years the barrier function has been envisaged as a two-compartment model where the corneocytes are likened to highly hydrophilic protein rich bricks joined by an intercellular mortar of hydrophobic lipids (9) .

In the context of contact dermatitis there are several points of interactions

when stimuli are applied on the skin surface. The skin response will depend on the penetration of the applied substance, its direct effects on stratum corneum and the other cell-populations of the epidermis, as well as indirect effects on proliferation and differentiation, the development of inflammation, a possible immunological response, and finally, possible systemic effects.

The keratinocytes have a rather uniform way of reacting to external chemical and physical stimuli. It is known that the epidermis responds to chemical stimuli with a hyperplastic growth (1,2,3,23). In epidermal carcinogenesis hyperplastic growth preceeds the development of the neoplasia (2). The control and regulation of this response is not fully understood and the control of epidermal cell proliferation is thus of major interest for the understanding of the physiology of both normal and pathologically altered skin.

Due to the dimensions of epidermis, it has previously been difficult to study physiological changes in the intact skin. With the introduction of microanalytic techniques such as the electron probe it has become possible to study the elemental composition of cells in frozen and freeze-dried sections of intact tissues and thus to obtain functionally and physiologically relevant information (15, 33).

It has been shown by several authors that the electron probe can be used to reveal information on the redistributions of elements under various pathological conditions (17) and it is also possible to differentiate between functionally different cell-populations in normal tissue (6, 14, 37, 43) and between sub-cellular compartments (7,26,38). Cell populations with a high mitotic activity are characterized by a high sodium content and sodium/potassium ratio (8,27,28,35,41,43) and transient changes in the sodium content might induce DNA-synthesis and mitosis (8, 32). In 1971 it was proposed by Cone (8) that the cell-membrane potential, i.e. the sodium/potassium ratio, is the main regulatory factor in the control of cell division in both normal proliferative and cancerous tissues. This hypothesis was recently discussed and supported in an extensive review on the use of bulk specimen x-ray microanalysis in cancer research (44).

The application of energy dispersive X-ray microanalysis (EDX) in dermatology has recently been reviewed elsewhere (10, 11, 12). One of the main goals in our work has been to establish a technique for the analysis of physiological alterations in epidermis at irritant contact dematitis. The purpose of the present review is to present how the EDX-technique can be used to obtain such information when the skin is exposed to different types of irritant stimuli.

Experimental and Methodological Considerations

The distribution of elements in normal epidermis was initially described using EDX of thin freeze-dried cryo-sections. In 1982 Wei et al.(40) applied the technique on normal guinea pig epidermis and in 1988 Warner et al. (39) analyzed normal human skin. It was demonstrated that the concentration of elements, e.g. sodium, potassium, phosphorous, and calcium, varied in different layers of epidermis.

The guinea pig is a frequently used animal system in experimental studies on contact reactions and was used in the studies discussed in this paper. Compared to human epidermal morphology, the epidermis of the guinea pig is thinner (5, 36) consisting of only two to four cell layers beneath the stratum corneum.

In the investigation of irritant reactions it is necessary to perform studies on both dose and time dependent variations.

The use of bulk specimens in this type of study offers some advantages. Bulk specimens are easier to prepare and analyze than thin sections. Hence, it is feasible to analyze a larger number of animals and of experimental variables such as time and concentration if bulk specimens are used. This tends to make the results more valid and useful from a biological point of view. The use of bulk specimens in a preliminary stage of the investigation can and does often, however, give rise to questions that require the use of thin sections for analysis at the subcellular level. However, such studies may then be limited to a particular set of experimental conditions as established by bulk specimen studies.

With thick sections (bulk specimens), 12-16 µm thick, for the EDX-analysis, a calculated spatial resolution of approximately 10 µm is obtained at an accelerating voltage of 20 kV (31). This resolution is determined by the spread of beam electrons in the specimen. This is in the range of a single cell cross section. In the stratified epidermis of the guinea pig we performed analyses at two levels , in stratum basale (or germinativum) and in stratum spinosum. The spatial resolution with this experimental system is then at the level of the epidermal stratum (19). Using this approach we have detected differences in the elemental content of the two epidermal layers in normal guinea pig epidermis (20) such as a higher magnesium and lower po-

tassium levels in stratum spinosum compared to the basal layer. While, given the relatively poor resolution of the analysis, a small contribution from the extracellular compartment cannot be excluded, comparison of data from thin sections and bulk specimens showed that the values for the intracellular concentrations agree well (12). If overrepresentation of the beam occurs, e.g., because of variations in section thickness, this is usally evident from variations in the concentrations of "bound" elements such as P and S. In such cases, data should be normalized to (one of) these elements (30).

Two factors of importance for the detection of elemental redistributions in thick sections of the skin are the time elapsed from the induction of the reaction studied and the rate of diffusion of ions into and out of the epidermis. This diffusion rate is very much dependent on the presence of an intact dermal circulation as shown in a study on thick sections from autolytic epidermis from normal guinea pig skin (20). After twenty four hours at room temperature there was no significant difference in the elemental composition between the normal and the autolytic epidermis.

Experimental Procedures

Throughout our studies the experimental substances have been applied to the back of the guinea pigs after clipping with an electric clipper. The animals were killed with an intraperitoneal overdose of barbiturate prior to tissue sampling. The tissue was immediately divided and further processed for EDX and/or light microscopy (LM) and transmission electron microscopy (TEM). For LM the specimens were fixed in buffered formaldehyde, embedded in paraffin and the sections were stained with haematoxylin and eosin. The TEM specimens were fixed in 2.5% glutaraldehyde in a phosphate buffer, postfixed in osmium tetroxide, dehydrated in ethanol and embedded in Epon. The ultrathin sections were stained with uranyl acetate and/or lead citrate and were viewed in a Philips 301 G electron microscope at 60 kV. For EDX the tissue was snap-frozen in liquid nitrogen and then stored in liquid nitrogen or in the refrigerator at -80° C. In the initial work (20), the freezing was done in Freon 13 sub-cooled with liquid nitrogen. Freeze-sectioning was done in a cryotome at -20° to -30° C. Sections were cut perpendicular to the skin surface.The sections were collected on a carbon holder (42), freeze-dried in the cryotome over night, and then coated with carbon.

The EDX-analysis was performed either in a JEOL 100C electron microscope fitted with a scanning attachment and a Kevex energy dispersive spectrometer (20) or in a JEOL 1200 EX scanning transmission electron microscope fitted with a Tracor energy dispersive X-ray analysis equipment (21, 22). Analysis was carried out in the spot mode. Physiologically important elements, i.e. Na, Mg, P, S, Cl, K, and Ca were analysed at two levels of epidermis, stratum germinativum and stratum spinosum. The morphological identification of epidermal strata in the cryosections in the scanning electron microscope was facilitated by examination of the adjacent stained sections in the light microscope (42). For each biopsy, four different spectra were analysed at each level of epidermis and the mean was calculated. The ratio of the characteristic counts (peak) to background counts (taken under the peak of interest) was calculated for all elements (29,30). Absolute quantitation of the elemental content was obtained by comparing the spectra from the specimens with those of a standard consisting of various mineral salts in a matrix of 20% gelatin and 5% glycerol. The standard was frozen and sectioned in the same way as the specimens (29, 30).

Results and Discussion

The effect of dinitrochlorobenzene - Prominent epidermal cell injury

This work was performed (20) with the objective to relate dose and time dependent elemental changes in epidermis to the ultrastructural alterations in the keratinocytes after the exposure to a toxic (irritant) substance. To produce a contact reaction dinitrochlorobenzene (DNCB) was dissolved in acetone and a 10% solution was applied on the back on each guinea pig in a group of three for 0, 3, 6, and 24 hours. In another group of three animals we applied 0.01%, 0.1%, and 1.0% DNCB solutions for 24 hours. On topical application, DNCB is toxic and allergenic and is therefore also used as an allergen in studies on skin immune response. The exposed skin was prepared for EDX and TEM. The changes of the elemental content in the keratinocytes are presented in Tables 1 and 2. In the skin exposed to 10% DNCB, there was a statistically significant variation in the phosphorus, potassium, and calcium levels (ANOVA, $p=0.05$) and in the other group there was a significant variation in phosphorus and calcium. The only sample with relatively marked increase in the sodium content was the skin exposed to 0.10% DNCB for 24 hours where the TEM analysis revealed minor signs of cell injury. At the ultrastructural level there were signs of increasing cell injury with increasing exposure time and

Table 1: The elemental content of the keratinocytes at different intervals after the exposure to 10% DNCB. Mean of stratum basale and stratum spinosum. mmol/kg dry weight. Mean and standard deviation (SD) are given.

Hours	Na	Mg	P	S	K	Ca
0	169 (55)	25 (3)	448 (3)	267 (23)	430 (16)	5 (3)
3	154 (52)	25 (6)	393 (61)	267 (5)	378 (19)	10 (2)
6	188 (44)	15 (6)	267 (38)	243 (16)	295 (15)	17 (4)
24	179 (26)	15 (5)	204 (28)	243 (9)	150 (8)	28 (7)

Table 2: The elemental content of the keratinocytes at 24 hours after the exposure to DNCB of different concentrations. Mean of stratum basale and stratum spinosum. mmol/kg dry weight. Mean and (SD) are given.

DNCB	Na	Mg	P	S	K	Ca
Contr.	131 (26)	12 (3)	386 (37)	247 (20)	437 (18)	4 (4)
0.01 %	185 (5)	13 (1)	400 (10)	264 (50)	415 (18)	6 (4)
0.10 %	147 (29)	9 (1)	309 (19)	213 (11)	304 (65)	11 (4)
1.00 %	159 (28)	5 (2)	173 (45)	223 (10)	294 (36)	17 (3)

Table 3: The elemental content of the keratinocytes 24 and 48 hours after the exposure to 5% sodium lauryl sulphate. mmol/kg dry weight. Mean and (SD) are given. (B=stratum basale; S=stratum spinosum).

Hours	Stratum	Na	Mg	P	S	K
0	B	133 (42)	20 (7)	370 (41)	277 (57)	282 (184)
	S	109 (46)	22 (4)	376 (25)	261 (62)	274 (235)
24	B	267 (136)	23 (6)	508 (158)	227 (15)	359 (98)
	S	261 (103)	28 (10)	532 (128)	213 (30)	362 (112)
48	B	209 (108)	17 (10)	401 (105)	248 (22)	327 (124)
	s	203 (70)	21 (10)	330 (157)	218 (24)	309 (148)

concentration. At 24 hours the epidermis was necrotic after 10% and 1% DNCB exposure. Concomitant with increasing cell injury there was a loss of magnesium, phosphorus, and potassium and an increase in calcium. These changes are in accordance with the elemental redistribution reported at cell injury or death in other tissue systems (17).

The effect of sodium lauryl sulphate - Mild irritant stimuli.

As a contrast to the previous study a mild irritant stimulus was produced by using a substance from another group of irritants (21). Sodium lauryl sulphate (SLS) is a detergent and has frequently been used as a model substance for the analysis of the skin response to this group of substances. A solution of 5% SLS in water was applied as a single dose on the skin of two groups of guinea pigs with five animals in each group. A third non-exposed group served as controls. Biopsies from one group were taken at 24 and from the other at 48 hours and were prepared for EDX, TEM, and LM.

The changes in the elemental content are shown in Table 3. The only statistically significant change (Dunnett's procedure;p=0.05; Dunnett's procedure allows a comparision between all groups assuming a normal distribution) was the increase in sodium found at 24 hours after the exposure. In the LM the number of basal and suprabasal keratinocytes were counted in 10 fields per biopsy at x1000. The LM analysis revealed a significant increase in the total number of keratinocytes at 48 hours. At the ultrastructural level, alterations were most pronounced at 24 hours with keratinocytes with rounded nuclei, cytoplasmic vacuoles, and the appearence of intracellular oedema. The results are in accordance with an initial membrane damage leading to increased sodium levels at 24 hours. The increased sodium may be associated with a stimulation of the keratinocytes resulting in an increased number of cells detected at 48 hours.

The effect of n-hexadecane - Epidermal hyperplasia

In order to study epidermal hyperplasia induced by substances known not to cause allergic reactions we have investigated the effect of n-hexadecane on guinea pig epidermis. This part deals with the elemental redistribution in the guinea pig epidermis during the development and the regression (24-192 hours) (22) of epidermal hyperplasia and includes new data on the induction phase (0-24 hours).

n-Hexadecane (Sigma Chemicals), a mineral oil, has been frequently used to study epidermal hyperplasia (4, 16, 25) . Using a pipette, a single dose of 0.5 ml n-hexadecane was applied to an area of 3 cm x 3 cm on the back of the guinea pigs. Skin biopsies were taken from groups of animals at 0, 6, 12, 24, 48, 96, and 192 hours following the application of the mineral oil. The biopsies were processed for LM and EDX as described above.
Energy dispersive X-ray microanalysis and LM were done according to the following schedule (nd=not done):

Time after application. Hours.	0	6	12	24	48	96	192
LM	x	nd	nd	x	x	x	x
EDX	x	x	x	x	x	x	nd
Number of guinea pigs	8	6	6	4	5	5	5

Light microscopy was performed at a magnification of x400 and the epidermal thickness (from the basal membrane to stratum corneum) was measured using an eye-piece grid with arbitrary units. On each biopsy the epidermal thickness was determined as the mean of ten separate measurements. The number of keratinocyte cell nuclei was determined as the mean of ten counts of cell nuclei along a grid line perpendicular to the dermal-epidermal junction.

For statistical analysis Wilcoxon's Rank sum test (p=0.05) was used to compare the epidermal thickness and the number of cell nuclei at 96 hours with the controls. The analysis on the elemental changes was performed on the mean values of the stratum germinativum and the stratum spinosum values using variance analysis (ANOVA; p=0.05).

The application of n-hexadecane resulted in a clearly detectable erythema at 24 hours with the addition of oedema at 48 hours. At 96 and 192 hours after the application the erythema had diminished and was replaced by a scaling of stratum corneum.

The application of n-hexadecane induced a transient epidermal hyperplasia with a maximal thickness of epidermis between 96 and 192 hours (Table 4). The increase in the epidermal thickness and the number of cell nuclei was statistically significant (p=0.05) at 96 hours and reached their maxima between 96 and 192 hours. The increase in epidermal thickness occurred prior to the increase in number of cell nuclei (Table 4) indicating the presence of an epidermal intra-cellular oedema in the interval 12-24 hours. This was supported by the LM analysis.

The result of the EDX-analysis is given in Table 5. The variance analysis showed significant changes (P=0.05) with

Table 4: The epidermal thickness (arbitrary units), the number of cell nuclei perpendicular to the dermal-epidermal junction and the Na/K ratio after a single application of n-hexadecane. Mean and (SD) are given. (nd=not done, B=stratum basale, S=stratum spinosum).

Hours	n	Thickness	Number of nuclei	Na/K ratio
0	4	0.96 (0.19)	1.34 (0.18)	B 0.72 (0.27)
				S 0.71 (0.32)
6	6	nd	nd	B 0.60 (0.19)
				S 0.67 (0.31)
12	6	nd	nd	B 0.80 (0.16)
				S 0.83 (0.16)
24	4	2.03 (0.21)	1.40 (0.25)	B 0.75 (0.05)
				S 0.65 (0.07)
48	5	2.98 (0.30)	2.40 (0.51)	B 0.55 (0.2)
				S 0.31 (0.15)
96	5	3.55 (0.80)	3.42 (0.41)	B 0.36 (0.04)
				S 0.30 (0.03)
192	5	1.96 (0.27)	1.90 (0.17)	B nd
				S nd

Table 5: The elemental content (mmol/kg dry weight) in the keratinocytes after a single exposure to n-hexadecane. Mean and (SD) are given.

STRATUM BASALE

Hours	n	Na	Mg	P	S	Cl	K	Ca	Na/K
0	4	196	21	523	269	318	271	15	0.72
		(36)	(7)	(67)	(53)	(50)	(82)	(4)	(0.27)
6	6	196	21	534	340	353	344	24	0.60
		(32)	(8)	(55)	(39)	(91)	(62)	(11)	(0.19)
12	6	210	18	539	313	348	275	28	0.80
		(129)	(4)	(80)	(62)	(104)	(63)	(8)	(0.16)
24	4	196	18	351	171	280	260	13	0.75
		(25)	(4)	(52)	(16)	(48)	(21)	(4)	(0.05)
48	5	180	28	497	204	268	329	13	0.55
		(22)	(4)	(96)	(27)	(45)	(87)	(4)	(0.23)
96	5	146	27	482	210	269	404	17	0.36
		(9)	(4)	(45)	(35)	(28)	(55)	(9)	(0.04)

STRATUM SPINOSUM

Hours	n	Na	Mg	P	S	Cl	K	Ca	Na/K
0	4	174	20	438	240	309	244	17	0.71
		(39)	(6)	(63)	(36)	(26)	(38)	(3)	(0.32)
6	6	180	23	448	294	328	300	21	0.67
		(42)	(9)	(80)	(49)	(81)	(76)	(7)	(0.31)
12	6	192	19	453	286	308	238	30	0.83
		(30)	(3)	(58)	(45)	(50)	(41)	(4)	(0.16)
24	4	178	26	401	213	292	273	15	0.65
		(21)	(3)	(45)	(20)	(18)	(27)	(4)	(0.07)
48	5	151	32	560	307	334	494	18	0.31
		(23)	(3)	(137)	(100)	(99)	(190)	(6)	(0.15)
96	5	128	37	487	234	277	428	15	0.30
		(15)	(2)	(45)	(16)	(26)	(40)	(3)	(0.03)

time for all elements except Cl. At 96 hours the sodium level was decreased, the potassium concentration was increased and the sodium/potassium ratio was thus lowered (Table 5). As can be seen in Table 5 there are variations in the sulphur values, possibly due to variations in section thickness or density. The elemental concentrations in epidermis have therefore been re-calculated assuming a constant sulphur content (S=100). These data are given in Table 6. The profile of the elemental changes in the interval 0 to 24 hours differed from that seen at 24-96 hours. In relation to S, the concentration of Mg, P, and K are low at 12 hours whereas the concentrations of these elements are increased at 24 hours and thereafter. In the case of Na the content was increased at 24 hours with a tendency towards decreased values (both absolute and in relation to S) at 96 hours.

The results of the LM and EDX-analyses are indicative of an initial cell injury initiating a hyperplastic response. During the hyperplastic response to chemical stimuli there is a transient expansion of the proliferative compartment in epidermis with an increased number of dividing supra-basal keratinocytes (1, 2). With the EDX-technique as applied in the present work we detected similar changes in the elemental content of the keratinocytes in both stratum germinativum and stratum spinosum but there appeared to be quantitative differences between the two strata. Considering the possibility of an expanded proliferative cell population in combination with the spatial resolution obtained, it is probable that the differences in elemental composition detected reflect changes associated with alterations in the functional state of the keratinocytes.

It has to be remembered that the hyperplastic epidermis is not at steady state. It is possible that the elemental composition found at 48 and 96 hours reflects the hyperplastic state of the tissue where there might be alterations in the qualitative and quantitative relation between nuclei and cytoplasm and quantitative changes in the cellular organelles, such as the ribosomes (2). It can be speculated whether the shift in the sodium/potassium ratio seen at 48 and 96 hours might influence the following regression of the hyperplastic growth seen as a decreased epidermal thickness at 192 hours (Table 4).

Table 6: The elemental content in the keratinocytes after a single application of n-hexadecane recalculated in relation to the sulphur content assuming S=100. Calculation performed on the means (cf. table 5). B=stratum basale, S=stratum spinosum.

Hours	n	stratum	Na	Mg	P	Cl	K	Ca
0	4	B	72	4	194	118	101	6
		S	73	8	181	128	102	7
6	6	B	58	6	157	109	101	7
		S	61	8	152	112	102	7
12	6	B	67	6	172	111	87	9
		S	67	7	158	107	83	10
24	4	B	114	10	205	163	152	8
		S	83	12	189	137	128	7
48	5	B	88	14	243	131	161	6
		S	49	10	182	108	161	6
96	5	B	69	13	228	128	192	8
		S	54	15	208	118	183	6

Conclusions

The application of EDX-analysis and bulk specimens for studies of irritant contact reactions is discussed in the present paper. It has been possible to demonstrate elemental changes compatible with cell injury, stimulation of cell growth and to follow the dynamics of a reactive epidermal hyperplasia. It also has been possible to differentiate between two layers of epidermis in the guinea pig skin and to detect differences in the epidermal response to three different substances belonging to different groups of irritant chemicals.

The changes induced by DNCB can be considered as typical for cell damage. The Na and Ca concentrations are increased, the concentrations of Mg, P and K are decreased. The increase in Na in the cells is relatively small, which could be due to the fact that in epidermal cells the Na concentration is high already under normal conditions. Epidermal cells share this property with e.g., chondrocytes, which also only have an indirect supply of oxygen.

The changes induced by SLS, on the other hand, are typical for a proliferative process with high rates of protein synthesis. The intracellular concentration of Mg, P, and K are increased. Much of the cellular phosphorus is present in nucleotides, the amount of which increases with proliferative and metabolic activity. Magnesium and K bind preferrentially to nucleic acid phosphate groups. The increase in Na concentration is not likely to be due to cell damage, and indeed, the increase is even stronger than in the case of DNCB. It has been suggested that call proliferation can be induced by active Na^+ uptake via the Na^+ - H^+ exchange mechanism. Such a mechanism could explain the relatively prominent increase in Na^+.

The situation after n-hexadecane is rather complex. It can be clearly seen that in relation to S (keratin) the concentrations of Mg, P, and K increase after 24 h. This is indicative of cell proliferation, and this notion is supported by the morphological findings. However, in contrast to what happens after SLS the proliferative changes are not accompanied by such a marked increase in cellular sodium levels. The EDX results give no indication of significant cell damage in the first 24 h after n-hexadecane, although the light microscopy suggests that some damage occurs. It should be noted that with DNCB, where morphological damage is prominent, only small changes in Na were found; hence, the effect of a much milder agent may be difficult to detect.

In conclusion, the application of EDX on thick sections can be a valuable tool in the investigation of the relationship between epidermal cell kinetics and irritant reactions and seems to be well suited for screening of different substances. Although the spatial resolution is low with thick sections, it is possible to gain valuable information with this method, especially in an initial stage of the work. For future work, it is necessary to perform analyses on thin sections to increase the resolution so as to make it possible to define subpopulations of keratinocytes and to look at intracellular redistributions of elements. This is especially important when rapid changes occur as the fluid volumes in epidermis are small and do not seem to provide diffusion volumes for rapid changes in the elemental content of the keratinocytes as measured in thick sections.

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References

1. Argyris TS (1968). Growth induced by damage. In Advances in Morphology 7: pp.1-43

2. Argyris TS (1981). The regulation of epidermal hyperplastic growth. CRC Crit Rev Toxicol 9:151-200.

3. Barnes FW, Seip WF, Burch CC (1968). Proliferation and resistance of epidermis in response to harmful stimuli. Proc Soc Exp Biol Med 129:584-593.

4. Birkin JA, White DA, Prottey C (1979). Epidermal metabolism in experimentally induced hyperplasia. Biochem Soc Trans 7: 408-409.

5. Brody I (1959). The keratinization of epidermal cells of normal guinea pig skin as revealed by electron microscopy. J Ultrastruc Res 2:482-511.

6. Cameron IL, Smith NKR, Pool TB (1979). Elemental concentration changes in mitotically active and postmitotic enterocytes. J Cell Biol 80:444-450.

7. Cameron IL, Hansen JT, Hunter KE (1986). Elemental concentration gradients between subcellular compartments. J Cell Sci 81:283-287.

8. Cone CD (1971). Unified theory on the basic mechanism of normal mitotic control and oncogenesis. J Theor Biol 30: 151-181.

9. Elias PM (1981). Lipids and the epidermal permeability barrier. Arch Dermatol Res 270:95-117.

10.Forslind B (1982). X-ray microanalysis in dermatology. Scanning Electron Microsc 1982; IV:1715-1724

11.Forslind B (1986). Particle probe analysis in the study of skin physiology. Scanning Electron Microsc 1986; III:1007-1014

12.Forslind B (1988). Clinical applications of scanning electron microscopy and energy dispersive X-ray analysis in dermatology : An up-date. Scanning Microscopy 2:959-976.

13.Goldstein LA (1983). Biochemistry and Physiology of the Skin, Vol. 1, Oxford University Press, pp. 3-63.

14.Gupta BL, Hall TA, Naftalin RJ (1978). Microprobe measurements of Na, K, and Cl concentration profiles in epithelial cells and intercellular spaces of rabbit ileum. Nature 272:70-73.

15.Hall TA (1979). Biological X-ray microanalysis.
J Microsc 117: 145-163.

16.Hoekstra WG, Phillips PH (1963). Effects of topically applied mineral oil fractions on the skin of guinea-pigs. J Invest Dermatol 40: 79-88.

17.Ingram P, Shelbourn JD, Roggli VL, eds.(1989). Microprobe analysis in medicine. Hemisphere, New York.

18.Lindberg M (1982). Studies on the cellular and subcellular reactions in epidermis at irritant and allergic reactions.Acta Dermato-Venereol (Sthlm), Suppl.105, pp. 1-45.

19.Lindberg M, Forslind B, Roomans GM (1983). Elemental changes at irritant reactions due to chromate and nickel in guinea-pig epidermis. Scanning Electron Microsc 1983; III:1243-1247.

20.Lindberg M, Roomans GM (1983). Elemental redistribution and ultrastructural changes in guinea pig epidermis after dinitrochlorobenzene (DNCB) exposure. J Invest Dermatol 81:303-308.

21.Lindberg M, Grundin TG (1987). Elemental changes in guinea-pig epidermis in the hyperplastic response to irritant stimuli. Br J Dermatol 116:477-483.

22.Lindberg M, Sagström S (1989). Changes in sodium-potassium ratio in guinea pig epidermis in n-hexadecane induced hyperplasia. Acta Derm Venereologica (Sthlm) 69: 369-372.

23.Marks F, Furstenberger G, Ganss M, Richter H, Seeman D (1983). Hyperplastic transformation: The response of mouse skin to irritation. Br J Dermatol 109:Suppl.25: 18-21.

24.Marks RM, Barton SP, Edwards C (1988). The physical nature of the skin. MTP Press Limited,Lancaster, 3-12.

25.Peters RF, White AM (1978). The relationship between cyclic adenosine 3,5-monophosphate and biochemical events in rat skin after the induction of epidermal hyperplasia using hexadecane. Br J Dermatol 98:301-314.

26.Pieri C, Zs.-Nagy I, Zs.-Nagy V, Giuli C, Bertoni-Freddari C (1977). Energy-dispersive X-Ray microanalysis of the electrolytes in biological bulk specimen. II. Age-dependent alterations in the monovalent ion contents of cell nucleus and cytoplasm in rat and brain cells. J Ultrastruct Res 59:320-341.

27.Pool TB, Cameron IL, Smith NKR, Sparks RL (1981).Intracellular sodium and growth control: A comparison of normal and transformed cells. In Cameron IL, Pool TB eds.: The Transformed Cell. New York: Academic Press,pp. 398-420.

28.Proll MA, Pool TB, Smith NKR (1979). Quantitative decreases in the in-

tracellular concentrations of sodium and other elements as Balb C/3T3 fibroblasts reach confluence. J Cell Biol 83:13A(abstract).

29.Roomans GM (1981). Quantitative electron probe X-ray microanalysis of biological bulk specimens. Scanning Electron Microsc 1981; II:345-356.

30.Roomans GM (1988). Quantitative X-ray microanalysis of biological specimens. J Electron Microsc Techn 9:19-43.

31.Roomans GM (1988). Introduction to X-ray microanalysis in biology. J Electron Microsc Techn 9:3-7.

32.Rozengurt E (1981). Na influx as a mitogenic signal for cultures fibroblastic cells. In Mozel LW, Schultz J,Scott WA, Werner R, eds.: Cellular Responses to Molecular Modulators. Miami Winter Symposia 18. New York: Academic Press, pp. 149-168.

33.Russ JC (1974). X-ray microanalysis in the biological sciences. J Submicrosc Cytol 6: 55-79.

34.Scheuplein RJ, Blank IH (1971). Permeability of the Skin. Physiol Rev 51:702-747.

35.Smith NKR, Sparks RL, Pool TB, Cameron JL (1978). Differences in the intracellular concentrations of elements in normal and cancerous liver cells as determined by X-ray microanalysis. Cancer Res 38:1952-1959.

36.Snell R (1965). An electron microscopic study of keratinization in the epidermal cells of the guinea pig. Z Zellforsch 65:829-846.

37.Thurau K, Dörge A, Rick R, Roloff C, Bech F, Mason J, Bauer R (1979). Intracellular electrolyte concentrations in epithelial tissue during various functional states. Scanning Electron Microsc 1979;II:733-738.

38.von Zglinicki T, Bimmler M (1986). The intracellular distribution of ions and water in rat liver and heart muscle. J Microsc 146:77-85.

39.Warner RR, Myes MC, Taylor DA (1988). Electron probe analysis of human skin: Elemental concentration profiles. J Invest Dermatol 90:78-85.

40.Wei X, Roomans GM, Forslind B (1982). Elemental distribution in guinea pig skin as revealed by x-ray microanalysis in the scanning tramnsmission electron microscope. J Invest Dermatol 79:167-169.

41.Wroblewski J (1987). Elemental changes associated with chondrocyte differentiation in rat growth plate. Histochemistry 87:145-149.

42.Wroblewski R, Roomans GM, Jansson E, Edström L (1978).Electron probe X-ray microanalysis of human muscle biopsies. Histochemistry 55: 281-292.

43.Zs-Nagy I, Toth L, Szallasi Z, Lamp I (1987). Energy-dispersive bulk specimen X-ray microanalytical measurement of the intracellular Na/K ratio in human laryngeal tumor. J Cancer Res Clin Oncol 113:197-202.

44.Zs-Nagy I (1989). A review on the use of bulk specimen x-ray microanalysis in cancer research. Scanning Microscopy 3:473-482.

Discussion with reviewers

C.W.Kischer: Do you mean that "intact circulation of the dermis" clears elements from the epidermis? If this is so, could you not monitor absorption of elements by measuring blood levels?
Author: If no circulation is present, diffusion of elements from the epidermis will be slow, because the concentration gradient dissipate. Although the blood in the dermis will clear a certain amount of elements from the epidermis, the increase in concentraton of such elements in the blood will be small and therefore difficult to measure.

R.R.Warner: You quote a spatial resolution of 10 µm at 20 kV, but state analyses were performed with a JEOL 100C or JEOL 1200EX. Were these instruments operated at 20 kV?
I.Zs.-Nagy: Why did you not try lower voltages, e.g. 10 kV?
Author: With a scanning attachment, the instruments can be operated at 20 kV; the JEOL 1200 EX can easily be operated at lower kV, but the JEOL 100 C only with difficulty, and then only at 10 kV, which we found slightly too low to get a spectrum with good statistics in a reasonable time.

R.R.Warner: The low Na/K ratios following long exposure times to hexadecane are surprising, and the author is not very clear in his discussion of how this might be due to changes between nucleus and cytoplasm. Would he please discuss the low Na/K ratio in more detail, and would he comment on the dry weight measurements for these time points?
Author: From Table 6 it can be seen that the decrease in the Na/K ratio is mainly due to the increase in K; the Na concentration changes only little. The increase in K is paralleled by an increase in Mg and P, indicative of the formation of nucleic acids involved in cell proliferation and protein synthesis. The application of dry weight measurments provides information on the elememtal content/distribution when the tissue water is removed. We consider this to be an adequate technique for our purposes. However, if there are major changes in the cellular water content at different time intervals this might influence the interpretation of the dry weight data. To

compensate for this, it would be better to perform the analysis on frozen hydrated specimens. This technique is, however, not an alternative at hand for us today.

Reviewer IV: If the cryo-sections are as thick as a single cell, then one must question elemental quantification due to absorption and fluorescence especially of sodium. Please comment.
Author: The peak-to-local background (background under the peak) method that we use for quantitative analysis provides an intrinsic correction for absorption (30). Secondary fluorescence is known to be negligible in bulk specimens consisting of soft tissue.

Reviewer IV: A well known problem arises by use of liquid nitrogen to quick freeze biopsies for later EDX analysis in that the freezing process is relatively slow thus allowing redistribution of cellular water and ions between the intracellular and extracellular environment. Such a freezing procedure is usally accompanied by cell shrinkage and an increase in the extracellular space as seen in the cryo-sections. Please comment.
Author: While indeed liquid nitrogen is not an optimal coolant, it appears to perform acceptably in our studies. It should be remembered that we are only interested in a very peripheral part of the specimen (the epidermis) and freezing rates are highest at the periphery of the specimen. Also, the stratum corneum and granulosum have low water content so that neither ice crystal formation nor ion/water fluxes appear to occur to an appreciable extent.