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THE FLUORESCENT PROFILES OF KERATOHYALIN GRANULES OF NEWBORN RAT EPIDERMIS WITH A NEW FLUORESCENT THIOL REAGENT (DACM)

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Abstract. A highly specific fluorescent thiol reagent, *N*-(7-Dimethylamino-4-methyl-coumarinyl)maleimide (DACM) has been used to examine the newborn rat skin in order to elucidate the cystine-rich keratohyalin granules or dense homogeneous deposits. After reduction of the disulphide bond with 2-mercaptoethanol, the periphery of the stratum corneum cells showed a very distinct fluorescence and the cytoplasm of the stratum corneum, granulosum and spinulosum showed moderate fluorescence. No fluorescence was observed, however, over the keratohyalin granules themselves. Intense, spotty fluorescence was observed from the remnants of nuclei and at the periphery of large keratohyalin granules of the stratum granulosum cells, which were assumed to be dense homogeneous deposits according to their distribution pattern. As the intense fluorescence elicited with DACM determines the abundance of cystine content in the material, cystine was found to be very scarce in keratohyalin granules, but abundant in dense homogeneous deposits and at the periphery of the stratum corneum cells.

Key words: DACM; Dense homogeneous deposits; Cystine-rich keratohyalin granules

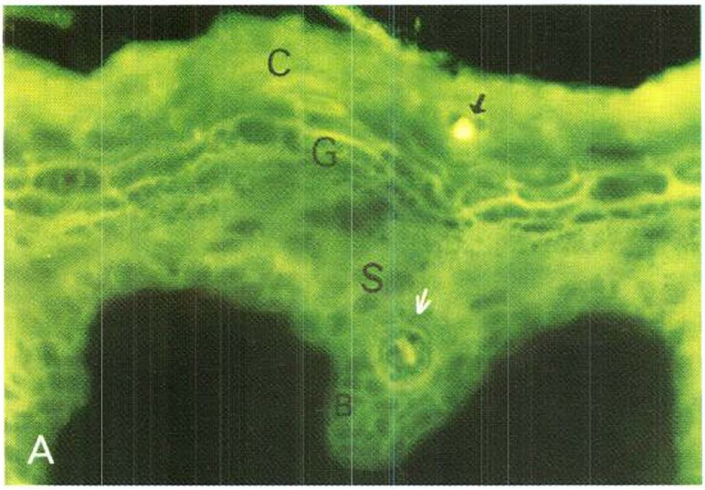
In rat epidermis, two kinds of keratohyalin granules can be observed by electron microscopy (3–5, 7). One type is large, and heterogeneous in shape, with a fine granular structure of moderate electron density (9). The other is small and round, and of greater electron density. These granules are usually located around the periphery of former granules and at the nuclear remnant of the stratum granulosum cells, and are more frequently observed in rat oral epithelium than the skin (5). This small granule, which was called a dense homogeneous deposit (DHD) by Fukuyama(4), reacted highly specifically with silver methenamine and blocking experiments indicated that sulfhydryl groups were responsible for this reaction (8). Fukuyama demonstrated the preferential incorporation of ^{3}H-cystine into this granule (DHD) and ^{3}H-histidine into the other type of keratohyalin granule (6). Matoltsy extracted keratohyalin granules with his refined method and showed that they had a high cystine content (12). These granules were very small and even in size (11). In contrast, the results of amino acid analysis by other investigators showed that the cystine content of extracted keratohyalin granule fractions was very low (1, 2, 14–16). This discrepancy in amino acid composition could be explained if Matoltsy had extracted preferentially dense homogeneous deposits and if other investigators had extracted another granule.

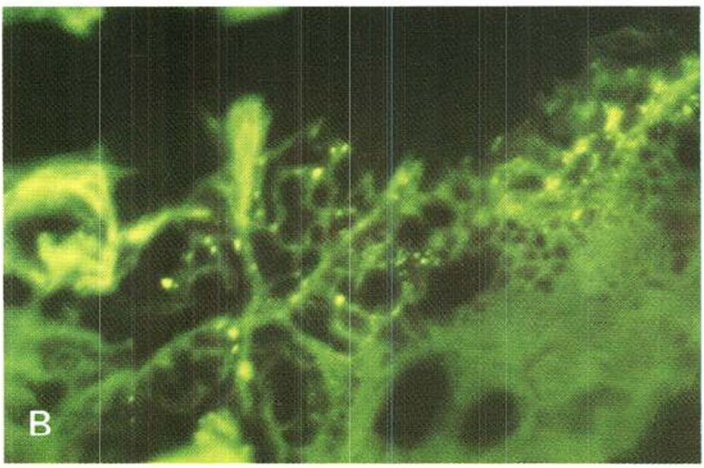
In this paper, we have tried to prove this assumption with the use of a highly specific fluorescent thiol reagent. This reagent is *N*-(7-Dimethylamino-4-methyl-coumarinyl) maleimide which was recently developed by Drs Takamitsu Sekine and Yuichi Kanaoka (10, 18).

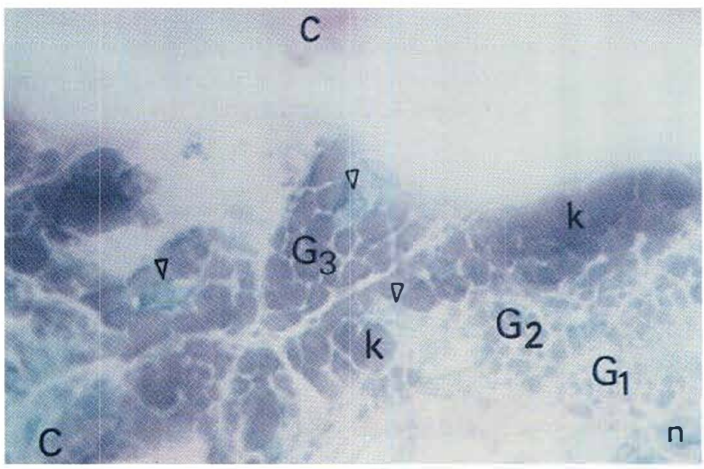
MATERIAL AND METHODS

Three-day-old Sprague-Dawley rat skin was used. From rat skin, 4 μm sections were prepared with a cryostat (Harris Manufacturing Co.) and fixed in cold 95% ethanol for 3 min.

One volume of 5 mM *N*-(7-Dimethylamino-4-methyl-coumarinyl)maleimide (DACM) in acetone solution was added to ten volumes of 10 mM phosphate buffer, pH 7.4. For free-sulphide detection, the specimens were incubated first in 10 mM phosphate buffer, pH 6.85, without 2-mercaptoethanol and were immediately rinsed in phosphate buffer, pH 7.4, again without mercaptoethanol. 450 μM DACM in 10 mM phosphate buffer, pH 7.4, was then placed on the specimen and incubated in a humid chamber at room temperature for 30 min. For both disulphide and sulphide bond detection, 4 μm sections were







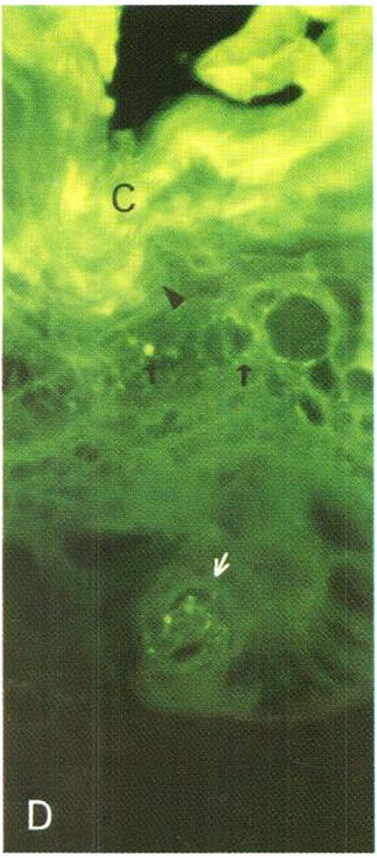


Fig. 1. A, B & D are specimens which were stained with DACM. C was stained with hematoxylin-eosin. (A) Without reduction of disulphide bond by 2-mercaptoethanol. *C:* stratum corneum, *G:* stratum granulosum, *S:* stratum spinulosum, *B:* stratum basale, ↕: a hair shaft, ↑: an interepidermal hair follicle. ×400. (B) After reduction of disulphide bond by 2-mercaptoethanol. Many dotted, strong fluorescences were observed ×400. (C) The same region as is shown in Fig. 1B. The same specimen was stained by hematoxylin-eosin after DACM staining. *C:* stratum corneum, G_{1-3}: stratum granulosum, G_3: the most maturated granular cell, *K:* keratohyalin granule, *n:* a nucleus, △: the remnant of the nucleus. ×400. (D) Many dotted fluorescences were observed in the stratum granulosum and in the intra-epidermal hair follicle. *C:* stratum corneum, ↑: the intra-epidermal hair follicle, ↕: dotted fluorescence, ▲: the fluorescence which was observed at the periphery of the stratum corneum cell.

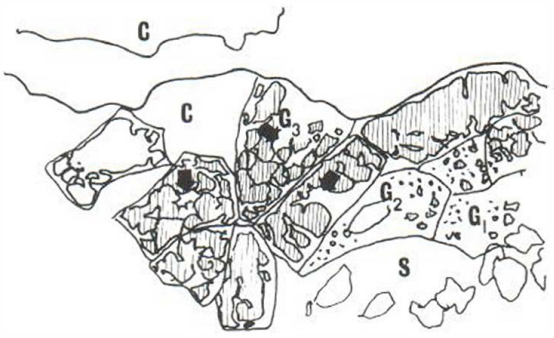


Fig. 2. Fig. 1C in schematic form. *C:* the stratum corneum, G_{1-3}: stratum granulosum cells. *S:* stratum spinulosum, ↟: the remnant of the nucleus.

preincubated in 10 mM phosphate buffer, pH 6.85, containing 1% 2-mercaptoethanol for 3 hours at room temperature. After this procedure, the slide was wiped with a filter paper, leaving the specimen covered in buffer. DACM was then placed on the specimen, followed by incubation at room temperature for 30 min in a humid chamber. After incubation the section was rinsed thoroughly in the same buffer. Fluorescence was examined under an Olympas Type FLM fluorescence microscope. The excitation wavelength was 400 nm and the emission wavelength was 480 nm. The same specimen was then stained with hematoxylin-eosin and examined.

RESULTS

Without reduction of disulphide bonds by 2-mercaptoethanol, the cytoplasm of the stratum spinulosum, granulosum and corneum showed very weak homogeneous fluorescence and weak fluorescence was also observed on the stratum corneum cells, whereas no fluorescence at all was observed over keratohyalin granules (Fig. 1A). After reduction of the disulphide bond with 2-mercaptoethanol, the periphery of the stratum corneum cells showed very distinct fluorescence and the cytoplasm of the stratum corneum, stratum granulosum and stratum spinulosum showed moderate fluorescence. No fluorescence was observed over keratohyalin granules themselves, but very strong, spotty fluorescence was observed over the remnants of nuclei and around the periphery of large keratohyalin granules of the stratum granulosum cells, and in the intra-epidermal follicular epithelium (Fig. 1B, D). The same specimens in which the fluorescence was examined, were stained by hematoxylin-eosin and the relationship with keratohyalin granules was examined (Fig. 1C, 2). Keratohyalin granules, both in the stratum granulosum and in the intra-epidermal follicular epithelium, consist largely of well-stained hematoxylin areas, but nothing was identified in the hematoxylin-eosin stained specimen where this spotty fluorescence was observed.

DISCUSSION

Whereas the Barnett-Seligman reagent was not found to be highly specific for sulphide or disulphide bonds in proteins, DACM is very sensitive and specific for both sulphide and disulphide bonds. It is valuable in the detection of such sulphur-rich materials such as DHD or sulphur-rich keratohyalin granules. Ogawa (13) has already examined human plantar skin using this reagent and found distinct fluorescence at the periphery of stratum corneum cells, but with no fluorescence over keratohyalin granules.

In this report, the dotted fluorescent materials, which are rich in sulphide or disulphide bond, were found at the periphery of large keratohyalin granules, but not over the keratohyalin granules themselves. These spotty fluorescent materials were most frequently observed both in the remnants of the nuclei of the stratum granulosum cells and in the intra-epidermal follicular epithelium. As dense homogeneous deposits were only found in rat skin and rat oral mucosa in which these deposits were located in the nucleus and at the periphery of keratohyalin granules, the distribution of these dotted, strongly fluorescent materials with DACM correspond well to the results of previous electron microscopic and radioautographic studies on DHD (4–7). Our data indicate that the materials derived from keratohyalin granules may be very poor in cystine, as the amount of sulphur-rich DACM-positive material was scarce. This means that the amino acid analyses of extracted keratohyalin granules indicating low cystine content (1, 2, 14–16), may be correct. As Walker et al. indicated in their recent paper (17), the incubation of rat skin in a buffer solution containing 0.5% trypsin caused dissolution of the usual type of keratohyalin granule. If this is correct and DHD are resistant to trypsin digestion, the keratohyalin granules, which Matoltsy collected by his unique method, might consist of DHD only and thus be cystine-rich.

DHD are visualized only by electron microscopy and cannot be seen in the light microscope. How-

ever, with the use of DACM, it is possible to visualize DHD even by light microscopy. Current studies are directed at the trypsin solubility of DHD in order to define their characteristics further.

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