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Quantitative Analysis of Langerhans' Cells in Epidermis at Irritant Contact Reactions Using Confocal Laser Scanning Microscopy

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Confocal laser scanning microscopy (CLSM) was used for quantitative analysis of CD1a+ cells in epidermis at irritant reactions. Sodium lauryl sulphate (2% and 4%) or non-anoic acid (20% and 80%) were applied to the skin of healthy volunteers under occlusion for 24 h. Skin biopsy specimens were taken after additional 24 h and were snap frozen. Freeze-sections, 25 µm thick, were stained with anti-CD1a antibodies (Leu-6) followed by FITC-labelled rabbit anti-mouse IgG. The sections were viewed and optically sectioned in the CLSM at four depth levels. The data was analysed using a threshold value for the fluorescence. The obtained result is presented as the proportion of specimen area having a fluorescence intensity above the threshold. The result demonstrates that the CLSM is a useful tool for obtaining not only structural information but also quantitative information from a defined tissue volume. In the present investigation it was possible to demonstrate variations in CD1a+ reactivity in epidermis at detergent-induced irritant reactions with a marked decrease in CD1a+ after 80% non-anoic acid exposure and only minor differences in the CD1a+ after 2% and 4% sodium lauryl sulphate exposure. ***Key words: Contact dermatitis; Immunology; Patch test.***

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Today it is well established that the skin is a heterogenous organ that provides a micromilieu for immunological and inflammatory events (1, 2). Langerhans' cells, keratinocytes and lymphocytes interact in a dose and time dependent manner during the induction and elicitation of contact allergic reactions. The keratinocytes also act as a key cell in non-specific inflammation in the skin (3, 4, 5). It has been shown that mild irritant stimuli such as occlusion with water and 0,5% sodium lauryl sulphate (SLS) induces morphological alterations in the epidermal Langerhans' cell population (6, 7). It is thus possible that minor single, or repetative, exposures of the skin to mild irritants might alter the micromilieu of the epidermis, affecting subsequent immunological events. It has also been shown in immunohistochemical and transmission electron microscopic studies (8, 9, 10) that different detergents induce different responses in the epidermis regarding Langerhans' cell morphology and number and in the expression of the intercellular adhesion molecule-1 (ICAM-1) on keratinocytes. To obtain a better understanding of the temporal, spatial and quantitative alterations in the different epidermal cell populations and their expression of surface markers, there is a need for a technique that allows both a morphological and quantitative analysis of specific structures in a given tissue volume.

The ability of a confocal laser scanning microscope (CLSM) to detect fluorescent signal from a uniformely thin "optical section" eliminates ambiguities arising from variable thickness of specimen tissue (11). The technique can also be applied for the three-dimensional reconstruction of cellular structures and has recently been applied for the reconstruction of epidermal Langerhans' cells stained for HLA-DR antigens (12). In the present study we have used CLSM to quantitatively determine alterations in the Langerhans' cell population at contact reactions induced by two different detergents, SLS and non-anoic acid (NAA).

MATERIALS AND METHODS

Subjects

Irritant reactions were induced in 4 healthy volunteers without any signs of skin diseases. All had given their informed consent. The investigation was approved by the Ethics Committee at Karolinska Hospital.

Patch testing

On each of two persons (2 males, age 26 and 52 years) 2% and 4% SLS in distilled water and distilled water alone were applied. These persons are referred to as subjects 1 and 2. On each of two persons (2 males, age 23 and 37 years) 20% and 80% NAA in isopropanol and isopropanol alone were applied. These persons are referred to as subjects 3 and 4. The test substances (fifteen microliter) were applied under occlusion for 24 h in small Finn-Chambers®, diameter 8 mm (13), on the gluteal area to ascertain non sun-exposed skin. In both groups unexposed skin served as control.

Skin biopsy specimens

Skin biopsies, diameter 3 mm, were taken at 48 h after dermal injection of local anesthesia (Lidocaine®, ASTRA, Södertälje, Sweden). The biopsies were immediately placed in transport medium (Histocon®, Histolab, Gothenburg Sweden) on ice and subsequently snap-frozen in chilled isopentane and stored at −70°C. The strength of the test reactions were graded visually at the time of biopsy.

Immunofluorescence staining

Vertical frozen sections were cut, 25 µm thick, in a cryotome at −20°C. The sections were acetone-fixed and incubated with mouse monoclonal anti-CD1a antibodies (Leu-6, working dilution 1/32, Becton Dickinson, Sunnyvale, CA, USA) for 30 min at room temperature, followed by washes in phosphate-buffered saline (PBS). Fluorescein-isothiocyanate (FITC)-labelled rabbit-anti-mouse IgG (working dilution 1/20, Dakopatts, Copenhagen, Denmark) was used as a secondary antibody. The sections were mounted with glycerol in PBS containing p-phenylenediamine (PPD) to reduce the fading of immunofluorescence (14). The coverslip was sealed with nailpolish to avoid oxidation of PPD leading to redbrown discolouring of the specimens. The dilutions of the antibodies were determined using sections from normal skin. Staining was not observed in controls without the primary antibodies.

Table I. *The mean fluorescence intensities (arbitrary scale; min = 0; max = 255) for the two subjects exposed to SLS for 24 h. Biopsies were taken at 48 h. Data obtained from 4 levels in the sections. Mean and standard deviation (SD) are given*

	Control	Water	2% SLS	4% SLS
Person 1	23,6	24,8	21,2	18,0
	(5,7)	(3,2)	(3,1)	(3,0)
Person 2	39,9	23,7	27,5	25,2
	(4,4)	(0,7)	(4,8)	(2,2)

The total number of measured optically sectioned areas in each case is 16

Microscopy

In the CLSM the recorded image represents a thin optical section of the specimen with almost all out-of-focus information eliminated. It is thus possible to get a three-dimensional (3D) recording of the specimen by scanning the object several times at different levels. A set of such images scanned at different depth in a specimen is referred to as a "stack". In this investigation each image consisted of 512 × 512 8-bit pixels.

The CLSM setup

In the present study two different microscopes were used, a PHOIBOS 1000 (a predecessor of the Sarastro 2000 instrument from Molecular Dynamics, Sunnyvale, California, USA) with photomultiplicator (PM)-voltage 860 V (subject 1), barrier filter 515 nm longpass, and beam splitter 510 nm longpass, and the CLSM at the Royal Institute of Technology with PM-voltage 860 V (subject 2) and 720 V (subjects 3 and 4), barrier filter 515 nm (subject 2) and 560 nm (subjects 3 and 4) longpass, and beamsplitter 510 nm longpass. To excite the FITC, the 488 nm line from an argon ion laser was used.

The two microscope setups have been described in detail elsewhere (15, 16). The CLSMs were built around light microscopes (Nikon Optiphot 1 or Zeiss Universal microscopes) with plano-apochromate 40X (N.A. 1.0) oil objectives.

Analysis of the biopsy specimens

Two freeze-sections were analyzed from each biopsy specimen. All material from one subject was stained and scanned at the same time under coded conditions. With both microscopes the stained histological sections were scanned to obtain 4 images (optical sections) of 512 × 512 pixels below each other, with three μm in between each section and with at least 2 such "stacks" per stained section yielding a minimum of 16 optical sections per biopsy specimen. For each analysis session the operating sensitivity of the CLSM was configured firstly by minimising laser output to a level where maximal fluorescence output from the specimen was still obtainable, whereafter the photomultiplier voltage was set to give maximum image brightness without saturating the 256-level display scale. Three different measuring methods were used:

Method 1: Practiced on person 1 and 2. Two areas chosen in the central parts on each of the 2 sections were scanned with the CLSM as described above. The average fluorescence intensity was measured in each digitized picture (optical section) on subject 1 with a built-in function in the PHOIBOS/4D program or with a software package, ISIS (Information System for Image Studies) on subject 2. For both persons the FITC-labelled CD1a+ cells in the epidermal area (excluding the stratum corneum) was measured on each optical section. Instead of a greyscale we used pseudo colours to be able to clearly see the basal membrane and the stratum corneum. A value for each optical section was thus obtained.

Table II: *The relative area of CD1a+ for the two persons exposed to SLS for 24 h. Biopsies were taken at 48 h. Data obtained from four levels in the sections. Mean and SD are given. Threshold value = the value selected on an arbitrary scale for the fluorescence intensities to consider a pixel positive (min = 0; max = 255)*

	Threshhold value	Control	Water	2% SLS	4% SLS
Person 1	20	0,44	0,51	0,39	0,30
		(0,12)	(0,08)	(0,07)	(0,06)
	40	0,13	0,16	0,09	0,07
		(0,06)	(0,05)	(0,02)	(0,02)
Person 2	50	0,26	0,02	0,04	0,02
		(0,1)	(0,01)	(0,03)	(0,01)

The total number of measured optically sectioned areas in each case is 16

Method 2: Practiced on persons 1 and 2. The same data as above was used but instead of the average fluorescence intensity the proportion of specimen area having a fluorescence intensity above some selected threshold value was measured. An algorithm was used to obtain a threshold value (17) for each subject. In this algorithm a bend point in the image histogram is used as the threshold value for the staining. The ISIS program was used to make a binary picture out of each of the scanned optical sections. All pixels above the threshold value received the value 1 and all pixels below received the value 0. The binary picture was then used to measure the relative area of the FITC-labelled CD1a+ cells in the epidermal area, excluding stratum corneum.

Method 3: Practiced on persons 3 and 4. As many epidermal fields as possible on the two histological sections, excluding the two fields at the both ends of the sections, were scanned with the CLSM as described above. From each of the optical sections a binary picture was obtained and measured by the ISIS program as in method 2. This was done for two thresholds on each subject, one which was held constant for both and one that was chosen specifically for each of the subjects

Table III: *The relative area of CD1a+ in epidermis of the two persons exposed to non-anoic acid (NAA) for 24 h. Biopsies were taken at 48 h. Data obtained from four levels in the sections. Mean and standard deviation SD are given. Threshold value = the value selected on an arbitrary scale for the fluorescence intensities to consider a pixel positive (min = 0; max = 255)*

	Threshold value	Control	Isoprop.	20% NAA	80% NAA
Person 3	20	0,29	0,35	0,51	0,09
		(0,08)	(0,11)	(0,13)	(0,05)
		n = 40	n = 32	n = 20	n = 36
	47	0,05	0,04	0,04	0,05
		(0,03)	(0,02)	(0,002)	(0,03)
		n = 40	n = 32	n = 20	n = 30
Person 4	20	0,60	0,70	0,19	0,09
		(0,08)	(0,13)	(0,06)	(0,11)
		n = 36	n = 32	n = 44	n = 40
	45	0,14	0,19	0,04	0,02
		(0,04)	(0,11)	(0,03)	(0,03)
		n = 36	n = 32	n = 44	n = 40

n = total number of measured optically sectioned areas

according to the algorithm described in method 2. We then obtained, as in method 2, a value of the relative area of positive fluorescence per optical section.

RESULTS

Epicutaneous testing with SLS resulted in a weak erythema after 2% SLS and erythema with oedema after 4% SLS in both persons. The NAA exposure resulted in similar reactions in the skin but the oedema was weaker after 80% NAA exposure compared to 4% SLS. Control skin and vehicle exposed skin were normal looking.

The result of the CLSM analysis is given in Tables I–III. In the four subjects it was possible to describe variations in the amount of CD1a reactivity in epidermis after exposure to the two detergents SLS and NAA. The exposure to 2% and 4% SLS caused minor changes in the CD1a reactivity (Tables I and II). In persons 3 and 4 (Table III) we found a decreased CD1a+ staining after exposure to 20% in one person and after exposure to 80% NAA in both volunteers. In person 3 there was a marked difference in the amount of CD1a+ (the relative area) after exposure to 20% NAA between the two applied threshold values (Table III). The influence of the threshold value on the relative area determination was demonstrated by choosing different values (Tables II and III).

DISCUSSION

In the present paper it is shown that different irritant stimuli do induce variations in the amount of CD1a reactivity in epidermis and that the CLSM can be a useful tool for the quantification of the labelled structures. It is also demonstrated that the result of the measurements (the fluorescence intensity), is dependent on several factors in the experimental system such as the setting of the background threshold. The choice of the threshold value is a crucial point for quantitative analysis as this discriminates between true staining and background. This is clearly seen when comparing two different thresholds for the same analysis (Tables II and III). The variations in the experimental (instrumental and immunohistochemical) settings were eliminated by using controls from the same individual and by staining and performing the analysis of all samples from one individual at the same time and with the laser beam and intensity well configured.

The study started out with the analysis of the mean fluorescence intensity in the optical sections (method 1, persons 1 and 2). By applying thresholding, we obtained a better detection of labelled structures and this technique was therefore applied for all specimens.

Another important question is which part(s) of the histological sections that should be used. Scanning several images below each other introduces possible errors such as absorption and scattering in the specimen, fading of the staining and an uneven penetration of the antibody. The obtained results in the present study are presented as the proportion of specimen area having a fluorescence intensity above the threshold and represents the mean of measurements from the 4 images scanned at different levels below the surface of the section. It was found by using different sampling techniques (see Material and methods) that the fields at the ends of the histological sections, revealed different fluorescent values than the more central part of the sections and these areas were thus excluded. This is in accordance with the findings of Mossberg et al. (17). The importance of adding PPD to the sections to reduce the fading of fluorescence under the laser beam has previously been shown (14, 17).

The enumeration of positively stained cells in the light microscope, e.g. Langerhans' cells, has often been used to describe changes in epidermis under pathological conditions such as contact dermatitis. The methods of counting cells in epidermis are connected with possible misinterpretations due to the technical limitations of conventional light microscopes and the thickness of the specimen sections (18, 19).

It is interesting that the quantification of the CD1a positivity can be performed within a specific tissue volume of epidermis using the CLSM and that this technique demonstrate the dose dependent variations in CD1a+ after application of SLS and NAA.

The CLSM provides a technique where the sampling of data can be performed as an automatic process. The data obtained permits both a quantitative and a structural analysis in the same tissue volume. This is an improvement compared with the conventional use of light microscopic techniques. This opens up new possibilities for the investigation of the time, volume, spatial and quantitative variations in the immune responses of the epidermis. It is notable that in some biopsies there seems to be a discrepancy between the number of CD1a+ cells and the relative amount of CD1a+ (10). This implies that alterations in the Langerhans' cell population defined as number of cells, might not reflect the true alteration in the amount of available surface structures.

In conclusion, it is possible to perform a quantification of the amount of CD1a positivity within a specific epidermal volume by using the CLSM. By combining the quantitative analysis with the possibility of performing three-dimensional reconstructions of cellular structures (12, 20) the CLSM provides a new and powerful technique for studies of immune reactions in the skin.

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