

INVESTIGATIVE REPORT

Stereological Quantification of Lymphocytes in Skin Biopsies from Atopic Dermatitis Patients

ANNE RINGER ELLINGSEN¹, FLEMMING BRANDT SØRENSEN¹, JYTTE OVERGAARD LARSEN³,
METTE SØNDERGAARD DELEURAN² and KRISTIAN THESTRUP-PEDERSEN²

Departments of ¹Pathology and ²Dermatology and Venereology, Aarhus University Hospital and ³Stereological Research Laboratory, Aarhus University, Denmark

Atopic dermatitis (AD) is histologically characterized by lymphocytic infiltration of the skin and quantitative assessment is required. This study introduces stereological techniques to quantify the number of lymphocytes in skin biopsies. Four-millimetre punch biopsies were taken from skin with active eczema in 8 adults with AD and from clinically normal skin from 4 of the patients. Five persons without allergy or skin disease served as controls. The mean number of lymphocytes in 4-mm skin biopsies was 469,000 and 124,000 in active eczema and in clinically normal skin, respectively. Compared with controls, the number of lymphocytes in biopsies increased by a factor of 6.8 in active eczema and a factor of 1.8 in clinically normal skin. If 20% of skin is affected by eczema the total number of lymphocytes located in the affected skin can be estimated to 1.27×10^{10} . A patient with clinically moderate AD has a considerable number of lymphocytes in the skin. Key words: counting frame; disector; fractionator; systematic random sampling.

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Anne Ringer Ellingsen, Department of Pathology, Kommunehospitalet, Aarhus University Hospital, Nørrebrogade 44, DK-8000 Aarhus C, Denmark.
E-mail: anne.ellingsen@iekf.au.dk

Characterization of the cellular infiltrate in atopic dermatitis (AD) skin has been the goal of several investigations. Various methods have been used in this context (1, 2), including attempts to quantify the number of lymphocytes in histological sections from normal (3, 4) and diseased skin (5, 6). The description of cytokine-driven T-cell lines from inflammatory skin of patients with AD (7–9) led to speculations about the number of lymphocytes in the skin, as the proliferative capacity of T-lymphocytes from skin biopsies grown *in vitro* is quite large.

Stereology can be used for obtaining unbiased estimates of the number of cells in a given tissue. The techniques involved and the mathematical principles of stereology have previously been described in detail (10–12).

In the present study, stereological techniques were used to quantify the number of lymphocytes in skin biopsies from patients with AD and from controls. A 3-dimensional (3D) stereological technique, the optical disector (11), and a robust and efficient sampling principle, the fractionator (13, 14), were used for the quantification.

MATERIAL AND METHODS

Patients and controls

Four-millimetre punch biopsy specimens were taken under local anaesthesia from inflammatory skin lesions of patients with AD, after informed consent. A total of 8 patients participated (7 males, 1 female; mean age 30 years, range 21–50 years). All patients had suffered from AD since childhood and had elevated serum immunoglobulin E (IgE) and several type I allergies.

The patients were grouped according to clinical manifestations, with mild affection defined as eczema in the face and large joint flexures ($n=2$), moderate affection with a more diffuse eczema, also including the trunk ($n=4$), and severe affection with a universal body distribution ($n=2$). The biopsies were taken from skin with active eczema and the locations were on the back ($n=5$), the arm ($n=2$) and the leg ($n=1$).

From 4 of the patients a biopsy was obtained from skin in the gluteal region, in which 3 had clinically normal looking skin and one had a very mild affection.

Four-millimetre punch biopsies from clinically normal skin were obtained from 5 healthy women in conjunction with plastic surgery of the mamma. None of the controls had a history of allergy or skin disease.

The study was approved by the local Ethics Committee of Aarhus County, Denmark.

Tissue processing

The 4-mm punch biopsies were handled with care to avoid deformations that could influence tissue volume and were fixed in 4% phosphate-buffered formaldehyde for 24 h. After fixation they were prepared for plastic embedding by dehydration in ascending series of ethanol, and then infiltrated with glycolmethacrylate (Technovit 7100, AX-lab, Denmark) for 6 days. Each biopsy was exhaustively serial-sectioned perpendicular to the epidermal surface using a Reichert-Jung 2050 Supercut Microtome with a microtome setting of 30 µm thickness. Plastic embedding made it possible to cut thick sections, which are optimal for 3D optical disector counting.

The sections were stained with a Giemsa stain modified for plastic-embedded tissue and coverslipped with Eukitt. Section thickness after tissue processing was measured using a Haidenhein microcator MT12 (Traunreut, Germany).

Theoretical aspects regarding cell counting in histological sections

Theoretically, cell numbers can be estimated in an unbiased way using a 3D approach. Profile counts on ordinary 2D histological sections will inevitably be biased as larger cells will have a greater probability of being hit by a section, because any cell sectioned will appear as a profile in the section with a probability related to the size and the shape of the cell. Counting of cells in sections is also biased if the fields selected for counting are chosen subjectively by the observer. Another problem is the edge effect introduced by traditionally used counting areas (10): how does the observer decide when to count cells at the edge of the area? This necessitates application of an unbiased counting frame, i.e. a set of geometrical criteria which ensures that cells are counted on the basis of their number, not their size, i.e. all profiles are counted with an equal probability. However, 2D profiles

seen in a single section represent a biased, non-representative sample of all 3D cells in the reference space.

The disector represents a simple probe for unbiased counting that samples cells with a uniform probability in 3D space, irrespective of their size, shape and orientation. In a disector 2 consecutive sections, either optical or physical, are used to define a sampling volume, i.e. a defined volume in which to count cells.

In a physical disector an unbiased counting frame is applied on 1 section and the correct count is obtained by comparison with the consecutive section. The sampling volume is the area of the counting frame multiplied by the distance between the upper surfaces of the 2 sections. A particle is counted if it appears in the lower reference section inside the counting frame and it does not appear in the upper section.

The optical disector principle is based on optical "sections" generated when moving the focal plane a known distance inside a thick physical section. The advantage of the optical disector is that it eliminates the time-consuming task of comparing separate physical sections, as well as making the identification of cells easier as they can be evaluated in 3 dimensions with respect to shape and size.

Aspects regarding estimates of total cell numbers in a specimen

When estimating the number of particles in a specimen, it is usually impractical to count all of the cells. Instead, the particle number can be estimated in a known fraction of the reference space. The fractionator principle provides sampling of particles uniformly at random with a known and predetermined probability, and then derives the total number, N_{total} , in the reference space from the number in the sample and the sampling probability.

The specimen is divided into slices and a predetermined fraction of the sections is sampled randomly, e.g. a sixth of all sections. Because any particle is contained in exactly one of the slices, according to the above disector criterion, every particle has the same probability of being sampled, i.e. the sampling is uniform. The total number can then be estimated as the product of the particle number in the sample and the inverse sampling probability. Furthermore, one can also decide to sample in uniformly random sampled subdivisions of the individual sections instead of the whole section. The total number of counted cells times all the inverse sampling fractions provides an unbiased estimate of cells, N_{total} .

Practical counting

Counting was performed by one person without knowledge of the clinical status of the patients; however, in most cases the morphology was indicative of the lesion in question. The degree of cellular infiltration in the skin was first evaluated qualitatively at low magnification and the biopsies were then grouped into either heavy or slight lymphocytic infiltration.

According to this grouping a specified fraction of the sections was sampled in a systematic, uniform random manner. These fractions, the section sampling fractions, f , were 1 out of 18 sections when heavy lymphocytic infiltration was present, 1 out of 9 if slight infiltration was seen, and 1 out of 6 in apparently normal skin.

The sampled sections were examined with an optical disector using computer-assisted microscopy (Olympus Castgrid®, Olympus Denmark A/S). In brief, live video images of the fields of vision in the microscope were transmitted by a video camera to a computer screen. The microscope was equipped with an electronic microcator which measured the focal plane displacement, and with stepping motors that controlled stage movements via the computer software.

The first section used for counting was chosen at random using a list of random numbers. The following sections were selected systematically according to the sampling fraction. Each sampled section was delineated at low magnification, $\times 70$, using the computer system to specify the inclusion area, which was the whole tissue section (Fig. 1).

Within this inclusion area the computer generated systematic, random fields of vision, as indicated in Fig. 1. The first field of vision was chosen at random by the program, and the following fields of visions were placed systematically, a predetermined, fixed distance apart, i.e. the selection was not influenced by the observer and all regions had equal probability of being sampled. The image of an unbiased counting frame with a specified area, A_{frame} , of $633 \mu\text{m}^2$ was superimposed on to the microscope images via the video-computer

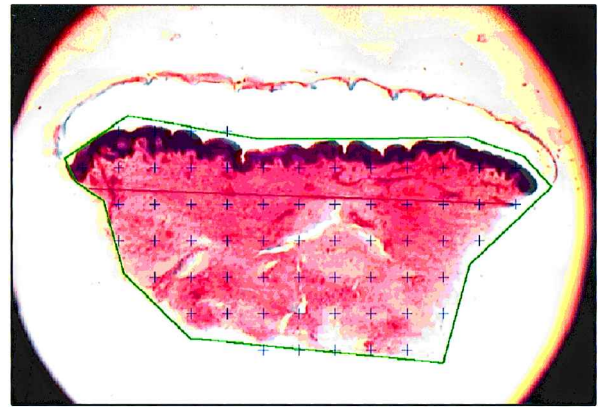


Fig. 1. Skin biopsy section showing the inclusion area and the principle of random, systematic sampling of fields of vision. The red line illustrates measurement of the horizontal width of a section, L_{base} , as explained in the Appendix.

interface. The counting frames were thereby placed within a specified distance ($dx = dy = 250 \mu\text{m}$), i.e. a known area sampling fraction was established.

The section area was estimated by point counting, simultaneously with the cell counting, as the point in the upper right corner of the counting frame, P_{area} , should hit cutaneous tissue to be included.

The mean total number of frames evaluated per biopsy was 762 (range 287–1,790). The actual counting was done at a magnification of $\times 2,200$. The counting frame was defined by red and green borders, as shown in Fig. 2, and only lymphocytes within the frame or touching the green inclusion lines were counted. Cells touching the red exclusion lines or their extensions were not counted according to the counting rules referred to above.

Within each frame the number of cells was counted in a 3D space as the focal plane was moved a fixed distance through the section. The distance evaluated was $20 \mu\text{m}$ (h , height of the disector) (Fig. 3), i.e. a known height sampling fraction was established. Within the counting volume, the disector volume V_{disector} , only cells appearing with a lymphocytic nucleus in focus were counted, Q^- .

Lymphocytes were defined by their cytological characteristics. They should have a centrally placed round nucleus with a condensed amorphous chromatin pattern surrounded by a scanty cytoplasm. Small lymphocytes, approximately $7\text{--}12 \mu\text{m}$ in diameter, were included, but larger, more blast-like cells were excluded because of uncertainty as to their origin and their similarity to histiocytes and stromal cells. Cells with folded or reniform nuclei characteristic of monocytes and cells with eccentric nuclei characteristic of plasma cells were ignored. The mean number of counted cells per biopsy was 113 (range 44–296).

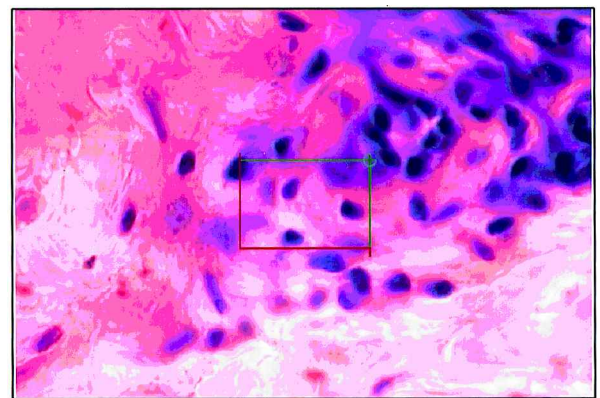


Fig. 2. An unbiased counting frame superimposed on to the microscope image. The position is $10 \mu\text{m}$ in the depth of a section. The cell in focus near the lower red line was counted. A total of 3 cells was counted in the disector volume.

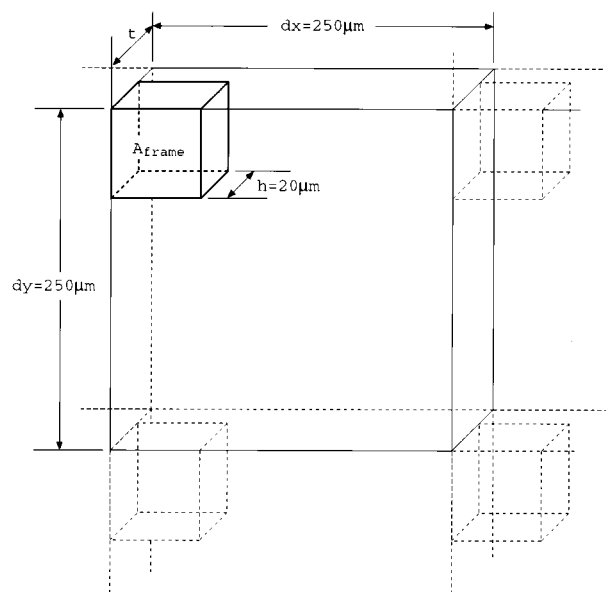


Fig. 3. Schematic presentation of 4 counting frames with the corresponding volumes, $V_{\text{dissector}}$.

Calculation of total number of lymphocytes

From a certain fraction of sections, fractions of cells were sampled, enabling the total number of lymphocytes, N_{total} , for each biopsy to be estimated as:

$$N = \Sigma Q^- \times \frac{1}{\text{Section sampling fraction}} \times \frac{1}{\text{Area sampling fraction}} \times \frac{1}{\text{Height sampling fraction}}$$

$$= \Sigma Q^- \times \frac{1}{f} \times \frac{\Sigma P_{\text{area}} \times dx \times dy}{\Sigma A_{\text{frame}}} \times \frac{t}{h}$$

Supplementary measurements were performed (see Appendix).

An estimated number of cells per original skin biopsy surface area (mm^2) and per volume (mm^3) could then be calculated. For comparisons, the estimated number of cells per basal cross sectional area (mm^2) and per width (mm) could also be calculated.

Statistical analysis

The biopsies were compared groupwise as control versus unaffected skin and unaffected skin versus affected skin. All groups were compared with a 2-tailed *t*-test after testing for inhomogeneity of variances (*F*-test). 2*p*-Values < 0.05 were regarded as statistically significant.

RESULTS

The results of the study are presented in Tables I and II and Fig. 4. The mean number of lymphocytes in 4-mm skin biopsies

Table I. Group means of the estimated unbiased parameters with the coefficient of variation ($CV = SD/\text{mean}$) in parentheses.

Clinical diagnosis	N_{total}	$N/A (\text{mm}^{-2})$
Non-atopics ($n = 5$)	68,987 (0.18)	5,488 (0.18)
Atopics, unaffected ($n = 4$)	123,512 (0.25)	9,825 (0.25)
Atopics, affected ($n = 8$)	469,241 (0.38)	37,326 (0.38)

N_{total} : number of lymphocytes per biopsy; N/A : number of lymphocytes per original skin biopsy surface area.

Table II. Estimated parameters given for comparison purposes: group means of the estimated parameters with the coefficient of variation ($CV = SD/\text{mean}$) in parentheses.

The parameters are dependent on the preparation procedures and the section thickness applied in the study.

Clinical diagnosis	$N/V (\text{mm}^{-3})$	$N/A_{\text{basal}} (\text{mm}^{-2})$	$N/L (\text{mm}^{-1})$
Non-atopics ($n = 5$)	3,600 (0.21)	10,036 (0.22)	351 (0.20)
Atopics, unaffected ($n = 4$)	11,761 (0.04)	20,470 (0.18)	698 (0.21)
Atopics, affected ($n = 8$)	30,834 (0.44)	60,224 (0.35)	2,038 (0.33)

N/V : number of lymphocytes per volume; N/A_{basal} : number of lymphocytes per basal cross-sectional area; N/L : number of lymphocytes per basal width, per section thickness.

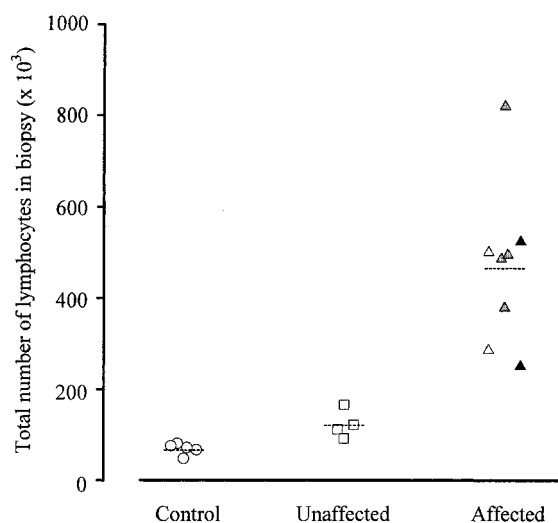


Fig. 4. Total number of lymphocytes in the biopsies. The horizontal bar indicates the group mean. In the affected group the triangles illustrate the clinical degree of affection: $\triangle$, mild; $\blacktriangle$, moderate; $\blacktriangle$, severe.

from control persons was 69,000 cells (median 72,000; range 48,300–81,200). In biopsies from AD patients it was 123,500 lymphocytes (117,000; 92,500–166,600) in clinically unaffected skin and 469,200 in active eczema (492,000; 252,200–821,700).

There were statistically significant differences between the groups. Patients with AD have about 80% more lymphocytes in normal-looking skin than control persons. In clinically affected skin the number of lymphocytes is increased by a factor of 6.8 over control skin and 3.8 over clinically unaffected skin. Tests for regional differences and gender differences were not performed because of the limited number of biopsies. The number of lymphocytes as a function of the clinically evaluated degree of affection did not indicate correlation between the clinical score and the estimated number of lymphocytes.

DISCUSSION

AD is a chronic inflammatory skin disease with a high prevalence (15, 16). The cellular infiltrate of skin affected by AD comprises primarily lymphocytes (17), most of which are CD4+ T-lymphocytes (1, 2, 5, 18–20). Some studies have

also reported increased numbers of eosinophil granulocytes (21), mast cells (22) and Langerhans' cells (2)/dendritic cells (23). However, these cells are not dominant features and all studies have found that the predominant infiltrating cell type is the lymphocyte, as also shown for the non-allergic type of AD, NAD (21).

So far, no studies have evaluated the total number of lymphocytes in skin biopsies from patients with AD by means of unbiased stereological techniques and most studies report sparsely on how counting was performed. Bos et al. (5) made calculations of 2D profiles/lymphocytes per 10 mm section width, but did not report where the width measurement was performed. Our experience is that it is difficult to measure the width, and the obtained number of counted cells is dependent on the section thickness. Other investigators (1, 21) report results per mm², which is a widely used format, but it is unclear whether the frame used for counting was unbiased and how the counting areas were selected. Regarding representativity of the evaluated tissue, Bos et al. (3) discuss the problem arising when the presence of larger vessels gave incidental high numbers of T-cells.

The approach used in this study to estimate the total number of lymphocytes in biopsies is not affected by stretching and shrinkage of the tissue during the tissue processing procedures and accordingly represents the proper way to evaluate the material.

The other results (Table II) are presented solely to give an impression of the values. One should be aware of the section thickness used and that the calculated number of cells per basal cross-sectional area includes all cells over and below the artificial plane applied when measuring the section width. The number of cells per volume was not reliable, as the biopsy volumes varied. This could be due to different amounts of dermal/subcutaneous tissue or different shrinkage of the tissue.

Indeed, when data are reported as densities, i.e. number of profiles per unit, the data also reflect the dimension of the reference parameter (volume, area, length).

The calculations per original skin biopsy surface area compared with calculations per (artificial) basal cross-sectional area after preparation of the biopsies reveal a difference in the range of a factor 1.6 to 2.1. This indicates possible influences from different sources on the final result, e.g. different stretching of the tissue when punching and handling the biopsy, different shrinkage of the tissue during preparation, or measurement difficulties due to the definition of measurement site. It is not possible to determine the crucial factor, and this is why the unbiased estimates are essential.

Even though an unbiased counting principle was used in this study, there are still possible sources of error. The major problem in this study design was the identification of lymphocytes, especially when judging larger, more blast-like cells. To reduce the impact of this possible source of bias in comparative studies, it is important that the same observer is employed throughout the study. So far it has not been possible to use immunohistochemical techniques on the thick plastic sections, but immunohistochemical identification of the cells certainly would make the counting easier.

The results show that even in clinically unaffected skin of patients with AD the number of lymphocytes is increased compared with skin from control persons. These findings confirm previous studies based on qualitative and semi-quantitative techniques.

Ordinary histological sections also show lymphocytes infiltrating clinically unaffected skin and this infiltration in non-lesioned skin has been reported previously (18, 24, 25). It may offer a pathophysiological explanation for the "flare-ups" that occur very easily in these patients. It can be estimated¹ that the number of skin-infiltrating lymphocytes in a patient with AD, macroscopically affecting 20% of the skin surface, is 1.27×10^{10} lymphocytes in the affected skin in addition to 1.34×10^{10} lymphocytes in the unaffected skin, compared with a total of 1.25×10^{10} lymphocytes in the peripheral blood. Thus, a patient with even a clinically moderate AD must have a substantial fraction of the lymphocyte pool located in the skin, in addition to lymphocytes in the spleen, intestines and lymph nodes.

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¹Number of lymphocytes located in affected and unaffected skin of a "standard" AD patient with active eczema in 20% of the surface area: Surface area = 1.70 m²; 20% affected skin = 0.34 m² = 340,000 mm²; 80% unaffected skin = 1.36 m² = 1,360,000 mm². Surface area of a 4-mm punch biopsy = $2^2 \times \pi$ mm².

Number of lymphocytes in affected skin =

$$\frac{340,000 \text{ mm}^2}{(2^2 \times \pi) \text{ mm}^2/\text{biopsy}} \times 469,200 \text{ lymphocytes biopsy} \\ = 1.27 \times 10^{10} \text{ lymphocytes.}$$

Number of lymphocytes in unaffected skin =

$$\frac{1,360,000 \text{ mm}^2}{(2^2 \times \pi) \text{ mm}^2/\text{biopsy}} \times 123,500 \text{ lymphocytes biopsy} \\ = 1.34 \times 10^{10} \text{ lymphocytes.}$$

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APPENDIX

Volume-estimation of biopsies

The total volume of the biopsy after histological processing and sectioning was estimated using Cavalieri's principle (12). Accordingly, the volume of a biopsy, V_{biopsy} , can be estimated, from a series of systematic random parallel sections separated by a known distance, as the total sum of section areas, $\Sigma A_{\text{section}}$, multiplied by the mean distance between the sections, T :

$$V_{\text{biopsy}} = \Sigma A_{\text{section}} \times T.$$

The section area was estimated from the number of points hitting tissue, P_{area} , times the area associated with each point: $A_{\text{section}} = \Sigma P_{\text{area}} \times A_{\text{point}}$, where $A_{\text{point}} = dx \times dy$.

The thickness of the sections was measured with the microcator at a final magnification of $\times 3,700$. The fraction of sections used for the thickness measurements was 1/18 for all biopsies at a fixed distance of 800 μm between the measuring sites along the x - and y -axes. A mean section-thickness for each biopsy could then be calculated (mean 34 μm ; range 28–39 μm).

The mean distance between sections, T , was the mean section thickness, t , multiplied by the inverse sampling fraction, $1/f$.

The total volume of each biopsy could then be estimated as:

$$V_{\text{biopsy}} = \Sigma A_{\text{section}} \times t \times 1/f.$$

Horizontal width and cross-sectional area estimation

Previous studies have reported the lymphocyte number per section width and section area and for comparative purposes *only* these parameters are included.

In sections sampled for measurements of thickness the distance was also measured from one side of the section to the other side, L_{basal} , i.e. the width of the biopsy, at the level of the superficial capillary plexus, where the majority of the inflammatory cells seemed to be located (Fig. 1). The distance was measured in micrometres at a magnification of $\times 70$.

The product of the sum of the section widths, ΣL_{basal} , and the distance between sections provided an estimate for the cross-sectional area of the biopsy at that level. Biopsy cross-sectional area, A_{basal} , was calculated as:

$$A_{\text{basal}} = \Sigma L_{\text{basal}} \times t \times 1/f.$$

The total section width, L_{total} , was estimated as the product of the sum of the section widths and the inverse sampling fraction, $L_{\text{total}} = \Sigma L_{\text{basal}} \times 1/f$.