Spatial Statistics for Epidermal Langerhans Cells —Effects of Protopic® Ointment 0.1% on the Spatial Distribution—

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Epidermal Langerhans cells (LCs) are dendritic cells abundant in the epidermis. LCs fulfill an essential role in skin immunology. We have applied spatial statistics to the study of LCs for the last 20 years. The immunosuppressive macrolide tacrolimus was discovered in 1984. Tacrolimus ointment has been available for atopic dermatitis patients in Japan since 1999 and in the U.S.A. since 2001. Three-week Protopic® Ointment 0.1% (0.1% tacrolimus ointment) application to mice reduced both the density and spatial regularity of LCs. The estimated potential model functions were similar in control and tacrolimus groups, but the diameter, σ, of the tacrolimus group was slightly larger than the control. Although studies using LC suspension such as flow cytometry analysis have become the mainstream, there is information that can only be obtained based on the spatial pattern of LCs. Spatial statistics will provide a new viewpoint in dendritic cell studies.
Key words: Spatial Statistics, Epidermal Langerhans Cells, Dendritic Cells, Tacrolimus, Protopic® Ointment

1. Introduction

Epidermal Langerhans cells have the central role in the skin's immune system. Normal Langerhans cells show a characteristic spatial pattern (Fig. 1). For the last twenty years, we have studied their spatial statistics based on the following hypothesis: "Epidermal Langerhans cells should develop an optimal spatial distribution. In the observed spatial pattern itself, there should be a clue."

The immunosuppressive macrolide tacrolimus was discovered in 1984 by Exploratory Research Laboratories, Fujisawa pharmaceutical Co., Ltd., Tsukuba, Japan. Tacrolimus is used to prevent graft rejection after organ transplantation in animals and human beings. In Japan, 0.1% tacrolimus ointment (Protopic® Ointment 0.1% for adult atopic dermatitis patients) has been used in medical services under health insurance since November 1999.

In this article, we would like to describe "how to apply spatial statistics for epidermal Langerhans cells", and "the observed effects of Protopic® Ointment 0.1% on the spatial distribution."

1.1 Epidermal Langerhans cells

Epidermal Langerhans cells are dendritic cells abundant in the epidermis. The human body is completely covered by a network of about 2×10^9 Langerhans cells (Romani *et al.*, 2006). The story of the Langerhans cell starts in 1868, when the medical student Paul Langerhans discovered a population of intraepidermal dendritic cells in human skin stained with gold chloride. In the more than 120 years since its discovery, the Langerhans cell has undergone a conceptual metamorphosis from a nerve cell to a melanocyte, to a histocyte, and finally to an antigen-presenting cell (Wolff, 1991).

Epidermal Langerhans cells serve as sentinel cells whose primary function is to survey the epidermal environment and initiate immune responses against microbial threats (Romani *et al.*, 2006). Epidermal Langerhans cells are bone marrow-derived cells, and they are antigen-presenting cells (Fig. 2). When presenting an antigen to T cells, epidermal Langerhans cells are known to detach from the epidermis to reach the regional lymph nodes through lymphatic vessels (Shimizu, 2007).

At the light microscopic level, epidermal Langerhans cells are difficult to detect in routinely stained sections; however, they appears as dendritic cells in sections stained with gold chloride, a stain specific for Langerhans cells (Odom *et al.*, 2000). They can also be stained with peroxidase-labeled monoclonal antibody CD1a or S100 (Odom *et al.*, 2000). Romani *et al.* (2006) reported that new experimental models and methods have invigorated the field of Langerhans cell study.

1.2 Tacrolimus ointment (Protopic® Ointment) and atopic dermatitis

The immunosuppressive macrolide tacrolimus (code number: FK-506) was discovered in 1984 by Exploratory Research Laboratories, Fujisawa pharmaceutical Co., Ltd., Tsukuba, Japan, during a search for immunosuppressive substances among natural products (Kino *et al.*, 1987b). FK-506 has been isolated from the fermentation broth of *Streptomyces tsukubaensis* No. 9993 as a colorless prism, and the molecular formula was determined to be $C_{44}H_{69}NO_{12}\cdot H_2O$ (Kino *et al.*, 1987a).

Fig. 1. ATPase-stained epidermal Langerhans cells (guinea pig).

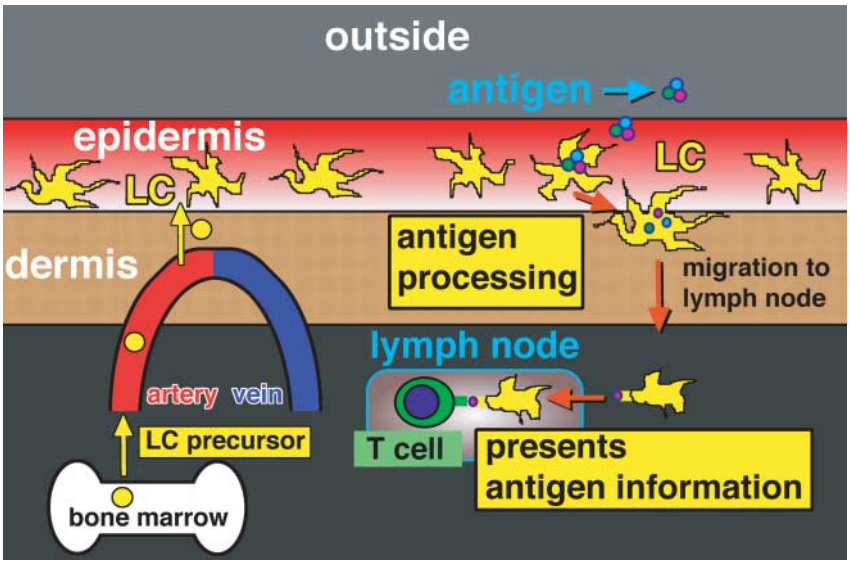


Fig. 2. Epidermal Langerhans cells (LC) are antigen-presenting cells derived from bone marrow.

Tacrolimus was used to prevent graft rejection after organ transplantation in animals and human beings (Panhans-Groß *et al.*, 2001). In the treatment of skin disease, oral tacrolimus has been used successfully in chronic inflammatory diseases such as psoriasis (Panhans-Groß *et al.*, 2001). Topical tacrolimus has been shown to inhibit cell-mediated hypersensitivity reactions in several animal models and allergic contact dermatitis in human beings (Panhans-Groß *et al.*, 2001). Several groups have reported that tacrolimus treatment results in phenotypic alternations of epidermal Langerhans cells (Panhans-Groß *et al.*, 2001; Wollenberg *et al.*, 2001).

Atopic dermatitis is one of the most common childhood skin diseases, affecting up to 25% of the population in the United States, Europe, and Japan (Hultsch *et al.*, 2005: the preceding paragraph was extracted from this article.). Topical corticosteroids have been the mainstay of treatment since the late 1950s. While providing excellent short-term efficacy, topical corticosteroid usage is limited by its potential adverse effects. These side effects include skin atrophy, striae, telangiectasia, acneiform eruptions, and the risk of absorption, leading to systemic effects such as hypothalamic-pituitary-adrenal axis suppression. Topical short-term treatment with even low potency corticosteroids eliminates epidermal Langerhans cells in the skin by inducing apoptosis. The recently introduced topical calcineurin inhibitors of 1% pimecrolimus cream and 0.03 and 0.1% tacrolimus ointment (Protopic®, Astellas Pharma Inc.) exhibit a more selective mechanism of action. Tacrolimus ointment has been available in the United States since 2001. Clinically, topical calcineurin inhibitors have been shown to be a safe and effective alternative to topical corticosteroids in almost 7 million patients (>5 million have received pimecrolimus >1.7 million tacrolimus).

In Japan, 0.1% tacrolimus ointment (Protopic® Ointment 0.1% for adult patients) has been used in medical service under health insurance since November 1999, and 0.03% tacrolimus ointment (Protopic® Ointment 0.03% for child patients) has been used since December 2003. Pimecrolimus cream was not yet authorized as a Japanese health insurance-covered medicine as of early 2009.

The following is the whole sentence of the mechanism of action from Protopic® prescribing information on Astellas Pharma US, Inc. (http://www.astellas.us/docs/protopic.pdf,

we read it on January 12, 2009: The quotation obtains the consent of Astellas Pharma Inc.). "CLINICAL PHARMACOLOGY; Mechanism of Action; The mechanism of action of tacrolimus in atopic dermatitis is not known. While the following have been observed, the clinical significance of these observations in atopic dermatitis is not known. It has been demonstrated that tacrolimus inhibits T-lymphocyte activation by first binding to an intracellular protein, FKBP-12. A complex of tacrolimus-FKBP-12, calcium, calmodulin, and calcineurin is then formed and the phosphatase activity of calcineurin is inhibited. This effect has been shown to prevent the dephosphorylation and translocation of nuclear factor of activated T-cells (NF-AT), a nuclear component thought to initiate gene transcription for the formation of lymphokines (such as interleukin-2, gamma interferon). Tacrolimus also inhibits the transcription for genes which encode IL-3, IL-4, IL-5, GM-CSF, and TNF-α, all of which are involved in the early stages of T-cell activation. Additionally, tacrolimus has been shown to inhibit the release of pre-formed mediators from skin mast cells and basophils, and to down regulate the expression of FcϵRI on Langerhans cells."

1.3 Spatial distribution of epidermal Langerhans cells

Several authors described density changes of epidermal Langerhans cells in different skin conditions (Bahmer and Lesch, 1987; Liu *et al.*, 1987; Oxholm *et al.*, 1987), and some authors reported changes of the regular distributions from a subjective point of view. About 20 years ago, we became aware that there was no systematic research on the spatial distribution of epidermal Langerhans cells. In certain fields, such as botany, ecology, forestry, zoology, etc., spatial distributions are important. Spatial data analysis has a long history (for review, see Diggle, 1983), and attractive mathematical methods have been developed.

We hypothesized that epidermal Langerhans cells adopted an optimal to facilitate their immunosurveillance based on an elegant system; and the pattern itself should provide clues to the system. Numahara *et al.* (1992), applied two spatial distribution indices to our study of Langerhans cells, and we compared the regularities in different skin conditions. As the results, we realized that the distributions in healthy skin were markedly regular. Successively, we found that the distribution of epidermal Langerhans cells in the guinea pig was completely regular, the pattern of Voronoi divisions fitted the territories, the random packing model simulated their bone marrow derivation, a repulsive interaction was demonstrated, and a repulsive potential function was estimated (Numahara *et al.*, 2001).

2. Review) How did We Approach Spatial Statistics for Epidermal Langerhans Cells?

2.1 Point map

A simple graphical representation of the pattern of objects as a point map is a very useful preliminary step towards understanding its properties (Illian *et al.*, 2008). After 1993, we performed on Macintosh® computers (Numahara *et al.*, 1994a; Numahara and Kojima, 1995) using the protocol show in Fig. 3. The cellular center coordinate chooses the nuclear center as a general rule.

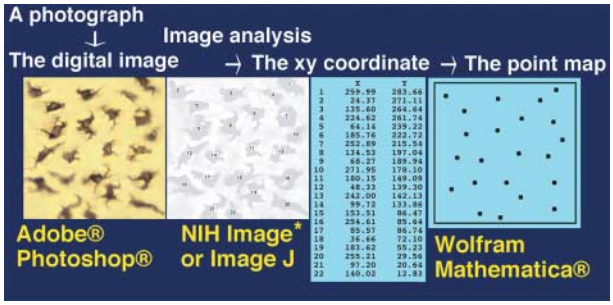


Fig. 3. Our protocol to produce a point map of the cells.

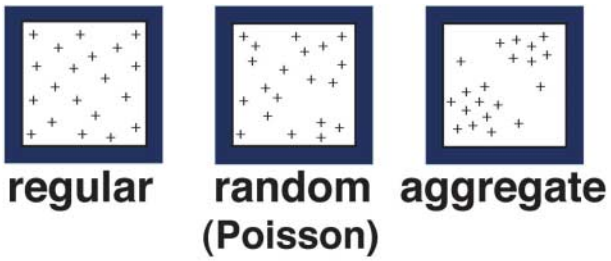


Fig. 4. Three simulated point patterns.

$$HSI = \frac{\sum_{i=1}^{N} r_{1i}^{\,2}}{\sum_{i=1}^{N} r_{2i}^{\,2}}$$

r_1: distance from a point chosen at random to its nearest individual
r_2: the distance from an individual chosen at random to the nearest individual
N: sample size (number of individuals)

regular pattern HSI < 1,
random pattern HSI = 1,
aggregate pattern HSI > 1

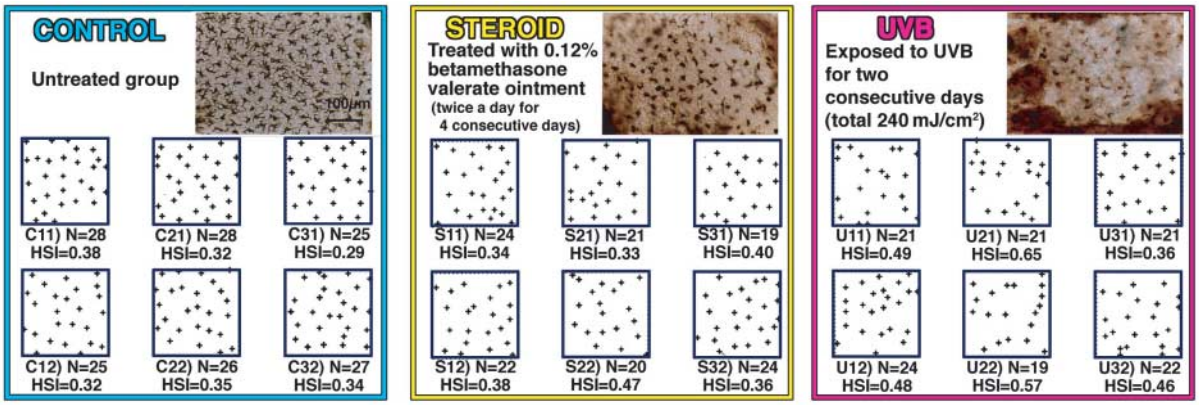
Fig. 5. Quantification of regularity. Hopkins-Skellam index (HSI).

The public domain NIH Image analysis was performed on a Macintosh computer (OS 9.2.2) (version 1.61, developed at the U.S. National Institutes of Health and available on http://rsb.info.nih.gov/nih-image/). Note: Windows, Linux, Unix, OS-2, and Mac users can use ImageJ which is similar to NIH Image. ImageJ runs on Linux, Mac OS 9, Mac OS X, Windows, and the Sharp Zaurus PDA (http://rsbweb.nih.gov/ij/).

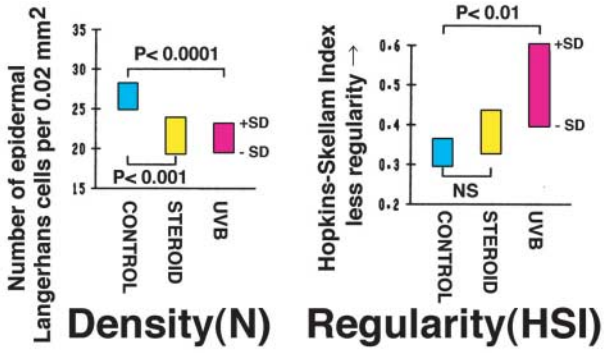
2.2 Quantification of spatial regularity, types, and the statistical test

Spatial point patterns are divided into three types (Fig. 4). A random pattern is formed by chance, and there is no interaction among the individual points. An aggregate pattern is formed by attractive interactions. When certain repulsive interactions occur among individuals, the individual points maintain a distance from each other and a regular pattern is formed.

To determine the type of distribution, we have applied the Hopkins-Skellam index (HSI) (Fig. 5) (Hopkins and Skellam, 1954). The HSI for an equilateral triangular lattice is $0.13888\cdots$, and the HSI for a square lattice is $0.1666\cdots$. T. Numahara wrote the Mathematica® (Wolfram Research,



(a)



(b)

Fig. 6. (a) Maps and Hopkins-Skellam index (HSI) of ATPase-stained epidermal Langerhans cells. N is the number of cells. Each group consisted of three guinea pigs (Hartley, 11 weeks-old, female). Each map is a square (one side of 200 μm). A revised figure, cited from Numahara *et al.* (1992). (b) The t-test for the results of (a). A revised figure, cited from Numahara *et al.* (1992).

Inc.) program to test for deviation in the observed value of HSI from unity. Analysis was performed on a Macintosh® computer (OSX). We show the program lists with Mathematica ® version 6.0 (Numahara *et al.*, 2007).

2.3 Applied example of the Hopkins-Skellam index

Numahara *et al.* (1992) introduced the Hopkins-Skellam index to study Langerhans cells of the guinea pig epidermis as a world first (Figs. 6(a) and (b)). Both topical steroid application and UVB exposure decreased the number of epidermal Langerhans cells to almost the same extent (Fig. 6(b)). The Hopkins-Skellam index revealed that the spatial regularity of Langerhans cells was significantly diminished in UVB-exposed skin, while the regularity remained in the topical steroid-applied skin (Fig. 6(b)).

The Langerhans cells in cultured epidermal specimens decrease with time. Numahara *et al.* (1994b) found that the Langerhans cell count decreases, but the regularity of the spatial arrangement was preserved by the incubation of an epidermal sheet for two days (Fig. 7). Even if epidermal Langerhans cells decrease, the residual cells may adjust to maintain the spatial regularity.

The mean of the Hopkins-Skellam index for the Langerhans cells in guinea pig epidermis was 0.334 with a standard deviation of 0.029 (Numahara *et al.*, 2001).

2.4 Voronoi division (or tessellation)

Numahara (1997, 2004) and Numahara *et al.* (2001) reported that patterns of the Voronoi division of epidermal Langerhans cells showed that each Voronoi polygon fitted the inside of epidermal Langerhans cells (Fig. 8). A Voronoi polygon is that part of a region that is nearer to that data point than any other. Voronoi polygons are frequently used in various fields, and are sometimes called the Dirichlet domain, Meijering cells, or S mosaics (Tanemura and Hasegawa, 1980).

T. Numaharawrote the Mathematica® program. Analysis was performed on a Macintosh® computer (OSX). We showed the program lists with Mathematica ® version 6.0 (Numahara *et al.*, 2007).

2.5 Geometrical models of territories

Tanemura and Hasegawa (1980) presented geometrical models for the establishment of territories of animals as follows:

(I) Adjustment model of territory: in the case of the synchronous settlement of territories, individual animals will

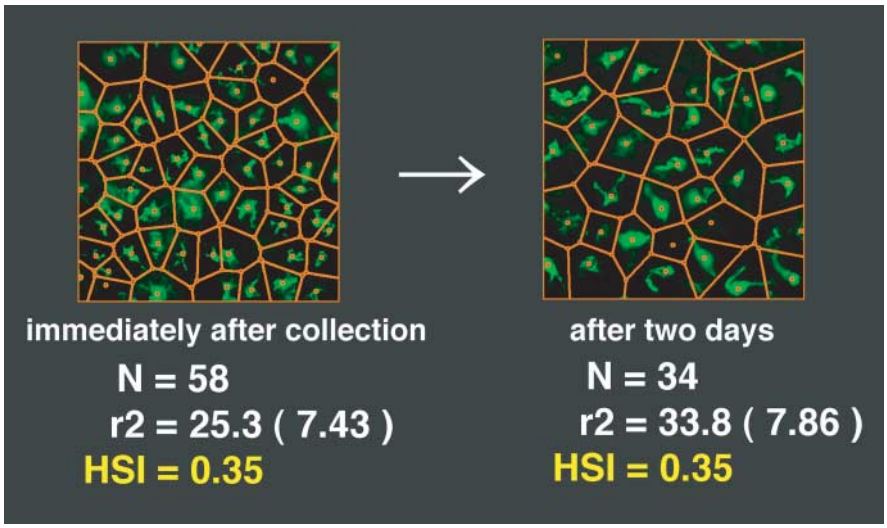


Fig. 7. Variation over time of Langerhans cells within an incubated epidermal sheet in MEM (stained with fluorescent-labeled monoclonal antibody for CD1a). N, r2 and HSI indicate the cell number, mean of nearest neighbors, and Hopkins-Skellam index, respectively. Each map is a 250-μm square. A revised figure, cited from Numahara *et al.* (1994b).

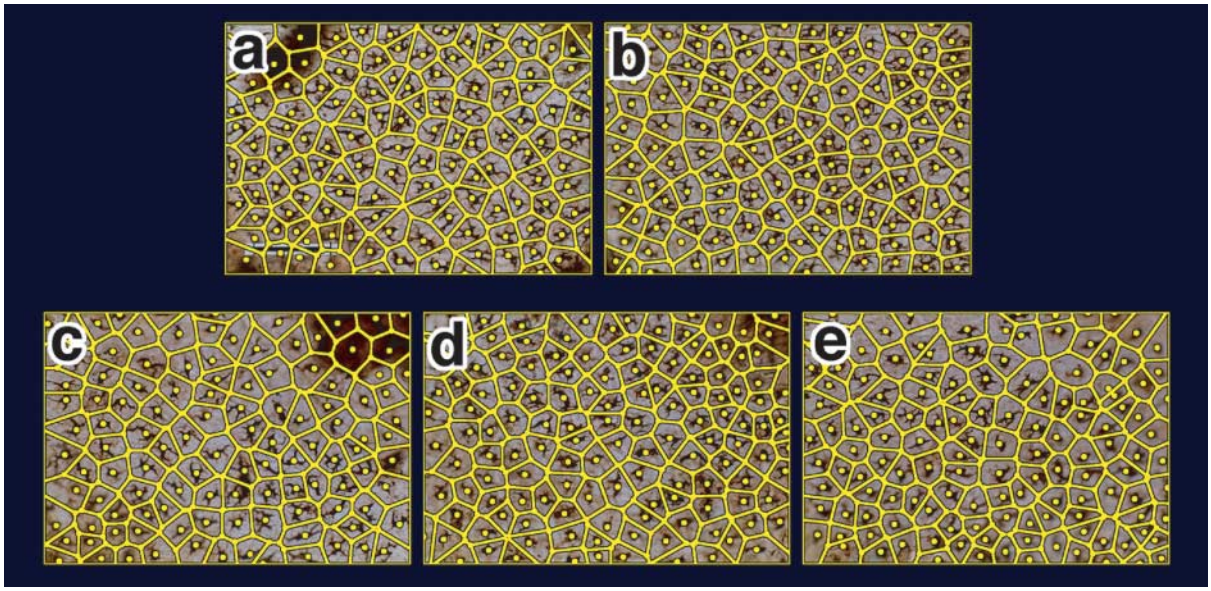


Fig. 8. Voronoi tessellations for the pattern of ATPase-stained epidermal Langerhans cells (guinea pigs). Each rectangle has an area of 400 $\times$ 250 μm. A revised figure, cited from Numahara *et al.* (2001).

adjust the position of their territorial centers until a stable set of boundaries is obtained.

(II) Random packing model of territory: a new individual arrives after the former occupants have established their territories; in asynchronous settlement, adjustment of the centers does not occur.

(III) Poisson model: a set of points distributed at random. Tanemura and Hasegawa (1980) also investigated distributions of the number of edges of the Voronoi polygons in the three models.

Numahara *et al.* (2001) counted and compared the number of edges of the 315 Voronoi polygons on the five epidermal Langerhans cell maps (Fig. 8) with the models. We chose the random packing model for the process of spatial distribution by statistical tests. Langerhans cells begin to appear in the epidermis by 7 weeks of gestation. Through pattern formation, a new Langerhans cell may arrive after the former Langerhans cells have established their coordinates. The natural course also reinforces the selected model.

2.6 Repulsive potential function

The main aim of point process statistics is to understand and describe the short-range interaction among the points (Illian *et al.*, 2008). Ogata and Tanemura (1984) discussed a class of repulsive potential functions, and provided an approximation method.

The model functions are:

(i) very-soft-core potential (V-S-C),

$$\Phi\sigma(r) = -\log[1 - \exp(r/\sigma)^2],$$

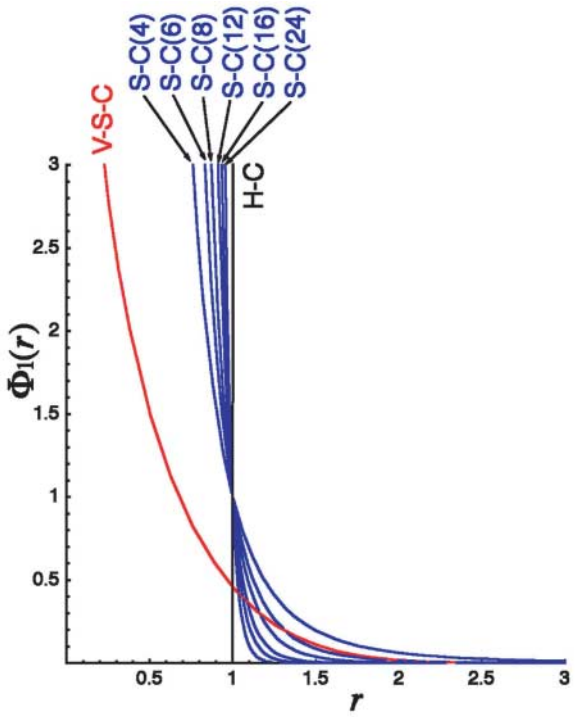


Fig. 9. The model repulsive potential functions. Ogata and Tanemura (1984).

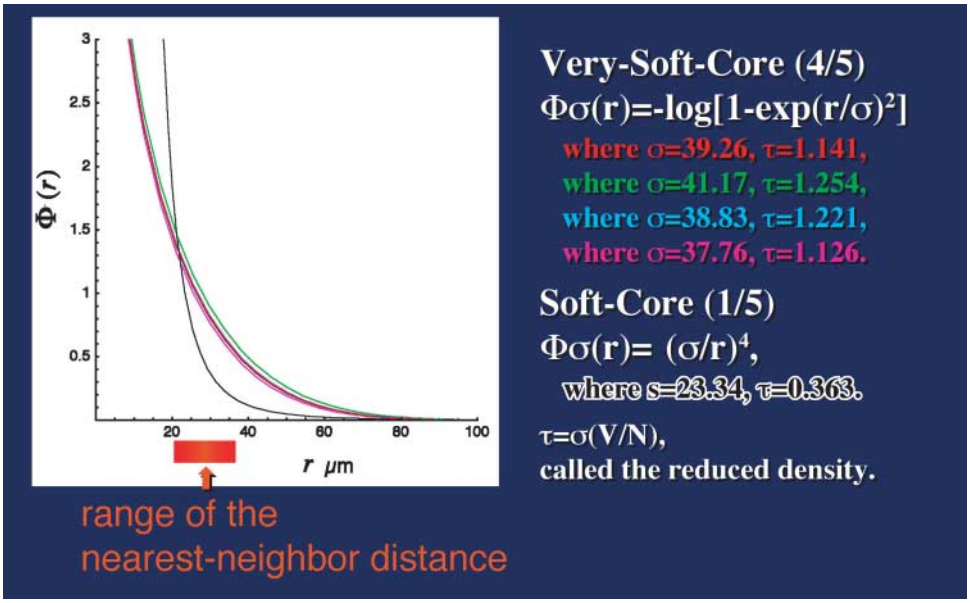


Fig. 10. Estimated repulsive potential functions of the Langerhans cells in the guinea pig epidermis. A revised figure, cited from Numahara *et al.* (2001).

(ii) soft-core potential (S-C(n)),

$$\Phi\sigma(r) = (\sigma/r)^n, n = 4, 6, 8, 12, 16, 24,$$

(iii) hard-core potential (H-C),

$$\Phi\sigma(r) = \infty \text{ for } r \leq \sigma, 0 \text{ for } r > \sigma.$$

The H-C model is analogous to packing iron balls into a box, for example; the V-S-C model applies to packing rubber balloons into a box; and the S-C model lies in between the H-C and V-S-C models (Fig. 9).

We have used a region with a periodic boundary in the estimation of potential function (Ogata and Tanemura, 1989). That is to say, the distance r_{ij} between points (x_i, y_i) and (x_j, y_j) on $V = [0, Tx] \times [0, Ty]$ is given by

$$r_{ij}^2 = \min\{|x_i - y_j|, Tx - |x_i - x_j|\}^2 + \min\{|y_i - y_j|, Ty - |y_i - y_j|\}^2.$$

This periodic boundary condition approximately realizes the state of an infinite particle system. During data analysis, a pair of points, i and j, crossing the predetermined periodic

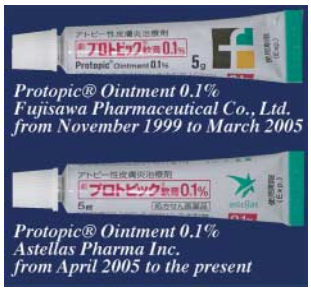


Fig. 11. 0.1% tacrolimus ointment (Protopic® Ointment 0.1%).

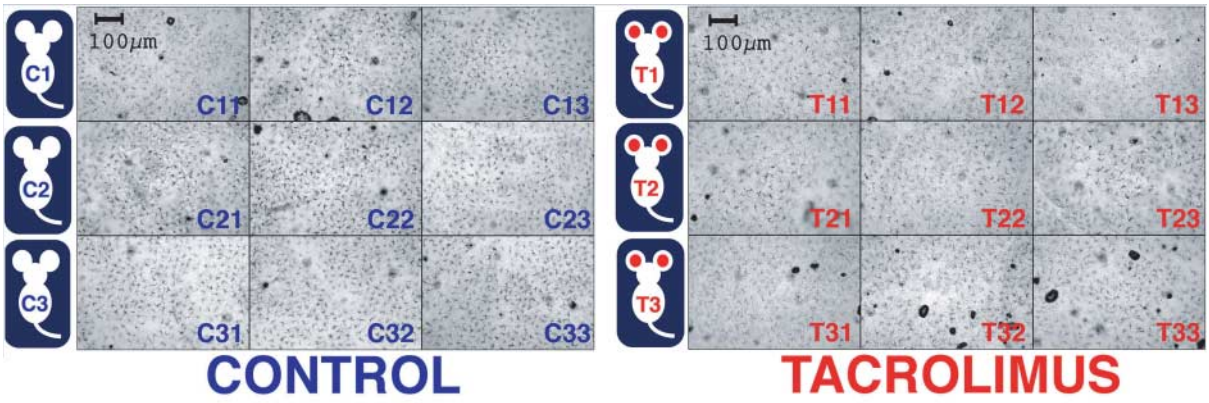


Fig. 12. The ADPase-stained murine (BALB/cAJcl) epidermal sheets. CONTROL: untreated. TACROLIMUS: every posterior auricle was treated with 0.1% tacrolimus ointment (Protopic® Ointment 0.1%) once a day for 3 weeks. The size of each rectangular window is 600 × 400 μm (3,000 × 2,000 pixels).

boundary could have a very short distance, r_{ij}, which affects the estimation of the potential for a given equilibrium point configuration. This can be avoided by moving the boundary slightly to change region V. The analysis was performed on a Sun Ultra Sparc JU1/170E (Compatible) computer using the Fortran 77 program; M. Tanemura conducted the programming for analysis.

We reported five estimated repulsive potential functions of Langerhans cells in the guinea pig epidermis (Numahara *et al.*, 2001). In four of them, V-S-C was the best fit model and S-C(4) was the second best fit model. In the remainder, S-C(4) was the best fit model. The mean of the nearest-neighbor distance between the Langerhans cells was less than 31 μm, and the estimated potential extended beyond 60 μm (Fig. 10). This result indicated that the interaction ranges overlap each other. The overlaps might be a safety system for immunosurveillance.

3. The Present Study) Effects of Protopic® Ointment 0.1% on the Spatial Distribution

3.1 Protopic® Ointment 0.1% (0.1% tacrolimus ointment)

Protopic® Ointment 0.1% (0.1% tacrolimus ointment) (Fig. 11) has been used for adult atopic dermatitis patients in Japan since November 1999. As of summer 2001, the effects on spatial patterns of LC had not been reported, and we confirmed this with the scientific advisory staff (Research division, Fujisawa Pharmaceutical Co.). So, we aimed to explore the effects on the spatial pattern. (On April 1st, 2005, Astellas Pharma Inc., Tokyo, Japan, was created through the merger of Yamanouchi Pharmaceutical Co., Ltd. and Fujisawa Pharmaceutical Co., Ltd.).

3.2 ADPase-stained murine (BALB/cA Jcl) epidermal sheets

ADPase-stained murine (BALB/cA Jcl) epidermal sheets were investigated (Fig. 12). Two mice groups, {T: TACROLIMUS} and {C: CONTROL}, were defined. Each of them consisted of three male mice (7 weeks old). Every posterior auricle of {T} was treated with Protopic® Ointment 0.1% once a day for 3 weeks, with a total dose of approximately 0.4 g each. {C} was the healthy control. They were individually caged in the experimental animal center of Kagawa Medical University. The experiments were carried out in autumn 2001. The animal ethics committee approved all experiments described in the present study.

3.3 Tissue processing

Our investigation required large flawless epidermal sheets, but mouse epidermis was extremely fragile and specimens would shred frequently during dermal-epidermal separation. We show the method we devised in Fig. 13.

3.4 Voronoi tessellation, density, and Hopkins-Skellam index

We show the Voronoi tessellation, density (N), and Hopkins-Skellam index (HSI) in Fig. 14. By the HSI, we quantify the regularity of the spatial pattern and can compare it closely. All the spatial point patterns were regular with level of significance 1.0×10^{-20} statistically. In other words, spatial regularity was extremely high.

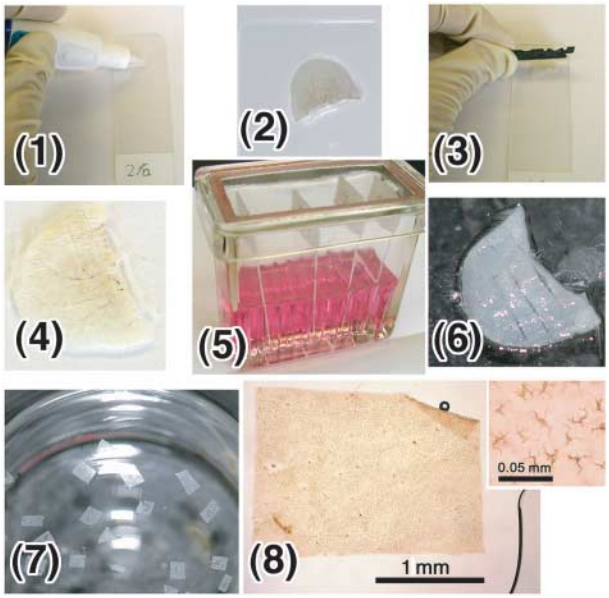


Fig. 13. Our procedure to obtain murine epidermal sheets. (1) Just after a mouse was sacrificed, we cut off both auricles with fine scissors. Immediately, we dropped Bond Aron Alpha High Speed EX® (Toagosei Co., Ltd., made of cyano acrylate) on a microslide glass (APS-coated SUPERFROST®, Matsunami Glass IND., LTD.). (2) Immediately, we glued the auricle and microslide glass, maintaining it as superficial and parallel with the posterior auricle (treated surface). (3), (4) The specimen was shaved gently with a razor blade (Feather-S blade®). Next, the specimen was gently cut in checkers pattern (each square had 2-to 3-mm sides). (5) The microslide glasses were immersed in 20 mM Na4-ethylendiamine tetraacetic acid in phosphate-buffered saline, pH 7.4, for 4 h at 37°C. (6) The epidermal sheets were separated from the dermis with fine forceps under a stereoscopic microscope. (7) Obtained epidermal sheets were kept flat by floating them on trismal buffer (20 mM trismal maleate, 0.2 M sucrose, pH 7.2) for 30 min. (8) One of the epidermal sheets (after ADPase histochemistry; a modification of the technique described by Chaker *et al.*, 1984).

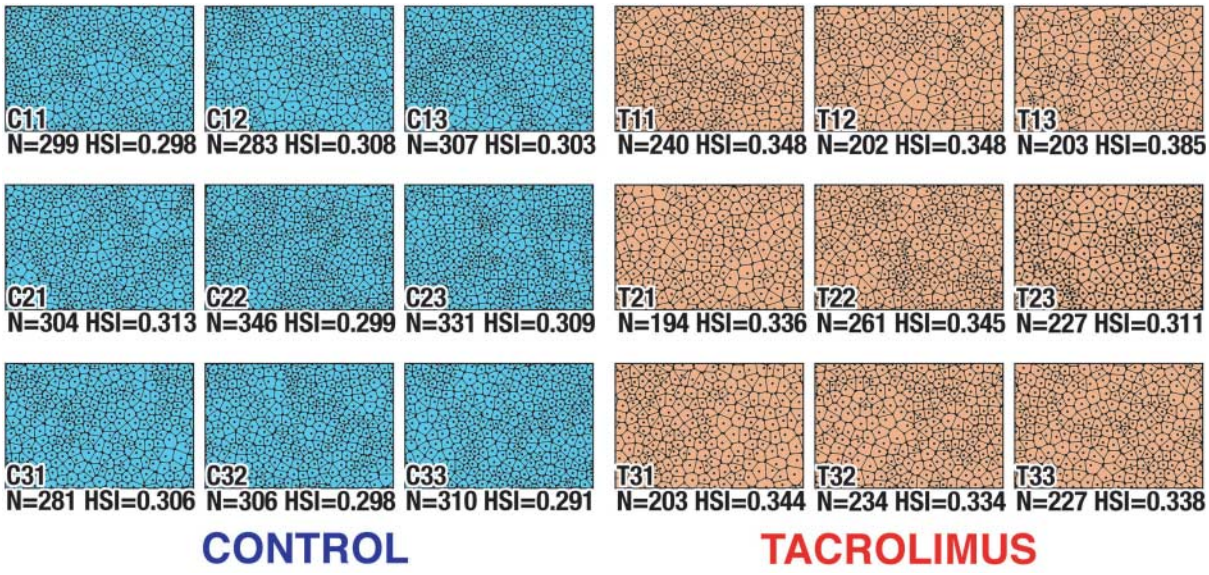


Fig. 14. The Voronoi tessellations for the murine epidermal Langerhans cells. CONTROL: untreated. TACROLIMUS: every posterior auricle was treated with 0.1% tacrolimus ointment (Protopic® Ointment 0.1%) once a day for 3 weeks. Each area is a rectangle (600 × 400 μm). N is the number of LCs. HSI is the Hopkins-Skellam index. (The regularity increases as the HSI decreases.)

3.5 Geometrical models of territories

The graph in Fig. 15 shows the three models and the observed data of epidermal Langerhans cells. The lines of CONTROL are near the random sequential packing model. The other models were dismissed with a level of significance of 0.05 by the Chi-square test.

3.6 Statistical tests of densities and spatial regularities (Fig. 16)

Three-week treatment course of Protopic® Ointment 0.1% application significantly reduced both the density and regularity ($p = 0.0012$ and $p = 0.0118$ by unpaired t-test; $p = 0.0495$ and $p = 0.0495$ by the Mann-Whitney U test, respectively).

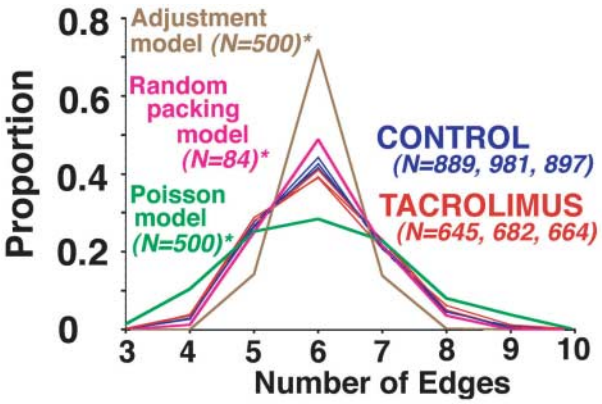


Fig. 15. Distributions of the number of edges of Voronoi polygons for epidermal Langerhans cells and three geometrical models*. CONTROL: untreated. TACROLIMUS: every posterior auricle was treated with 0.1% tacrolimus ointment (Protopic® Ointment 0.1%) once a day for 3 weeks.

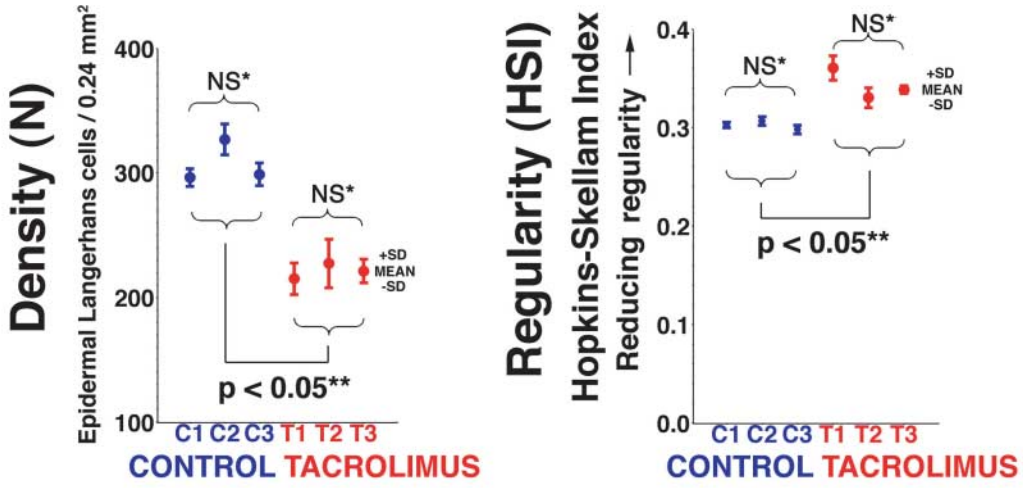


Fig. 16. Tests for the densities and spatial regularities of epidermal Langerhans cells. CONTROL: untreated. TACROLIMUS: every posterior auricle was treated with 0.1% tacrolimus ointment (Protopic® Ointment 0.1%) once a day for 3 weeks. NS*: by the Kruskal-Wallis test. $p < 0.05$**: by the Mann-Whitney U test.

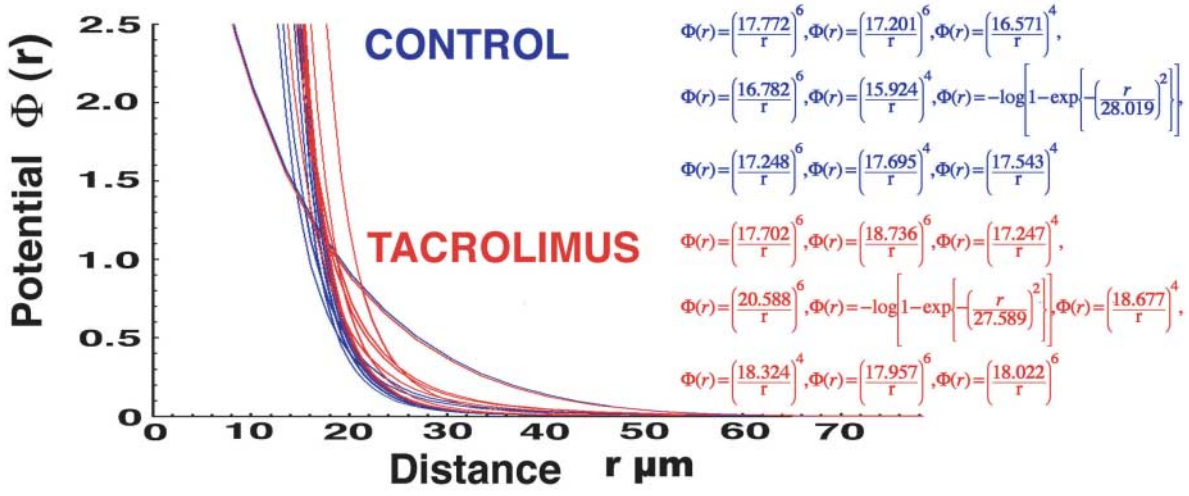


Fig. 17. The estimated potential functions of epidermal Langerhans cells. CONTROL: untreated. TACROLIMUS: every posterior auricle was treated with 0.1% tacrolimus ointment (Protopic® Ointment 0.1%) once a day for 3 weeks. The estimated model functions were very similar between-groups. However the chart shows that some diameters in the tacrolimus group became large.

CONTROL: {N = 307.4 (16.99), HSI = 0.303 (0.005)}
TACROLIMUS: {N = 221.2 (6.17), HSI = 0.344 (0.016)}.

3.7 The estimated potential model functions

The estimated potential model functions (Fig. 17) were similar in the CONTROL and TACROLIMUS groups. In both groups, the soft-core potential (S-C(n)) model,

$$\Phi\sigma(r) = (\sigma/r)^n, n = 4 \text{ or } 6,$$

was chosen except for in one case. The diameter (i.e., σ) of the TACROLIMUS group was slightly larger than in the CONTROL. With an exception, the very-soft-core potential (V-S-C) model was the best fit model and soft-core potential (S-C(4)) model was the second best fit model.

4. Conclusion

Epidermal Langerhans cells serve as sentinel cells whose primary function is to survey the epidermal environment and initiate immune responses against microbial threats (Romani *et al.*, 2006). After initial invasion, the antigen is engulfed by Langerhans cells (Fig. 2). The Langerhans cells should be arranged in the epidermis ideally to perform immunosurveillance efficiently. The distribution of Langerhans cells should reflect their surveillance function. We reviewed our research on spatial statistics for epidermal Langerhans cells in Sec. 2. Why have we applied spatial statistics? The following description of Ripley (1981) expresses our thoughts well. "The human eye and brain form a marvelous mechanism with which we analyze and recognize patterns, yet they are subjective, likely to tire, and so to err. The explosion in computing power available to the average researcher now makes it possible to do routinely the intricate computations needed to explore complex spatial patterns."

In our present study, a three-week treatment course of Protopic® Ointment 0.1% (0.1% tacrolimus ointment) application to mice (BALB/cA Jcl) significantly reduced ($p < 0.05$) both the density of epidermal Langerhans cells and the spatial regularities: {CONTROL: N = 307.4 (16.99), HSI = 0.303 (0.005)}, {TACROLIMUS: N = 221.2 (6.17), HSI = 0.344 (0.016)}, where N indicates the number of Langerhans cells, and HSI indicates the Hopkins-Skellam index. The estimated potential model functions were similar in the control and tacrolimus groups, even though the diameter, σ, of the tacrolimus group was slightly larger than the control. The results suggested that tacrolimus modified the spatial pattern of epidermal Langerhans cells.

It is still unknown how Protopic® Ointment 0.1% altered the spatial distributions. Tacrolimus has been shown to down-regulate the expression of FcϵRI on Langerhans cells (ASTELLAS PHARMA US, Inc., 2009). Tacrolimus also inhibits the transcription of genes that encode GM-CSF, TNF-α and some cytokines (ASTELLAS PHARMA US, Inc., 2009). GM-CSF is a colony-stimulating factor, and it activates granulocytes, macrophages, T cells, and Langerhans cells (Shimizu, 2007). TNF-α is a multifunctional cytokine, and one of its functions is modulation of the activation and migration of Langerhans cells (Shimizu, 2007). These factors may modulate the spatial distributions of Langerhans cells.

There were some weak points regarding our research. Firstly, we used untreated mice instead of vehicle-treated mice as the control group. Thus, it remains unknown whether vehicle itself had an impact on the spatial distribution of epidermal Langerhans cells. Secondly, a retrial for confirmation has not been performed to assess whether the results that we observed are universal or not.

Around 20 years ago, it was a standard procedure to observe changes in the form and density of stained epidermal Langerhans cells on epidermal sheets. We adopted the view that the spatial pattern of epidermal Langerhans cells itself should provide a clue to the skin's immune system over the last 20 years. Regrettably, we feel that studies using Langerhans cell suspensions such as flow cytometry have become the mainstream. Of course, the phenotyping of epidermal Langerhans cells using flow cytometry is a powerful and effective tool for analyzing the characteristics of the cells. There should be the information that is not obtained unless it comes out of the spatial pattern. Spatial data analysis has made marked progress with the recent development of computer technology. Illian *et al.* (2008) described the process as follows: "The patterns formed by the objects are analyzed in many scientific disciplines; hence a great variety of objects may be considered such as atoms, molecules, biological cells, animals, plants, trees, particles, pores, or stars and galaxies. Point process statistics is perhaps the most developed and beautiful branch of the modern field of spatial statistics. In addition to the classical fields of application such as archaeology, astronomy, particle physics and forestry, today their fields such as ecology, biology, medicine, and materials science extensively apply point process methods." Spatial statistics will provide a new viewpoint in epidermal Langerhans cell studies. Spatial statistics (or point process statistics) will be very helpful in the future for studying epidermal Langerhans cells (or dendritic cells).

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Note added in proof. In 2009, we improved the Mathematica®, program under the ISM Cooperative Research Program (2009-ISM-CRP-2006). The Computation time was shortened to a one-80th when we introduced NearestFunction (Mathematica®, version 7.0) into our Hopkins Skellam index calculation program. We show the improvement program list in the following article.

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