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Quantification of Birbeck's Granules in Human Langerhans' Cells

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A proper understanding of the function of Birbeck's granules requires that they should be counted under various conditions. We now present a simple quantification method which does not depend on specific assumptions concerning size, shape or location of the particles counted and can, moreover, be applied for counting other subcellular structures as well. A population of 69 serially sectioned human Langerhans' cells had an average of 290 granules and no cell contained fewer than 96 granules, indicating that as they degrade, granules are continuously formed. Suprabasal Langerhans' cells contained significantly more granules than did the basal cells. ***Key words: Human skin; Epidermis; Electron microscopy; Serial sections; Stereology.***

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The finding that the epidermal Langerhans' cells (LC) are immunocompetent (for review, see Ref. 1) but also have non-immunological defence functions (2) is of great importance for the understanding of this cell system, though it leaves several problems unsolved. The unique organelle of these cells, the Birbeck's granule (BG), has attracted much interest, as it has been correctly assumed that the clarification of its functional role would contribute much to the elucidation of LC function.

It is virtually impossible to count each individual BG as this involves mapping of all granules. Mapping is extremely difficult and time-consuming, especially when there is a dense population of BG in several sections of an LC. The situation is further complicated by the fact that, under certain conditions, the BG are highly electron-lucent and invisible, unless goniometry is applied (Fig. 1). BG are thin, rounded plates, composed only of sheets of cytomembrane enclosing material that appears to emanate from the glycocalix (3). Thus, a BG attains maximal contrast when the plane of the plate is parallel to the electron beam. As the angle to the beam increases, the BG becomes indistinct and can disappear, even for experienced electron microscopists (see Ref. 2 for references).

We now present a simplified method that makes the counting of BG a realistic undertaking. The method is non-parametric in the sense that no parametric model is assumed for the distribution of size and shape of the BG.

MATERIALS AND METHODS

Material for counting

Twenty-four punch biopsies (3 mm) of clinically normal forearm skin were obtained from 3 adult volunteers without local anesthesia or other pretreatment. The biopsies were taken in all seasons in the course of 2 years. Each sample was divided into three pieces and processed for electron microscopy as described elsewhere (for references, see Ref. 2). A series of 200 consecutive sections (each about 200 μm long and covering the thickness of the epidermis) was taken from each biopsy and distributed on

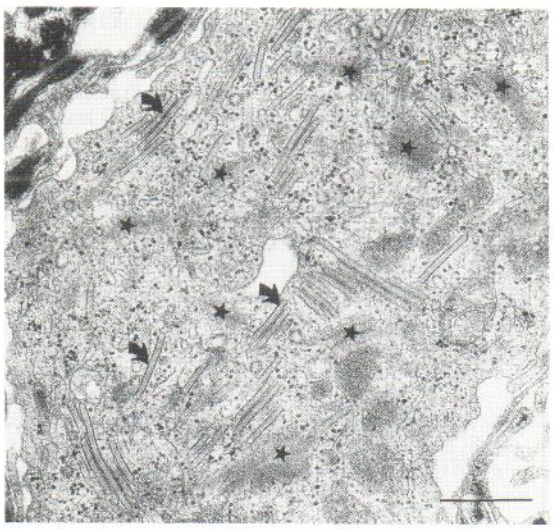

Fig. 1. Section through a Langerhans cell's BG-rich region. Some of the BG plates are near perpendicular or perpendicular to the section plane (*arrows*), others are more indistinct while oblique sectioned (*stars*). Bar = 0.5 μm.

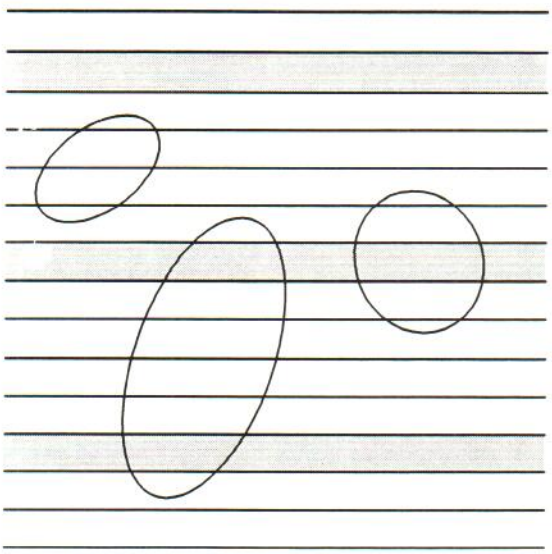

Fig. 2. Random distribution of BG in the section series.

10 grids. After calibration of the microscope, perpendicularly re-sectioned sections were used to determine the mean thickness of the sections, which was found to be 50 nm (S.D. = 5.1).

All non-keratinocytes which appeared within the section series were mapped and scrutinized until a definite identification was secured. The BG were counted at a magnification of 8300 × 10 in every fifth section.

Method for counting BG

The BG consist of two sandwiched flat and rounded cytomembrane parts with a round outlines (3) (also see Ref. 2 for further references) and it is highly improbable that they can appear more than once in a section. We shall make two basic assumptions:

1. Any BG will appear in a given section once at most.
2. One distribution of size and shape of BG applies to all LC.

Every fifth section is used in our counting routine. Due to the varying form, size and spatial orientation of the granules and their location within a series of consecutive sections, some of the BG will then appear in none of the sections, some appear in one, some in two, or even three of the sections examined, as shown in Fig. 2.

The total number of BG appearances thus observed in N_{obs}. Due to assumption 2, the ratio between the mean number of BG with the bottom (or top) apex in a random section and the mean number of BG appearing in a random section is a constant, R, irrespective of the BG density within the LC. Hence, the total number, N, of BG can be estimated as:

$$N=N_{obs}*5r$$

where r is an estimate of R. The estimate r is obtained using random disectors (4) of a random sample of LC. One

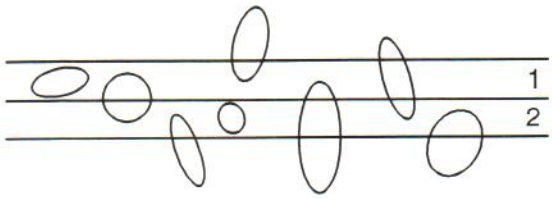

Fig. 3. Principle for the disector method, composed of two adjacent sections.
n_1 = number of BG observed in section 1
n_1' = number of BG observed in section 1 but not in section 2
n_2 = number of BG observed in section 2
n_2' = number of BG observed in section 2 but not in section 1
In this example $n_1 = 5$, $n_1' = 2$, $n_2 = 6$, $n_2' = 3$.

disector is defined here as a limited cytoplasmic area containing a maximum of 20 BG and the corresponding area in the next serial section (Fig. 3); the disectors were randomly sampled from each of 22 randomly chosen LC. Using the notation from Fig. 3, n_1'/n_1 and n_2'/n_2 are two estimates of R, which may be pooled into the estimate $(n_1'+n_2')/(n_1+n_2)$. Pooling these estimates from all LC sampled, the estimate

$$r=\Sigma(n_1'+n_2')/\Sigma(n_1+n_2)$$

is obtained, where Σ denotes the sum over all LC.

Table I. *Disector counting from a random sample of 22 LC*

The values of n_1 and n_2 are assessed without tilting, n_1' and n_2' with tilting in two perpendicular directions.

LC	n_1	n_1'	n_2	n_2'
1	5	2	5	3
2	13	1	13	0
3	16	1	15	4
4	17	3	13	0
5	14	2	15	1
6	7	0	8	1
7	4	0	5	2
8	14	2	16	3
9	8	3	9	2
10	11	2	10	2
11	12	1	15	1
12	3	2	8	3
13	4	0	6	3
14	10	3	7	2
15	6	0	6	1
16	12	5	16	5
17	6	1	5	0
18	5	0	4	0
19	4	2	5	3
20	6	1	7	1
21	3	2	6	0
22	3	1	3	0
Total	183	34	197	37

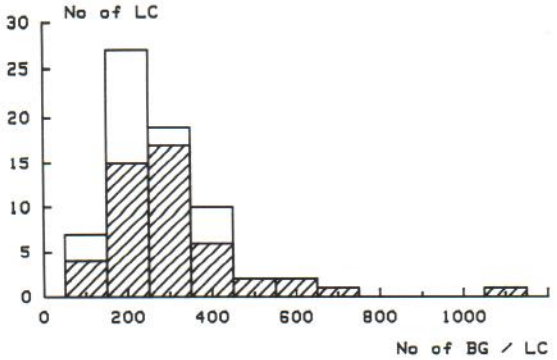

Fig. 4. Histogram illustrating the number of BG (N) per LC. The average number is 290. Min = 96, max = 1114, S.D. = 152. □, = basal LC; ▨, = suprabasal LC.

As explained in the Introduction, some of the BG are indistinct or invisible, depending on their position in relation to the electron beam. Hence, an 'individual error' is introduced if the cumbersome goniometry is avoided when BG are counted, since the experience of the microscopist determines how far such indistinct structures can be identified as BG.

In the counting routine, N_{obs} is determined without using goniometry. However, estimating r as described above, we have used goniometry for the assessments of n_1' and n_2' – but not for the assessment of n_1 and n_2. Hence a correction for the 'individual error' is built into our method. This is readily seen from the relation

$$r=\Sigma(n_1+n_2)_{g+}/\Sigma(n_1+n_2)_{g-} \times \Sigma(n_1'+n_2')_{g+}/\Sigma(n_1+n_2)_{g+}$$

where the first ratio is a correction factor for not using goniometry when counting N_{obs}, and the second factor is an application of the disector counting method. The subscripts $_{g+}$ and $_{g-}$ indicate counting with vis-à-vis without goniometry.

Disector counts from a random sample of 22 LC are reported in Table I. The values of n_1 and n_2 were assessed without tilting, whereas the values of n_1' and n_2' are assessed with tilting in two perpendicular directions. Using the data in Table I we have:

$$r=(34+37)/(183+197)=0.19$$

and the correction factor $5r=0.93$.

Out of 22 LC sampled, 7 belonged to the basal cells and 15 to the suprabasal. Using the basal cells only, $r=0.197$ or $5r=0.99$ were obtained. Using the suprabasal cells only, one obtains $r=0.182$ or $5r=0.91$. The discrepancy between these estimates does not indicate any violation of assumption 2 above, though the samples were rather small, so no conclusion should be drawn.

A confidence interval for $5r$ was computed according to Tukey's Jackknife method (5). This method yielded the same estimate of $5r$ as above, 0.93, with a standard error of 0.14. An approximate 95% confidence interval for $5r$ is (0.64; 1.22), and an approximate 80% confidence interval is (0.75; 1.11).

RESULTS

General electron microscopic observation

A total of 71 LC had their entire cell body localized within the section series. Twenty-one cells (called basal LC below) had either a considerable part of the cell body (not necessarily containing the nucleus), or the whole cell body in the basal layer. Nine of these cell bodies, of which 6 were found in one individual, were entirely confined to this layer and apposing the basal lamina. Of the cells within the spinous layer, 7 had a pronounced high-levelled position with part of the cell bodies reaching the innermost granular cells.

Two suprabasal LC were deemed abnormal (widened perinuclear space and rough endoplasmic reticulum, remarkably long, undulating or curved, and sometimes even branched BG) and were excluded from the results below.

Most of the BG are invariably found in the portion of the cytoplasm harbouring Golgi apparatus and the centrosomes. The intracellular distribution of granules implies that these may be absent in many (sometimes up to 30–40) consecutive sections, whereas one single section through the organelle-rich cytoplasm could contain over 100 BG. A total of about 20,000 BG were examined in this material.

Lastly, 5 exocytic cells and no 'indeterminate cells' were found.

Application of the counting method

As described earlier, the distribution of BG was investigated by counting all granules in every fifth section without tilting. Sixty-nine LC (21 basal LC and 48 suprabasal cells) lying entirely within the series sections were examined.

The average number of BG counted (N_{obs}) for

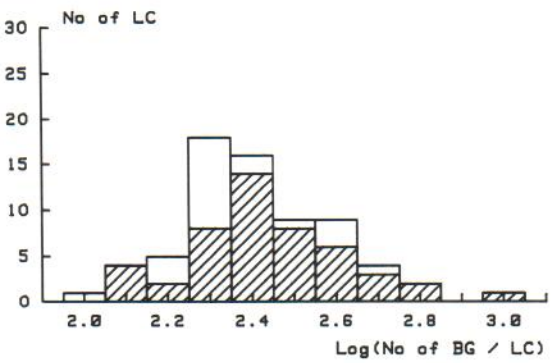

Fig. 5. Same as Fig. 4, but in the logarithm scale.

each of the 69 LC was 311 (S.D. = 163), for the 21 basal LC 259 (min = 103, max = 483, S.D. = 101) and for the 48 suprabasal cells, 334 (min = 132, max = 1198, S.D. = 180). The 7 extremely high-levelled LC among the suprabasal cells contained an average of 327 BG (min = 132, max = 620, S.D. = 204).

The distribution of the estimated number of BG (N) per LC is illustrated by a histogram in Fig. 4. The histogram in Fig. 5 is based on the logarithm of the number of BG per LC in the same material. Obviously, the log transformation yields a more normal distribution. The log transformation also stabilized the variance. The variance ratio between the suprabasal material and the basal LC material was reduced from 3.2 with the linear scale to 1.3 with the log scale. We therefore recommend that comparisons of BG counts in different materials be made using a t-test or analysis of variance based on log counts. There is, of course, no need to compute N for such comparisons, as it differs from N_{obs} by a constant factor only, or by a constant term on the log scale.

The number of BG per LC for each individual was compared by means of a one-way analysis of variance of log(BG-count). There were no statistically significant differences between individuals at the 5% level for either basal LC or suprabasal cells. However, a t-test of the difference in log(BG-count) between the 21 basal LC and the 48 suprabasal cells showed significance at the 5% level (two-tailed).

DISCUSSION

The presence of BG in the LC is the result of a unique and functionally interesting cells membrane activity, 'cytomembrane-sandwiching', which involves the superimposing of discrete cytomembrane parts (3). Although the turnover time of BG is still unknown, it is reasonable to assume that the number of granules in an LC reflects the intensity of this particular cytomembrane activity. A counting method for BG is an obvious requirement for the further elucidation of BG function.

The quantification methods which theoretically could be used for counting BG involve difficult and time-consuming procedures. For example, the use of the disector method (4) would make it necessary to map all the BG in hundreds of section pairs, which includes the use of goniometry. Such mapping of BG is impossible in practice.

The method presented here is in contrast both convenient and appropriate for counting BG. No extensive mapping of BG is required. In addition, our method is based on few geometric assumptions. First, any BG is assumed to appear in a given section once at most, which is certainly true if the BG are convex, as is the case if they are elliptic plates.

Second, there is assumed to be one distribution of size and shape common for all LC. If this assumption is false, separate correction factors should be derived for different samples of LC, e.g. for basal LC and for suprabasal LC, and, possibly, for each individual separately. The validity of this assumption is difficult to verify. An attractive feature of our method is that it is non-parametric in the sense that no parametric model for the distribution of size and shape of the BG is assumed. As a consequence, the calculations are rather simple, and the results should be more reliable than when a parametric model is assumed.

The quantification method presented here can also be used for other subcellular structures, provided that their shape prevents them from appearing more than once in one and the same section.

The final equation, $N=N_{obs}*5r$ implies that the observed BG count should be corrected by a constant factor, depending on the observer and on the thickness of the sections. Correcting the BG count is therefore only a matter of changing the scale.

A large number of LC were observed in this study and all were found to carry BG, the minimum number in a LC being 96. This demonstrates that the formation of BG is continuous and keeps pace with degradation. It should be emphasized in this context that all non-keratinocytes in all section series were identified.

The statistically significant difference in number of BG in basal LC and in suprabasal LC is intriguing. The nature of the stimulus that induces formation of BG is unknown and it cannot yet be stated with certainty whether it acts on the internal or external side of the cytomembrane. Exposure to digitonin in vitro (3) or to other drugs either in vitro or in vivo (6) rapidly provokes cytomembrane-sandwiching in the LC. Such findings indicate that granule formation can at least be triggered by external factors. It is far from clear how an extracellular factor in the epidermis could exert a differential effect on high- and low-levelled LC, since all LC seem to be exposed to the same environment within the epidermis. Uniformity of this environment would seem probable as, first, the intercellular space is large and

allows for effective transport of substances (2) in the intercellular fluid and, second, the dendritic tree of the LC often forms an extensive network and thus makes effective contact with the various levels of the living epidermis. Also, the cells are capable of moving around in the interstices and can, under certain conditions, rapidly rearrange their positions (7). It is possible, although highly hypothetical, that the LC pass through active phases which would be reflected by an increased production of BG when the LC travel to higher epidermal levels, followed by temporary and relative inactivation when they return towards the basal part of the epidermis. Even though the observation of a differential number of BG in basal vs. suprabasal LC cannot yet be satisfactorily explained. This study confirms in a concrete way an earlier supposition that suprabasal LC are richer in BG than are basal LC (8, 9, 10).

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