Morphology of Pinocytotic Vesicles in the Capillary Endothelium of Rabbit Lungs using Automated Equipment

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SUMMARY  Pinocytotic vesicles are one of several anatomic factors relevant to the permeability for macromolecules of capillary endothelia. We quantified the number and distribution of pinocytotic vesicles present in the endothelium of pulmonary capillaries. Several similar studies have been reported in the past for endothelia of systemic capillaries, but the technical difficulties involved in this difficult application of morphometry never have been discussed. In the present work, an on-line microprocessor was used to assist with the processing of the point-counting data. The average figures from nine rabbit lungs for parameters thought to be relevant to characterize the population of vesicles are: thickness of the endothelium (TAUe), 0.192 µm; number of vesicles per volume unit of cell (N/Sv), 131 vesicles/µm²; vesicular load of each cell front (N/S), 186 vesicles/µm², and (N/Sv), 181 vesicles/µm². The number of vesicles per volume unit (N/Sv) is smaller than that which has been described for systemic capillaries.

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ONE OF the mechanisms for transport of macromolecules across endothelial cells is the pinocytotic (also called plasmalemmal or cytopemptic) vesicle. Pinocytotic vesicles first were described in detail by Bruns and Palade (1968a). They have an average diameter of 70 nm and functionally represent a nonhydraulic pathway thought to be driven by Brownian forces (Shea and Karnovsky, 1966; Casley-Smith, 1969; Shea et al., 1969; Green and Casley-Smith, 1972; Weinbaum and Caro, 1976; Rubin, 1977). This mechanism coexists with a system of pores thought to be isolated at the endothelial junctions; filtration through the junctions is driven by forces represented in the Starling equation (see review by Renkin, 1977). Simionescu et al. (1975), by using relatively small tracers, demonstrated the occurrence of chains of vesicles and of true transendothelial channels in the endothelium of small vessels of the rat diaphragm, particularly in the venular segment of capillaries (Simionescu et al., 1978). These could be regarded as pores. Such channels never have been shown in the lung. Tracer studies using electron microscopy have shown that pinocytotic vesicles can take up tracers and transport and release them across endothelial cells (Bruns and Palade, 1968a, 1968b; Karnovsky, 1967; Simionescu et al., 1975 and 1978, Wissig and Williams, 1978; Casley-Smith and Chin, 1971). This matter has been reviewed in detail by Palade et al. (1979).

A similar vesicular transport across squamous epithelial alveolar cells also has been demonstrated (Schneeberger and Karnovsky, 1968, 1971; Pietra et al., 1968; Gil, 1978). Despite this morphological evidence, the precise role of vesicular transport in the lung has remained controversial. Staub (1979) has stressed that the steady state lymph-to-plasma protein ratio and lymph flow in the sheep lung can be accounted for within the framework of the Starling forces with a system of pores of different diameters (Blake and Staub, 1976) without any need to invoke vesicular transport.

In further studies to clarify the significance of vesicular transport in relation to that of other pathways, several authors have attempted to quantify different segments of the systemic capillary network. Bruns and Palade (1968) and Simionescu et al. (1978) correlated counts of vesicles with an index of labeling and location in attempts to define such functionally relevant parameters as mean transcellular transit time, probability that collision of a vesicle with a membrane results in fusion, and even cytoplasmic viscosity (Shea et al., 1969; Green and Casley-Smith, 1972). It has been claimed that in the lung, the number of vesicles increases during development of both hydrostatic (DeFouw and Berendsen, 1978) and oncotic edema (DeFouw and Berendsen, 1979). This is surprising because it would establish an apparent link between vesicular transport and hydraulic factors. Clearly, it is necessary to have reliable baseline data on the number of vesicles and to have a reliable method for counting them. Unfortunately, the published studies do not deal adequately with the technical difficulties inherent in morphometry of vesicles. Some studies

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work with three-dimensional reconstructions or sections through the surface, methods which tend to reduce the size of the sample; others prefer to compute the volume of the vesicles and others do not discuss sample size. Some workers have not made a distinction between profiles (traces of vesicles in the two-dimensional sections) and vesicles in the original three-dimensional space; finally, others have computed the volume density but not the number of vesicles.

Morphometry of pinocytotic vesicles involves a difficult application of stereological techniques for different reasons: (1) difficulties with random sampling because the small dimensions of the vesicles in general command high magnification micrographs and thus reduction of the amount of tissue sampled; (2) inhomogeneous distribution, which adds to the sampling problem; (3) distribution along two different surfaces, and (4) the need to use a small tissue constituent such as the endothelial cell as a restricted reference system.

The following is a systematic study of pinocytotic vesicles in the endothelial cells of pulmonary alveolar capillaries. Segmental differences have been reported in certain systemic networks (Simionescu et al., 1978; Palade et al., 1979), but this matter never has been studied systematically in the pulmonary circulation. In the absence of reliable data, the prevailing opinion is that the vesicular population of all pulmonary capillary endothelia is homogeneous. Our own observations are compatible with this assumption.

Methods

Morphometry

Morphometry was performed following the general principles outlined by Weibel (1972). Random photographs of thin sections were overlaid with a coherent grid consisting of points, lines, and test area (see Fig. 1). We counted test points inside profiles, intersections of test lines with boundary lines of profiles, or the number of profiles inside a test area.

Instrumentation: Application and Analysis Programs

Because of pattern recognition problems inherent in the automatic image analyzers, the collection of point counts generally must be done manually. Subsequently, a fully automatic processing of data is desirable. Large computers are not suitable for this purpose because morphometric analysis is characterized by the steady generation of a comparatively low number of point counting data during a long period of time. The on-line instrumentation and analysis programs described here are based on the Tektronix 4051 computer system equipped with 16,000 words of memory, a 300K byte magnetic tape transport, a storage display and keyboard.

The application programs for electron microscopic morphometry utilize these features and peripherals of the 4051. An analysis program provides the identification of the experimental data. The current file can be interrupted, stored on the magnetic tape transport, then later reinitialized to augment the set of counts. A set of 15 keys is available; each is defined independently and can represent any definition of structures. The number of keys for stroke counts could be increased if required. The sampling grid used is replicated on the video display screen to provide visual feedback of the current location of the point-counting position (Fig. 2). Current values of the mean and standard deviation of the point-counting frames are available for verification of the results. The point count data input provides error checking of the key struck, blank fills for nonvalid information in the field, and backspacing to delete and reenter information. Figure 2 is a hard-print copy of the video display at the end of the analysis of the field shown in Figure 1. The profiles of the epithelial (0) and endothelial (2) cells have been drawn on the grid display for clarity.

The point-counting information per frame is stored on the magnetic tape and analyzed by a separate set of statistical programs. These programs verify the presence of all data necessary to calculate the morphometric variables. A particular frame may be deleted from the data set or additional frames concatenated to it. Unvariable statistical information is displayed as tabular lists of graphical plots. The derived variables are analyzed by linear regression analysis directly from the magnetic tape storage.

Procedure

At the onset of the procedure, it was necessary to identify the object, assign a storage file location, select one test grid among several available, enter the scale factor, and define how many random fields would be required to make up a "frame." "Frame" here means simply the number of fields pooled together to form a random sample on which the stereological formulas were applied. In our experiments, a frame invariably was comprised of three fields. Finally, we accounted for every single point of the grid. Since we were interested in only a restricted part of the tissue, profiles of endothelial cells, points lying on not-needed structures were eliminated by pressing the blank key ("b"). After all the volumetric point positions of the displayed grid were accounted for, we counted the intersections or vesicular profiles. Current mean values for the volumetric counts and their standard deviations were permanently displayed on the screen. Current counts recorded by the other keys were also visible. This was needed to indicate if the means and the standard deviation were improving with the addition of more photographs or not. Raw data counts were stored on magnetic tape. The analysis program applied the formulas described below and computed
FIGURE 1  Electron micrograph of lung parenchyma of rabbit with superimposed multiple purpose M168 grid. This is representative of the image seen on the screen of the projector used. Note the variation of thickness of the endothelial cell. BP, blood plasma; A, air space; Ep, a squamous alveolar epithelial cell; En, a capillary endothelial cell; d, test line.
parameters locally in each frame. Subsequently, frame results were averaged. Wherever appropriate, the analysis program provided use of additional formulas for computing secondary relationships from the morphometric results. At no time did the analysis pool raw counts from the whole organ.

Selection of Relevant Parameters

\((N_{v_m}^c)\), numerical density or number of vesicles in each \(\text{cm}^2\) of endothelial cell; \((N/S)_v\) and \((N/S)_i\), vesicular load or number of vesicles in each \(\text{cm}^2\) of surface which must be determined separately for two surfaces, the vascular or luminal (b) and the interstitial or abluminal (i) surfaces.

To arrive at these results other intermediate parameters must be computed. Some of these must be shown to give the reader a suitable description of the tissue sampled. These parameters are: \((V_{v_m}^c)\), volume density of endothelial cells in the tissue; \(S_{v_m}\) and \(S_{i_m}\), surface densities of the endothelial cell on the vascular (b) and interstitial (i) surfaces; \(\text{TAU}_m\), arithmetic mean thickness of the endothelial cells.

Experimental Methods

New Zealand White rabbits (2.5 kg) were anesthetized with pentobarbital (50 mg/kg) iv. After cannulation of the trachea, bilateral pneumothorax was produced by puncturing the abdominal side of the diaphragm and blunt extension of the puncture to open the pleural cavities. Without opening the thoracic cage, the lungs with 3% glutaraldehyde in phosphate buffer adjusted to 330 mOsm at a constant pressure of 20 cm H\(_2\)O. After the flow of fixative stopped, the trachea was ligated, the thorax was opened, and the lungs were removed and placed in fixative for at least 2 additional hours.

Then the lung was diced and postfixed with osmium in collidine buffer following standard procedures to ensure that blocks from at least six different areas were embedded and accordingly labeled (stratified sampling).

At least five blocks from each rabbit were sectioned for electron microscopy. Ultrathin sections were of silver-gold interference color corresponding to a thickness of 85 nm. Every effort was made to prepare sections of identical thickness. Seventy or more micrographs were taken at the same magnification. Additionally, a carbon-grating replica (21,600 lines/cm) was photographed for calibration purposes. The final magnification at the time of the point counting was around 15,100x. Nonbiased micrographs were taken by selecting tissue from technically optimal parts of the section at a low power and then turning to the appropriate magnification to take a photograph.

The negative film strip was contact-printed in a light box, and the positive 35-mm film was projected on the screen of a desktop projector fitted with a multipurpose test system M168 (Weibel, 1979). Figure 1 is representative of how a field was seen on the screen. Figure 2 shows the situation at the end of the counts of this particular field. The following counts were needed: (1) volumetry points: Pen, count of end points inside profiles of endothelial cells; Pt, count of end points over all other tissue components excluding air and blood; (2) intersections: Ienb, count of intersections of test lines with...
the boundaries of the luminal or vascular surface of endothelial cells; Ieni, count of intersections with the abluminal or interstitial surface of endothelial cells; (3) vesicle counts: Nenb, profiles of pinocytotic vesicles abutting on vascular surface of endothelium; Neni, profiles on interstitial surfaces of endothelial cells. All the profiles of vesicles recognized were assigned to one of these two groups. It was assumed that all vesicles were related to one of the two surfaces. When no material attachment could be seen, they were assigned to the nearest surface. The validity of this assumption will be discussed later. The smallest profiles that could be identified positively had a diameter of roughly one-third of the equatorial vesicular diameter of 70 nm.

Formulas

The equation for the volume density of the endothelial cells is $V_{ven} = \frac{Pen}{Pt + Pen}$. The asterisk denotes that the reference system of this parameter is a restricted volume, in this case, only tissue without air or blood compartments rather than the whole organ. $V_{ven}$ is the percentage of tissue volume taken up by the capillary endothelium; $V_{ven}$ would have been the percentage of total volume taken up by the endothelium in the whole lung including air spaces.

The equations for the surface densities of both surfaces, vascular and interstitial in the analyzed micrographs are:

\[ S_{vb} = 2 \frac{Ienb}{L_T}; \]
\[ S_{vi} = 2 \frac{Ieni}{L_T}; \]

where $L_T$ is the calibrated total length of the test lines.

According to Weibel and Knight (1964), the equation for the mean arithmetic thickness of the endothelial cell is:

\[ TAU_e = \frac{d \cdot Pen}{2 \cdot (Ienb + Ieni)} \]

where $d$ is the length of one of the short test lines (see Figures 1 and 2).

The equation for the numerical density of vesicles inside endothelial cells is:

\[ N_{ven} = \frac{N_s^*}{D + T - 2h} \]

where $N_s^*$ (areal density of profiles) is the number of profiles counted per unit of area of the test grid. In general, $N_A$ is:

\[ N_A = \frac{Nenb + Neni}{A_T} \]

where $A_T$ is the area of the test system; in the case of the multipurpose grid M168, with $P_T$ being the total number of grid points:

\[ A_T = P_T \cdot \frac{\sqrt{3}}{2} \cdot d^2. \]

$A_T$ must be multiplied by $Pen/P_T$ to account for the fact that we are counting vesicles only on the parts of the grid over endothelial cells. Thus:

\[ A = \frac{\sqrt{3}}{2} \cdot d^2 \cdot Pen. \]

$D$ is the true mean diameter of the vesicles and not of the profiles ($D$). The assumption is made that the vesicles can be regarded as spheres. We used the value commonly reported in the literature:

\[ D = 70 \text{ nm} = 7 \times 10^{-6} \text{ cm}. \]

$T$ is the thickness of the plastic sections analyzed which was found to be

\[ T = 85 \text{ nm} = 8.5 \times 10^{-6} \text{ cm}. \]

$h$ is the depth of penetration into the sections of the smallest vesicles that can still be identified. We could identify profiles of a diameter down to one-third of the equatorial diameter. If $R$ is the equatorial radius of the vesicles and $r$ is the radius of the smallest recognizable vesicle, the Pythagorean theorem can be used to compute the fraction of $R$ that does not penetrate into the section and then

\[ h = R - \sqrt{R^2 - r^2}. \]

$T$ and $h$ are introduced to correct for section thickness effects which in this case are substantial because the section is thicker than the diameter of the vesicles. The equation for the final diameter corrected for section thickness effects is

\[ \bar{D} + T - 2h = 1.5 \times 10^{-5} \text{ cm} \]

which is to be entered in the final formula for the desired $N_{ven}$:

\[ N_{ven} = \frac{Nenb + Neni}{(\bar{D} + T - 2h) \cdot (\sqrt{3}/2) \cdot Pen \cdot d^2} \]

The computation of vesicular loads for both sides is performed in the following two steps: (1) the numerical density is distributed between the two sides according to the location of vesicular profiles:

\[ (N_{ven})_b = \frac{Nenb}{Nenb + Neni} \]

and

\[ (N_{ven})_i = \frac{Neni}{Nenb + Neni} \]

and (2) these values are divided by their respective surface densities, $S_{vb}$ and $S_{vi}$, previously computed.
Results

The results are summarized in Table 1. The summary line shows an average of the sample values. To interpret these data, understanding of the sampling technique is needed. The use of photographs taken in a strictly random manner, such as would have been needed for the characterization of the volume density of endothelium in the whole lung (V_num) or the total surface density of the endothelial cell fronts (S_num), was not practical; at the high magnification required, the number of empty photographs (air spaces) or of photographs with very small amounts of tissue would have been excessive. Therefore, we selected at low power optically fixed areas of tissue that contained alveolar capillaries, centered them, and then increased the power to the previously chosen magnification level at which the vesicles became well visible. Although this is not a real random sampling technique, we believe it is unbiased with regard to the vesicular population of the endothelium. However, parameters such as the volume density of endothelium and the surface density are not representative of the whole lung; they simply describe the sample studied and were used for local calculations. The most important information is the numerical densities of vesicles inside the containing endothelial cell. Translating our results into μm units (1 cm = 10^7 μm), the endothelium examined had a thickness of 0.19 μm and contained 131 vesicles/μm²; the vascular surface had approximately 196 vesicles/μm²; the interstitial surface had only 181 vesicles/μm². Side by side and assuming spherical size, all the vesicles related to the luminal front would occupy a surface of 0.75 μm²/μm² of cell surface and those of the interstitial front 0.69 μm². In general, the vesicular load is similar to that reported by Bruns and Palade (1968) for heart muscle capillaries but the relative significance of both fronts is reversed (106/μm² for vascular front; 117/μm² for tissue front); however, the numerical densities reported by these authors were much higher (≈883/μm³ in 0.1- to 0.2-μm-thick endothelium). Similarly, Casley-Smith (1969) found in endothelium of a thickness of 0.25 μm a load of 125 vesicles/μm² but a numerical density of 1900/μm³. Smith et al. (1978) found for an endothelial thickness of 0.25 μm numerical densities of 912 and 956/μm³ and for 0.17-μm-thick vascular capillaries 1171/μm³, but they did not report vesicular loads per cell front.

The standard deviations of the mean of our data per animal are relatively high. This poses some statistical problems. All the animals except the last show a higher vesicular load on the vascular side. This difference is evidently not significant. By the same token, small increases in the amount of vesicles or a shift of their location could not reliably be detected unless they were very large. Yet comparison of the individual averages shows that they are similar to each other. Accordingly, the standard deviation of the summary data is small. This encouraged us to pool all the frames of all the animals into a sample pool of large size (105 frames, each consisting of 3 fields) and attempt a correlation analysis in the hope that the slope of the regression line would provide a reliable basis for comparison with experimental groups. Regression plots by the least square method can be performed automatically by the microprocessor used for the analysis. The best correlation coefficient (Figure 3) was found between the numerical density and the endothelial thickness (r = 0.539). Other authors interested in this problem had already intuitively correlated the amount of vesicles with the endothelial thickness, which appears to be the item most relevant to vesicular transport function. Attempts to improve r by single or double log plotting failed because the correlation coefficient remained unaffected. For Figure 3, the slope of the linear plot (b) is = -0.041 with a se(b) of 0.16 and 95% confidence limits of ±0.314. In a log-log plot of the same correlation, the relevant factors were: b, 6.411; se(b), 0.851; and 95% confidence limits, ±1.699. In a log Y/log X plot, b is 0.232, se(b) is ±0.031, and the 95% confidence limits are ±0.06. The correlation coefficient r in the three cases was virtually unchanged. We concluded that these alternative anal-

Table 1. Morphometry of Vesicles in Capillary Endothelium of Nine Rabbit Lungs

| Animal | V_num (cm²) | S_num (cm²) | N_num (cm²) | N/Sa (cm²) | N/Sb (cm²) | Tau (cm²) |
|--------|-------------|-------------|-------------|------------|------------|-----------|
| 1      | 0.388 (0.111) | 735 (1776) | 1.138 (0.064) | 1.730 | 1.332 | 1.740 (0.997) |
| 2      | 0.346 (0.067) | 8040 (1801) | 1.833 (0.839) | 1.749 | 1.629 | 1.835 (1.004) |
| 3      | 0.283 (0.100) | 6530 (1200) | 1.423 (0.589) | 2.269 | 2.185 | 1.826 (1.004) |
| 4      | 0.330 (0.178) | 5846 (2000) | 1.458 (0.489) | 2.959 | 2.214 | 1.962 (1.004) |
| 5      | 0.369 (0.076) | 7059 (1845) | 1.563 (0.756) | 2.342 | 2.025 | 2.210 (1.144) |
| 6      | 0.287 (0.154) | 7305 (2334) | 0.948 (0.361) | 1.599 | 1.599 | 2.258 (0.893) |
| 7      | 0.283 (0.067) | 8413 (1961) | 1.482 (0.268) | 1.872 | 1.638 | 1.874 (0.642) |
| 8      | 0.387 (0.086) | 6948 (1690) | 1.225 (0.547) | 2.131 | 1.431 | 2.218 (1.062) |
| 9      | 0.418 (0.134) | 7299 (1643) | 1.140 (0.384) | 1.333 | 2.448 | 1.889 (1.236) |
| Summary | 0.348 (0.057) | 7195 (733.6) | 1.309 (0.203) | 1.955 (0.504) | 1.809 (0.422) | 1.929 (0.342) |

* Figures in parentheses are sd, and all are no have the same power of ten as the average they qualify.
* All values × 10^4; † All values ×10^6; ‡ All values ×10^3.

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yses did not represent an improvement and that locally there is a modest correlation between the number of vesicles and the thickness of the cell. This can be used for predicting the numerical density after measurements of the endothelial thickness, which are substantially easier.

**Discussion**

This work represents the first attempt to apply modern standard morphometric techniques to the quantification of vesicular content of endothelial cells. The full complexity of the problem has been critically discussed. The analysis frame by frame without pooling of primary counts and without use of a large computer has been rendered possible by the introduction of microprocessors. From this point of view, our work cannot be compared with previous publications in which less elaborate techniques were used or a detailed discussion of the authors' methods was not provided. Meaningful comparisons are not possible. Especially striking is that numerical densities in our study are much lower than those reported in different segments of the systemic capillaries by others. This is not necessarily a conflict, since both systemic and pulmonary capillary networks differ in many functional aspects and therefore numerical densities need not be identical. The thickness of pulmonary endothelial cells is very variable; occasionally, the cell is so thin that no space for vesicles is left; other areas contain space for at least two vesicles; other areas are still thicker. Visual inspection of the random photographs shows a heterogeneous distribution of the vesicles with comparatively large areas without vesicles and with clusters of vesicles in other areas. In view of this situation, use of a very large sample of material is essential.

We worked with comparatively thick sections (T = 85 nm). For comparison, Simionescu et al. (1978) had used 60-nm sections. Despite our efforts, it is difficult to guarantee that the thickness was really constant. This is a potential problem because a thickness value must be entered in the formula for numerical densities, and it is unclear whether the consequences of deviations in both directions cancel out.

Another factor is possible changes in the vesicular diameter, D as used in the formula is the true average diameter of the vesicles. We simply assumed this to be 70 nm, a value generally accepted in the literature. Studies have shown that diameter values in fact are scattered (Bruns and Palade, 1968; Casley-Smith, 1969). We used strict criteria in the identification of vesicles. We did not count profiles of vesicles with irregular appearance of a diameter less than one-third of the equatorial 70 nm [which corresponds to profiles of vesicles with a depth of penetration into the section of less 1 nm.] Strictly, the formula \( N_V = N_s/(D + T - 2h) \) holds only for perfectly spherical bodies, which the vesicles are not. Therefore, all data are to be taken as best possible estimates. The estimates of other authors were of vesicles after administration of tracers presenting the possibility that these either increase the number of vesicles or render more of them visible. The lungs of the rabbits in our study had not been subjected to any experimental manipulations. One of the assumptions that we made in
the present study must be discussed: We assigned every vesicle to one of the surfaces and did not count any of them as being in transit completely surrounded by cytoplasm. This assumption was arbitrary and prompted by the obvious difficulty in ensuring that a given vesicle seen without attachments to either cell front is in fact free. In our material, the average cell thickness was only 0.19 μm, which should be compared to an equatorial diameter of the vesicles of 0.07 μm. It has been suggested that diffusion times are very short: Casley-Smith (1969) suggested as little as ~0.5 seconds; later work by Green and Casley-Smith (1972) gave more widely varying transit times; Shea et al. (1969) estimated the transit time to be ~1 second. The fixation time and fixation related events add a new uncertainty. We believe that our assumption was permissible and likely to conform to reality.

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