ELECTRON MICROSCOPE STUDIES OF THE MICROVILLI OF HELA CELLS

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ABSTRACT

Microvilli of HeLa cells cultured in vitro were preserved for electron microscopic examination at different stages of routine cultivation procedures. By a double-embedding technique, vertical sectioning for electron microscopy was possible. It revealed that, although the microvilli were present on all sides of the cell in the dispersed stage and in the attached stage, they were not present on the bottom of the cell when it was stretched on the surface of the dish. When the cells were grown in dense colonies, they were found on top of each other, and microvilli were present on all sides, except on the bottom surface of those cells in contact with the dish. We achieved a more dramatic demonstration of the microvilli by developing a surface-replica technique which retains their spatial arrangement and permits characterization of the distribution of their number, length, and diameter.

INTRODUCTION

Electron microscopic studies of cytoplasmic projections of mammalian cells cultured in vitro are less extensive than the studies of the microvilli of in vivo cells. It is generally accepted that the microvilli of the epithelial cells of the jejunum function to increase the surface area through which absorption can occur (2). Microvilli of granulocytes are believed to function in the aggregation during phagocytosis (11).

Although investigators have presented evidence that microvilli of the surface of cultured mammalian cells may play a role in functions such as hemadsorption (12), cell aggregation (10), and cell fusion (8), the electron microscopic characterization of them is limited (13, 16) and no speculation on their singular function has been advanced. For certain studies in our laboratory on the attachment and spreading reactions (3) during in vitro cultivation of HeLa cell, it was desirable to examine the changes in the cell surface during the reaction.

In our examination we found that the surface of the HeLa cell has many cytoplasmic projections. In this report we have characterized these projections and have examined their changes when the cells were cultured by the commonly used techniques of monolayer cultivation.

MATERIALS AND METHODS

Cell Cultures, Media, and Culture Methods

Cell stocks of HeLa S3 (15) were maintained as monolayers according to the methods described by Ham and Puck (7). The growth medium consisted of nutrient solution F-10 supplemented with 5% fetal bovine serum, 10% calf serum, and 0.01% Panmede (Paines and Byrne, Greenford, England) (6); in some experiments the F-10 was supplemented with 1 mg/ml of fetuin (4). Monolayer cultures were dispersed with 0.05% trypsin (7) or 0.05% ethylenediaminetetraacetic acid (EDTA) adjusted to pH 7.0 in 0.85% NaCl.

Electron Microscopy

VERTICAL SECTION TECHNIQUE: Flat-bottomed vials, ½ inch in diameter, were prepared by depositing onto the bottom of the vials a vacuum-

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evaporated layer of palladium approximately 100 A in thickness. Dispersed cell preparations were inoculated into growth media 5 mm deep in the vials and were incubated for different time intervals. Fixation of the cells in the glass vials was carried out in 5% glutaraldehyde for 15 min followed by postfixation for 5 min in 1% osmium tetroxide (14). The samples were dehydrated in an alcohol over silica gel. Final embedding was in Epon or cross-linked methacrylate. The glass was shattered from the polymer plug by a 5–10 sec immersion of the vial in liquid nitrogen. By this procedure the metal film was left with the polymer. Pieces of the polymer block were reembedded in the same polymer in gelatin capsules turned 90° from the original plane. The trimmed block was sectioned with glass knives on a Porter-Blum ultramicrotome; vertical sections were yielded through both the cells and the metal film.

SURFACE REPLICA TECHNIQUE: Cell preparations were grown on 3 × 1 inches glass microscope slides in Petri dishes. An inoculum of about 5×10^4 dispersed cells in the complete growth medium spread over the surface of the slide was incubated at 37° C for 12–15 hr. The medium was removed, and 5% glutaraldehyde at pH 7.4 was introduced with rapid agitation for the first 15 sec, to remove debris from the cell surface. The fixative was then allowed to stand for 15 min. This fixation was followed by postfixation in 1% osmium tetroxide and then the slide was washed in distilled water. For some preparations the slide was soaked for ½ hr in 10% aqueous dimethyl sulfoxide, was plunged into liquid nitrogen, and then was placed on a brass block which had been precooled in liquid nitrogen; for other preparations the slide was placed directly on the precooled brass block. The brass block with the frozen slide preparation was placed in the metal evaporation unit and freeze-dried in vacuum. The preparation was shadowed with gold-palladium at an angle of 15° or 45° and then was coated with 300–500 A of carbon from rods positioned directly above. Overnight immersion of the slide in 10% potassium hydroxide removed most organic material. When the alkali was removed and 2% acetic acid was carefully introduced, the film floated away from the slide and was picked up on Formvar-coated grids introduced from beneath the replica.

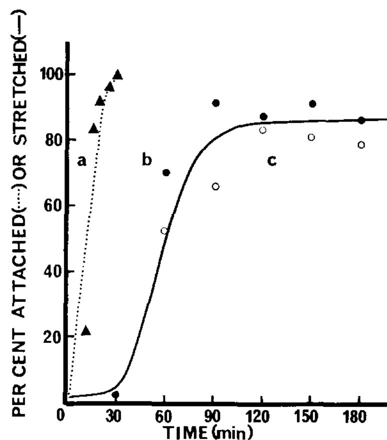

FIGURE 1 Rates of attachment and spreading of HeLa cells. Monolayer cultures were dispersed with 0.05% trypsin or 0.05% EDTA and counted, and 5×10^4 cells were inoculated into growth medium in replicate 60-mm plastic Petri dishes. After different time intervals at 37°C the dishes were rinsed, fixed, and stained (7). The cells in 10 fields selected at random in the light microscope were counted. ▲ per cent of inoculum cells dispersed by trypsin which were attached. ● per cent of inoculum cells dispersed by trypsin which were spread. ○ per cent of inoculum cells dispersed by EDTA which were spread *a*, *b*, and *c*, indicate stages examined by electron microscopy.

RESULTS

Time Sequence of the Cell Reactions

The rate of attachment of the dispersed cells to the surface of the dish was measured by using a light microscope to count the number of cells adhering after different intervals of incubation at 37°C in growth medium. The cultures were washed, fixed, and stained (7) and the total cells remaining, in 10 fields selected at random in the microscope, were counted. These results are shown by the dotted line in Fig. 1. It shows that the cells attached at a rate which was comparable to the settling of spheres of that size according to Stokes's law.

In the same way the spreading of the cells was measured by using a light microscope but only those cells with the spread appearance (3) were scored in the 10 fields selected at random. The results are shown by the solid line in Fig. 1. This reaction was much slower than attachment and required about 2 hr for maximum spreading. The rate was independent whether the inoculum cells were dispersed with EDTA or with trypsin. The results indicated three different early stages for examination by electron microscopy: (*a*) the dispersed stage before 5 min incubation, (*b*) the attached but not spread stage after 15–45 min incubation, and (*c*) the spread stage, after 2 hr incubation.

Electron Microscopy of Vertical Sections

Specimens of the cells in the three stages noted above were prepared for examination with the

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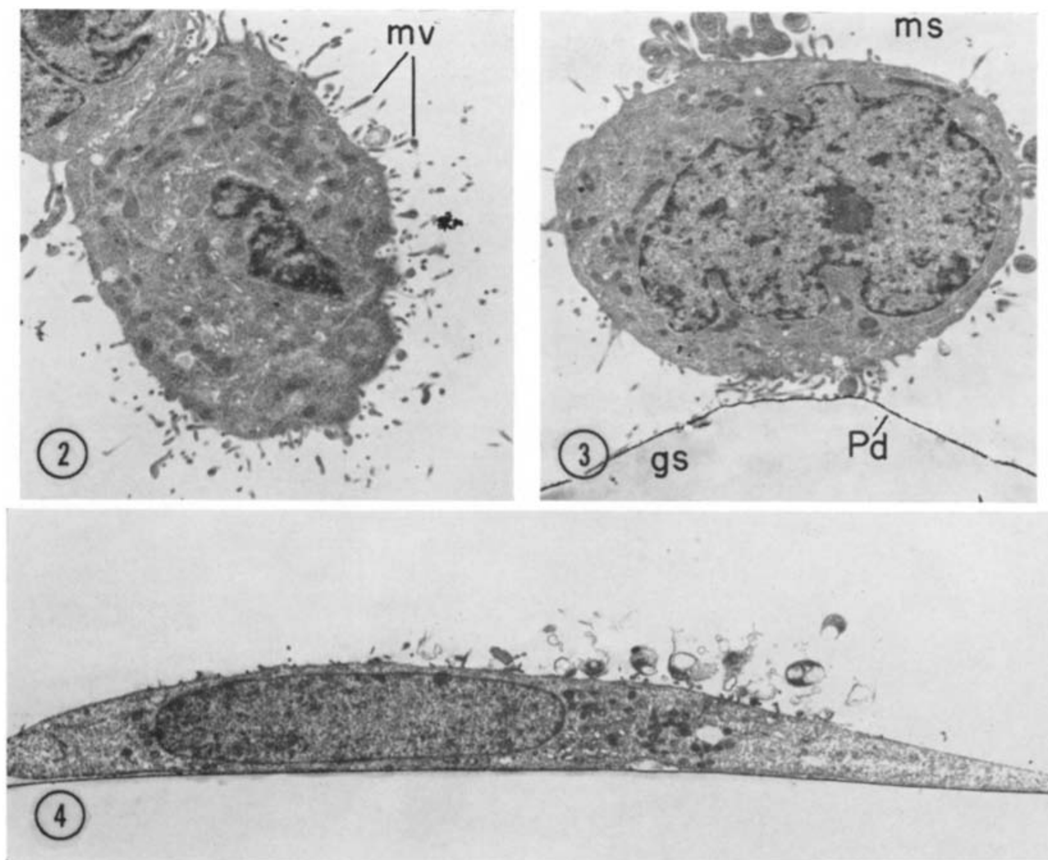

FIGURE 2 Electron micrograph of trypsin-dispersed cells before attachment. (*mv*) microvilli × 4,400.

FIGURE 3 Electron micrograph of a vertical section, prepared according to Materials and Methods, of cell attached but not spread after 30 min incubation at 37°C in growth medium. *ms*, medium side; *gs*, glass side; *Pd*, palladium film. × 3,400.

FIGURE 4 Same as Fig. 3, but showing spreading of cell after 12 hr incubation. × 2,400.

electron microscope by the vertical-sectioning technique described in Materials and Methods. Typical results of the appearance of the cells in the dispersed stage are shown in Fig. 2. When prepared carefully, HeLa cells dispersed by either trypsin or EDTA had microvilli on all sides. After 30 min incubation in growth medium the cells were in the attached stage and, as illustrated in Fig. 3, microvilli were present between the main body of the cell and the palladium film. In the third stage, after sufficient time had been allowed for complete spreading of the cells, very few microvilli were found on the bottom surface of the cells as shown in Fig. 4 although they were present on the top. The attachment was by close apposition of the unit membrane of the cell and the metal film. This is illustrated at higher magnification by the micrograph in Fig. 5 in which the separation of the unit membrane from the palladium is approximately equal to the membrane's own thickness, even though the irregularities of the surface are relatively gross. From an examination of many serial sections it was determined that this close approach of the cell to the underlying substrate was over large areas of contact.

When cells were grown into dense colonies or monolayers and examined by the vertical-sectioning technique, cells were found on top of each other and microvilli were present on all sides of the cells except the bottom surface of those cells in

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contact with the metal film. The appearance of these microvilli at intermediate magnifications is shown in Fig. 6. A large fraction of the microvilli were cut in cross-sections with these thin-sectioning techniques. This high proportion of transverse sections in ultramicrotomy has been discussed by Crawley and Harris (1). No difference was detected between preparations dispersed with trypsin or EDTA.

Electron Microscopy of Surface Replicas

Although the vertical-section technique permitted a satisfactory examination of the changes in the microvilli during the incubation reactions, their complete characterization required the development of the surface-replica technique described in the Materials and Methods. The results of this method of preparation are illustrated in Figs. 7–8 at low and intermediate magnification, respectively. In preparations quick-frozen from 10% dimethyl sulfoxide, as shown in Fig. 7, the microvilli were not upright and it was possible to measure their distribution in terms of number, length, and diameter. The results of these measurements are presented in Table I and the histograms shown in Fig. 9. Table I shows that the average density of microvilli on the surface of the HeLa cell is about 13 per 100 μ^2. As shown in Fig. 9 the microvilli have an average length of between 2 and 4 μ, but their length was found to range from the lower limit of detection to over 7 μ. The average diameter of the microvilli was between 800 and 1200 A and agreed with the values obtained from vertical sections.

The replica technique revealed many reticulated patches on the surface of the cell; one such patch

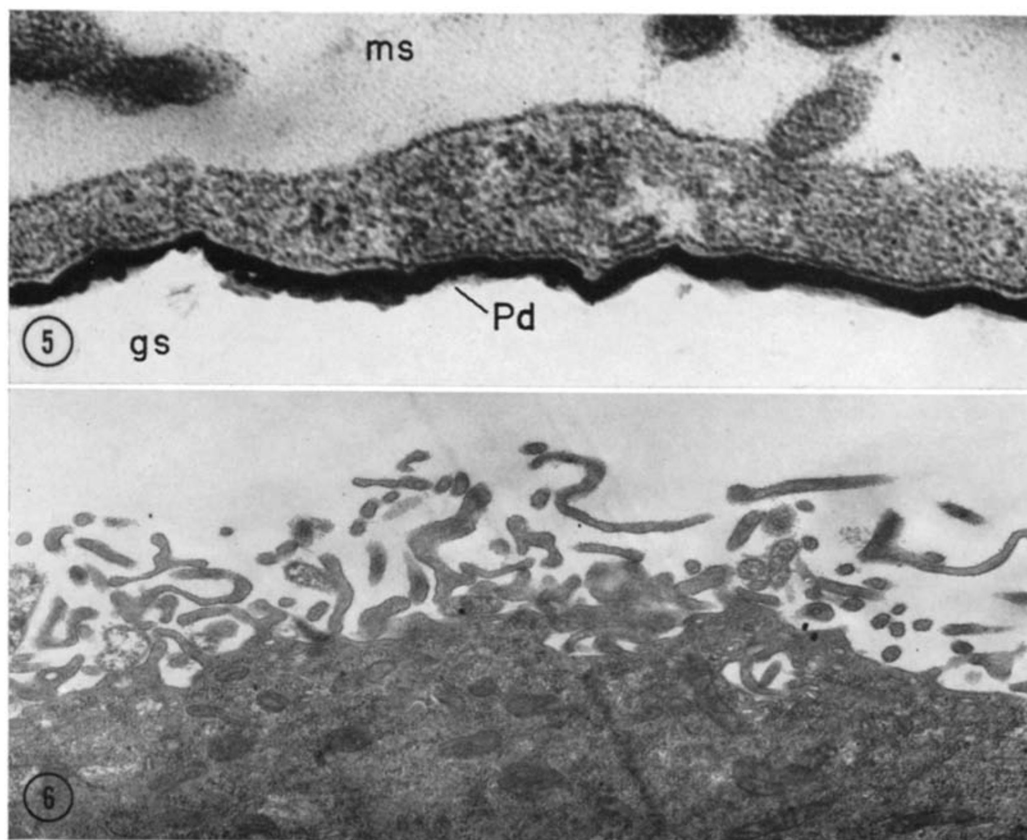

FIGURE 5 Electron micrograph of a vertical section near the periphery of a cell after 48 hr incubation. *ms*, medium side; *gs*, glass side; *Pd*, palladium film. × 140,000.

FIGURE 6 Same as Fig. 4, but showing only the upper surface of the cell after 48 hr incubation. × 16,400.

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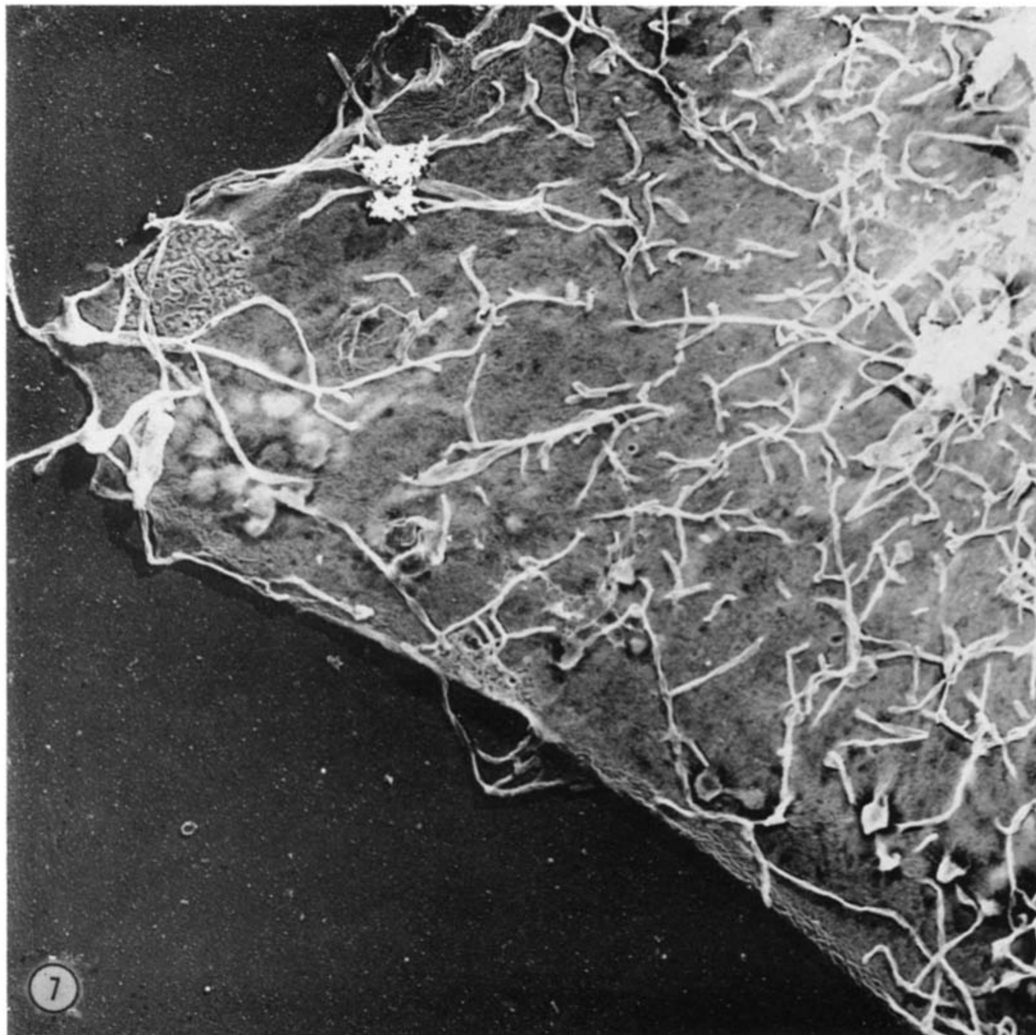

FIGURE 7 Electron micrograph showing microvilli in a preparation made by the replica technique, described in Materials and Methods, in which quick-freezing in liquid nitrogen is used. × 3,800.

is shown in the upper left quadrant of Fig. 7. No microvilli appeared to emanate from these areas, although microvilli were found over all parts of the cells including the nuclear part.

DISCUSSION

The presence of a large number of microvilli on the top surface of the spread cell suggests their implication in the transport of nutrients into the cell and the release of synthesized materials from the cell into the medium. Marcus (12) proposed this method for the release of hemagglutinin from virus-infected HeLa cells into the medium; his estimate of 200–400 as the number of microvilli on the surface of the spread cell was based on hemadsorption studies. We know from direct counts given in Table I that the average number of microvilli per 100 μ^2 is 13.4 and that the area of the spread HeLa cell is about 1600 μ^2 (12); therefore, we obtain a value of just over 200 microvilli per cell.

The single unit membrane of the cell was found to be close (see Fig. 5) to the underlying palladium surface; very few microvilli were found on the

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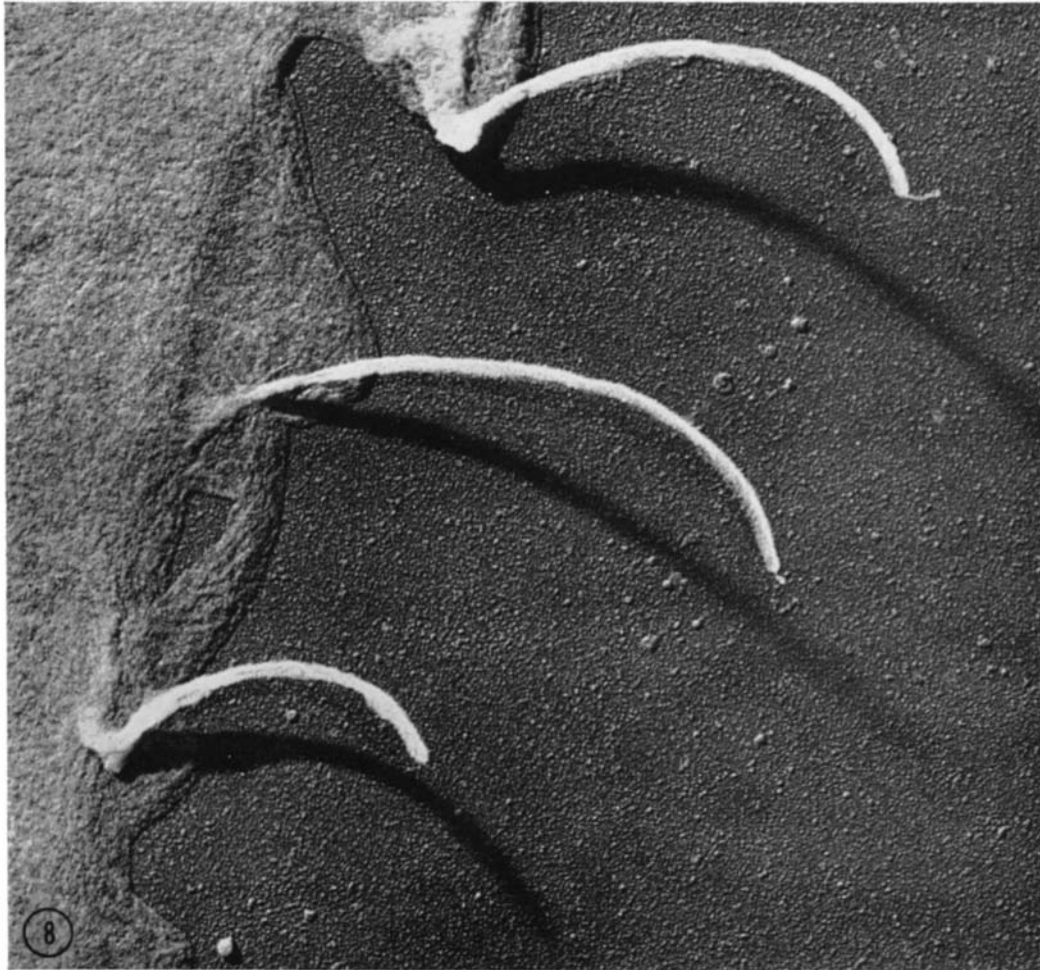

FIGURE 8 Electron micrograph of a specimen prepared by the replica technique in which freezing on a brass block is used, which preserves the spatial arrangement of microvilli. × 20,400.

bottom of the cell. An interesting proposal which one might make on the basis of these observations is that the microvilli play a role in the propagation of the cell in culture by acting as attachment organs and by retracting during the spreading reaction. In support of this concept, we found that cells which would not attach after being heated to 42°C for 10 min had their microvilli drawn into their surface. However, an electron microscopic examination of cultured CHL1 and 3T3 cells, which differ widely in their response to contact inhibition (5) but undergo these same attachment and spreading reactions, failed to reveal microvilli.

Microexudates are considered to exist outside the "trypsin barrier" of cultured cells and to separate the cells from each other and from the attachment surface (9). The relationship of these cell exudates to the requirements of medium, (3) for the spreading reaction is as yet not understood. Preliminary studies with these methods have shown that no microvilli are left on the dish surface when the cell is removed with trypsin or EDTA, but a substance remains (12) which can be detected by immunological methods.

Structurally the cytoplasmic projections of the HeLa cells resemble more closely the microvilli of granulocytes than the microvilli of the brush borders. If the microvilli of granulocytes function

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TABLE I

Density of Microvilli on the Cell Surface

Micrograph*	No. of microvilli	Area	Avg No./100 μ^2
		μ^2	
197a	147	1400	10.4
197d	133	1071	12.2
210b	259	1400	18.5
210d	228	1400	16.2
210e	226	1095	20.6
211b	317	3400	9.4
Total	1310	9766	13.4

* Measurements made on micrographs like the one shown in Fig. 7.

in phagocytosis (11), perhaps this suggests that the microvilli of HeLa cells function in pinocytosis. An examination of several cell types in culture by these methods has been undertaken with this idea in mind.

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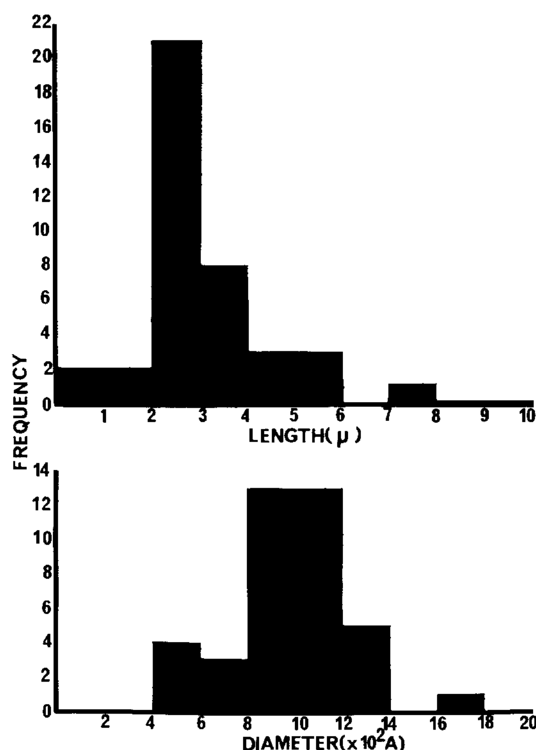

FIGURE 9 Histograms of the dimensions of the microvilli determined from approximately 40 measurements of electron micrographs like Figs. 7–8. The upper diagram shows the length determinations and the lower diagram the diameter determinations.

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