AN INVERSE RELATION BETWEEN CELL DENSITY AND THE NUMBER OF MICROVILLI IN CULTURES OF BHK21 HAMSTER FIBROBLASTS

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SUMMARY

The relationship between culture density and the number of microvilli on the cell surface has been studied using BHK21 cells in established monolayer culture. The number of microvilli can be estimated quantitatively by a scanning electron-microscope technique. It can be increased by applying specific antiserum, but whether antibody is applied or not a significant number of cells regularly bear microvilli. This number is characteristic of the culture density. In sparse cultures above a certain minimum density it is relatively high, and in confluent cultures it is much reduced. These results indicate that microvilli may be inhibited by cell contact.

INTRODUCTION

Microvilli are well-known structures on the surfaces of cells, particularly since the advent of surface replica techniques and scanning electron microscopy, which allow the study of the entire surface (Fisher & Cooper, 1967; Follett & O'Neill, 1969). However, knowledge of their structure and function, or the mechanism of their formation, remains fragmentary. It is known that they are tubular outgrowths of the plasma membrane, about 0.1 μm in external diameter. They have also been found to enclose small numbers of fibrils, each between 4 and 6 nm in diameter (Goldman & Follett, 1969). Fibrils of this type also form part of a matted network immediately underlying the plasma membrane and it is possible that microvilli arise by the outgrowth of such fibrils along an axis normal to the plane of the membrane.

It is clear that microvilli, because of their extremely large surface area (no membranous structure has a radius of curvature considerably smaller than this), offer a way of disposing of plasma membrane when it is temporarily in excess; for example, when cells round up for division. In fact it has been shown that rounded cells bear numerous microvilli, which become progressively less numerous when the cells are allowed to spread out on a solid substratum (Follett & Goldman, 1970). Counts of the number of microvilli allow estimates to be made of their total surface area and it has been shown that this area may be as much as the change in apparent surface area which occurs on rounding up.

In addition, it seems possible that microvilli may be involved in exchange of materials with the medium (because of the increased surface area they provide, and because of their known presence in such structures as the intestinal brush border). For theoretical reasons, it is also likely that fine surface projections of this type may promote adhesion between cells, if only by limiting the mechanical work involved in draining the gap between the approaching surfaces. Finally, it seems possible that they may be involved in cell movement, and a mechanism of cell movement involving the extension, attachment and retraction of fine pseudopodia has been proposed (Wolpert & Gingell, 1968). In general, there is not at present sufficient evidence to judge any of these hypotheses.

There has recently appeared some evidence indicating that the occurrence of microvilli may be influenced by cell contact. It has been observed that the bonding of erythrocytes to BHK 21 tissue culture cells by immune adherence is restricted to sparsely distributed cells. In confluent cell sheets, under otherwise identical conditions, the number of attached erythrocytes is conspicuously reduced (O'Neill, 1968). In order to explain this finding, it was suggested that immune adherence to tissue cells was dependent on the presence of microvilli, and that microvilli were absent from contact-inhibited cells.

The experiments we are about to describe represent an attempt to investigate further the conditions which govern the appearance of microvilli on BHK 21 cells, in particular in relation to culture density.

METHODS

Cell culture

The methods used in these experiments were largely those described in Follett & O'Neill (1969) and in O'Neill (1968). The cells were BHK21/C13 (Stoker & Macepherson, 1964), obtained from a short passage stock. They were recovered from the frozen state and discarded after 1 month's further growth. Cells were grown in Eagle's medium containing 2-fold nutrients and 10% calf serum and 10% tryptose phosphate broth on 18-mm round coverglasses in 60-mm Petri dishes. The same batch of serum was used for the entire series of experiments. Cells were plated at varying densities as indicated, and incubated for 24 h at 37 °C in an atmosphere of 5% CO₂ in air before use. Cultures termed 'sparse' were plated at 3500 cells per cm², and cultures termed 'confluent' at 70,000 cells per cm². These figures correspond to 1 x 10⁵ and 2 x 10⁶ cells per dish respectively. BHK cells show some delay before growth is resumed after trypsinization. Since this period is believed to be considerably less than 24 h, the culture densities actually observed may have been slightly in excess of these figures.

Preparation for electron microscopy

Care was taken to maintain the temperature at 37 °C until the moment of fixation. At this time, medium was removed from the cultures, and glutaraldehyde in phosphate buffer (pH 7.4) at room temperature added without delay. After 15 min, they were washed 3 times in phosphate-buffered saline and then fixed for 5 min in buffered osmium tetroxide (Zetterqvist, 1961). In some instances a glutaraldehyde preparation containing sucrose (0.25 M) and calcium (0.1 mM) in phosphate buffer was used. After fixation the coverglasses were washed 5 times in distilled water, frozen by plunging rapidly into Arcton 22 at its melting point (−160 °C) and stored in a freezer cooled by liquid nitrogen. A brass block with a flat surface, weighing 1 kg, was cooled to equilibrium in liquid nitrogen, and then transferred to the chamber of a vacuum-coating unit. The coverglasses were then placed on this block and the chamber immediately evacuated. One hour after evacuation they were uniformly coated with a minimum of 40 nm of carbon.
To prepare replicas, the coverglasses were then shadowed at an angle of 30° with Ni/Pd, removed from the vacuum and extracted with 50% KOH at room temperature for 4 days. They were then briefly extracted with 5% acetic acid, and the loosened film floated on to a clean water surface. Areas of the film were then picked up on grids, blotted dry and examined in a Siemens Elmiskop Ia electron microscope.

To prepare cells for the scanning microscope, the carbon-coated coverglasses were given a second uniform coating of approximately 20 nm of gold, withdrawn from the vacuum and stored over desiccant. These coverglasses were examined without any further treatment in a Stereoscan scanning electron microscope (manufactured and operated by Cambridge Scientific Instruments Ltd., Cambridge, England).

**Counting microvilli**

Cells were examined for microvilli on their upper surfaces either in the scanning microscope or, as replicas, in the transmission electron microscope. In some experiments cells were scored either as positive (bearing microvilli) or negative (bearing no microvilli). A minimum of 5 microvilli were counted on a cell before it was recorded as positive. In other experiments the actual numbers of microvilli on each cell were recorded. In either case, areas of the culture were selected at random. Each figure was derived from counting a minimum of 100 cells. In the transmission electron microscope the field of view was limited; only cells with at least 4/5 of the total area visible were counted.

**Mitotic index**

The mitotic index was measured by fixing coverslips (prepared at the same time and in the same way as material for electron microscopy) for 30 min in methanol, followed by staining for 5 min in Giemsa stain. Nuclei were counted, and mitotic index quoted as the percentage represented by mitotic figures of the total number of nuclei. Over 1000 cells were counted in each determination.

**Antibody treatment**

In some cases cells were treated with antibody. The conditions used were exactly those previously recorded for immune adherence experiments on monolayers (O'Neill, 1968). Coverglasses were treated with rabbit anti-BHK21/C13 serum number A8938 (previously heated for 30 min at 56°C) at a concentration of 1%, v/v, in phosphate-buffered saline (pH 7.4) containing 0.2% bovine serum albumin (fraction V powder, Sigma Chemical Co.), 0.1 mM Ca²⁺ and 0.5 mM Mg²⁺, at 37°C for 20 min. This was followed by washing 3 times in the same warm saline. Controls were treated with saline from which the antibody had been omitted.

**RESULTS**

**Appearance of cultures at different densities**

Cells were examined both by scanning electron microscopy and as replicas in the transmission electron microscope. The replica technique gave good resolution and required only a conventional microscope, but the field of view was very small. In addition, replicas could be made only of smooth cell sheets and even these showed distortion due to collapse of the film. The scanning microscope did not suffer from these disadvantages and allowed data to be collected rapidly.

Cells were examined most frequently at two standard culture densities. The first was 3500 cells/cm² (‘sparse’). This was the density which had been found previously to be the optimum for immune adherence (O'Neill, 1968). The second was 70000 cells/cm² (‘confluent’), which was the density at which a continuous cell sheet first appeared. It is important to note that, in ‘sparse’ cultures, cell contacts were by no means in-
frequent. Although only about 10% of the substratum was covered by cells, small
groups of cells were common. In addition, in 'confluent' cultures, areas of completely
contiguous cell sheet were rare. Small gaps of a few microns between the cells were
common, although cells were occasionally piled up on one another. At higher densities
piling-up became more common, and since such very dense cultures were difficult to
observe we have not studied them.

In sparse cultures the cells appeared flattened and multipolar. While most of the
cells had smooth featureless surfaces, some bore microvilli (Fig. 3). In confluent cul-
tures, cells had a fusiform bipolar shape and were oriented parallel to one another
(Fig. 4). In this case the surfaces were more often ridged, and the ridges were generally
axially oriented. However, even when the ridges were irregular (Fig. 5) their
appearance was clearly distinct from microvilli (Fig. 6).

Microvilli were characteristically straight and of constant diameter (approximately
80–100 nm). In replica preparations, they appeared either as electron-dense rods or as
outlines (Fig. 7). This difference in appearance may depend on whether the cyto-
plasm within them is removed during the extraction process, and in some cases inter-
mediate stages can be seen where the terminal part remains dense while the base
appears in outline only. Other features were also seen on replicas, including depressions
which were tentatively identified as the mouths of endocytosis channels.

It was apparent that, while microvilli were not present on the majority of cells in
sparse culture, they were regularly found on a small proportion of the cells. In con-
fluent cultures, this proportion was much smaller. Cells which were rounded up, and
presumably in the course of division, were seen in all cultures examined. In all cases
they bore extremely numerous microvilli, often more than 200, and were attached to
the substratum by elongated processes of similar diameter. Such cells were relatively
infrequent and never formed more than a minor proportion of the total number of
cells bearing microvilli. Occasionally, in gaps in confluent cultures, processes were
seen with a diameter similar to microvilli but which were over 10 μm in length. They
were oriented along the axes of the cells (which in these cultures are characteristi-
cally parallel) and ran between the cell and the substratum. This type of process was
not counted. In fortunate cases, areas of completely contiguous cells were seen (Fig. 8). Such cells were completely devoid of microvilli.

*Estimation of the number of cells bearing microvilli*

Replicas of cultures at various cell densities were observed, and the number of cells
bearing microvilli recorded. Counting 100 cells in each case, 10 estimations from dif-
ferent areas of the same sparse coverglass were compared. The average of these 10
figures was 30%. The range was ± 2%, which is well within the statistical variation to
be expected. Different cultures prepared on the same day also showed good agreement,
but there was considerable variation from day to day. Over the whole series of experi-
ments the proportion of positive cells in sparse cultures was between 20 and 40%.

A group of cultures at 5 different densities was prepared, and examined for positive
cells after 24 h in culture. The results of this experiment appear as Fig. 1. At very low
culture densities (1750 cells/cm²) the proportion was close to 20%, but rose to 40%.
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when sparse density was reached. As the density was further increased the proportion fell, and continued to fall progressively until, at confluent density, only 11% of the cells were positive. In a second group of cultures, prepared at the same time, the proportion of mitotic figures was estimated. At sparse and lower densities the mitotic index proved to be 1.3%. At all higher densities the mitotic index was close to 3.0%. Thus the fall in the proportion of cells bearing microvilli as the density was increased from sparse to confluent was not related to the growth rate.

Fig. 1. Relation between number of cells bearing microvilli, cell density, and mitotic index. ×, Percentage of positive cells; O, mitotic index.

Estimation of the number of microvilli

Actual numbers of microvilli on individual cells were also counted. For this purpose only sparse and confluent cultures were used. There was a wide variation in the number of microvilli on individual cells, covering the range from zero to over one hundred. Such wide variation was sometimes found even between cells in close contact, but in these cases at least one side of the cell was always free and in contact with the substratum only. As was found for the number of positive cells, the average number of microvilli per cell in a culture also varied from day to day. Over the whole series of
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experiments the average for sparse culture varied between 10 and 20 per cell. Again, variation between cultures prepared on the same day was very much less. All the experiments reported below represent comparisons between sparse and confluent cultures prepared at the same time.

![Histograms A and B](image)

**Fig. 2.** Distribution of microvilli at 2 culture densities, without and with antibody treatment: A, confluent; B, sparse; C, antibody-treated, confluent; D, antibody-treated, sparse. The percentage of cells bearing no microvilli is plotted in a separate column to the left in each case. Remaining columns represent cells bearing 1-10, 11-20, 21-30 and so on.

The results of a series of experiments of this type are plotted in Fig. 2 and analysed numerically in Tables 1 and 2. Histograms A and B of Fig. 2 and Table 1 show that there was an approximately 4-fold difference in the number of cells bearing more than
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10 microvilli between the 2 culture densities. In addition, they show a similar or greater difference in the average number of microvilli per cell, and also a (somewhat smaller) difference in the maximum number of microvilli observed on any one cell. They also show that this increase in number in sparse cultures is not contributed to by the class of the cells bearing one to ten microvilli, which in fact decreases. Histograms c and d, and Table 2, refer to a group of observations performed on cells which had been treated with specific antiserum. The concentration of the antiserum used was one half of that at which cytotoxic effects were known to appear; complement was not present, and the cells remained viable as judged by plating efficiency estimates. It can be seen

Table 1. Distribution of microvilli between sparse and confluent cultures
(Analysis of the data given in Fig. 2 a, b. Data obtained from use of the scanning microscope are included for comparison.)

<table>
<thead>
<tr>
<th></th>
<th>Sparse</th>
<th>Confluent</th>
<th>Sparse/Confluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum number observed on 1 cell:</td>
<td>152</td>
<td>70</td>
<td>2.2</td>
</tr>
<tr>
<td>Maximum number observed on 1 cell: (scanning microscope):</td>
<td>224</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Proportion of cells bearing more than 10 microvilli:</td>
<td>36%</td>
<td>11%</td>
<td>3.2</td>
</tr>
<tr>
<td>Proportion of cells bearing microvilli (scanning microscope):</td>
<td>37%</td>
<td>13%</td>
<td>2.85</td>
</tr>
<tr>
<td>Average number per cell:</td>
<td>18.4</td>
<td>4.6</td>
<td>4.0</td>
</tr>
</tbody>
</table>

Table 2. Distribution of microvilli in cultures treated with antiserum
(Analysis of the data given in Fig. 2 c, d.)

<table>
<thead>
<tr>
<th></th>
<th>Sparse</th>
<th>Confluent</th>
<th>Sparse/Confluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum number observed on 1 cell:</td>
<td>218</td>
<td>120</td>
<td>1.8</td>
</tr>
<tr>
<td>Proportion of cells bearing more than 10 microvilli:</td>
<td>68%</td>
<td>19%</td>
<td>3.58</td>
</tr>
<tr>
<td>Average number per cell:</td>
<td>43</td>
<td>10</td>
<td>4.3</td>
</tr>
<tr>
<td>Ratio between the average number on antibody-treated and control cells:</td>
<td>2.34</td>
<td>2.17</td>
<td>—</td>
</tr>
</tbody>
</table>

that the effect of antibody was to more than double the number of microvilli in each case. Normal rabbit serum did not have this effect. Comparing different experiments, it also appeared that the number of microvilli on cultures treated with antibody showed less variation from day to day. Although the total numbers of microvilli were so greatly increased, the relation between sparse and confluent densities remained unchanged. While nearly 20% of the cells at confluent density were now positive, the number of positive cells at sparse density was now nearly 70%.
DISCUSSION

It had previously been shown that BHK cells when in the rounded form, either during division or after trypsinization, bear microvilli in large numbers. Follett & Goldman (1970) have, for example, shown that immediately after attachment to the substratum in recently trypsinized BHK cultures a high proportion (over 90%) of the cells bear microvilli. As spreading progresses during the next 10 h the number of cells bearing microvilli falls to the low value, about 30%, characteristic of these cultures. It was concluded from these results that the reduction in apparent surface area which must accompany rounding up is directly associated with the appearance of microvilli, and that the microvilli act as organelles for the storage of excess plasma membrane when cells adopt the rounded form. Thus, since cells round up before dividing, the proportion of cells bearing microvilli in an established culture would be expected to be directly related to the rate of division in the culture.

These conclusions are in general confirmed by the work reported here. However, we have also found two other quite different parameters relating to the number of microvilli. Culture density, in particular, appears to have an important effect on the number of microvilli in different cultures. At very low densities we have shown that few cells bear microvilli. Mitotic indices show that the reduction in number at very low densities is related to the reduction in cell growth rate. Thus the mitotic index at the lowest density studied is half the maximum observed, and the number of cells bearing microvilli is also half the maximum. Increase in the growth rate when moderate cell densities are reached is well known as the feeder effect. It seems likely that many cell activities are inhibited at very low densities, and production of microvilli may be another instance of this. However, at most of the densities studied, there is a regular inverse relationship between cell density and the number of cells bearing microvilli. This fall in the number of positive cells at higher cell densities cannot be accounted for by changes in culture growth rate. It is already known that BHK cells do not show density-dependent inhibition of growth until several monolayer equivalents have been reached (House & Stoker, 1966). In the cultures used in the present experiments, the mitotic index remained high, and relatively constant, at all cell densities between $7 \times 10^3$ and $7 \times 10^4$ cells/cm². At the lower of these two densities, however, the proportion of cells bearing microvilli is nearly 3 times greater.

These observations do not refer to rounded, dividing, cells. Even in confluent cultures, rounded cells showed dense coverings of microvilli.

In addition to the effect of cell density on microvilli, it has been found that specific antiserum affects the number of microvilli. Applications of low concentrations of antibody increased the number of microvilli more than 2-fold. It is not known how this comes about; perhaps the simplest explanation is that antibody reduces the adhesiveness of cells. In this way the increased number of microvilli could be a simple consequence of the cells being slightly more rounded. It is equally possible that the antibody has some more specific effect on the cell surface, and it is for example known that cell processes are immobilized in HeLa cells treated with antibody (Carey & Pettengill 1967). However, it is clear that antibody does not alter the relation between microvilli
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and cell density. In both normal and antibody-treated cultures the number of microvilli is 4 times greater under sparse conditions.

These results support the idea that microvilli are necessary for immune adherence between cells. It had been reported (O'Neill, 1968) that confluent areas of BHK cultures showed no immune adherence under conditions in which in other areas the reaction was intense. We find here that most cells treated with antibody bear microvilli in sparse culture, and that they are absent from confluent areas. In addition, the numbers of microvilli observed are of the same order of magnitude as the number of red cells which bind to individual BHK cells by immune adherence.

It is reasonable to suppose that the reason for this relationship is that the antigen is localized at the tips of the microvilli. Such a localization is particularly likely in the case of HeLa cells infected with Newcastle disease virus, in which haemadsorption has been shown to take place at the tips of fine processes (Marcus, 1962). However, in the present case, immunofluorescence experiments (C. H. O'Neill, in preparation) show that antibody to BHK cells distributes uniformly over the whole cell surface. It is not in fact necessary to invoke localization of the antigen in order to explain the relationship observed. Adhesion of any sort requires the close apposition of the adhering surfaces, and close apposition of cells is energetically more probable when the areas involved are small. This is because the volume of medium draining from the gap, and the total electrostatic repulsive force, are both reduced (Pethica, 1961; Curtis, 1967). If microvilli have significant mechanical strength, or are actively extending, then the probability of close apposition would be still more favoured. While it is not clear to what extent intercellular adhesion in general is controlled by microvilli, these considerations offer an adequate explanation for the close similarity which we have observed between the distribution of microvilli and of immune adherence.

It is not known by what mechanism microvilli become less frequent in confluent cultures, and several alternative hypotheses are possible. Firstly, there may well be many differences in metabolism between sparse and confluent cultures, and in particular many constituents of the medium may approach exhaustion. We have not examined the possibility of induction of microvilli by fresh medium, and it seems unlikely to be a factor in these experiments in view of the brief time for which the cells were cultured. Secondly, an analogy can be drawn between the induction of microvilli by antibody and their induction by other potentially injurious agents such as digitonin (Graham et al. 1967), or virus (Harris, Watkins, Ford & Schoefl, 1966). It is possible, therefore, to argue that adverse conditions in general produce microvilli, and that for some reason cells in sparse culture experience adverse conditions. Thirdly, the known presence of microvilli in absorptive organs indicates that they are functionally involved in absorptive processes, even if only to increase the total free surface area. It is possible to argue that the lower number of microvilli in confluent cell cultures reflects a decreased requirement for absorption.

However, the weight of the evidence indicates that, all other things being equal, the number of microvilli is inversely related to the number of cell contacts. It does not support the arguments listed above and suggests a direct, local effect on the surfaces of
cells coming into contact. The phenomenon shows many parallels with contact inhibition of movement (Abercrombie & Heaysman, 1954). We do not as yet have any evidence for the precise relationship between inhibition of microvilli and inhibition of movement. It may simply be that moving cells are less well spread. It is equally possible that some of the microvilli we observe are locomotory pseudopodia. For example, pseudopodia which fail to attach may persist for a time on the surfaces of these cells.

We wish to thank Miss Carole Laird and Mr Alistair Munro for their very competent technical assistance. Dr I. A. Macpherson kindly supplied a culture of BHK21 cells. We are also indebted to the Cambridge Scientific Instruments Ltd. and to various members of their advisory service for an opportunity to use their ‘Stereoscan’ instrument, and for permission to publish Figs. 3–6.

REFERENCES


(Received 31 January 1970)
Culture density and microvilli

Figs. 3 and 4. For legend see following page.
Fig. 3. Single *BHK* cell from sparse culture, viewed in the scanning microscope. Note flattened tripolar shape with a detectable convexity over the nucleus. Cracks around the edges (marked) are presumably artifacts due to freezing. Numerous microvilli can be seen. $\times 4000$.

Fig. 4. Similar area of confluent cell culture, showing fusiform shape of cells. Some areas of bare substratum remain. Elongated processes extend from the cells in an axial direction, and the cell surface is ridged in some areas, but no microvilli can be seen. $\times 3500$.

Fig. 5. High-power scanning electron micrograph of portion of a cell's surface showing irregular ridges. Compare with Fig. 6. $\times 9500$.

Fig. 6. High-power scanning electron micrograph showing microvilli. Note regular shape with parallel sides, rounded tips, and characteristic width. $\times 13000$. 

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Fig. 7. High-power transmission electron micrograph of portion of a cell's surface showing microvilli. The greater resolution obtainable with this technique is apparent. Some microvilli appear as hollow tubes with one end closed. Others, presumably less fully extracted by the alkali treatment, appear as electron-dense rods. In either case the appearance is characteristic. In addition, apertures can be seen in the cell surface, which may be the mouths of pinocytosis channels. ×15500.

Fig. 8. High-power micrograph of portion of a confluent cell culture. Portions of at least 4 cells can be seen, in complete contact with one another. Note the absence of microvilli. Some longitudinal ridging of the cell surfaces may reflect structural elements underlying it. All the cells bear nodular elements; their significance is not known. ×12500.
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