Europ. J. Cancer Vol. 15, pp. 843–855.

0014-2964/79/0601-0843 $02.00/0

The Surface Structure of 7,12-Dimethylbenz(a)Anthracene Transformed C3H/10T½ Cells. A Quantitative Scanning Electron Microscopical Study*

HARALD J. K. SAXHOLM† and ALBRECHT REITH‡

†*Fellow of the Norwegian Cancer Society, Institute of Pathology, University of Oslo, Rikshospitalet, Oslo* 1, *Norway*
‡*Norsk Hydro's Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, Oslo* 3, *Norway*

Abstract—*The mouse embryo fibroblast cells, termed C3H/10T½, developed three types of morphologically altered foci after exposure to 7,12-dimethylbenz(a) anthracene. The types* III *and* II *cells showed oncogenic potential, whereas the type* I cells *remained non-oncogenic. By scanning electron microscopy the concentration of short microvilli was found to increase with increasing cell culture passage for all three types. Only on types* II *and* III *cells were long microvilli observed. There was a significant correlation between the oncogenic potential and the presence of long microvilli. This observation may be of value in screening for oncogenic transformation of cells in culture.*

INTRODUCTION

MOUSE embryo fibroblast C3H/10T½ cells have been exposed to 7,12-dimethylbenz(a)anthracene (DMBA). Morphologically transformed cells of three types have been tested for their ability to form tumors in immunosuppressed mice. Scanning electron microscopy (SEM) is an excellent method for detecting morphological alterations of the plasma membrane of cells in culture.

In this study we present a positive correlation between the oncogenic potential of DMBA transformed cells and the surface concentration of long microvilli. The ability of a cell line to grow as a transplanted tumor is called oncogenic potential and this parameter is quantitated as the inverse mean tumor development time. The significance of these findings for carcinogenesis is discussed.

MATERIALS AND METHODS

Cell source and culture conditions

The origin and method of culture of the C3H/10T½ clone 8 cells have previously been described [1]. Three transformed cell lineages obtained after exposure of the cells to DMBA were designed types I, II and III respectively, according to their growth pattern. The oncogenic potential was weak for type II and stronger for type III cells, and increased with increasing number of passages in culture [2]. Non-synchronized cells in the sub-confluent late logarithmic phase of growth were used throughout the study [3].

Preparation of palladium covered agar

Cover glasses were defatted in 50% acetone–alcohol and dipped in 1% agar. A copper grid was used to mask the agar before layering of the palladium by evaporation.

Preparation of cells for electron microscopy

The cells were grown on cover slips and fixed at 37°C for 60 min by adding very carefully dropwise 2% glutaraldehyde at 37°C, buffered with 0.1 M cacodylate and 0.1 M sucrose, pH 7.2, to the culture medium. The cells were dehydrated by alcohol and taken through the critical point drying by liquid CO_2 [4], using a Balzers Critical Point Drier. The cells were then coated with a thin film of gold (40 nm) using a Polaron sputter

Accepted 22 August 1978.
*This work was supported in part by a grant from the Norwegian Cancer Society.

apparatus. The specimens were viewed and photographed with a Philips PSEM 500 scanning electron microscope operated at 25 kV at a tilt angle of 20°C, or as otherwise stated.

Evaluation of micrographs

For quantitative evaluation of microvilli and blebs on the cell surface, micrographs of 50 strictly randomly chosen cells, photographed at a primary magnification of ×1250, were viewed on 2 times magnified copies. The evaluation was undertaken by a person not knowing the oncogenic potential of the coded cells. The concentration of microvilli on the cell surface was described in four classes: those with none −, with few +, many ++ (still possible to count), and innumerable +++ (counting no longer possible) microvilli. Microvilli were called "long" when the length was more than three times the diameter.

RESULTS

The results are shown in Tables 1 and 2 and in Fig. 1 and characteristic pictures are

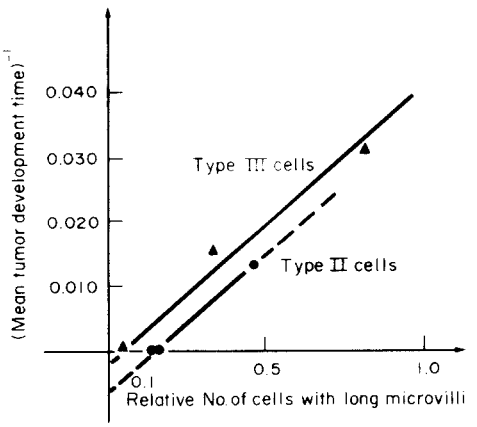

Fig. 1. The correlation between the oncogenic potential (ordinate) and the fraction of cells with long microvilli (abscissa) for the types II *and* III *cells. Regression coefficients were* 1.00 *and* 0.99 *respectively.*

shown in Figs. 2–7. Low power magnification revealed the growth pattern of transformed and oncogenic cells with the multilayered piling-up and cord-like arrangement. The cells were spindle-like and had a rather compact body (Fig. 2a). Non-transformed cells in contrast were flattened with large thin projections (Fig. 2b), but the most distinct differences were seen on the cells' surface.

Amount of microvilli on the cells

Table 1 shows that an increase in microvilli concentration was seen with increasing cell culture passage for all three types. For the non-transformed C3H/10T½ cells the pattern was practically unchanged at several passages tested (passages 10 and 20 shown in the table). The type III cells had the highest concentration of microvilli throughout. The number of cells without microvilli decreased with increasing passage for all three types. Thus 40 of the 50 cells studied were without microvilli in the type I cells. With increasing passages even the non-oncogenic type I cells showed a high fraction with an extremely high concentration of microvilli, similar to the corresponding passages of the oncogenic types II and III cells.

Appearance of microvilli on the cells

In fully transformed cells both short and long microvilli can easily be discerned (Fig. 3a), whereas non-transformed cells display a smooth membrane surface (Fig. 3b). Higher passages of the non-oncogenic type I cells displayed, short, stubby microvilli (Fig. 4b). In contrast, oncogenic type III cells exhibited both long and short microvilli (Fig. 4a). These differences were reproducible and seen in cells cultured on glass, plastic or metal. In addition, the fraction of cells not exhibiting microvilli diminished with increasing passage number except for the non-transformed cells (Table 1). Type III cells were the most homogeneous (Fig. 5a), whereas in type II (Fig. 5b)—and even more expressed in type I, a certain fraction of cells did not exhibit microvilli at all.

Only on the type II and the type III cells were "long" microvilli observed. The non-oncogenic passages of these cells displayed a very low concentration of long microvilli, randomly dispersed among the much higher concentration of short microvilli. The oncogenic passages of the type II and the type III cells had a very high concentration of long microvilli. The long microvilli were 3–4 times as long as the short ones (Fig. 4b). In Fig. 1 the oncogenic potential of types II and III cells is plotted aginst fraction of cells with long microvilli. There is a statistically significant ($r > 0.99$) correlation between oncogenic potential and the presence of long microvilli.

Amount of blebs on the cells (Table 2)

Most of the cells in the four groups were without blebs. However, in each case quite a few of the cells had a low concentration of blebs. Higher concentration of blebs appeared

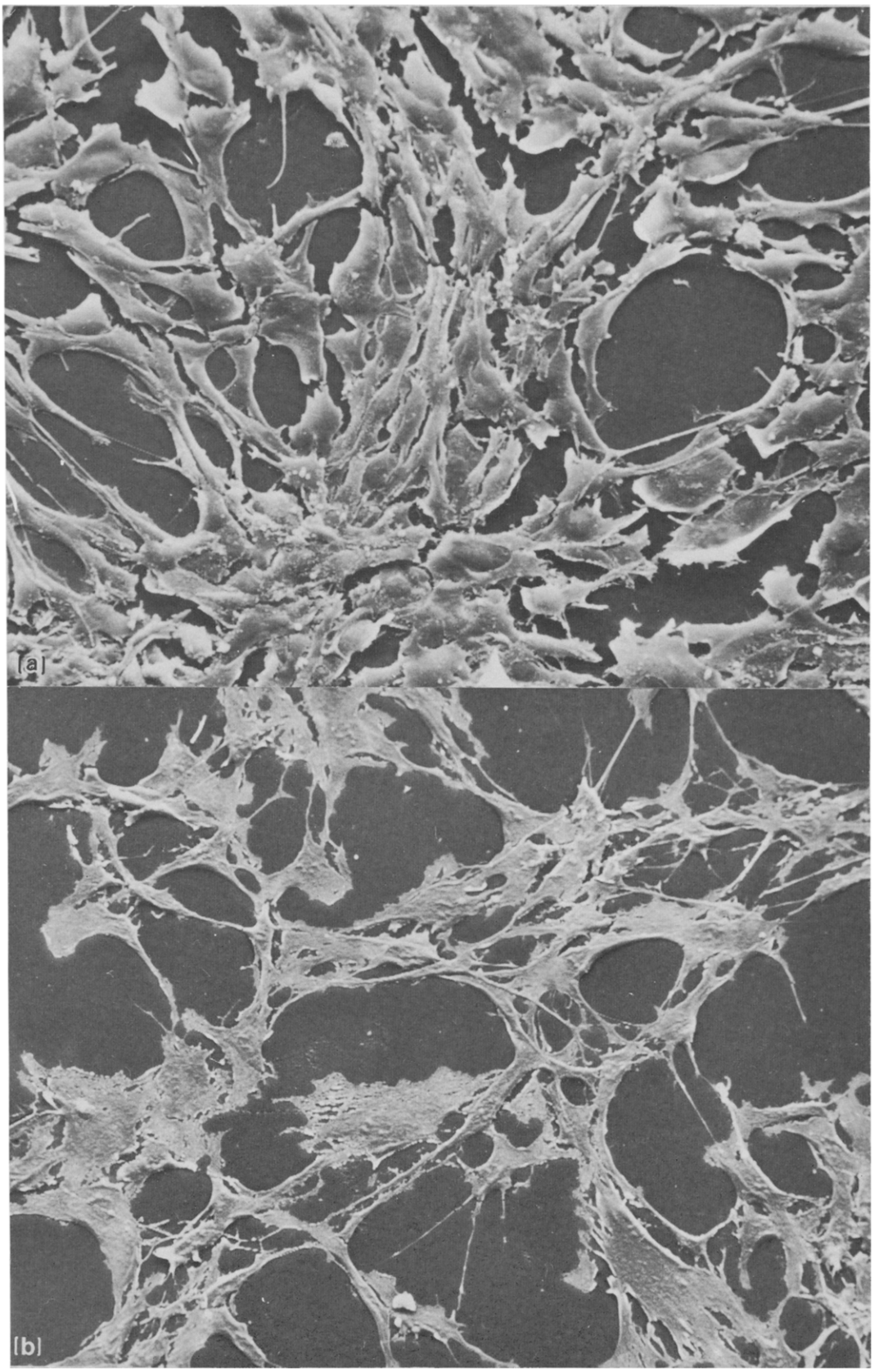

Fig. 2. Survey view of the growth pattern of transformed and oncogenic and non-transformed cells (tilt angle 45°). (a) The oncogenic type III, *passage* 61, *is characterized by a multilayered piling-up and cord-like arrangement. (b) The non-transformed cells are flattened with large thin projections.* × 150.

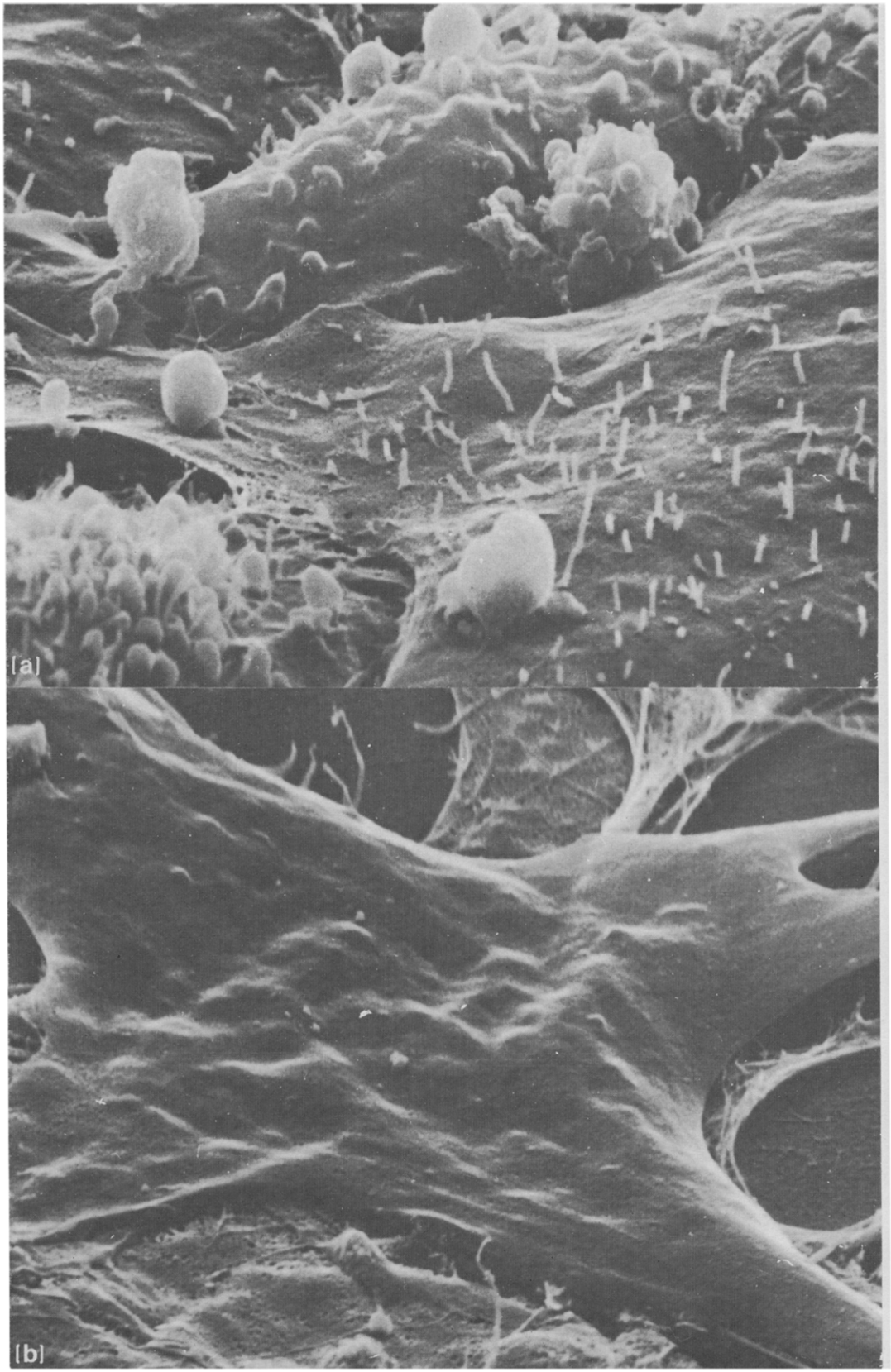

Fig. 3. Survey view of the cell surface of transformed and non-transformed cells taken at a tilt angle of 67°. (a) The cells display microvilli of different length and blebs on their surface. (b) Non-transformed cells in contrast exhibit smooth membrane surface. ×8300.

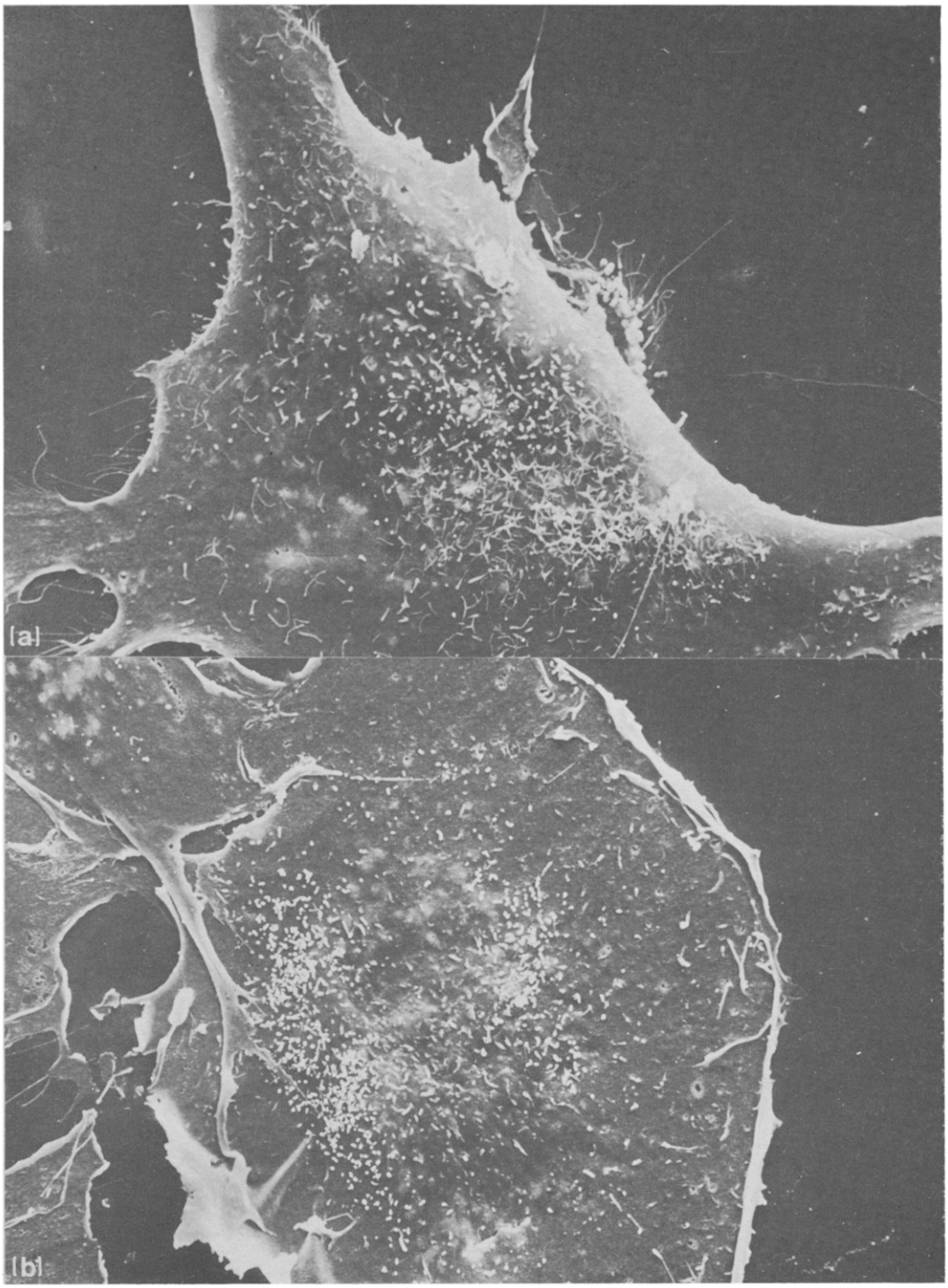

Fig. 4. *Comparison of small and long microvilli.* (*a*) *Long and small microvilli on an oncogenic type* II *cell, passage* 32. (*b*) *Small, stubby microvilli on a non-oncogenic type* I *cell, passage* 31. × 2700.

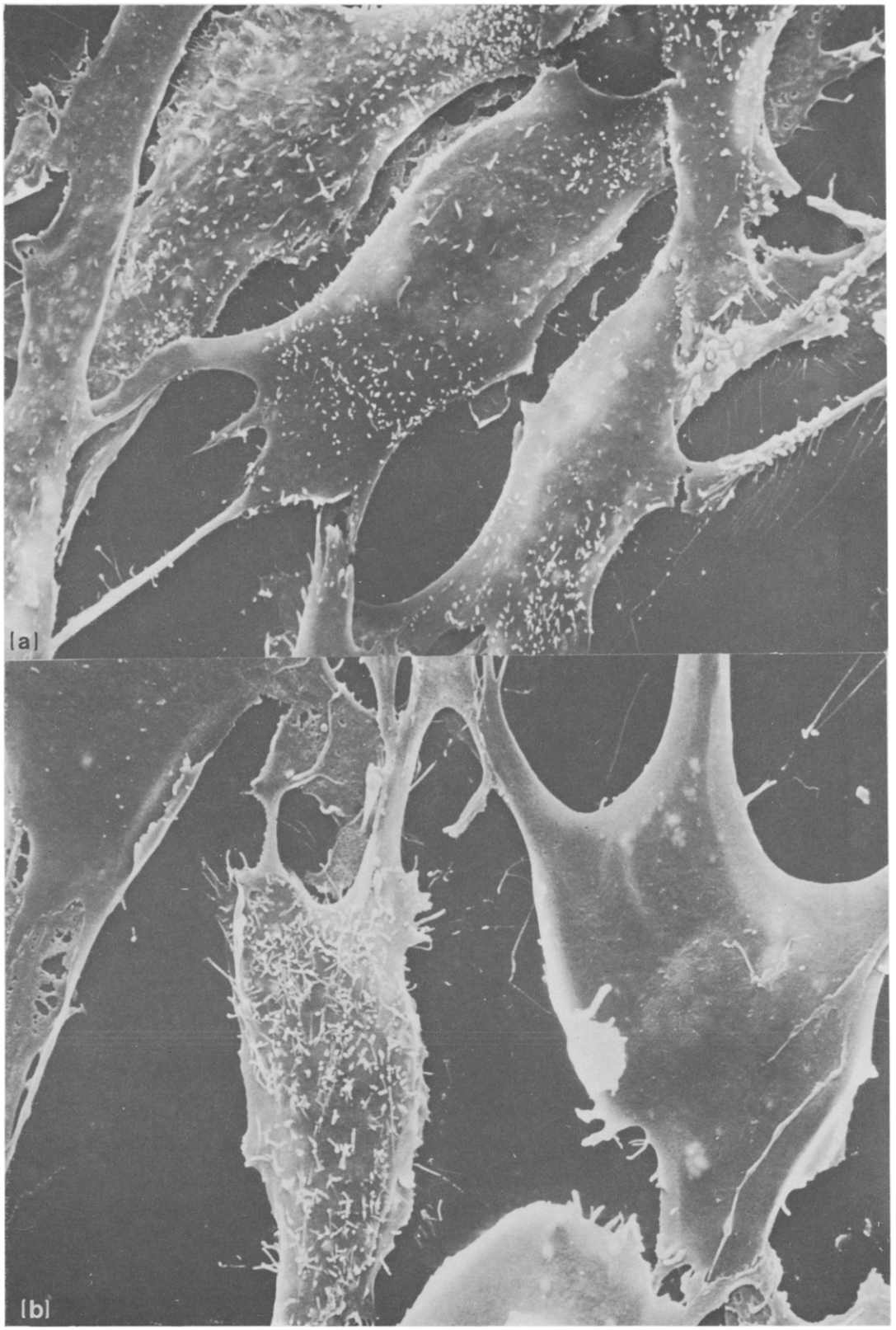

Fig. 5. *Differences in concentration of microvilli in type III and type II cell populations at passage 31 or 32 respectively.* (*a*) *All cells of the oncogenic type III exhibit microvilli.* (*b*) *For the type II, only the cell in the center displays innumerable microvilli, whereas the other cells exhibit only very few.* × 1000.

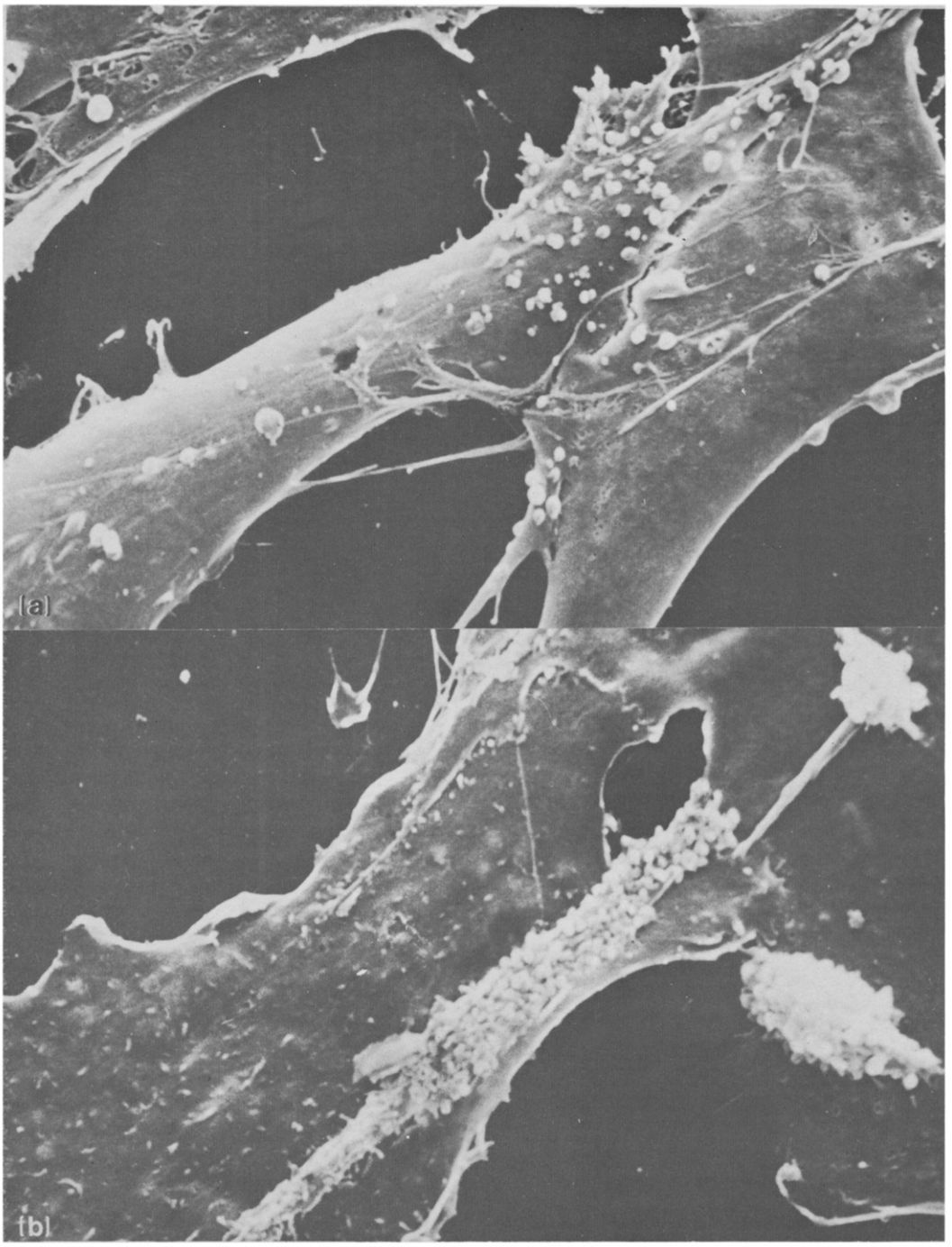

Fig. 6 (*a and b*). *Typical blebbing feature of different cell types.* (*a*) *Non-transformed* $10T\frac{1}{2}$ *cells.* (*b*) *Type* I *passage* 31.

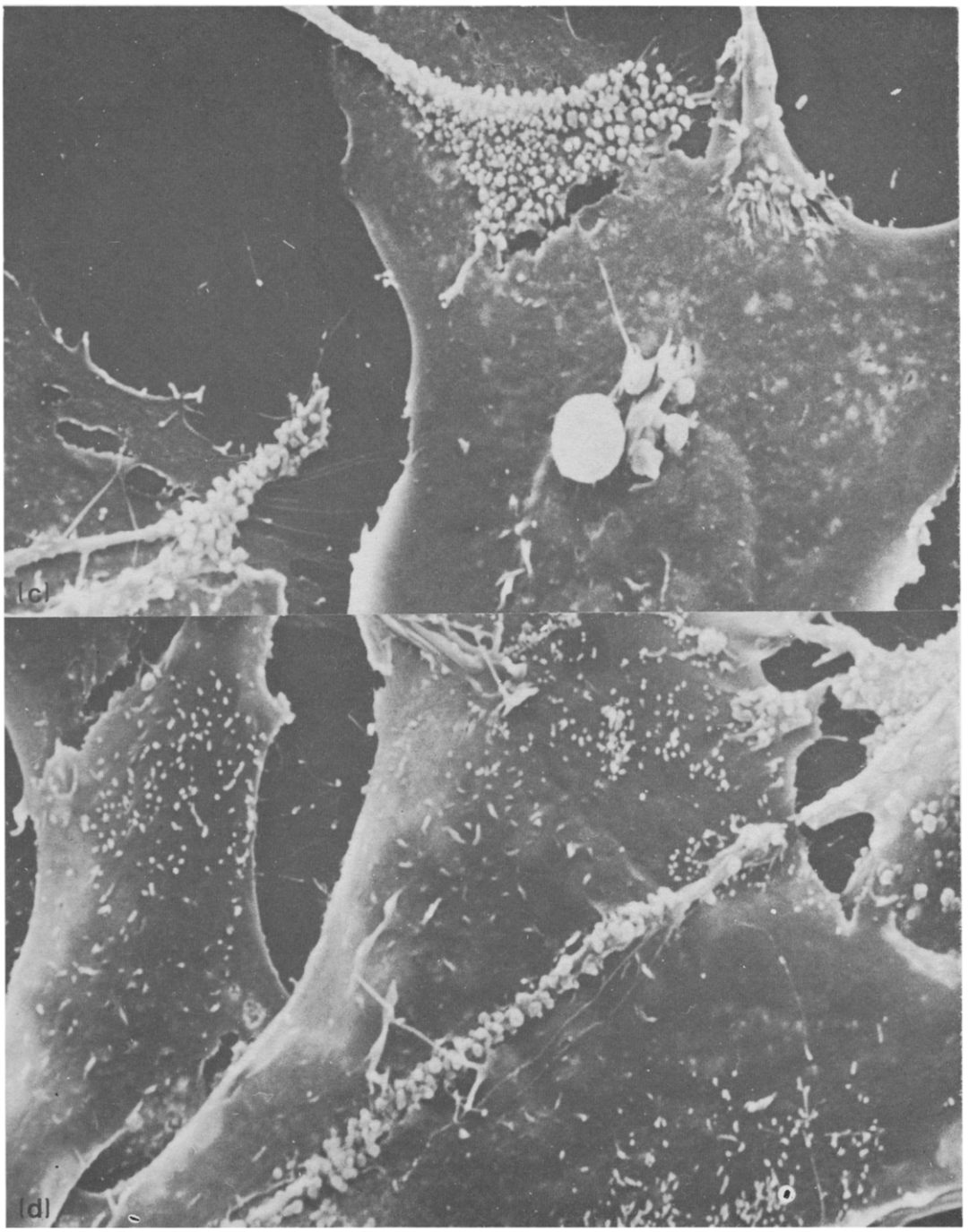

Fig. 6 (c and d). Type III passage 31. × 1500.

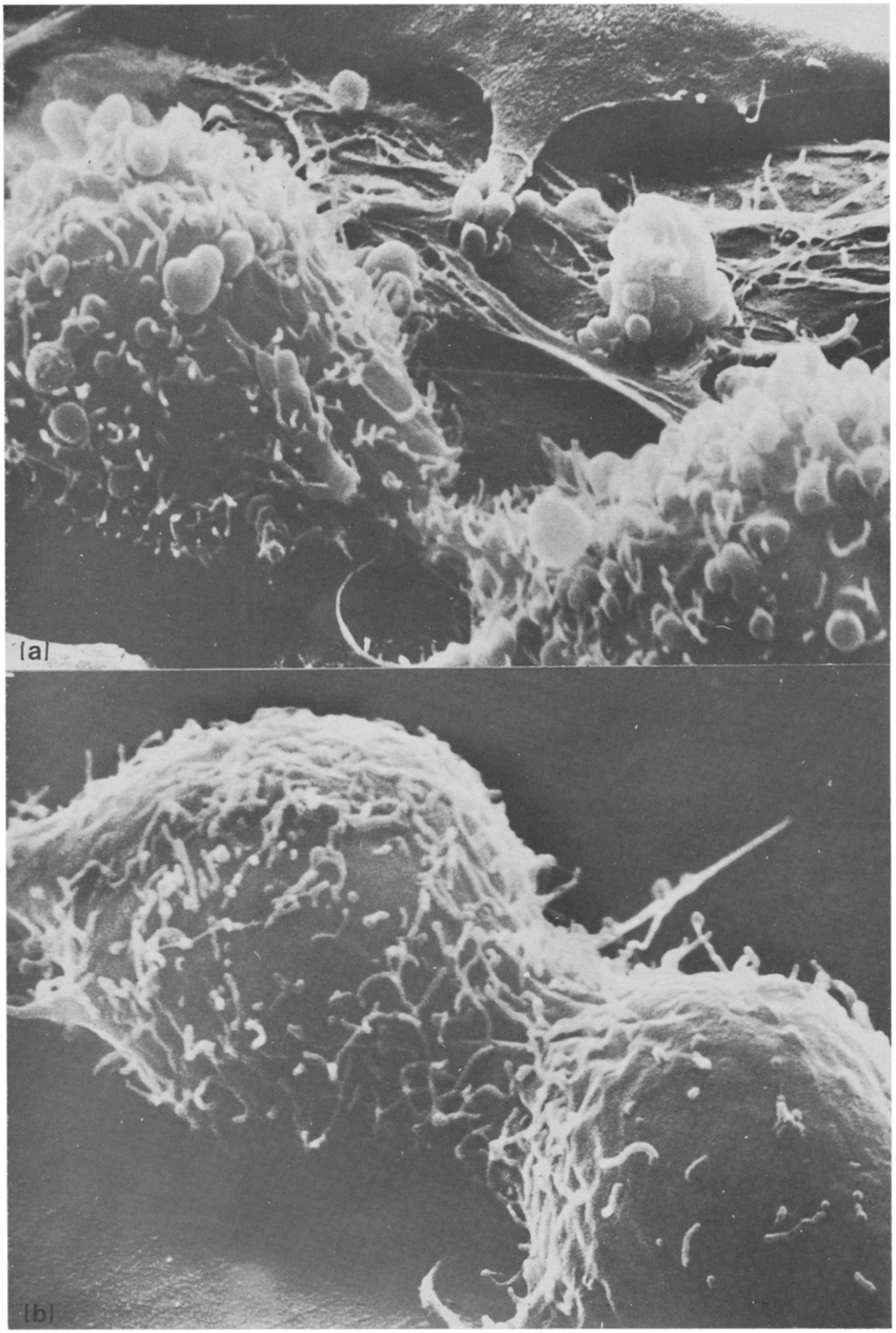

Fig. 7. Mitosis in transformed and non-transformed cells. (*a*) *Type* III *cells, passage* 61, *exhibit microvilli and blebbing activity on both daughter cells.* (*b*) *Non-transformed cells have a similar surface although only cells exhibiting microvilli are demonstrated here.* × 8300.

Table 1. *Amount, appearance and distribution of microvilli on C3H/10T½ cells and on DMBA transformed types* I, II *and* III *cells*

Cells	Passage	Oncogenic potential	Amount of microvilli				Appearance of short/long microvilli			Distribution of microvilli total cell/nuclear region		
			+++	++	+	−	+++	++	+	+++	++	+
C3H/10T½	10	no	1	1	8	40	1/0	1/0	8/0	0/1	0/1	1/7
	20	no	0	1	12	37	0/0	1/0	12/0	0/0	1/0	10/2
Type I	11	no	0	1	9	40	0/0	1/0	9/0	0/0	1/0	1/8
	14	no	0	5	20	25	0/0	5/0	20/0	0/0	3/2	12/8
	32	no	21	16	12	1	21/0	16/0	12/0	21/0	16/0	5/7
Type II	6	no	7	12	15	16	3/4	8/4	15/0	7/0	11/1	9/6
	17	no	3	16	26	5	0/3	12/4	26/0	2/1	9/7	16/10
	32	yes	15	18	17	0	3/12	7/11	17/0	13/2	12/6	8/9
Type III	10	no	2	18	29	1	2/0	16/2	29/0	2/0	18/0	26/3
	31	yes	28	14	8	0	15/13	10/4	8/0	25/3	12/2	3/5
	65	yes	26	18	6	0	0/26	3/15	2/4	26/0	18/1	6/1

The C3H/10T½ cells and the DMBA transformed types I, II and III cells were prepared for scanning electron microscopy from the subconfluent culture in late logarithmic phase of growth (Methods). The oncogenic potential of these cells had been determined earlier [2]. The amount and appearance of microvilli were quantitatively evaluated on 50 strictly randomly chosen cells at the various culture passages for the different cell types. The concentration of microvilli is described as none −, few +, many + + (still possible to count) and innumerable + + + (counting no longer possible). As to their appearance, the ratio between the number of cells that display short and the number of cells that display long microvilli, is shown in each of the three categories. The distribution over the total cell or the nuclear region only is shown for the three concentration categories.

Table 2. Amount of blebs on C3H/10T½ and on DMBA transformed types I, II *and* III *cells*

Cells	Passage	Amount of blebs on the cells +++	++	+	−
C3H/10T½	10	0	0	11	39
	20	0	1	13	36
	11	0	1	20	29
Type I	14	1	9	20	20
	32	1	1	9	39
	6	1	3	14	32
Type II	17	1	2	13	34
	32	0	0	41	9
	10	0	2	6	42
Type III	31	2	18	14	16
	65	10	12	8	20

The C3H/10T½ cells and the DMBA transformed types I, II and III cells were prepared as above. The amount of blebs was quantitatively evaluated in each group of 50 strictly randomly chosen cells at the various culture passages for the different cell types. The concentration of blebs is described as for the microvilli (legend Table 1).

sporadically on certain areas of the cell surface (Fig. 6). No clear-cut pattern of bleb distribution could be established, except that all the transformed cells had significantly more blebs than the non-transformed cells. During mitosis microvilli and blebs were observed in the non-transformed as well as in all the transformed cell types (Fig. 7a and b).

DISCUSSION

Our study has shown that in chemically transformed fibroblast cells there is a positive correlation between a high concentration of long microvilli and the oncogenic potential. It is too early to say whether this is a general phenomenon. We suggest that this correlation may be useful in scoring for oncogenic transformation of cells in culture.

Whether the long microvilli may be related to the ability to infiltrate and metastasize, which are the ultimate malignant criteria, is too early to say.

Whereas studies by Porter [5] concerned spontaneously and virally transformed Balb/3T3 cells, Malick and Langenbach [6] reported on the membrane morphology of chemically transformed culture cells. Both types of transformation displayed general similarities, such as microvilli, blebs and ruffles. These surface structures were also found upon transformation of hamster embryo cells in culture by X-irradiation [7]. Some studies [6] related microvilli and marginal ruffles to transformation by methylcholanthrene (MCA) and blebs together with cytoplasmic strands to transformation by DMBA.

Thus, the relationship between cancer cells and an increased number of microvilli was described [5–7], but no distinction between short and long microvilli was made. Our study shows that the development of the oncogenic potential is related to the development and concentration of the long microvilli. High number of short microvilli only, as seen in the type I cells, was not correlated to oncogenic potential.

Hamster embryo fibroblasts in culture have few cells with unevenly distributed microvilli [8] of variable length. However, virally transformed hamster fibroblasts showed a larger number of microvilli [8], but some evidence [9] has also demonstrated the lack of microvilli in SV-40 transformed fibroblasts. For BALB/3T3 cells a tumorigenic transformant of spontaneous origin developed a large number of microvilli and blebs [5]. MCA-transformed C3H/10T½ cells displayed microvilli of variable length, whereas the DMBA-transformed cells were characterized by blebs and so-called cytoplasmic strands [6]. In accordance with our finding of an increase in microvilli concentration of DMBA-transformed cells as a function of passage in culture, Malick and Langenbach [6] observed an increase in the number of microvilli after inoculation and establishment of cells from the resulting fibrosarcomas in culture. However, they did not report an increase in the microvilli concentration of transformed cells carried in culture only.

When investigating the role of surface morphology in agglutination by Con A, it was found that for surface cultures 4 of 10 investigated cell lines which had a high agglutinability were also characterized by many microvilli [10].

Neither the mechanism of the formation of microvilli nor their biological significance is well understood at this time. It is thought that they may be connected with nutrition as they are more frequent on free surfaces of cells and increase the surface area substantially [11]. Microvilli and blebs have also been reported to be membrane storage sites during mitosis [3]. Microvilli are not only found on cells specialized for absorption, e.g., intestinal or liver cells, but also observed in cells with secretory activity, e.g., parietal cells in the stomach, and in sensory cells. This indicates that microvilli may not function identically

and that their membranes may have molecular specializations for special functions [12]. It has been reported for rapidly proliferating cells and for transformed cells which have a high amount of microvilli and a low concentration of dibutyryl (3′5′) cyclic adenosine monophosphate (cAMP), that increasing cAMP leads to a decrease or disappearance of microvilli [13]. This observation has been confirmed for SV-3T3 cells and VLM cells [14].

It has been shown that microvilli formation was an early surface change of the urinary bladder epithelium in rats, mice and hamsters, but not in guinea pigs, following the administration of *N*-butyl-*N*(4-hydroxybutyl)nitrosamine [15].

Acknowledgements—The authors want to express gratitude to Mrs. Gunhild Aker for excellent work with the cell cultures and to Mrs. Ruth Puntervold for excellent work with the scanning electron microscopy. We especially want to thank Dr. Sigvald B. Refsum and Dr. Olav H. Iversen for stimulating interest. Part of the study was presented at the 69th Annual Meeting of the American Association for Cancer Research, Washington D.C., April 1978.

REFERENCES

1. C. A. Reznikoff, D. W. Brankow and C. Heidelberger, Establishment and characterization of a clonal line of C3H mouse embryo cells sensitive to postconfluence inhibition of division. *Cancer Res.* **33,** 3231 (1973).
2. H. J. K. Saxholm, The oncogenic potential of three different 7,12-dimethylbenz(a)anthracene transformed C3H/10T½ cell clones at various passages and the importance of the mode of immunosuppression. *Europ. J. Cancer* (In Press).
3. L. E. Allred and K. R. Porter, Cell morphology and the cell cycle. In *Growth Kinetics and Biochemical Regulation of Normal and Malignant Cells.* (Edited by B. Drewinko and R. M. Humphrey) p. 295–315. Williams and Wilkins. Baltimore (1977).
4. T. F. Anderson, Techniques for the preservation of the 3-dimensional structure in preparing specimens for the electron microscope. *Trans. N.Y. Acad. Sciences* **13,** 130 (1951).
5. K. R. Porter, G. J. Todaro and V. Fonte, A scanning electron microscope study of surface features of viral and spontaneous transformants of mouse BALB/33 cells. *J. Cell Biol.* **59,** 633 (1973).
6. L. E. Malick and R. Langenbach, Scanning electron microscopy of *in vitro* chemically transformed mouse embryo cells. *J. Cell Biol.* **68,** 654 (1976).
7. C. Borek and C. M. Fenoglio, Scanning electron microscopy of surface features of hamster embryo cells transformed *in vitro* by X-irradiation. *Cancer Res.* **36,** 1325 (1976).
8. R. Glaser, V. Mumaw, R. Farrugia and B. Munger, Scanning electron microscopy of the surfaces of hamster embryo cells transformed by herpes simplex virus. *Cancer Res.* **37,** 4420 (1977).
9. N. G. Collard and J. H. M. Temmink, Surface morphology and agglutinability with concanavalin A in normal and transformed murine fibroblasts. *J. Cell Biol.* **68,** 101 (1976).
10. T. C. Wright, T. E. Ukena and M. J. Karnovsky, Relation of lectin-induced and spontaneous adhesion to tumorigenicity, In *Membrane Anomalies of Tumor Cells, Progress in Experimental Tumor Research.* (Edited by D. F. H. Wallach). Vol. 22, S. Karger, Basel (1978).
11. E. J. Ambrose and D. M. Easty, *Cell Biology.* Nelson, London, (1970).
12. H. S. Bennett, The cell surface: components and configurations. In *Handbook of Molecular Cytology.* (Edited by A. Lima de Faria). North Holland, Amsterdam. (1969).
13. J. Otten, G. S. Johnson and I. Pastan, Cyclic AMP levels in fibroblasts: relationship to growth rate and contact inhibition of growth. *Biochem. biophys. Res. Commun.* **44,** 1192 (1971).
14. J. Z. Borysenko, T. E. Ukena and M. J. Karnovsky, Effects of db-cAMP and theophylline in concanavalin A binding site distribution on transformed and protease-treated cell lines.*Expt. Cell Res.* **107,** 253 (1977).
15. T. Shirai, G. Murasaki, M. Tatematsu, H. Tsuda, S. Fukushima and N. Ito, Early surface changes in the urinary bladder epithelium of different animal species induced by *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine, *Gann* **68,** 203 (1977).