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# Human Prostatic Carcinoma in Cell Culture: Preliminary Report on the Development and Characterization of an Epithelial Cell Line (EB 33)*

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Summary. The permanent epithelial cell-line EB 33 was developed from a human prostatic carcinoma. First attempts were made to characterize this strain by functional, morphological and kinetic parameters. The doubling time was found to be quite slow and to remain constant at 50.0 h during the exponential phase of growth over many passages. High acid phosphatase activity in the cytoplasm was found by histochemical means in comparison to HeLa cells. Electron microscopic studies suggested the epithelial origin of the cell-line. The karyotype was near triploid. Successful heterotransplantation into "nude mice" was achieved reproducibly. Histological examination of the heterotransplants revealed solid epithelial tumors with a rapid rate of growth. The findings reported suggest the prostatic epithelial origin of the cell-line EB 33. Their origin from the carcinomatous part of the explanted tissue remains still unproven. Future aspects for the development of an experimental model for human prostatic carcinoma are discussed.

Key words: Prostatic carcinoma, permanent cell line, heterotransplantation, chromosome analysis, electron microscopy.

## Introduction

Human prostatic adenoma and carcinoma have been subject to in vitro investigation by the second author and his associates for more than four years. During this period 73 adenomas and 38 carcinomas have been cultured with the ultimate goal of creating a model for further investigation of this puzzling tumor, namely of its hormone dependence. Knowing well the limitations of cell-culture techniques in the exploration of human neoplasms functional characterization in vitro has always been strongly emphasized. It has been shown in the past that a high level of acid phosphatase activity and its responsiveness to androgen withdrawal is a useful marker and allows identification of prostatic epithelial cells in culture.

Previous reports on this subject (7, 8) also included a technique for initiation of primary cultures, the characterization of cells in culture as epithelial cells of prostatic origin by means of organ culture and histochemical techniques, and the development and use of an androgen-free tissue-culture medium.

In the present study preliminary results on the development of the first permanent epithelial cell-line from human prostatic adenocarcinoma and on the characterization of the cells by means of growth kinetics, histochemical determination of acid phosphatase activity, electron microscopy, heterotransplantation and karyotyping are presented.

## Materials and Methods

On June 4, 1973 the 63 year old patient J. R. underwent total perineal prostatectomy after a needle biopsy of the prostate had shown a moderately differentiated adenocarcinoma of the prostate.

The tumor had two different histological patterns as can be seen in Fig. 1. Fig. 1a shows an adenocarcinoma forming large glands with quite well differentiated cells and a small amount of nuclear variation. Fig. 1b represents the less differentiated part of the carcinoma with cells growing in solid cords inbetween the muscular bundles.

The palpable nodule of carcinoma in the surgical specimen measured about 1.5 cm in diameter. The tumor had not infiltrated the seminal vesicles or the fibrous prostatic capsule.

Primary culture. This tumor was the 33rd human carcinoma of the prostate cultured in this laboratory and was named EB 33. Primary culture was initiated on the day of operation. A large piece of tumor (about 3 g) was minced with iridectomy scissors as fine as possible under sterile conditions in

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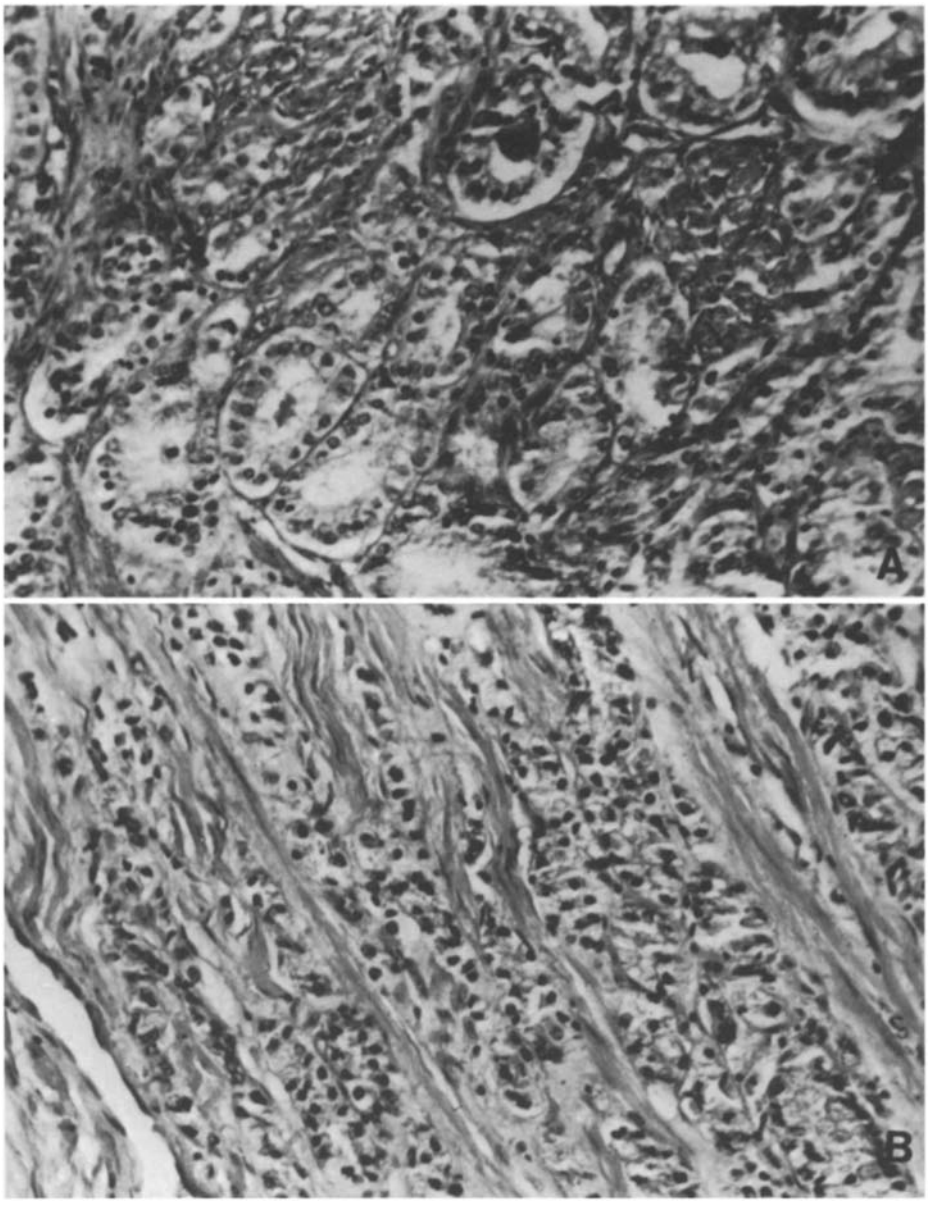


Fig. 1. Histology of surgical prostatic specimen, patient J.R., total prostatectomy on June 4, 1973, Urologische Universitätsklinik Würzburg. A) Adenocarcinoma with large glands and a slight amount of nuclear variation. B) Solid part of the tumor with cells infiltrating the musculature in cords and sheets

a laminar flow sterile working bench. The tissue was then plated in large Falcon plastic petri dishes (ordering number 3007) and suspended in 5 ml of Ham's F 10 tissue culture medium with 12.5% horse serum (HS) and 2.5% fetal calf serum (FCS). The cultures were incubated at 37 °C in an atmosphere of air and $CO_2$. The pH in the incubator was maintained between 7.2 and 7.4.

Subcultures and growth kinetics. Subculturing is achieved by treatment of the cultures with 0.1% viokase in a calcium- and magnesium-free phosphate buffer solution. Incubation time varied between 3 and 10 min.

Hemocytometer counts are carried out at the time of each subculture, and the total number of cells per plate is calculated. Two stock-bottles (Falcon 3024) are always prepared and used for continuous growth curves. These cultures are started with an inoculate of about $10^5$ cells in 15 ml of medium with testosterone 1.0 µg/ml in 0.3% ETOH.

For growth curves with daily counts the small Falcon petri dishes are used (ordering number 3002). $2.0 \times 10^4$ cells are usually plated and fed with 5 ml of medium. Two plates are subcultured every day and four hemocytometer counts are obtained from each plate and plotted on semi-logarithmic paper.

As a control of hemocytometer counting several

fields of the size of a microscopic low power field are always marked on the bottom of the dishes with a self-made branding iron. Daily counts in 3 such fields correlate well with the number of cells found in the hemocytometer if there are no pipetting mistakes. This useful and time-saving control will naturally only indicate the proportion of growth and will not produce absolute counts.

Histochemical determination of acid phosphatase. For histochemical determination of acid phosphatase confluent cultures are fixed with citrate buffered acetone and stained according to the Sigma technique (12) with naphthol As-MX (p-nitrophenyl phosphate) as substrate and Fast Blue RR as the coupling dye.

The incubation time was always 3 h. HeLa cells and human fibroblasts were used as controls.

Electron microscopy. For electron microscopic examination several confluent cultures of the 5th passage of EB 33 were fixed with a mixture of $OsO_4$ (1.5%) and glutaraldehyde (2.5%) in 0.075 M cacodylate buffer, pH 7.4 for 30 min, dehydrated and imbedded in Epon. Thin sections cut parallel to the bottom of the petri dish were examined with the Siemens Elmiskop IA.

Heterotransplantation. Successful heterotransplantation of EB 33 cells into 2-3 weeks old male or female "nude mice" (2, 5) was achieved by subcutaneous injection of $10^5$ to $10^6$ cells. "Nude mice" are bred in this laboratory quite successfully with the precautions of sterile cages, low fat diet, sterile acidified drinking water and early separation of hairless animals from the haired mates. A laminar flow sterile cage rack may be used but does not seem to be essential for successful breeding. 24 h prior to transplantation the animals are conditioned with 25 mg of testosterone enanthate subcutaneously.

Karyotyping. The karyotype of the EB 33 strain was first determined in the 7th subculture. The cultures were incubated with 0.05 ug/ml medium of Vinblastin sulfate for 6-10 h. Metaphase cells were harvested and treated according to a standard protocol (3). Giemsa stains were carried out. Microphotography was then done with oil immersion and phase contrast in monochromatic light using the Zeiss Photomicroscope II. Chromosome counts were obtained from 31 different cells.

## Results

The development of the strain EB 33. Primary cultures of the moderately differentiated human prostatic adeno-carcinoma EB 33 were established on the 4th of June, 1973. One week later it was noticeable with the naked eye that several pieces of tissue in each plate became round and smooth whereas others remained unchanged. Some pieces were stuck to the bottom of the petri dish. On the 10th day outgrowth of cells was observed through the phase contrast microscope in 8 of 13 plates. Morphologically the cells were not different from any of the earlier primary cultures of human prostatic carcinoma tissue. However, during the following days the colonies grew unusually large and in some plates developed a confluent monolayer. On the 16th

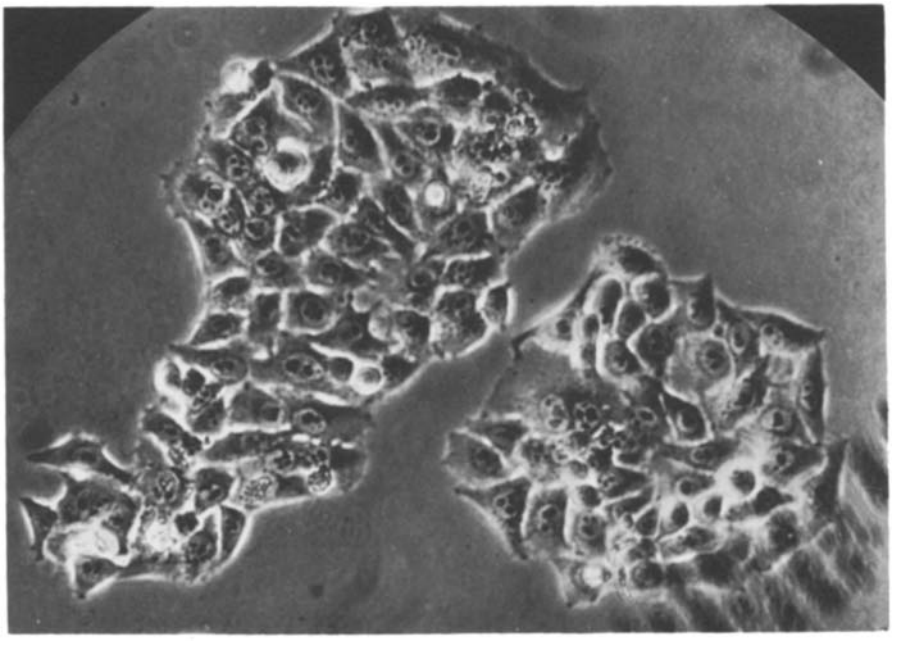

Fig. 2. Phase contrast photomicrograph of EB 33 cells, 4th passage. Cells are polygonal in shape and show varying numbers of prominent nucleoli

day $1.5 \times 10^4$ cells were obtained and subcultured into one small plate with 5 ml of medium containing 1.0 µg of testosterone per ml. Some small pieces of tissue were allowed to slip into the subculture. Continuous exponential growth resulted. Weekly subcultures were done and cells were frozen away in liquid nitrogen.

Morphologically, as observed with the phase-contrast microscope, only one epithelial-like cell type was identifiable as shown in Fig. 2. The cells are polygonal in shape and usually have up to four quite prominent nucleoli. They are not different from many earlier subcultures of other prostatic carcinomas.

The further development is illustrated in chart 1 which shows the continous growth curve obtained from the stock bottles up to the 18th passage. Each plotted figure represents the average of two hemocytometer counts. The identical slope of almost all curves indicates a constant rate of growth from the very beginning up to now. The remaining plates of EB 33 primary cultures were subcultured two days later on the 18th day in culture. It is quite remarkable that in spite of identical growth and subculture conditions these cells did not grow but died within several weeks. This suggests that the time of subculture of primary cultures of human prostatic carcinoma may be of great importance for continuous growth.

Growth kinetics of EB 33. The growth curve shown in chart 2 allows a more detailed analysis of the growth of cells of the 17th passage of EB 33. Daily counts in 2 separate plates were done. HeLa cells served as a control. $2 \times 10^4$ cells were plated in each of 40 small dishes. After an initial lag phase all cells enter the phase of exponential growth. It is evident that EB 33 cells from the 17th passage grow much slower than HeLa cells. The doubling time from the 3rd day on was calculated to be 50.0 h for the EB 33 cells and 8.9 h for the HeLa cells. The HeLa cells reached saturation at a density of $8.2 \times 10^5$ cells per plate on the 7th day. EB 33 was still growing in a logarithmic way on the $11^{th}$ day after subculture. Saturation density could not be determined from this experiment but was higher than $2 \times 10^5$ cells per plate.

Chart 3 shows the results of a growth experiment that determines saturation densities of the 20th passage of EB 33 cells for 3 different plating densities. ($10^3$, $10^4$, $10^5$ cells per small plate). The diagram shows that saturation density is highest with $10^4$ cells plated and reaches $8.97 \times 10^5$ cells

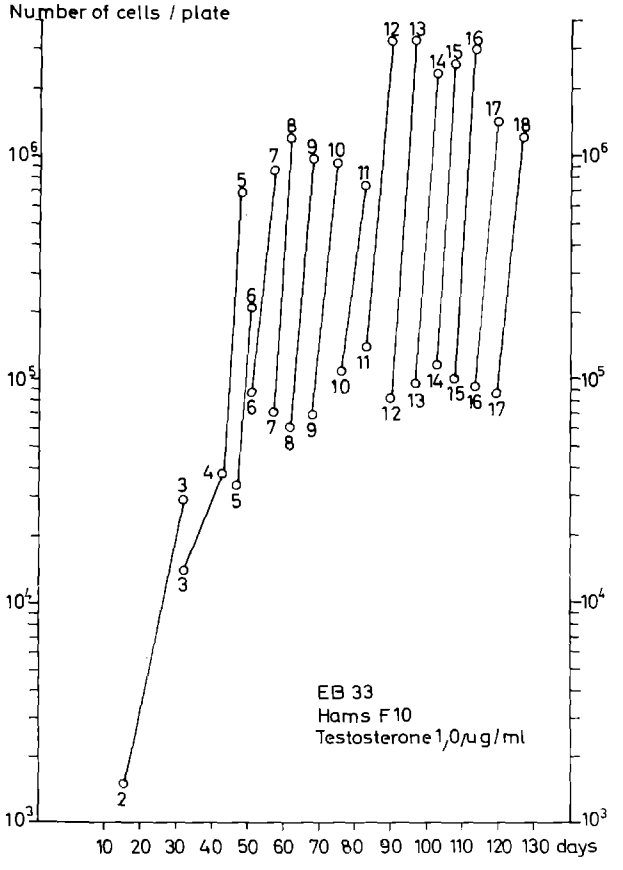


Chart 1. Continuous growth curve of the epithelial prostatic cell-line EB 33 from passage 2-18

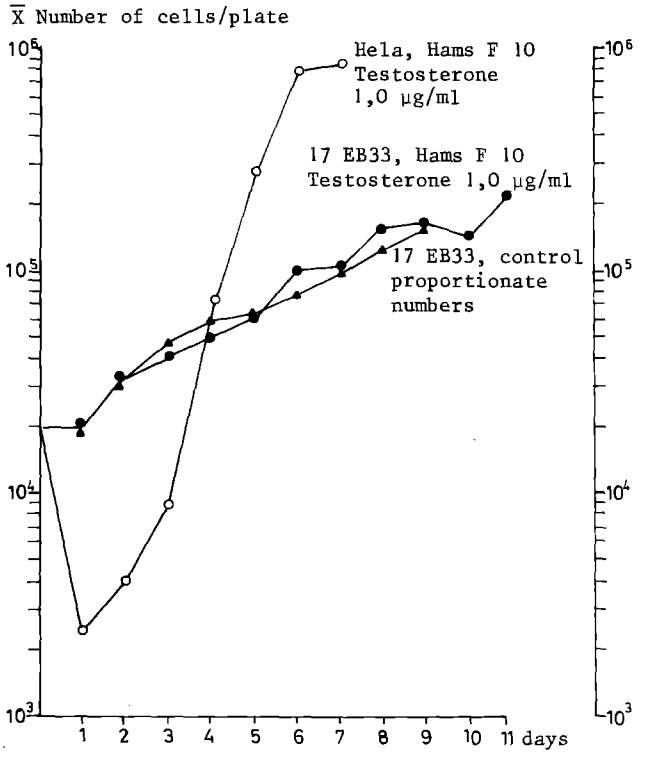


Chart 2. Growth curve of EB 33 cells and HeLa cells illustrating the lesser rate of growth of the EB 33 cells. A doubling time of 50.0 h can be calculated for the prostatic cells

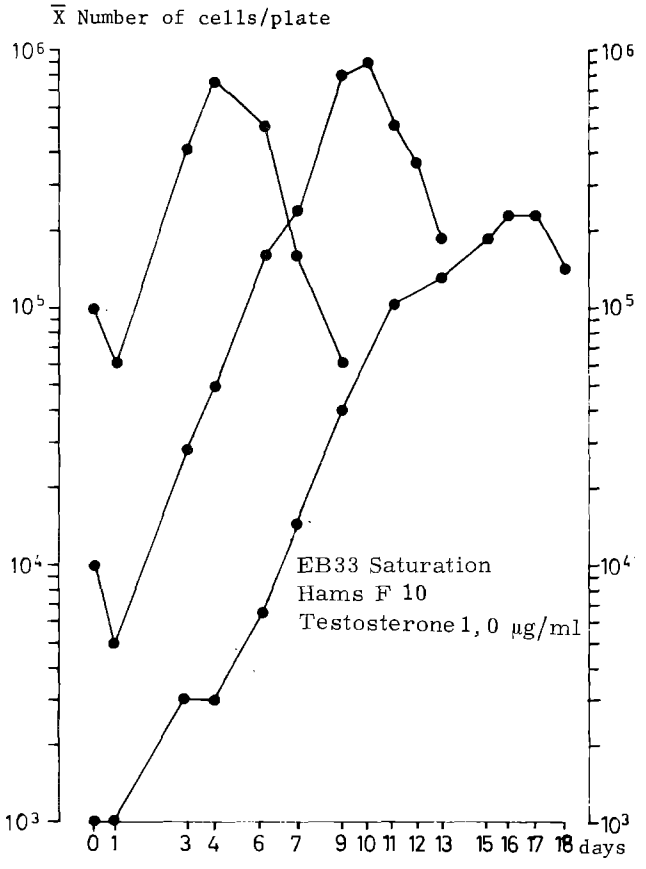


Chart 3. Time and density of saturation in relation to plating densitiy. The highest saturation density is reached after 10 days when $10^4$ cells are plated

after 10 days. The diagram is otherwise self-explanatory.

Histochemical staining of acid phosphatase. The amount of acid phosphatase present in epithelial-like cells from prostatic adenoma and carcinoma has previously served for their characterization as cells of epithelial prostatic origin (12). For gross visualization and comparison of acid phosphatase the same histochemical technique has been used on confluent cultures of EB 33 and HeLa cells for comparison. The blue stain indicative for the enzyme activity was much darker in cultures of EB 33, however marked variation was observed within the same cell population. Biochemical studies and experiments on inducibility of acid phosphatase by withdrawal and addition of androgens as previously described for primary and secondary cultures of non permanent cells were not yet carried out and will be subject to a separate publication.

The results obtained with EB 33 cells indicate that these cells contain much more acid phosphatase than other permanent strains. This finding may confirm the origin of EB 33 cells from prostatic epithelial cells, not necessarily from cancer cells.

Electron microscopy of EB 33. Electron microscopic examination of cells from the 5th passage of EB 33 was undertaken.

The cells show prominent nucleoli, and several areas of condensed chromatin within the nuclei (Fig. 3). The cytoplasm contains many free ribosomes and a few rough cisternas of endoplasmic reticulum. Glycogen is found mostly in its monoparticular form but sometimes it is aggregated to smaller or larger fields. Dense bodies (lysosomes) of different size occur preferentially close to the Golgi-fields. The surface of the cells often shows villous-like projections (Fig. 4) suggesting the epithelial origin of the cells.

Heterotransplantation. Heterotransplantation of EB 33 cells into nude mice was to date attempted 16 times. 12 of the animals were female, 4 were male. 3 transplants did not take in 2 females and one male animal. This results in a failure rate of only 18.8%. The failures remain unexplained. Nodules of tumor were first noticeable between 6 and 26 days after transplantation with an average of 2-3 weeks.

In Fig. 5 the nude mouse number 8 is shown 41 days after injection of $10^6$ EB 33 cells subcutaneously on her back and on her right side. Two large tumors have grown. They were first palpable 12 days after transplantation.

Most tumors were harvested for repassaging in cell culture and for histological examination. Distant metastases were never observed at autopsy. The longest time of survival with a tumor transplant was 127 days.

The histological picture of the transplanted tumors is quite uniform and differs greatly from the original surgical specimen. As can be seen in Fig. 6 a and b the transplanted tumor is a solid carcinoma with many mitoses indicating a rapid rate of growth.

Chromosome analysis. Chromosome counts revealed that most cells of the 7th passage of EB 33 had 65 chromosomes. Table 1 shows the results of 31 counts done on fotographs of 31 different cells. Fig. 7 gives an example of an analysis of a cell of 7 EB 33 with 67 chromosomes. It can be seen that all chromosomes except A 1, the largest somatosome are tripled. This makes the karyotype near triploid.

## Discussion

There is no question about the urgent necessity of an experimental model for the study of human prostatic carcinoma as it is the second most frequent neoplasm in men in the older age groups.

In the past the usefulness of studying any human carcinoma in cell culture has been seriously doubted. Unfortunately most of the well studied perma-

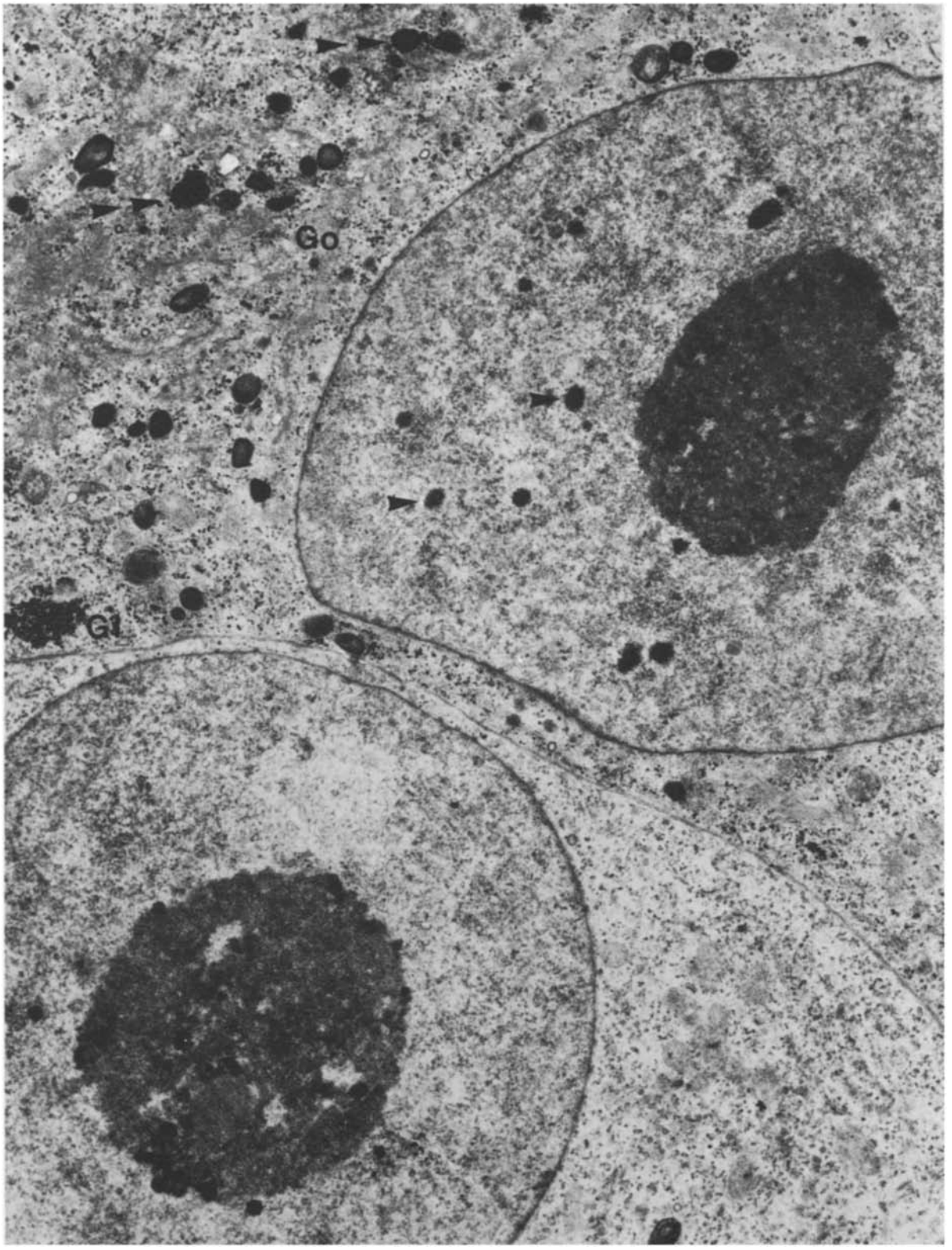


Fig. 3. Electron photomicrograph of EB 33 cells, 5th passage, magnification of original x 7000. Two adjacent cells and in one case condensed areas of chromatin ▶
Dense bodies are found in the upper cell ▶ ▶
close to the Golgi fields (Go). A small field of glycogen (Gl) is seen

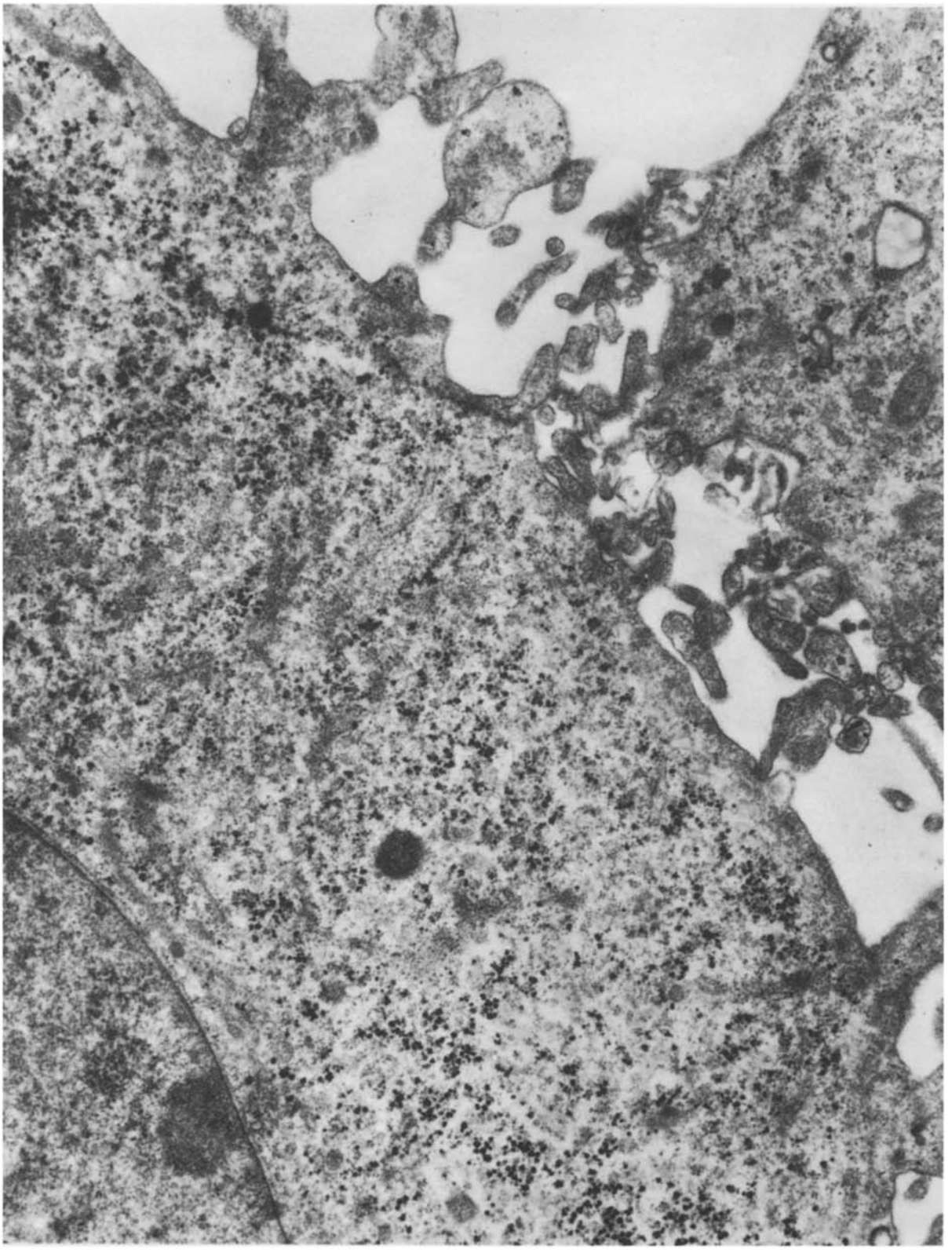

Fig. 4. Electron photomicrograph of EB 33 cells, 5th passage, magnification of original x 27 000. The free surface of a cell showing villous-like projections. Many glycogen particles are scattered throughout the cytoplasm

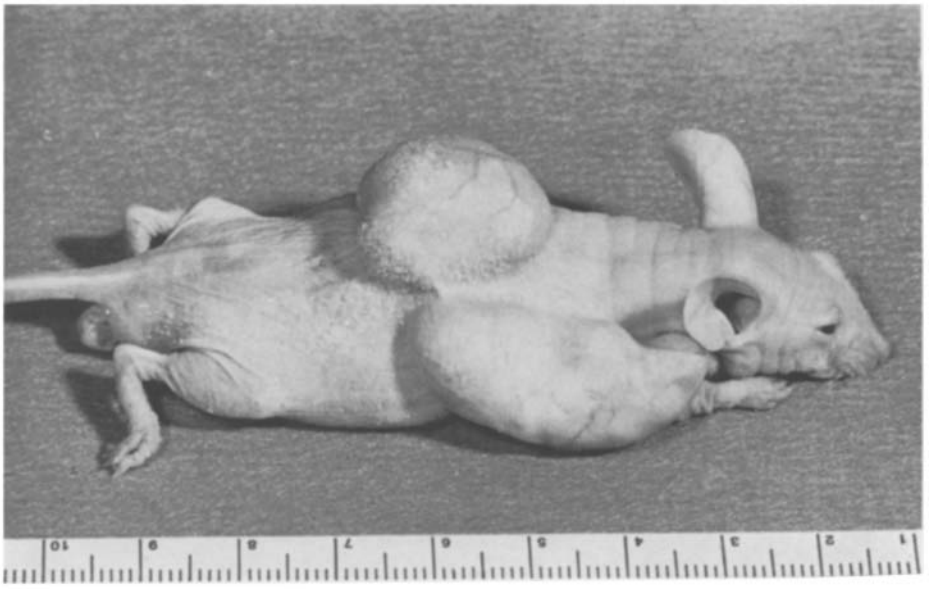

Fig. 5. Nude mouse No. 8, 8 weeks old, 6 weeks after transplantation of $1.0 \times 10^6$ EB 33 cells subcutaneously. Two large tumors have grown and were first palpable after 6 days

nent strains of tumor cells have lost the functional and immunological characteristics of their tissues of origin. Profound changes in ploidy occur. Lieberman and Ove (4) found a striking similarity of the enzyme pattern of 4 different permanent strains of tumor cells. Growth kinetics undergo fundamental changes in vitro. The term "dedifferentiation" is commonly used for these alterations.

On the other hand examples of maintenance of differentiated function for varying periods of time are not infrequent even in the older literature. Sato and his co-workers were able to explain and limit the phenomenon of dedifferentiation by their concept of "selective overgrowth". They demonstrated by immunological techniques that most cells in primary cultures of one day old rat-livers were not parenchymal cells, and that lack of identifiable functional activity was due to overgrowth of non parenchymal cell elements (9). This does not preclude the possibility of later loss of function of the few parenchymal cells present in their cultures. Sato deduced that biological selection of parenchymal cells by culture techniques or by animal passages is necessary and will lead to the maintenance or recovery of differentiated function in cell culture under proper culture conditions. This concept has proven to be true, and its application to hormone producing or endocrine dependent animal tumors has resulted in a number of cell-strains that remained functional indefinitely (1, 10).

These concepts must be applied to the study of human tumors in tissue culture as well. The well-known process of dedifferentiation and overgrowth will otherwise lead to erroneous results and conclusions that may reflect a particular cell culture set up correctly but will have nothing in common with the original tumor. Valuable results will only be obtained if functional characteristics can be established that will identify tumor cells in vivo and in vitro reproducibly.

In the past it has been difficult to apply all of these concepts to our cultures of human prostatic cancer. The large amount of acid phosphatase activity in the epithelial cells of primary and secondary cultures, and its reducibility in an androgen-free enviroment has been useful for their identification and characterization. However the lack of permanent growth and the impossibility of repassaging the cells through the original host or through a laboratory animal prevented the use of the most efficient techniques for selection of functionally active tumor cells. Therefore the development of the first permanent strain of epithelial cells from a human prostatic adenocarcinoma and the solution of the transplantation problem by the use of nude mice seem to present a major break-through for further exploration of this tumor.

It remains unexplained to us why 32 human prostatic carcinomas did not grow permanently as the tumor EB 33 did. The experience with EB 33 may suggest that the time of the first subculture is critical. Furthermore the phenomenon of heavy initial growth in the primary culture seems to be a property of some individual tumors.

The growth kinetics of EB 33 are compared to HeLa cells grown in the same medium as a control. The unusually long doubling time of 50.0 h of the line EB 33, as compared to 8.9 h for the HeLa cells, may suggest, that the latter have reached a state of much more marked dedifferentiation. Saturation densities are in the same range for both strains.

The kinetic studies presented are the baseline

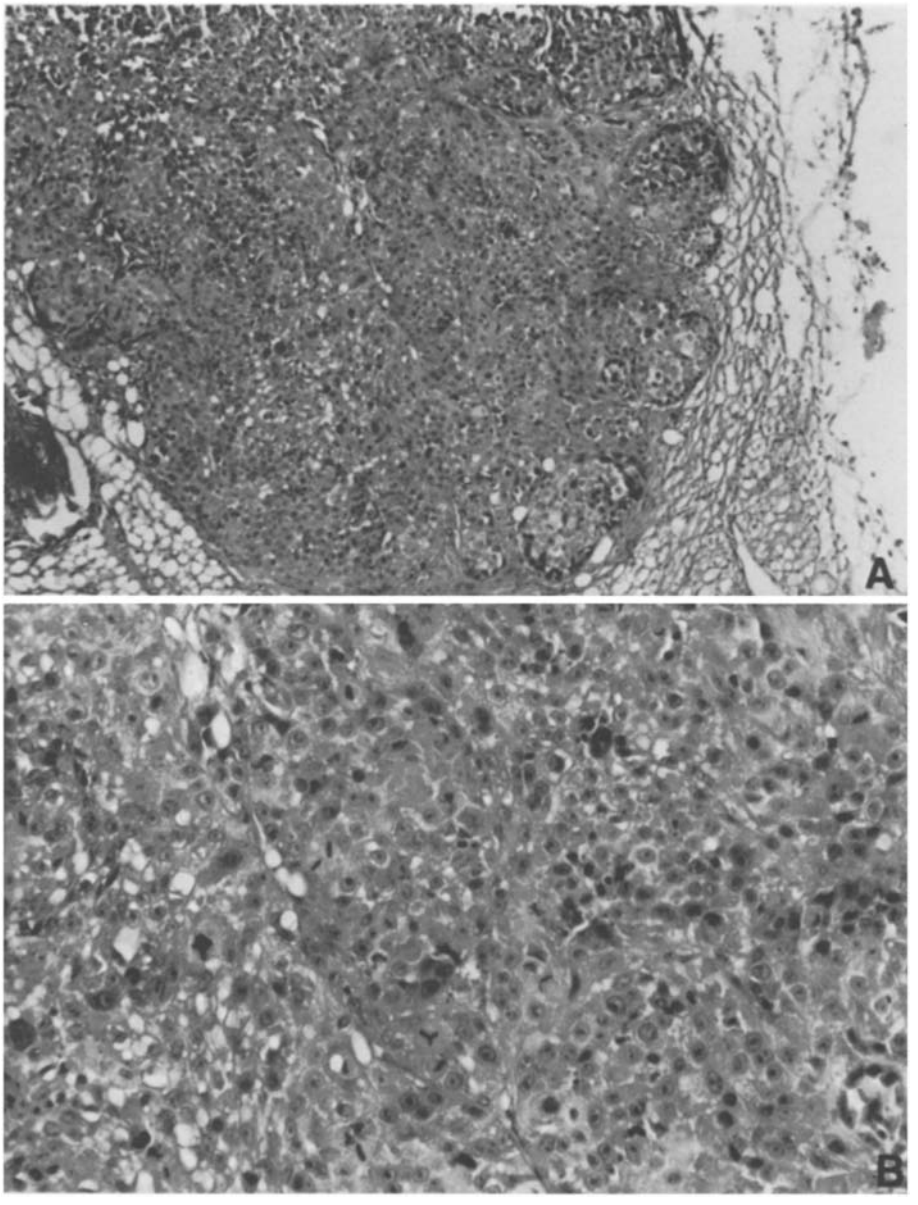


Fig. 6. Histology of a tumor grown from EB 33 cells in nude mouse No. 8. A) Low power magnification showing nodular pattern of growth and surrounding connective Tissue. B) High power magnification showing cellular details of the solid carcinoma. Many mitoses can be seen

for a series of growth experiments in various hormonally defined media. First results indicate the possibility of the presence of an androgen dependent sub-population. Cloning experiments and selection experiments with BUdR according to the technique described by Puck et al. (6), and modified by Sato et al. (11) are also in progress. The results will be the subject of forthcoming publications. The goal of these studies is the isolation of androgen dependent sublines that may help to clarify the complex endocrinological behaviour of this tumor.

Electron microscopy, histochemical determination of acid phosphatase activity and chromosomal analysis are continously used as parameters for changes that may occur during the current experiments. Their detailed discussion is deferred until more material is accumulated. It may be mentionned that to our knowledge this is the first complete chromosomal analysis of cells from human prostatic cancer ever published.

The microscopic morphology of the EB 33 cells, electron microscopy, their high acid phosphatase activity and the epithelial tumors growing in nude mice after transplantation prove the origin of the strain from prostatic epithelium.

Additional evidence must be presented to prove the origin of EB 33 from the carcinomatous part of the surgical specimen plated. The initial lag phase of growth in primary cultures of prostatic adenoma may last up to 12 days. As organ culture experi-

Table 1. Chromosome counts of 31 cells of the 7th passage of EB 33 cells ranging from 60 to 67 with a modal value of 65

| No. of cells | 60 | 61 | 62 | 63 | 64 | 65 | 66 | 67 |
|---|---|---|---|---|---|---|---|---|
| 1 | X | | | | | | | |
| 2 | | | | | | | | |
| 3 | | | | X | | | X | |
| 4 | | X | X | | | | | X |
| 5 | | | | | X | | | |
| 6 | | | | | | | | |
| 7 | | | | | | X | | |

ments have shown, at this time metaplastic prostatic epithelial cells completely fill the spaces of the former acini and ducts, then epithelialize the surface of the explanted tissue and eventually migrate out on the culture-plate to form a monolayer by numerical growth (13). This clarifies the origin of the epithelial cells growing in primary cultures of adenoma of the prostate. To our knowledge time course organ culture experiments of human prostatic carcinoma have never been reported. Such experiments are now being done in this laboratory and should solve the pending problem.

Initially it was expected that heterotransplantation of EB 33 cells into nude mice would prove the strain to be of carcinomatous origin. However, when cells of the permanent strain MA 160, the only permanent epithelial line that originated from a human prostatic adenoma (14), were transplanted into nude mice in an identical fashion the growth of tumors resulted. On histological examination of both, the tumors growing from EB 33 and MA 160 cells presented no obvious differences. In both instances solid carcinomas with a rapid growth rate and no similarity to the tumor of origin resulted.

In this respect the presented results of heterotransplantation were disappointing but not unexpected to us. However, final conclusions would be premature because the results of transplantation after selection experiments, transplantation from primary and organ cultures and directly from surgical specimens are still pending and will be reported later.

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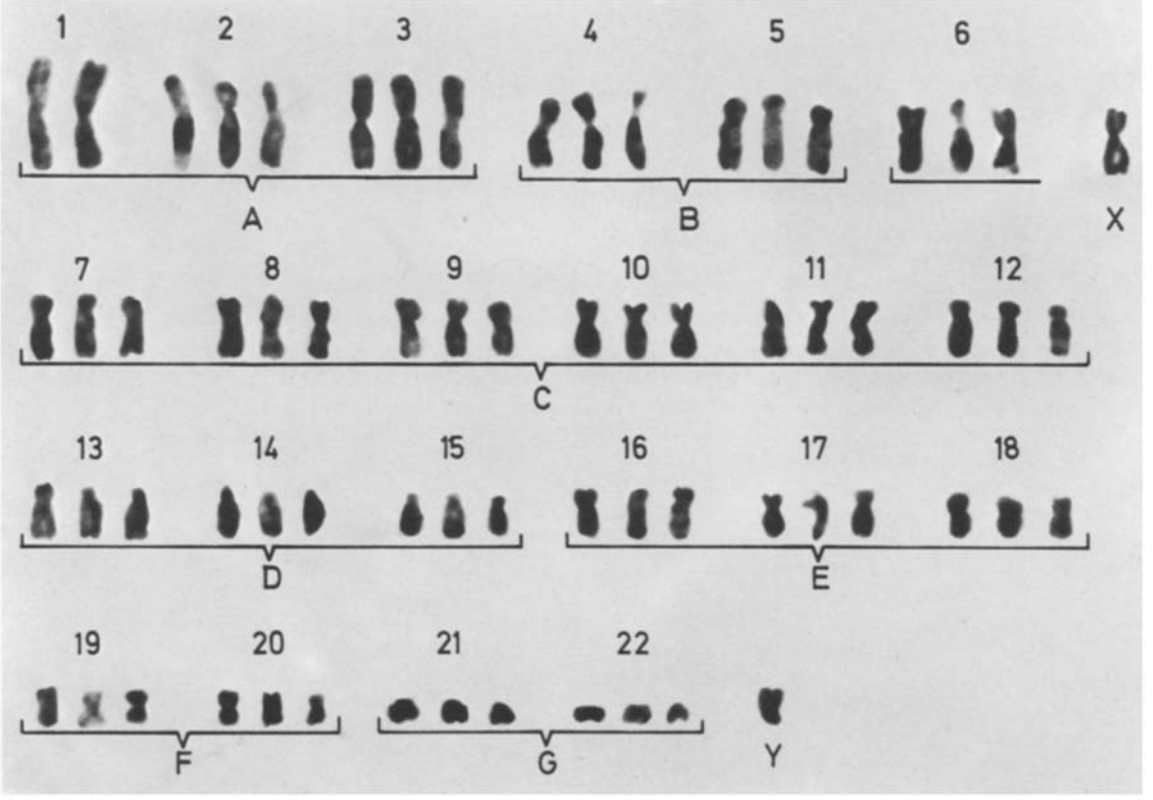


Fig. 7. Chromosomal analysis of a EB 33 cell from the 7th passage. 67 chromosomes were found. The cell is near triploid, one of the chromosomes IA, the largest somatosome is missing

References

1. Buonassisi, V., Sato, G., Cohen, A. I.: Hormone-producing cultures of adrenal and pituitary tumor origin. Proc. nat. Acad. Sci. (Wash.) 48, 1184-1190 (1962)
2. Flanagan, S. P.: "Nude", a new hairless gene with pleiotrophic effects in the mouse. Genet. Res. 8, 295-309 (1966)
3. Furuyama, J.: Methods of chromosomal analysis. J. clin. Sci. (Osaka) 6, 656-664 (1970)
4. Lieberman, I., Ove, P.: Enzyme activity levels in mammalian cell cultures. J. Biol. Chem. 233, 634-640 (1958)
5. Pantelouris, E. M.: Absence of thymus in a mouse mutant. Nature 217, 370-371 (1968)
6. Puck, T., Kao, F.: Genetics of somatic mammalian cells, VII. Induction and isolation of nutritional mutants in Chinese hamster cells. Proc. nat. Acad. Sci. (Wash.) 60, 1275-1281 (1968)
7. Schroeder, F. H., Sato, G., Gittes, R. H.: Human prostatic adenocarcinoma: Growth in monolayer tissue culture. J. Urol. 106, 734-739 (1971)
8. Schroeder, F. H., Mackensen, S. J.: Human prostatic adenoma and carcinoma in cell culture: The effects of androgen-free culture medium. Invest. Urology (accepted for publication)
9. Sato, G., Zaroff, L., Millis, S. E.: Tissue culture populations and their relation to the tissue of origin. Proc. nat. Acad. Sci. (Wash.) 46, 963-972 (1960)
10. Sato, G. H., Yasumara, Y.: Retention of differentiated function in dispersed cell culture. Trans. N. Y. Acad. Sci. 28, 1063-1079 (1966)
11. Sato, G., Augusti-Tocco, G., Posner, M.: Hormone-secreting and hormone-responsive cell cultures. In: Recent Progress in Hormone Research, Vol. 26 p. 539-545, New York 1970
12. Sigma Technical Bulletin No. 85. The histochemical demonstration of acid and alkaline phosphatase. St. Louis 1969
13. Stonington, O. G., Hemmingsen, H.: Culture of cells as a monolayer derived from the epithelium of the human prostate: A new cell growth technique. J. Urol. 106, 393-400 (1971)
14. Vincent, M.: Strain MA 160, Catalogue of Microbiological Assoc., Bethesda, Md., USA

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