Belg. J. Zool. — Volume 120 (1990) — issue 1 — pages 21-35 — Brussels 1990

(Manuscript received on 28 April 1989)

EVALUATION OF METHODS FOR FIBROBLAST ELIMINATION IN MIXED CULTURES

by

T. W. BRIERS (1) (*), T. M. VANDEPUTTE (1)
and M. E. DE BROE (2)

(1) Innogenetics n.v.,
Industriepark Zwijnaarde 7, box 4, B-9710 Gent (Belgium)
(2) Department of Nephrology and Hypertension,
University Hospital Antwerp,
Wilrijkstraat 10, B-2520 Edegem (Belgium)

SUMMARY

The usefulness of inhibitors of fibroblast growth in cultures for the selective culture of neoplastic epithelial cells was assessed using a model system. Two cell populations, permanent neoplastic epithelial HeLa cells and finite fibroblastic FLOW-4000 cells, were mixed, and cultured in 24 well plates. A fibroblast elimination treatment was performed for one set of 24 well plates, the other 24 well plates were used as controls. While the total cell population was determined by counting, the measurement of alkaline phosphate activity of HeLa cells was used for the estimation of the fraction of HeLa cells in the mixed culture ; in this way cell growth kinetics could be determined for the two subpopulations. The percentage of HeLa cells in the mixed cultures was evaluated every day until confluency of the culture. Using this model, several schedules for the inhibition of fibroblast growth were tested : Falcon Primaria culture plates, D-valine, putrescine, cytosine arabinoside, geneticin, and an anti-fibroblast antibody. Geneticin was useful for the selective elimination of fibroblasts in a mixed culture as well as for a series of primary cultures.

Key words : cell culture, epithelial cells, fibroblasts.

INTRODUCTION

The selective elimination of fibroblasts from primary cultures of normal or neoplastic tissues has been an exasperating problem for scientists involved in experiments using epithelial cells from normal or neoplastic tissues. Usually primary cultures start off nicely as mixed cultures of fibroblasts and epithelial cells, but after a few passages one ends up with a culture of solely fibroblasts. Several

(*) To whom requests for reprints should be addressed.

reports have described methods for the selective culture of epithelial cells from normal or neoplastic tissues (OWENS, 1975, FOGH, 1975, BASHOR, 1979). These procedures do not completely eliminate fibroblasts, while others employ complex technical procedures (e.g. organ perfusion, cloning, alternate animal passage and tissue culture, mechanical destruction of undesired cell types, enzymatic treatments, selection by antibodies) which limit general usefullness. As a result, success of a fibroblast elimination schedule depends largely on the skill of the technician.

There is great need for simple and accurate methods for the selection of epithelial or other cell types out of cultures. In fundamental research and in clinical studies, the use of laboratory animals is becoming more stringent. For the oncologist and the clinician, cell culture of neplastic cells is useful only in practice if within a short time span the neoplastic cells can be selected out of the culture (WELANDER, 1983). These cells can then be used for the selection of therapeutics, antibodies to tumor-associated antigens, or even for more complicated procedures such as the production of antigenic tumor cell variants (BOON, 1983).

Techniques for the selection of epithelial cells have been tested on primary cultures with as the main measurement the visual observation of the elimination of fibroblasts. It was our goal to develop a quantitative method for the measurement of fibroblast elimination from mixed cultures. We describe a method to tests the usefulness of potential inhibitors of fibroblast growth in a culture of a permanent neoplastic cell line and a finite fibroblast line.

METHODS AND MATERIALS

Cell culture

A permanent neoplastic cell line, HeLa (epithelial cancer, human cervix), and a finite fibropblastic line FLOW-4000 (human kidney, FLOW inc.) were cultures in Earle's modified Eagle's medium supplemented with 10 % heat inactivated fetal calf serum (FCS) and 1 % non-essential amino acids. Antibiotics were not added to the growth medium. Subculture of confluent cultures at split ratios of 1:10 (FLOW-4000) and 1:30 (HeLa) was accomplished with a calcium and magnesium free phosphate buffered saline which contained trypsin (0.25 %, w/v) and EDTA (0.04 %, w/v). The subculture interval was usually 7 days.

Fibroblast inhibition assay

After trypsinization, epithelial HeLa and fibroblastic FLOW-4000 cells were mixed at a concentration of 1×10^4 cells/ml each or 5×10^3 cells/ml for HeLa cells and 1.5×10^4 cells/ml for FLOW-4000 cells. The final cellular concentration was 2×10^4 cells/ml. One ml cell suspension aliquots were plated in each well of 24 well plates (Costar, Mark II 3424). The cells were allowed to adhere to the plastic surface. The second day the fibroblast inhibitor was added to the culture wells and left in the well for a selected time.

Daily, treated and non-treated culture wells ($n = 4$) were trypsinized and the total cellular population was measured by a direct enumeration of cells employing a hemocytometer. This procedure was repeated until the cultures were confluent. The HeLa subpopulation was estimated in an indirect way by a measurement of the alkaline phophatase (AP) activity of HeLa cells using the formula (the method for the determination of the AP activity is given below):

$$\frac{AP_m - (\text{total cell count}) \times AP_{FLOW}}{AP_{HeLa} - AP_{FLOW}} = \text{HeLa cell count } (\times 10^4)$$

AP_m, AP activity of the mixed culture;

AP_{FLOW}, AP activity of a FLOW-4000 culture expressed per 10^4 cells;

AP_{HeLa}, AP activity of a HeLa culture expressed per 10^4 cells.

Control plates of HeLa and of FLOW-4000 cells were used to measure daily the AP activity of the HeLa cells and the background activity of the FLOW-4000 cells. FLOW-4000 cells were used for the experiments only between passage 2 and 5; therefore new batches were regularly established from cryopreserved cells.

Determination of the statistical significance between treated and non-treated cultures was analyzed by the parametric Students't test and the non-parametric MANN-WITNEY method.

Morphology

Cells growing in tissue culture containers were examined by standard light microscopy. Alternatively, cells were grown on tissue culture chamber slides (Miles Lab.; LABTEK 4808), fixed, and stained by standard histological staining procedures.

Determination of AP activity

The cells of the mixed or control cultures were suspended in isotonic saline after trypsinization. The AP activity was measured for intact cell suspensions (5×10^3 to 1×10^4 cells/ml). The total AP activity was determined kinetically by the method of VAN BELLE *et al.* (1977). One unit of enzyme activity is defined as the liberation of 1 µmol nitrophenol ($E_{405\,nm} = 14{,}600$ liters $mol^{-1}\,cm^{-1}$) from 4-nitrophenyl phosphate per min at 37° C when incubated in a solution containing 5 *m*M 4-nitrophenyl phosphate in 0.1 M N-ethylamino ethanol buffer (pH 10.2).

Products and treatments

Primaria culture plates (24 well plate, Becton Dickinson, Falcon 3847) with a surface modified polystyrene that inhibits fibroblast growth was used instead of Costar 24 well plates. Putrescine (1,4 diaminobutane, Janssen Chimica 23-1400-1; 34 and 56 mg/L) and cytosine arabinoside (Janssen Chimica 22-718-20; 3 mg/L) were added to the 24 well plates on day 1, and left in the growth medium for the duration of the experiment. For D-valine medium (Gibco BRL, 041-2570), the normal

growth medium was changed on day 1 by D-valine medium supplemented with 5 % FCS, dialysed for 2 days at 4° C against an Earle's salt solution ; the growth medium of the control plate was also supplemented with 5 % FCS. Geneticin (G418 sulfate, Sigma G5013) was added on day 1 at a concentration of 1 mg/L growth medium and left for two to three days ; then the wells were washed and normal growth medium was replaced. The monoclonal antibody to fibroblasts (LICR LON/FIB 86.3) was a gift from Dr. Edwards (University of Cambridge, Department of Pathology). Anti-fibroblast containing ascites was diluted in Earle's modefied Eagle's medium (1:50 to 1:1000). The cells were washed and the anti-fibroblast containing medium was added. After 1 hr at 37° C, the cells were rinsed and rabbit complement put on, diluted 1:5 to 1:50 in medium. The plate was again incubated for 1 hr at 37° C. Finally, the complement was removed, the cells rinsed with medium and growth medium replaced.

RESULTS

AP activity of the cell lines

The neoplastic epithelial cell line, HeLa, contained a large amount of AP activity (3.01 ± 1.02 U AP/mg protein). This AP activity was mainly membranous and was found on the plasma membrane (this was verified using histochemistry for total AP on electron microscopical level, results not shown). The AP activity of HeLa cells was 266 ± 10 *n*U/cell. In the fibroblast line there was only a negligeable amount present on the plasma membrane, 4.7 ± 1.1 *n*U/cell. This difference was used to estimate the amount of HeLa cells in culture.

To be able to use the measurement of AP activity of HeLa cells for the estimation of the amount of HeLa cells in the mixed culture, it was essential to evaluate the effect of the dilution of the cell suspension on the total AP activity (Fig. 1A), and to test the effect of the cell density during the culturing period on the total AP activity (Fig. 1B). There was a linear correlation between the dilution of the cell suspension and the AP activity measured. The dilution used in the experimental setup was always around 5×10^3 to 1×10^4 cells/ml. The increasing cell density during the culturing period had only a small effect on the total AP activity (slope = − 0.0011). For that reason, HeLa cells were seeded in 24 well plates at the same concentration as in the experimental setup, and AP activity was determined daily.

Elimination of fibroblasts out of mixed cultures

In the assay, presented in Fig. 2, two plastic growth surfaces where compared for their effect on the growth of epithelial cells and fibroblasts in mixed cultures. For the total populations as well as for HeLa cells and FLOW-4000 cells, the growth

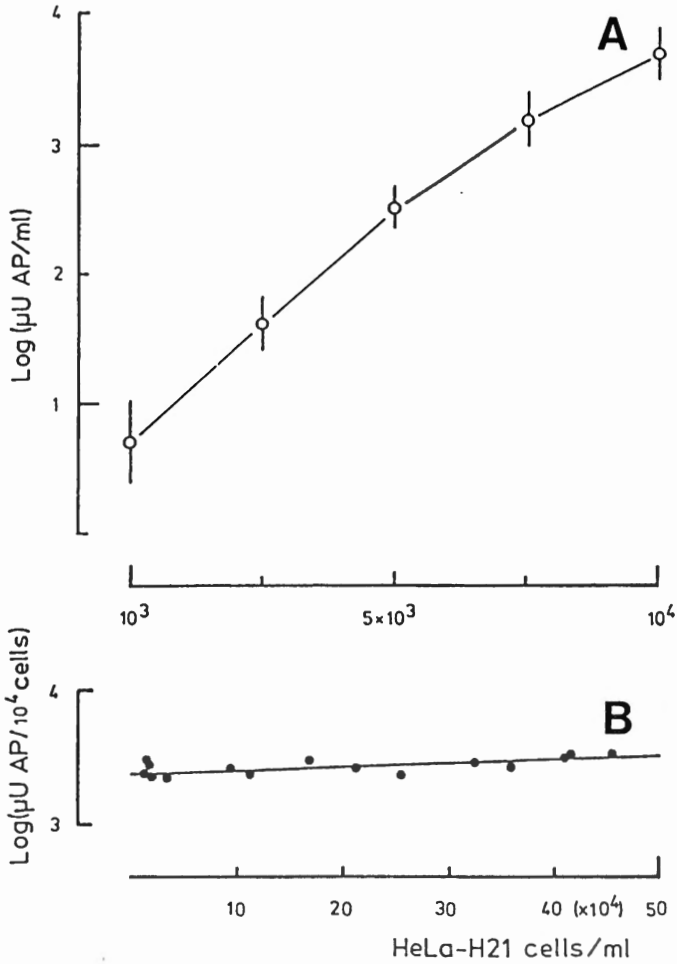

Fig. 1. — (A) Kinetic measurement of the total alkaline phosphatase activity of different HeLa cell dilutions (Mean ± SD, $n = 4$). (B) Kinetic measurement of the total alkaline phosphatase activity in function of cell density of HeLa cells. Cell density is expressed as the amount of HeLa cells of one well in one ml.
Linear regression : $y = 3.4 - 0.0011X$, $R = 0.93$.

curves had a normal sigmoidal pattern. Falcon Primaria plates with a modified polystyrene surface were found to be not superior to Costar plates for the inhibition of the growth rate of FLOW-4000. On the contrary, proliferation of FLOW-4000 cells was more pronounced in the Falcon Primaria, although this increase was not significant. This is also clearly depicted in Fig. 3.

Fig. 3 summarizes the results of experiments in which several methods were compared : adding putrescine (two concentrations were tested : 34 mg/L and 56 mg/L)

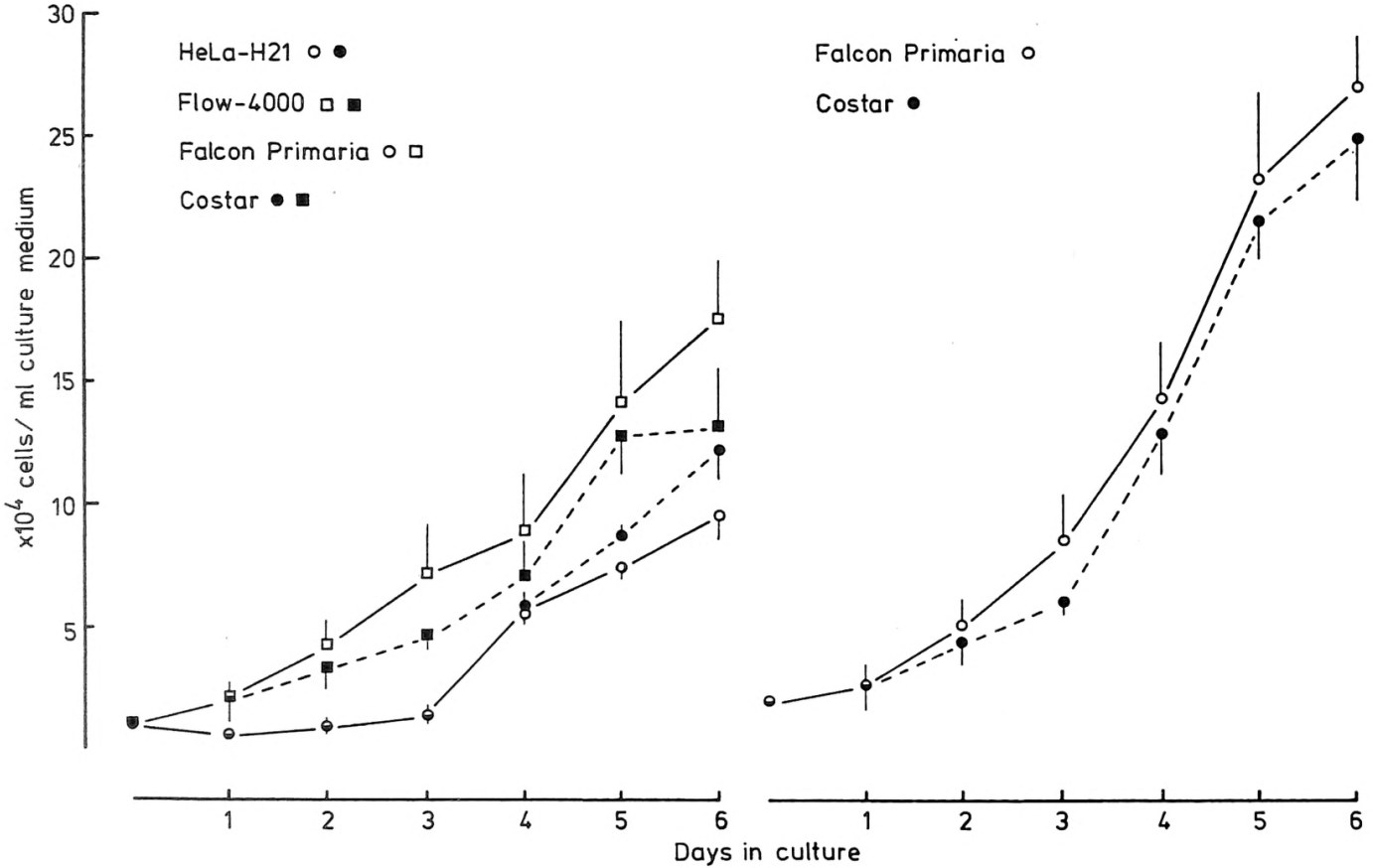

Fig. 2. — Comparison of the cell growth of a mixed culture of epithelial cells, HeLa, and fibroblasts, FLOW-4000, for two different plastic surfaces : the 24-well plate of Costar and the 24-well plate of Falcon primaria (inhibits fibroblast growth). HeLa and FLOW-4000 were each seeded at a concentration of 1×10^4 cells/well. (A) cell counts per day per cell type ; (B) total cell counts per day (mean ± SD, $n = 4$).

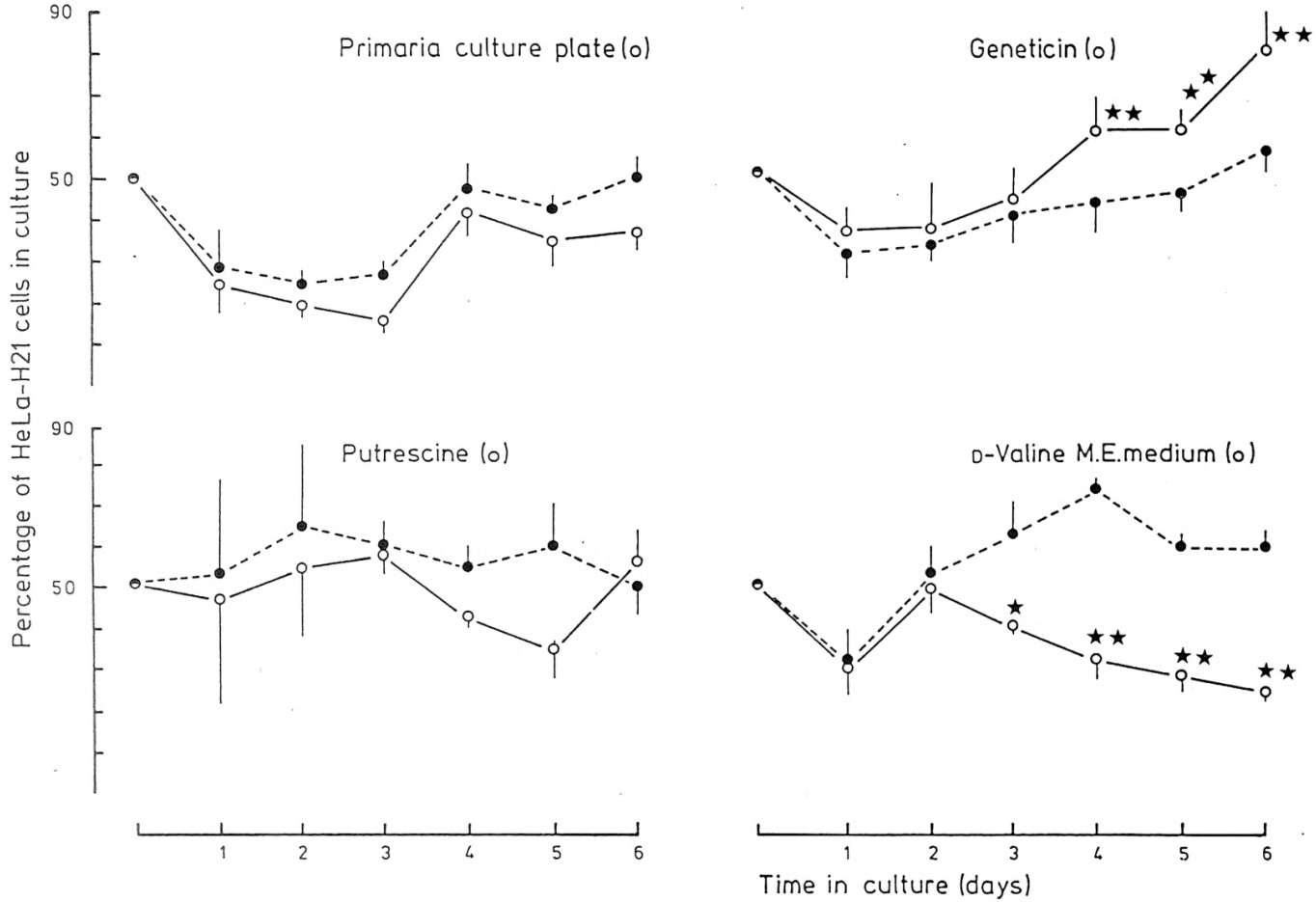

Fig. 3. — Percentage of proliferating HeLa cells in a mixed culture of HeLa and FLOW-4000 cells for different treatments (○) as compared with non-treated control cultures (●). Cells were seeded at a concentration of 1×10^4 cells per well for each of the two cell types. Putrescine (56 mg/L) and D-Valine growth medium were added for the whole duration of the experiment. Geneticin (1 mg/L) was added on day 1, and was discarded on day 3 (mean ± SD, n = 4 to 8 ; * $p < 0.05$, ** $p < 0.025$).

to the growth medium, replacing L-valine in the growth medium by its D-enantiomer, and adding geneticin to the medium for a few days. The data are presented as the percentage of HeLa cells in the mixed culture per day. Geneticin was the only product able to reduce significantly the growth of fibroblasts. When geneticin was added for two days to a mixed culture of HeLa and FLOW-4000 cells (1×10^4 cells/ml for the two cell types), the percentage of HeLa cells was significantly ($p < 0.025$) higher from day 4 on when compared with the control cultures. Growth medium that contained D-valine instead of L-valine had an adverse effect, since it reduced the growth of both subpopulations (for HeLa cells from 3 on and for FLOW-4000 cells from day 4 on). For the total populations this effect became measurable from day 4 on (for D-valine cultures, day 3 [9.31 ± 0.45] $\times 10^4$ cells and day 4 [12.81 ± 2.00] $\times 10^4$ cells ; for control cultures, day 3 [11.69 ± 1.95] $\times 10^4$ cells and day 4 [27.94 ± 3.37] $\times 10^4$ cells). However, this reduced growth rate was more pronounced for the HeLa subpopulation (Fig. 3).

Cytosine arabinoside at the concentration of 3 mg/L was toxic for both cell types (for the total cell populations, on day 1 [1.19 ± 0.12] $\times 10^4$ cells *versus* the control [2.05 ± 0.51] $\times 10^4$ cells and on day 2 [2.03 ± 0.45] $\times 10^4$ cells *versus* the control [8.64 ± 0.37] $\times 10^4$ cells). The treated cultures were extinct after 4 to 5 days. The other treatments had no or little adverse effects on the cell growth kinetics. The curves of treated and control cultures were quite comparable (as for the results shown in Fig. 2) and the elimination of part of one subpopulation resulted in an increase of the other.

As geneticin was able to inhibit the growth rate of fibroblasts in mixed cultures, we investigated if this was also true for cultures where the percentage of epithelial cells was lower than 50 %. Therefore, we reduced the amount of HeLa cells to 25 % of the total cell population (2×10^4 cells/well, or 5×10^3 HeLa cells/well), and tested this aminoglycoside for two different time spans, two and three days (Fig. 4). There was a significant increase of the HeLa population in the treated cultures for both incubation times when compared with the non-treated controls. However, prolongation of the treatment did not benefit the increase of epithelial cells. Proliferation of the HeLa population only increased after a lag period of one day when the geneticin treatment was ended. Before treatment, the epithelial HeLa cells appeared as small colonies, while the fibroblasts occupied most of the growth surface. After treatment, the fibroblasts could only be found sparingly as small clusters of cells that did not grow well between large colonies of proliferating HeLa cells.

Selective antibodies to undesired cell types would be the perfect tool to select cells in a rapid and non-toxic way. Using tissue culture chamber slides, we tested several parameters for the elimination of fibroblasts by an anti-fibroblast antibody (LICR LON/FIB 86.3). Different dilutions of the anti-fibroblast antibody containing ascites (1:50 to 1:1000), and of the complement (1:5 to 1:50) were tried. The combination of 1:200 for LICR LON/FIB 86.3 and of 1:10 for the complement resulted in maximal lysis of fibroblasts. A minimal incubation period of 30 min. at 37° C was needed for an optimal effect. Treatments of cultures on days 1, 2 or 4 gave

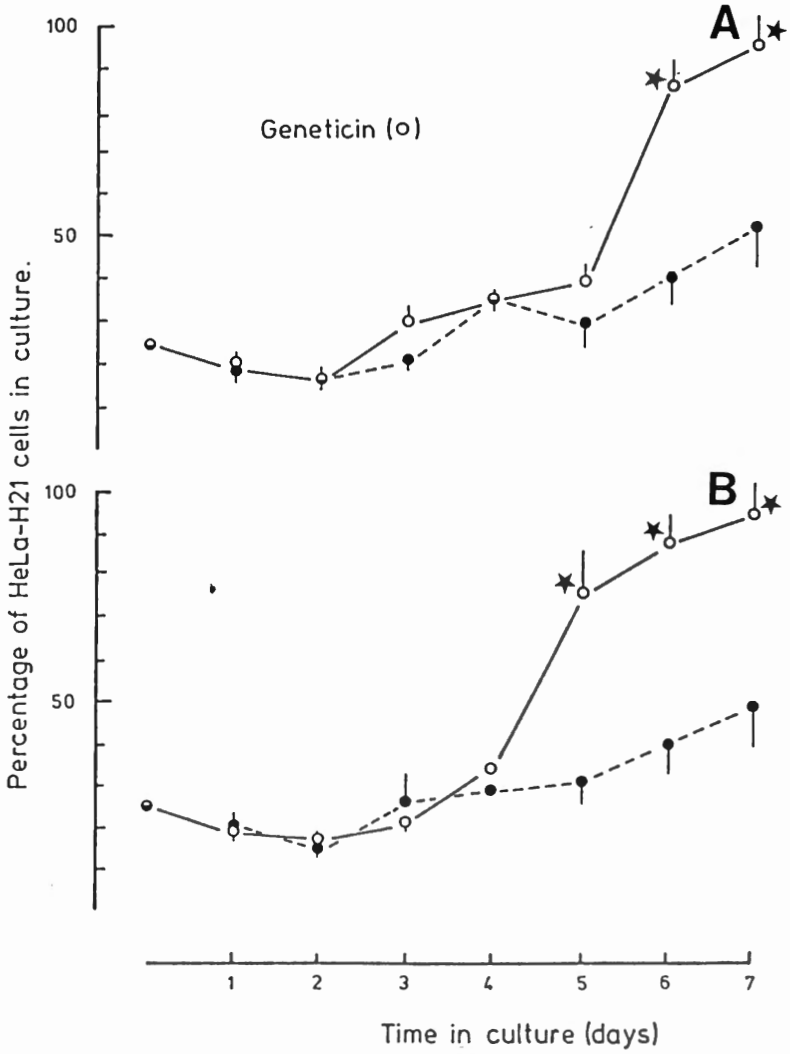

Fig. 4. — Percentage of proliferating HeLa cells in a mixed culture of HeLa and FLOW-4000 cells for a treatment of Geneticin (○) as compared with non treated control cultures (●). Cells were seeded at a concentration of 0.5×10^4 HeLa cells/well and 1.5×10^4 FLOW-4000 cells/well. Geneticin (1 mg/L) was added on day 1 and was discared on day 4 (A), or day 3 (B) (mean ± SD, $n = 4$; $^* p < 0.025$).

comparable results. LICR LON/FIB 86.3 was clearly able to destroy a large part of the fibroblast population out of mixed cultures. The HeLa cells remained as polygonal cells, while most of the elongated, often spindle shaped fibroblasts disappeared from the bottom of the culture well leaving empty spaces. However, the whole FLOW-4000 population was never completely eliminated. Consequently,

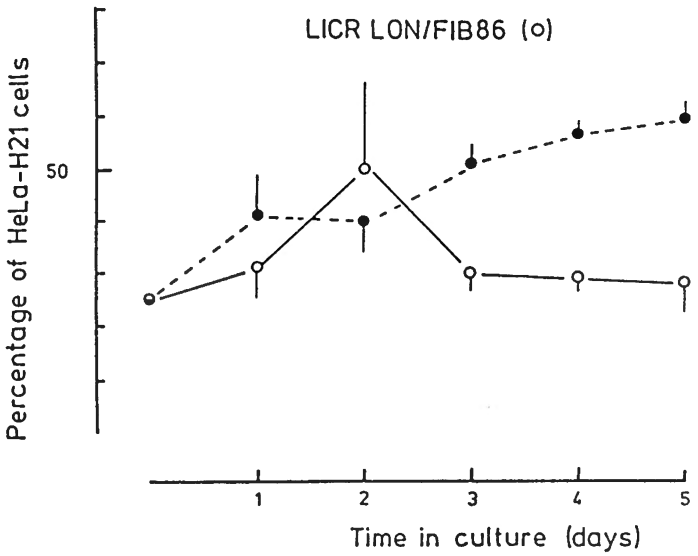

Fig. 5. — Percentage of proliferating HeLa cells in a mixed culture of HeLa and FLOW-4000 cells, treated with anti-fibroblast antibody and complement (○) as compared with non-treated control cultures (●). Cells were seeded at a concentration of 5×10^3 HeLa and 1.5×10^4 FLOW-4000 cells/well. Treatment was performed on day 1 : anti-fibroblast Moab (LICR LON/FIB 86.3) for 1 hr at 37° C followed by rabbit serum 1:10 diluted in Earle's modified Eagle's medium for 1 hr at 37° C (mean ± SD).

when we tried the antibody on our model with a treatment on day 1, we were not able to show a continuous inhibitory effect on FLOW-4000 cells after an additional four days of culture (Fig. 5). On the contrary, the surviving FLOW-4000 cells, which were clearly reduced in number the day after the treatment, proliferated again after a lag period of one day (for the treated cultures, on day 1 $[4.37 \pm 0.47] \times 10^4$ cells, on day 2 $[2.42 \pm 0.27] \times 10^4$ cells and on day 3 $[5.71 \pm 1.65] \times 10^4$ cells ; control cultures, on day 1 $[3.74 \pm 0.60] \times 10^4$ cells, on day 2 $[7.10 \pm 1.93] \times 10^4$ cells and on day 3 $[9.65 \pm 2.19] \times 10^4$ cells), and overgrew the HeLa population quite rapidly.

Elimination of fibroblast-like cells out of primary cultures

Geneticin has been used routinely for the selection of epithelial cells (neoplastic and normal ; ovary, testis, kidney, lung ; Tab. 1). In contrast to collagenase and putrescine each treatment of geneticin resulted in a clear elimination of most of the fibroblast-like population present in the culture flasks. Two treatments were often needed, and the whole duration of the selection took from 2 to 4 weeks. The

cultures could usually be kept for about 3 months but storage in liquid nitrogen was only possible in some casses (3) where the amount of cells was sufficient.

TABLE 1

Elimination of fibroblast-like cells out of primary cultures of human tissues

Tissue (1)	Treatment (2)	Number of experiments	Result (3)	Days in culture
Ovary CA	collagenase	2	F + E islands	200, 150
	geneticin	2	mainly E islands	120, 90
Parathyroid AD	collagenase	2	F + small E islands	100, 60
	putrescine	2	F + E islands	50, 60
Testes CA	geneticin	1	only E	110 (ST) (4)
Kidney CA	geneticin	3	only E	120, 160 (ST) 100 (ST)
Lung	geneticin	6	only E islands	150, 103, 120, 130, 90, 100

(1) Tissue, CA, carcinoma, AD, adenoma.

(2) Treatment, collagenase, 0.5 g/L added to the growth medium when fibroblast-like cells started to dominate and was continued until they started to die, putrescine, 0.56 mg/L growth medium, geneticin, 1 mg/L growth medium, treatments of two days.

(3) Results, F, fibroblast-like cells, E. Epithelial-like cells.

(4) ST, Stored in liquid nitrogen.

DISCUSSION

Short-term cultures of neoplastic human cells contain an abundance of stromal cells which may disturb refined studies. It is very peculiar that in the majority of primary cultures set up from human tumors, both stromal and neoplastic cells die after a lilited number of cell generations, resulting in eventual extinction of these cultures. The success rate of establishment of neoplastic cell cultures has been low (e.g. IOACHUM *et al.*, 1974 ; LEIBOVITZ *et al.*, 1976 ; MCBAIN *et al.*, 1984 ; KIRKLAND and BAILEY, 1986). This can largely be explained by an overgrowth of fibroblasts, or by the finite life of neoplastic cells.

The state of the art of the establishment of neoplastic epithelial cell cultures involves cloning, alternate animal passage and tissue culture, enzymatic treatments (collagenase, trypsin), substrate modification, mechanical destruction of undesired cell types, density gradients, etc. (FOGH, 1975 ; OWENS, 1975 , BASHOR, 1979 ; FRESHNEY, 1983), or combinations of these techniques (KEDAR *et al.*, 1982 ; DANES, 1985 ; KIRKLAND and BAILEY, 1986 ; etc.).

An apparently elegant method was developed by GILBERT and MIGEON (1975). They exchanged L-valine by D-valine in the growth medium resulting in a survival of cells that contained D-amino acid oxidase, an enzyme that only occurs in specialized epithelium of many mammals but not in fibroblasts (GILBERT and MIGEON, 1975 ; ORGEBIN-CRIST *et al.*, 1984). In our model system, growth medium that contained D-valine reduced the growth of the HeLa population more than the growth of FLOW-4000 cells. Apparently, the neoplastic epithelial line HeLa does not produce D-amino acid oxidase, and is therefore more susceptible to growth media without L-valine. This may also be true for other neoplastic epithelial cell types. Therefore, the application of D-valine containing media for the selection of epithelial cells is only useful for epithelia of specific origin. This may also be true for putrescine and cytosine arabinoside products being used for the isolation of bovine parathyroid cells (BRANDI *et al.*, 1986) and the culture of rat and rabbit epididymal cells (ORGEBIN-CRIST *et al.*, 1984), respectively.

Another method was introduced by HALABAN and ALFANO (1984), who selectively eliminated fibroblasts from cultures of normal human melanocytes with geneticin. Geneticin (G-418 sulphate) is an aminoglycoside related to gentamicin and is used as a selective agent in molecular genetics. In our model as well as in primary cultures of cells of different tissues, geneticin was a reliable tool for the elimination of fibroblasts. The fibroblasts are possibly eliminated because geneticin is taken up, what is accompanied by an increase of lysosomal activity (intracellular staining for acid phosphatase) and a deterioration of the cell by bursting of lysosomes. The analogue gentamicin has been shown to be taken up by fibroblasts in culture and this uptake is accompanied by a marked increase in the total volume of lysosomes (AUBERT-TULKENS *et al.*, 1979). This increase may be an indication of an early lysosomal change, a phenomenon which has been correlated with nephrotoxic effects of aminoglycosides (GIULIANO, 1986).

Antibodies to (un)desired cells may in the future prove to be an ideal tool for the selection of certain cell types. The main problems will be the development of monoclonal antibodies with sufficient affinity and specificity, and of accurate methods to perform the separation (complement activation, magnetic beads, chromatography, growth surface coating, e.g.). In some cases the production of suitable antibodies may prove not to be to difficult as for the immune elimination of host fibroblasts, when the goal is the cultivation of human tumor cells transplanted in nude mice (OKABE *et al.*, 1983). It is also possible to select cell types that express a specific antigen on their cell surface, like brush border enzymes of proximal tubular cells of the kidney (SMITH and GARCIA-PEREZ, 1985), or tumor-associated antigens (TRELEAVEN *et al.*, 1984). The use of tumor-associated antigens for the selection of neoplastic cells has the disadvantage that interesting cell types may be lost, because of the heterogeneity of most tumors for the expression of such antigens. Therefore, the idea of eliminating the fibroblast is very attractive.

Using the monoclonal antibody LICR LON/FIB 86 EDWARDS *et al.* (1980) and GUSTERSON *et al.* (1981) were able to selectively culture epthelial cells from human squamous carcinoma and keratinocytes. We were also able to show morphologically the elimination of a part of the fibroblast fraction out of mixed cultures on

tissue culture chamber slides. This result is in agreement with the findings of EDWARDS *et al.* (1980), who could only kill part of the fibroblast population after each treatment. In our experiments, the remaining FLOW-4000 cells seemed to recover rapidly. A second reason for the failure may be the short time interval between seeding and treatment (24 hrs), as not sufficient antigen may be present on the FLOW-4000 plasma membrane. This short time after seeding was used (1) to be able to compare the results with previous experiments and (2) to allow the antibody an easy access to the fibroblasts present. GUSTERSON *et al.* (1981) pointed out that the use of reseeded populations of cells facilitated access of the antibody to fibroblasts otherwise trapped beneath the epithelial cells. The complement used may also be a reason for the uncomplete elimination of the fibroblast population. The antibody LICR LON/FIB 86 only killed well with rabbit serum, but this serum was somewhat toxic on its own, and the optimal dilution used by these authors was 1:20 to 1:40. The serum dilution that we used for complement activation was 1:10. The toxic effect of the rabbit serum on HeLa cells may have reduced its proliferation, although no toxic effect was observed on control cultures.

ACKNOWLEDGEMENTS

This work was supported by grants from the Fonds voor Kankeronderzoek of the Spaar- en Lijfrentekas, Nationale Loterij-FGWO (grant 9.0005.84), and a research grant from te University of Antwerp. T. W. Briers gratefully acknowledges a research fellowship (navorsersbeurs) of the Institute for research in Industry and Agriculture (I.W.O.N.L., Belgium). The authors thank Dr. P. A. Edwards (Department of Pathology, University of Cambridge, UK) for providing the anti-fibroblast antibody, LICR LON/FIB 86.3, and Dr. W. Fiers and Dr. A. Van de Voorde (Laboratory for Molecular Biology, University of Ghent, Belgium) for providing the HeLa cell line. They also thank Dr. Z. Bernemann for critically evaluating the manuscript.

ABREVIATIONS USED

FCS, fetal calf serum.

AP, alkaline phosphatase.

REFERENCES

AUBERT-TULKENS, G., F. VAN HOOF and P. TULKENS (1979), « Gentamicin-induced lysosomal phospholipidosis in cultured rat fibroblasts. Quantitative ultrastructural and biochemical study », *Lab. Invest.*, **40**, 481-491.

BASHOR, H. (1979), « Dispersion and disruption of tissues », *Meth. Enzymol.*, **58**, 119-132.

BOON, T. (1983), « Antigenic tumor cells variants obtained with mutagens ». Adv. Cancer Res., **39**, 121-151.

BRANDI, L. M., L. A. FITZPATRICK, H. G. COON and G.D. AMBACH (1986), « Bovine parathyroid cells : cultures maintained for more than 140 population doublings », *Proc. Natl. Acad. Sci USA,* **83,** 1709-1713.

DANES, B. S. (1985), « Long-term-cultured colon epithelial cell lines from individuals with and without colon cancer genotypes », *J. Natl. Cancer Inst.*, **75,** 261-267.

EDWARDS, P. A. W., D. M. EASTY and C. S. FOSTER (1980), « Selective culture of epithelial cells from a human squamous carcinoma using a monoclonal antibody to kill fibroblasts », *Cell Biol. Int. Reports,* **4,** 917-922.

FOGH, J. (1975), *Human tumor cells in vitro,* Plenum press, New York-London, 557 p.

FRESHNEY, R. I. (1983), *Culture of Human cells,* Alan R. Liss Inc., New York, 248 p.

GIULIANO R. A. (1986), « Renal handling of aminoglycosides : cortical uptake and release as an approach to the understanding of nephotoxicity », Doc. Thesis, U.I.A., Antwerpen (Belgium).

GILBERT, S. F. and B. R. MIGEON (1975), « D-valine as a selective agent for normal human and rodent epithelial cells in culture », *Cell,* **5,** 11-17.

GUSTERSON, B. A., P. A. W. EDWARDS, C. S. FOSTER and A. M. NEVILLE (1981), « The selective culture of keratocytes using a cytotoxic antifibroblast monoclonal antibody », *Br. J. Dermatol.,* **105,** 273-277.

HALABAN, A. and F. D. ALFANO (1984), « Selective elimination of fibroblasts from cultures of normal human melanocytes », *In vitro,* **20,** 447-450.

IOACHIM, H. L., M. SABBATH, B. ANDERSSON and H.R.K. BARBER (1974), « Tissue culture of ovarian carcinomas », *Lab. Invest.,* **31,** 381-390.

KEDAR, E., B. L. IKEJIRI, G. D. BONNARD and R. B. HERBERMAN (1982), « A rapid technique for isolation of viable tumor cells from solid tumors : use of the tumor cells for induction and measurement of cell mediated cytotoxic responses », *Eur. J. Cancer Clin. Oncol.,* **18,** 991-1000.

KIRKLAND, S. C. and I. G. BAILEY (1986), « Establishment and characterization of six human colorectal adenocarcinoma cell lines », *Br. J. Cancer,* **53,** 779-785.

LEIBOVITZ, A., J. C. STINSON, W. B. III MCCOMBS, C. E. MCCOY, K. E. MAZUR and N. D. MARBRY (1976), « Classification of human colorectal adenocarcinoma cell lines », *Cancer Res.,* **36,** 4562-4569.

MCBAIN, J. A., J. L. WEESE, L. F. MEISNER, W. H. WOLBERY and Y. K. V. WILLSON (1984), « Establishment and characterization of human colorectal cancer cell lines », *Cancer Res.,* **44,** 5813-5821.

OKABE, T., N. SASAKI, M. MATSUZAKI, Y. IMAI, Y. KANEKO, F. MATSUZAKI, F. TAKUTU and T. TSUSHIMA (1983), « Establishment and characterization of a new human functional cell line from a choriocarcinoma », *Cancer Res.,* **43,** 4920-4926.

ORGEBIN-CRIST, M. C., J. JONES-DAVIES, P. STOREY and G. E. OLSON, (1984), « Effect of D-valine and cytosine arabinoside on H^3-thymidine incorporation in rat and rabbit epididymal epithelial cell cultures », *In vitro,* **20,** 45-52.

OWENS, R. B. (1975), « Selective cultivation of mammalian epithelial cells », *Methods Cell Biol.,* **9,** 341-355.

SMITH, W. L. and A. GARCIA-PEREZ (1985), « Immunodissection : use of monoclonal antibodies to isolate specific types of renal cells », *Am. J. Physiol.,* **248,** F1-F7.

TRELEAVEN, J. G., F. M. GIBSON, J. UGELSTAD, A. REMBAUM, T. PHILIP and G. B. CAINE (1984), « Removal of neuroblastoma cells from bone marrow with monoclonal antibodies conjugated to magnetic microspheres », *Lancet*, **i**, 70-73.

VAN BELLE, H., M. E. DE BROE and J. WIEME (1977), « 1-*p*-Bromotetramisole, a new reagent for use in measuring placental or intestinal isoenzymes of alkaline phosphatase in human serum », *Clin. Chem.*, **23**, 454-459.

WELANDER, C. E. (1983), « In vitro testing of ovarian carcinomas : Basis for planning therapy », in : *Carcinoma of the ovary*, Ed. H. G. Bender and L. Beck), p. 51-66. Gustav Fischer Verlag, Stuttgart-New York.