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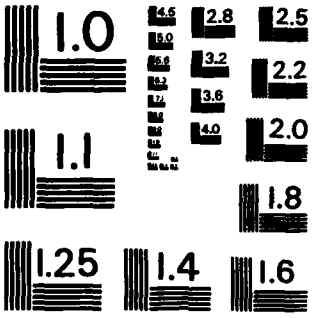

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19. ABSTRACT (Continue on reverse if necessary and identify by block number)

In the first year, optimal culture conditions for in vivo transformed cells and for normal granulation tissue cells were determined. In addition, selective growth condition for in vivo transformed cells were compared.

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The influences of media, sera, pH and pO_2 on growth of freshly isolated cells derived from fibrosarcoma or fro, a 4 day old granuloma tissue were investigated. The low pO_2 found in vivo (30-40 mmHg) stimulated in vitro growth of transformed as well as normal cells in culture. The stimulus was much higher in tumor cells (2-5 fold increase) than in normal cells (1. 2-1. 4 fold). With a selected set of growth modifying chemicals (TPA, dexamethasone Vitamin E, glutathion, insulin) this stimulus could not be further enhanced.

Optimal conditions for growth of transformed cells in living hosts (nude mice and rats) were investigated. It was found, that species, strain, and inoculum size influenced growth of →

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Christpher T. Lind, Lt, Col, USAF	(202) 767-5021	NL

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tumor cells. Furthermore it was observed that co-injection of normal cells shortened the lag time. The in vitro handling of transformed cell did not affected their growth in nude mice.

Selective growth conditions for transformed cells were evaluated with 14 tumor cell population grew out of tumors. In vitro, growth in Ca^{2+} depleted medium and anchorage independent growth in soft agar were the most reliable conditions.

In the second year, we started to analyze freshly isolated cells exposed in vivo in the graulation tissue to the established genotoxic agent N-methyl-N-nitro-N-nitrosoguanidine (MNNG) at a dose which induces a high yeild of mutations and which gives rise to fibrosarcoma development at the site of injection.

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Interim Scientific Report of Grant AFOSR-82-0338

1. 9. 83 - 31. 8. 84

Project Title: Correlation of mutagenic, carcinogenic and cocarcinogenic effects of chemical substances (Granuloma Pouch Assay).

0015J

1. PREFACE

The AFSC grant supports a substantial part of a research project at the Institute of Toxicology, Swiss Federal Institute of Technology and University of Zürich in Switzerland. In this project, new methods for the detection and characterization of mutagenic and carcinogenic chemicals are developed. The concept is based on an in vivo/in vitro approach in rats. The part supported by the grant deals with the detection of malignant or premalignant cells in vitro after exposure in vivo to a carcinogen.

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3. REPORT OF SUPPORTED WORK PERFORMED

3.1. General concept

Mutagenic and carcinogenic chemicals can be detected in vitro using tests with microorganisms and mammalian cells and in vivo in life-time rodent bioassays. A number of mechanisms are identified which either enhance or retard the biological reactions that are involved in DNA damage, mutation and tumor development. DNA damage can be repaired, and cells which were transformed by chemical, viral or physical insults remain quiescent under the control of the surrounding tissue. These secondary modifying mechanisms can be influenced by chemicals and other endogenous and exogenous factors. In order to arrive at a better understanding of these factors which affect the development of an initial DNA lesion into a transformed phenotype, experiments were performed in the Granuloma Pouch Assay, an in vivo model developed in our Institute[7]. In this system, mutagenic events and in vivo transformed cells can be detected in a granulation tissue. Growth of this tissue is induced by the injection of 25 ml of air under the rat skin. This defined cell population consisting of fibroblasts, endothelial cells and macrophages can be exposed to test compounds. Given the chemical locally, a direct interaction with the growing cells can be achieved or when administered systemically, the chemical reach the granuloma tissue in unchanged or in metabolized form via the blood system[4].

Cells harvested from the granuloma tissue by enzymical dissociation grow very well in tissue culture and are therefore accessible for further analysis or treatment. So far, mutagenic events like DNA- strand breakage[2], chromosomal aberrations, sister chromatid exchanges[6] and gene mutations (growth in selective media) were evaluated[3,5].

The in vivo transformed cells can be obtained in long term experiments, in which induced fibrosarcomas can be detected by palpation[9,10]. Cells isolated from these fibrosarcomas can again be cultured in vitro and serve as reference cultures for transformed cells[1].

Because many insults are possible which affect the complex process from the initial DNA lesion to a lethal tumor, without direct interaction with the genome, it is not justified to limit the detection of

carcinogenic chemicals to their direct genotoxic activity alone. Therefore, the following concept was chosen: Target cells are exposed in vivo to a carcinogen. Subsequently, after isolation of these cells, manipulations in vitro should help to overcome the extended latency period observed in long-term carcinogenicity tests. The benefit of this approach is twofold: One is the better understanding of processes involved in the expression of the transformed phenotype and the other is the application of selected manipulations in an efficient short term test for the detection of preneoplastic or neoplastic cells.

3.2. Summary of work performed in the first year.

In the first year, optimal culture conditions for in vivo transformed cells and for normal granulation tissue cells were determined. In addition, selective growth condition for in vivo transformed cells were compared.

The influences of media, sera, pH and pO_2 on growth of freshly isolated cells derived from fibrosarcoma or from a 4 day old granuloma tissue were investigated. The low pO_2 found in vivo (30- 40 mm Hg) stimulated in vitro growth of transformed as well as normal cells in culture [8]. The stimulus was much higher in tumor cells (2-5 fold increase) than in normal cells (1.2-1.4 fold). With a selected set of growth modifying chemicals (TPA, dexamethasone, Vitamin E, glutathion, insulin) this stimulus could not be further enhanced.

Optimal conditions for growth of transformed cells in living hosts (nude mice and rats) were investigated. It was found, that species, strain, and inoculum size influenced growth of tumor cells. Furthermore it was observed that co-injection of normal cells shortened the lag time[1]. The in vitro handling of transformed cells did not affected their growth in nude mice.

Selective growth conditions for transformed cells were evaluated with 14 tumor cell populations which differed in their phenotype. In living hosts all isolated cell populations grew out to tumors. In vitro, growth in Ca^{2+} depleted medium and anchorage independent growth in soft agar were the most reliable conditions.

3.3. Work performed in the second year

In the second year, we started to analyze freshly isolated cells exposed in vivo in the granulation tissue to the established genotoxic agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) at a dose which induces a high yield of mutations and which gives rise to fibrosarcoma development at the site of injection.

3.3.1. Detection of preneoplastic cells

In a pilot study, rats were subjected to three different treatment schedules. Group I received croton oil injected into the air pouch, group II croton oil and MNNG and group III vitamin E. Four hours, two, six and twelve days after treatment, animals were killed and granuloma cells isolated. Subsequently growth of cells was observed in Ca^{2+} depleted medium, in soft agar and in living hosts. Cells were not further treated or cultured in vitro.

In nude mice none of the injected freshly isolated cell populations grew out to a tumor. In soft agar cultures, surprisingly, cells from all three groups formed clones in soft agar at day six and twelve (group I 0.35% and 0.4%, group II 0.4% and 0.5%, group III 0.15% and 0.2%). In Ca^{2+} depleted medium, the neoplastic index (NI) (cells able to form colonies in Ca^{2+} deficient medium/ cells able to form colonies in normal culture medium) was enhanced only in the carcinogen treated cells isolated 4 hours after treatment (NI of group I: 0.11, group II 0.23 and group III 0.01). Established control values are 0.08. This result indicates that the NI most likely reflects an acute membrane damage, either by croton oil alone or by MNNG and croton oil.

Therefore in a second broader study, group I received the active, but less toxic component of croton oil, 12-O-tetradecanoyl-phorbol-13-acetate, group II again MNNG and group III was without treatment. The latency period in vivo was extended. Cells were isolated 2, 4, 15, 21, 28 and 35 days after treatment. In addition, cells were further cultured in vitro under physiological pO_2 ($5\%O_2$) for three weeks and subsequently subjected to the set of selective growth conditions and manipulations as proposed in the request for the second year.

3.3.1.1. Selective growth conditions

Ca^{2+} depleted medium: NI from freshly isolated cells was below 0.4 and did not differ between the three groups. In subcultured cells, again there was no difference found associated with the different treatment regime. However, with increasing latency period in vivo, there was a tendency toward increased NI in the range between 0.3 and 0.7.
Soft agar: Colony formation in soft agar differed between the three groups. In group II, clearly more cells formed clones.
However, surprisingly, also cells from group I (TPA treatment) and group III (untreated) showed a low colony formation capacity (see table 1). In subcultured cells, in all groups no enhanced colony formation was found. Coculture with mouse cells (see 3.3.3.) did not improve the expected specific response in the carcinogen treated group. Interestingly, in cocultures with normal rat cells, the colony formation in all three groups was reduced, most drastically in cells from untreated animals (Table 1). It is planned to use this selective response in further experiments.
Growth in living hosts: None of the tested freshly isolated carcinogen exposed cell populations gave rise to a tumor in nude mice. This, even after a latency period similar to the longterm carcinogenicity study.

3.3.1.2. Enhancement of malignancy

It was speculated that prolonged culture of carcinogen treated cells in vitro might promote their malignancy. Therefore, cells were cultured for three weeks in vitro and again subjected to selctive growth conditions as mentioned above. No effect of the treatment in vivo was detectable (table 1), this in contrast to data obtained with established cells lines. An experiment using hyperoxic conditions (31% O_2) as a second insult is now under way.

From the results of the vo studies, the following conclusions were made:
1) Cells treated in vivo with a short pulse of a carcinogen cannot express their transformed phenotype in a single step. Because it was proven in our system that the carcinogen exposed cells cary gene mutations, we conclude that this type of genetic alteration is not sufficient for the expression of malignant growth characteristics.

2) The chemically treated cells do not express their neoplastic henotype within the first 35 days in vivo after treatment. For technical reasons, however, it is most suitable to isolate cells 14 days after treatment.
3) The second insult necessary for the expression of the transformed phenotype is not provided simply by culturing cells in vitro. This result is in contrast to what was found with established cell lines and confirms that such cells of established cell lines are already in a preneoplastic state.
4) Special attention has to be payed on individual cell populations which can be lost during isolation of the cells after treatment or during in vitro manipulations.

As a consequence for the work in the forthcomming year the interest is focused on three subjects:

a) The substrate provided to the freshly isolated carcinogen-treated cells has to be evaluated.
b) The second insult in vitro has to be modified.
c) A new endpoint for preneoplastic/neoplastic cells has to be selected which detects changes on an enzymic level and not only on growth behavior.

3.3.2. Chromosomal rearrangements.

Because the carcinogenic activity of chemicals correlate best with their clastogenic activity, it is essentiel to analyse variations in the DNA content of individual cells. The most efficient method is the use of a flow cytometer. In September 1983, a flow cytometer and the data analysis system were delivered. Handling (adjustements) of the apparatus was trained. The most suitable staining method and cell type which can be used as a standard were evaluated. The influence of fixation procedures and of sample storage was determined for particular cell populations. Optimal DNA measurements were performed with the fluoresence stain DAPI (4' 6-diamino- 2-phenyl indoledihydrochloride) combined with a protease (pepsin) and a detergent pretreatment of the cells. As a reference cell population, we selected thymocytes recovered from rat thymus of untreated animals. This cell population was easy to isolate, homogeneous and exerts a low aggregation frequency. In our standard the coefficient of

variations, a measurement of the dispersion of the DNA content in individual cells, was below 1.5% (see figure 1). With this value, differences in the DNA content as low as an individual large chromosome can theoretically be detected. Reproducible histogramms with low CV were also obtained from freshly isolated granulation tissue cells.

In contrast the CV of cultured untreated cells was often higher than 2.5%, too high for the detection of a discrete clastogenic effect. This problem arises most likely from unspecific stainings in the cytoplasm. Experiments are now under way to improve the accuracy of DNA-measurements in cells cultured in vitro, since this is a prerequisite for the detection of presumably preneoplastic cells.

3.3.3. Mechanism of growth control

We attempted to characterize further the growth stimulus found in coinjection experiments in vivo in living hosts. This physiological stimulus provided by normal cells could be used for the specific accumulation of preneoplastic cells in vitro in soft agar cultures.

As found in vivo, the coculture of tumor cells with the normal granuloma cells enhanced their cloning efficiency and growth rate[1]. The stimulus was dependent of the number of normal cells added (figure 2). Further analysis revealed that the cocultured cells stay alive during coculture period, do not divide but synthesize and release an agar diffusible factor which stimulated growth of tumor cells (figure 3). Furthermore, the mouse cells isolated from the tissue which surrounds the injected tumor cells in nude mice, showed an even higher growth stimulating activity than the rat fibroblast cells [1]. Further characterization of the factor led to the conclusion that this physiological stimulus can only partially be replaced by a single hormone-like growth factor such as insulin. Thus, although these interactions were found between in vivo transformed cells and normal cells, it is most likely that this growth stimulus is a suitable method for growth promotion of chemically treated cells (preneoplastic) or to shorten the expression of neoplastic cell by this in vitro manipulation.

3.3.4. Effect of asbestos, a presumably nongenotoxic carcinogen

The interaction of asbestos fibres with freshly isolated granuloma cells was further characterized. The cytotoxicity increased with enhanced fibre concentrations. With an electron microsocopy study it was confirmed that the fibres were ingested into the cytoplasm. The genotoxic activity was evaluated on three different levels: Nuclear size, sister-chromatid exchanges and gene mutations.

Exposure to the fibres resulted in enlarged nuclei and increased frequency of tetraploid methaphases. Sister chromatid exchanges were increased at the highest tolerated fibre concentration. No gene mutations were so far inducible at the 6- TG^r locus. This leads to the conclusion that asbestos fibres react through an indirect pathway e.g. via membrane damages.

In order to analyse mechanisms which lead to the enlarged nuclei (tetraploidy), the application of a spindle specific stain was not succesful. Therefore, in a pilot study, monoclonal antibodies to tubu ' were used in order to detect the suspected damages of microtubuli. Allthough the resolution was satifactory, no dramatic effect could be detected so far. As a possible pathway we speculate that the fibres induce lipid peroxidation. The investigations to test this hypothesis are now under way.

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3.4. Personnel

Dr. S. Fritschi left the Institute. Instead Dr. E. Sommer, with a profound expertise in cell culture type of work, joined our research group.

Schwerzenbach, 26.10.84 Peter Maier Ph.D.

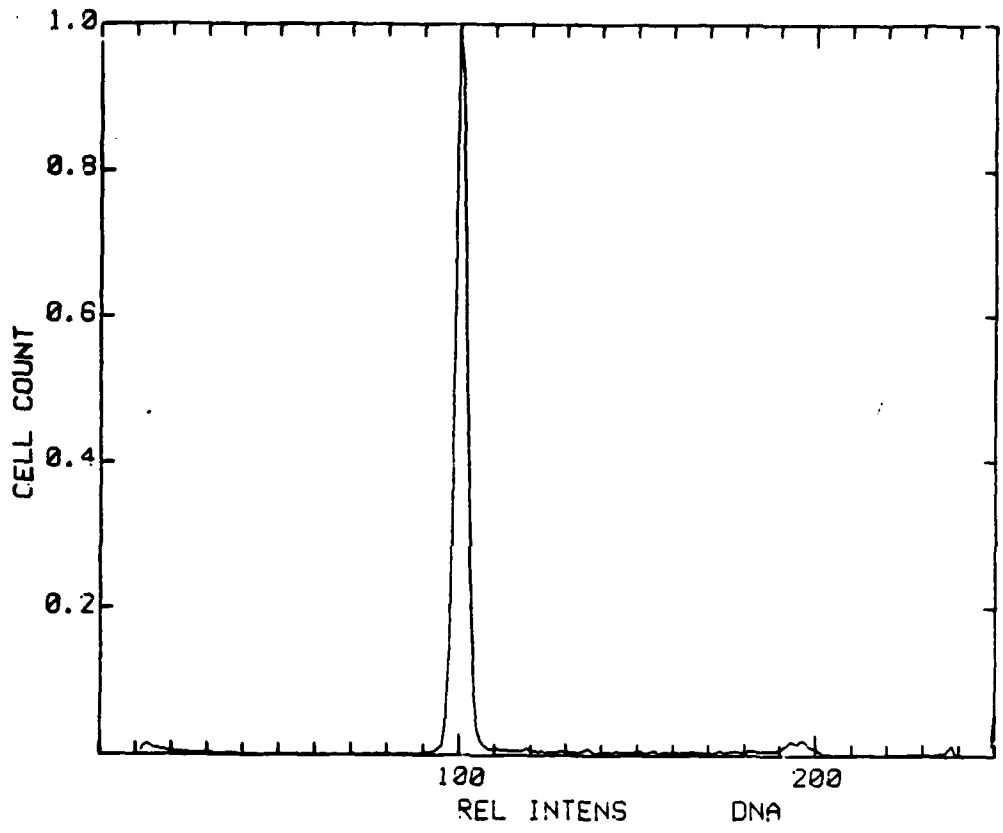
Table 1

Clone formation of freshly isolated (P) and cultured (SC) granuloma cells cocultured with normal mouse and rat cells.

Isolation of cells (days after treatment)	Type of cell population	group I (TPA)			group II (TPA+MNNG)			group III (control)		
		I	I+M21	I+K	II	II+M21	II+K	III	III+M21	III+K
4	P	±	+	±	+	++	+	-	+	-
	SC	±	±	-	±	+	-	±	±	-
14	P	+	++	±	++	++	±	+	++	±
	SC	-	-	-	-	-	-	-	-	-
21	P	+	++	+	+		-			
	SC	+	+	±	±	+	±	-	+	-
28	P	+	+	-	++	+	+			
	SC	-	+		±	±				
35	P	±	+		+	-	+			
	SC	-	±	-	±	+	-			

- no clones; ± clones with < 30 cells: + clones with> 30 cells;
++ clones with>100 cells.
M21: coculture with mouse cells
K : coculture with rat cells

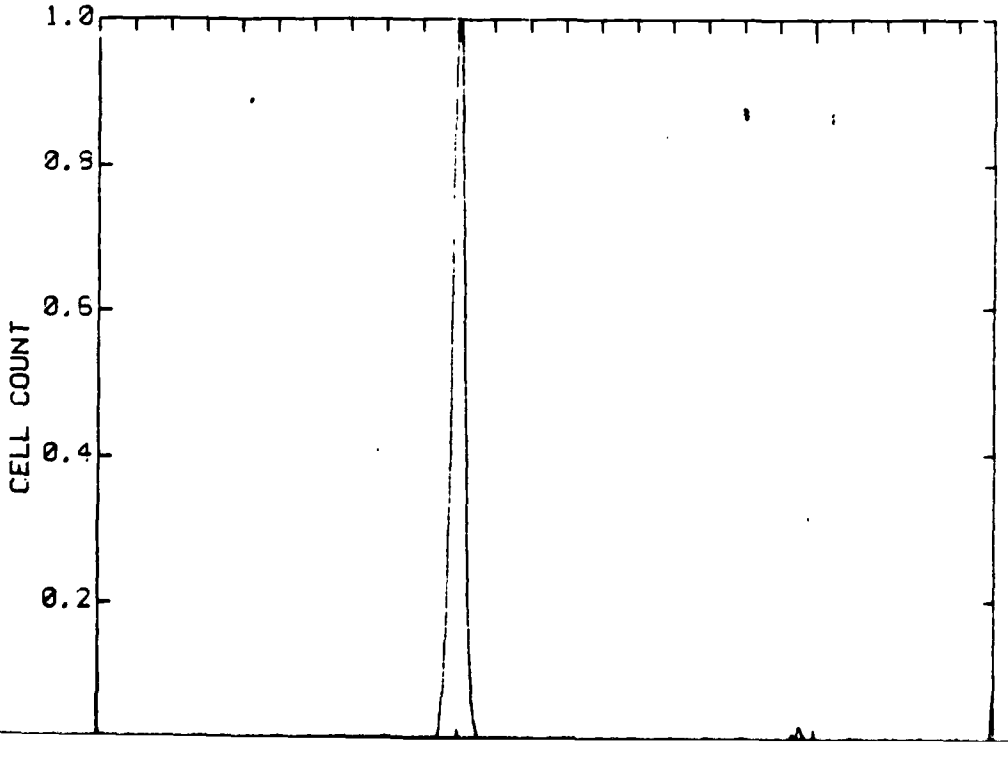
DATE: 24.10.84 FILE: RATTE HIST: 7 SAMPLE: THYMO/♀.3WO



PAR : DNA
PM1 : 3.68
PM2 :
A1 :
A2 :
A3 :
DAPI pH6.5
TOTAL:
14320. CELLS

Figure 1. DNA-histogram of thymocytes isolated from a male and female Sprague-Dawley rat.

DATE: 24.10.84 FILE: RATTE HIST: 8 SAMPLE: THYMO/♂.3WO



PAR : DNA
PM1 : 3.68
PM2 :
A1 :
A2 :
A3 :
DAPI pH6.5
TOTAL:
3477. CELLS

Figure 2. Effect of co-culture of variable amounts of normal cells (MC57,SGC) on the cloning efficieny of tumor cells in soft agar.

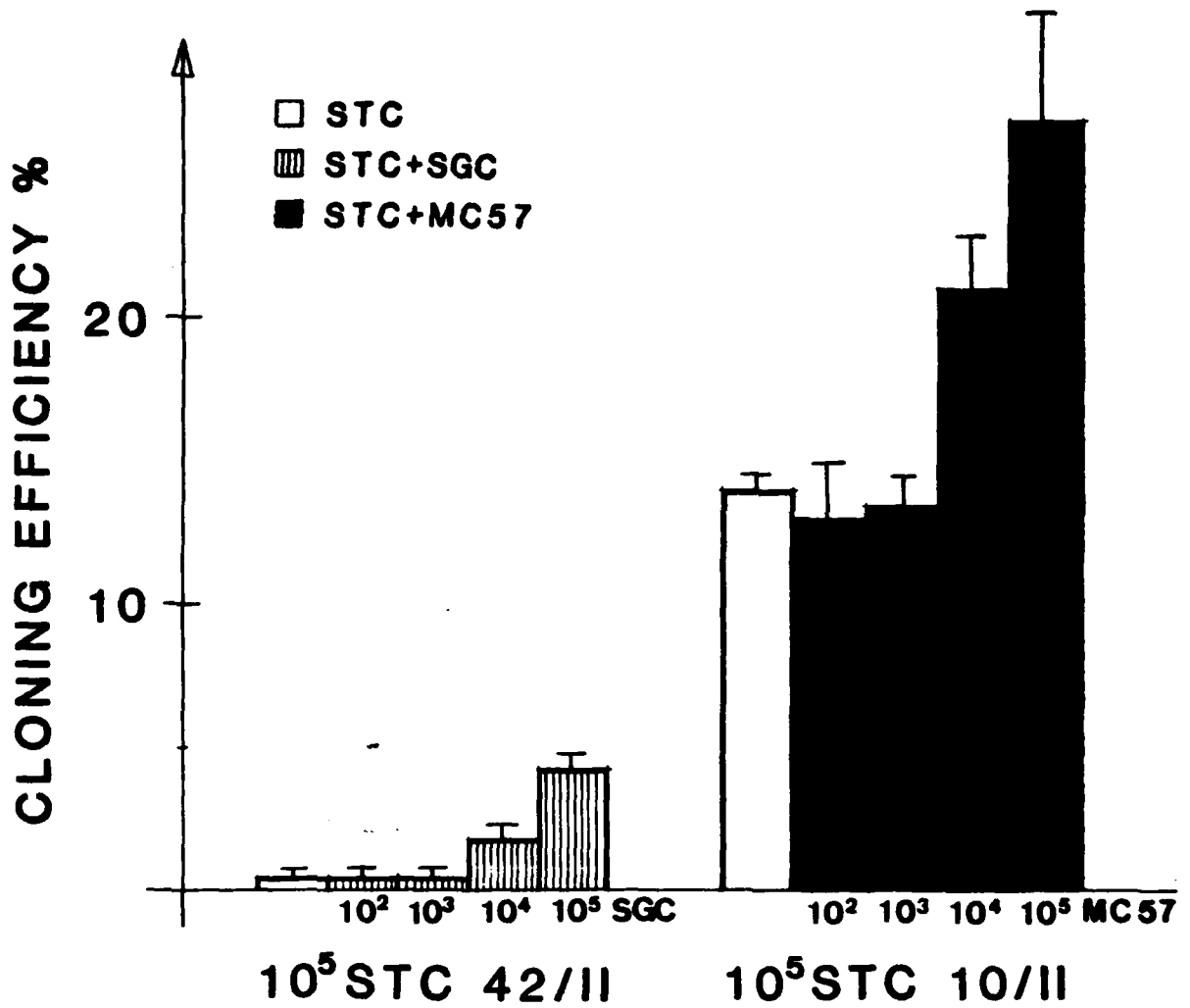
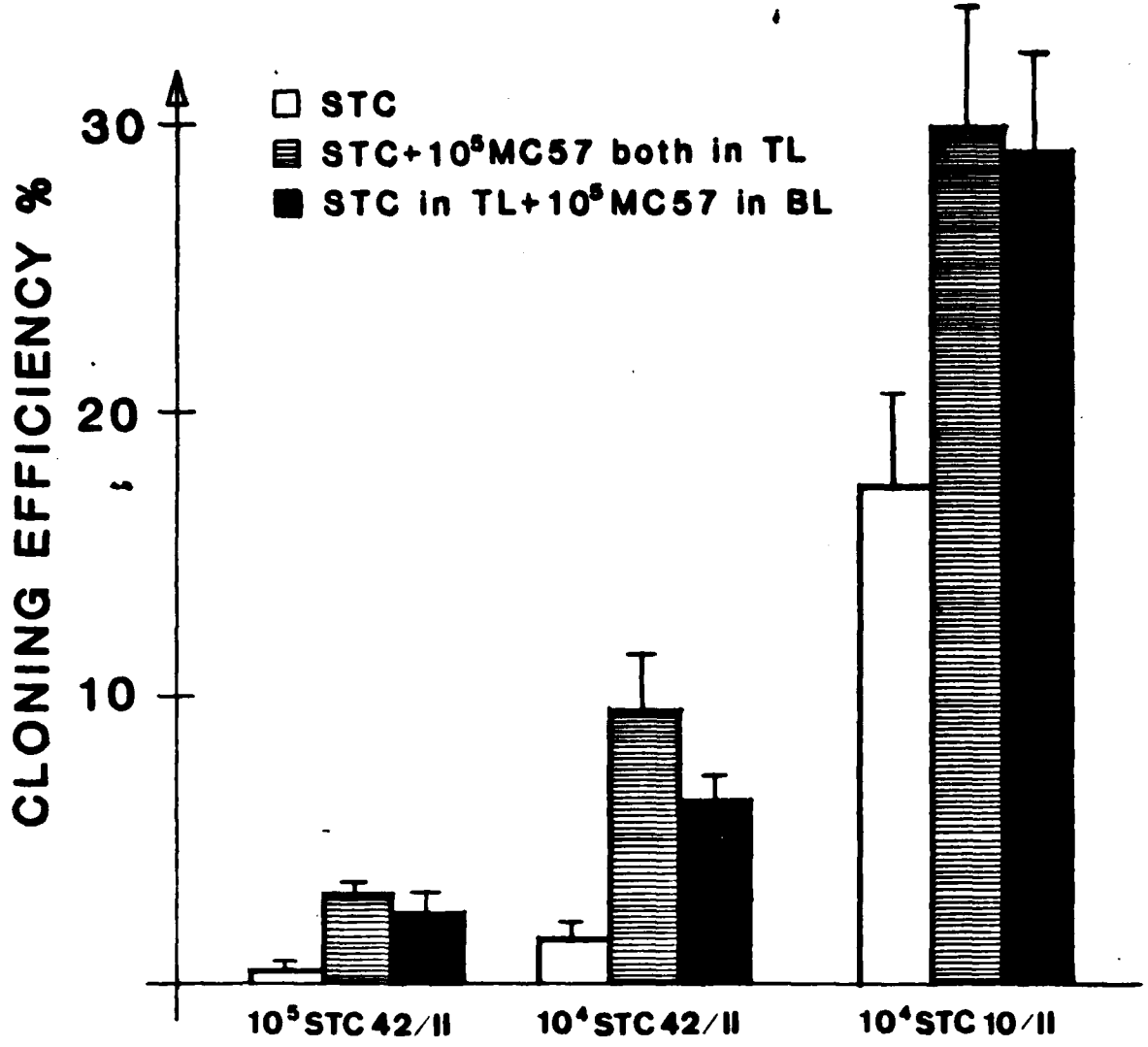


Figure 3. Growth stimulation of normal MC57 cells co-cultured in separate agar layers (TL = top agar layer BL = basal agar layer).



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