

**Passive Antitumor Defense System: Hypothesis and  
Experimental Results**

**Ph.D. Thesis**

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*To my parents who gave me the possibility  
to become a biochemist,  
to my wife, to my daughter Gabi and to my colleagues who helped me  
to work as a biochemist  
and  
to my son Peti who wants  
to be biochemist*

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## **ABBREVIATIONS**

**KS** Kaposi's sarcoma

**NHL** non-Hodgkin's lymphoma

**LAK** lymphokine-activated killer (cells)

**PET** positron emission tomography

**PADS** passive antitumour defence system

**MTT** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

**PBS** phosphate-buffered saline: 140 mM NaCl, 3 mM KCl, 8 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub> (pH 7.1)

**TAE** 40 mM Tris-acetate, 1 mM EDTA

**ATCC** American Type Culture Collection

# INTRODUCTION

## **The ineffectiveness of the known immune system to prevent the development of tumours**

It is well known that full-blown AIDS is associated with substantial loss of virtually all cellular and humoral immune responses [1-5]. The degree of AIDS-related immunological impairment is well demonstrated by rejection-free renal graft survival in a patient with AIDS despite the significant and prolonged withdrawal of the usual immunosuppressive agents used in renal transplants [6]. Because of the global defects of the known immune system in AIDS, the incidence of all tumours should increase in AIDS populations if the known immune system were the only mechanism to prevent the development of tumours. However, contrary to expectations the incidence of only some kinds of tumours, mainly KS and NHL, has significantly increased [7, 8]. Taking into consideration that during the period 1981-89 a significant, continuous fall was observed in the percentage of AIDS-related KS in European AIDS cases ( $p$ -trend $<0.0001$ ) [9], and that large differences can be observed in tumour incidence between homosexual and heterosexual, haemophilic, and injection drug user AIDS patients [2, 10, 11], and that cohort studies indicate that KS occurs at a relatively constant rate before immune suppression becomes profound (starting 1-2 years after HIV infection) and the incidence does not increase as the immune function declines [12], and that an increase in the incidence of KS and NHL has not yet been described in children with AIDS who have also been treated and then examined regularly over an extended period [13], it can be stated that even in the case of the above-mentioned tumours the reason for the high incidence is not the defects of the immune mechanism but other agents [10]. To sum up, despite global abnormalities in the immune system in AIDS the incidence of only a few kinds of tumour increases what is more the degree of immunosuppression seems not to be a critical factor in the development of even these tumours. These findings strongly support that the known immune system has no significant role in the mechanism preventing tumour development.

This statement is further supported by the following observations: The majority of clinically relevant tumours are not or are only weakly immunogenic [2, 14]. The tumours possess different active and passive mechanisms of immune escape including Fas ligand expression by the tumour, immunosuppressive cytokines, inhibitory neuropeptides, etc. as active mechanisms and inadequate expression of adhesion and costimulatory molecules (e.g., B-7) as passive mechanisms [15]. There is no increase in common tumours (carcinomas of the breast, colon, lung, prostate, etc.) in primarily immunodeficient children or in immunosuppressed adults (usually transplant recipients) [16]. In immunosuppressed organ allograft recipients only some kinds of malignancies, primarily KS, have a higher incidence than in normal populations [2, 17]. Patients with selective immune deficiency disease (e.g., lepromatous

leprosy) show no evidence of increase in tumours [18]. The frequency of spontaneous or induced solid tumours in animals whose immunity is suppressed by anti lymphocyte serum or in congenitally immunodeficient animals is not different from that in immunologically intact animals [18].

It can be raised as an objection that the above-mentioned discrepancies in the range and frequency of tumours can be explained by the short survival time of patients, namely, the other kind of tumours cannot develop during this short interval. However, according to recent opinion it is likely that most human cancers originate within two years or less of detection [19]. At the same time, virtually all HIV-infected patients already suffer from a gradual deterioration of the immune system during the period (median, 10 years) of clinical latency [20] and many organ transplant patients (e.g., in the case of kidney transplant) have more than ten years survival times [2]. On the other hand in clinical reports a variety of other malignancies in HIV-infected persons [7, 21] and in immunosuppressed organ allograft recipients [2, 18] have been noted but their frequency is no higher than in the rest of the population. All of these preclude the short survival time as the reason for the discrepancies of incidence and range of tumours in patients having immune deficiency.

Another objection may be that in AIDS patients and in organ transplant recipients the majority of the tumour types are eliminated by the remaining part of the immune system. However, the observations that the remaining part of the immune system is unable to reject renal graft in AIDS patients [6] and that the low levels of immune reaction facilitate rather than inhibit tumour growth (enhancement) [22-25] contradict this statement.

The explanation that natural killer cells and macrophages have a tumour preventive role in the above cases is refuted by many publications [18, 26, 27]. According to the most recent evidence, the impairment of natural-killer-mediated cytotoxicity by plasma of cancer patients is correlated to their nucleosome concentrations because the nucleosomes released from dead cells may allow the tumour cells to escape natural-killer-mediated lysis [28]. It has been strongly suggested that activated macrophages may even promote tumour growth [29, 30]. The observations made in connection with AIDS, including the diminished cytotoxic capability and pool sizes of natural killer and LAK cells, abnormal function of monocytes and macrophages [1-5], etc. and the change of tumour incidence independently from these supply the strongest evidence against the general tumour preventive role of natural killer cells and macrophages *in vivo*.

### **Possible Explanations for the Anomalies of Tumour Incidence**

Despite the ineffectiveness of the known immune system the majority of people do not die from tumours. There are two possibilities to explain this contradiction. One is that the rise of a cancer cell is a rare singular event but that

a tumour may develop from every cell arising. In other words, there is no defence mechanism against tumours and the frequency of cancer cell formation equals the frequency of tumour development. This assumption includes the high vitality of these cancer cells in any conditions. However, this contradicts the observations that the cell death rate is still high within non-necrotic tumour tissue [31] and that 70 % to 90 % of newly-produced tumour cells in humans die spontaneously by a mechanism that is as yet poorly understood [19]. On the other hand, while from a rapidly-growing tumour 1 cm in size millions of tumour cells can be shed into the circulation every day, only a very small percentage (<0.01 %) of circulating tumour cells initiates metastatic colonies [32].

The other possibility is that cancer cell formation is quite common but that the majority of cells are not able to multiply in order to produce a tumour because they die shortly after they arise. The reasons for this may either be entirely random effects or the action of a systematic defence system. Were cell death an absolutely accidental event and were it to occur independently of any kind of defence mechanism, the simultaneous development of a number of primary tumours in organs should be a relatively frequent occurrence. However, the development of even double synchronous primary tumours is rather rare [33, 34].

Thus, the fact that tumours do not develop in the majority of the population during their lifetime can only be explained by the existence of other, up to the present unknown, defence system or defence systems.

### **The Passive Antitumour Defence System: a Hypothesis**

It is obvious that the components of a general defence mechanism (a “surveillance”) must be in the circulatory system. It is well known, that different small substances of the circulatory system (amino acids, monosaccharides, nucleobases, vitamins, membrane permeable intermediates of the cell metabolism, etc.) can reach and enter both normal and tumour cells because their presence is fundamental for cell functions. Their uptake by normal cells is regulated, but there is abundant evidence that the uptake of the majority of these substances by tumour cells is increased, unregulated and proportional to their availability [35-41]. A net flux of them occurs towards the tumour cells and they may attain high levels relative to the levels in surrounding normal tissues [37, 39]. These observations are well known, widely accepted, what is more, some techniques of tumour detection (e.g., PET) use this feature of cancer cells [41-44].

According to our hypothesis, this feature, i.e., the increased, unregulated uptake of some of these substances may be fatal for arising tumour cells, when the number of cells is still low and there are sufficient quantities of substances in the environment of cells that the cells can accumulate them. It can be assumed that some of these substances may be toxic for cancer cells and can kill them if their concentrations can reach high level in the cells. To

our hypothesis that happens with arising tumour cells in the majority of the population during their lifetime if the number of cells arisen is not too high (absence of strong carcinogenic effects) or the concentrations of the required substances are not too low (healthy subjects, balanced food intake). Otherwise, the number of tumour cells arising can exceed a critical value (critical cell number) at which the divisions of the cells overcompensate the killing of cells by the mentioned substances, and it is most likely that a tumour develops. The killing of tumour cells by the given substances form the Passive Antitumour Defence System.

The many fundamental changes undergone by a normal cell in order to become a cancer cell have been well-demonstrated [45-47]. Cancer cells have marked and characteristic enzymatic and metabolic imbalance. Their various control factors, membrane permeability, transport systems, enzyme amounts, enzyme activity, enzyme regulation, different metabolic pathways, isozyme composition, etc. alter considerably compared with normal tissues. Accordingly tumour cells differ largely from normal cells. However they differ only in degree, not in kind. To the best of our knowledge, there is no absolute, qualitative difference between normal and tumour cells at all. This precludes the possibility that a single substance can kill the tumour cells selectively, only more than one co-operating molecules can be effective.

The defence mechanism can be interpreted so that the arising aberrant cells are in a metabolic respect incompatible with their environment. Thus, certain substances of this adversary environment are when abundant fatal to them and destroy these deviant cells. Obviously, the various kinds of tumour cells differ from normal cells in numerous ways, and therefore the type and amount of the substances effective against them also differ to a certain extent. However, the physiological mixture of the substances in the blood is probably generally effective against all kinds of cancer cells. These substances are able to perform a continuous and a relatively constant level of "surveillance" because in normal conditions their concentration in the blood is retained within a narrow range by physiologically regulated anabolism, catabolism, reabsorption and excretion.

The function of these "killer" molecules is generally not the protection of the organism. They play such a protective role only when tumour cells arise and exist. This dual role is very similar to the protective role of substances (i.e., fatty acids, porphyrins, lactic acid, etc.) which are bactericidal to certain pathogenic micro-organisms [18]. Only the killing of tumour cells needs the collective, simultaneous, synergistic effect of more than one substance, because the altered-self cells do not differ to such an extent from the non-altered cells, like the nonself pathogenic micro-organisms.

In the following set of experiments we tried to evidence the above hypothesis.



## **MATERIALS AND METHODS**

### **Materials**

Adenine, L-arginine, L-phenylalanine, L-histidine, L-tryptophan, L-tyrosine, L-methionine, 2-deoxy-D-ribose, oxalacetic acid, L(-)malic acid, d-biotin, pyridoxine, riboflavin, L-ascorbic acid sodium salt, D(+)-mannose, sodium bicarbonate, adonitol, N-acetyl-D-glucosamine, *myo*-inositol, D(+)-glucosamine, DL-6,8-thioctic acid, glutathione, taurine (all of them were cell culture tested biochemicals) were purchased from Sigma Chemical Co. (St. Louis, MO) or recently from Sigma-Aldrich Co. (Budapest, Hungary). The same substances were also obtained from Serva Feinbiochemica (D-6900 Heidelberg 1, FRG) and from Reanal (Budapest, Hungary). Dulbecco's modified Eagle's medium, Medium 199, RPMI-1640, trypan blue, MTT, adenosine, D(+)galactose, folic acid, hypoxanthine, D-pantothenic acid hemicalcium salt, orotic acid monosodium salt, hippuric acid sodium salt, p-aminobenzoic acid, L-carnitine, DL-isocitric acid, cis-aconitic acid, pyruvic acid, uridine, cobalamin, allantoin, L(-)fucose, betaine, levulinic acid, propionic acid sodium salt, 4-nitrophenylphosphate (Sigma tablets No. 104-105), and catalase (No. C-40) were purchased also from Sigma Chemical Co. or recently from Sigma-Aldrich Co. Amino acid Kit (AS-30), D-mannitol, nicotinamide, and thiamine hydrochloride were obtained from Serva Feinbiochemica GmbH & Co. (Heidelberg, Germany), creatine, creatinine, succinic acid disodium salt, L-cysteine hydrochloride, D(-)-ribose from FLUKA AG (Buchs, SG, Switzerland) and fetal calf serum from Sebak GmbH (Aidenbach, Germany). All other chemicals were of the purest grade available from Reanal (Budapest, Hungary) or recently from Reanal Finechemical Co. (Budapest, Hungary).

### **Mice**

Six- to eight-week-old female BALB/c mice and BALB/c (nu/nu) mice were obtained from Department of Immunology and Biotechnology, University Medical School of Pécs (Pécs, Hungary). All mice were housed in plastic cages, 5 mice/cage, and provided with food and water *ad libitum*. Nude mice were maintained under specific pathogen-free conditions and all manipulations with the animals were performed by sterile technique.

### **Tumour Cells and Culture**

The Sp2/0-Ag14 mouse myeloma, the EL4 mouse lymphoma, the A20 mouse B cell lymphoma, the Jurkat human acute T cell leukaemia cell lines were kindly provided by Prof. Dr. Péter Németh (Department of Immunology and Biotechnology, University Medical School of Pécs). The K562 human erythroleukemia, the HeLa human cervix epithelioid carcinoma, the HEp-2 human larynx epidermoid carcinoma, the Hep G2 human hepatocellular carcinoma, the MCF7 human breast adenocarcinoma, the LLC-MK<sub>2</sub> rhesus monkey (*Macaca mulatta*) kidney, the MDCK canine (*Canis familiaris*) kidney, and the Vero African green monkey kidney cell lines were generously provided by Prof. Dr. Júlia Szekeres (Department of Microbiology, University Medical School of Pécs). The Caco-2 human colon adenocarcinoma cell line was kind gift of Dr. György Szucs (Department of Virology & Laboratory ÁNTSZ, County Institute of National Public Health Service, Pécs) and the MCF7/ADR Adriamycin-resistant human breast cancer cell line [48] was kindly supplied by Dr. Endre Kálmán (Department of Pathology, University Medical School of Pécs).

The Sp2/0-Ag14, EL4, A20, Jurkat, MCF7, Hep G2, and K562 cells were cultured in RPMI 1640 medium containing L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), and 10 % fetal calf serum. The HeLa, MCF7/ADR, MDCK, and LLC-MK<sub>2</sub> cells were grown in Dulbecco's modified Eagle's medium containing 10 % fetal calf serum. HEp-2 cells were grown in the same medium containing 5 % fetal calf serum. Vero cells were grown in Dulbecco's modified Eagle's medium and Medium 199 (1:1) containing 10 % fetal calf serum. Caco-2 cell line was cultured in Medium 199 containing 10 % fetal calf serum. The cells were incubated in a humidified atmosphere of 5 % CO<sub>2</sub> at 37 °C. All cell lines were free of *Mycoplasma*.

### **Cytotoxicity Assay**

The toxicity was assessed by adding the tested compounds dissolved directly in the applied medium in the indicated concentrations specified under figures to cultures in 96-well micro plates. Because ascorbic acid has short half-life in culture [49] it was added to culture once every 24 h [49] in the indicated amounts. To avoid the effect of pH, the solutions - except for ascorbic acid - and mediums were incubated overnight in a humidified atmosphere of 5 % CO<sub>2</sub> at 37 °C, before use. Immediately before use, 5 mM solution of ascorbic acid was made by using the appropriate medium incubated overnight and this solution was further diluted with the same medium to the working concentration. In the case of Sp2/0-Ag14, EL4, A20, Jurkat, and K562 lines, the logarithmically growing cells were harvested from the medium and resuspended to a final concentration of 4x10<sup>4</sup> cells of Sp2/0-Ag14, EL4, Jurkat, and

$2 \times 10^4$  of K562, A20 in 250  $\mu\text{l}$  appropriate medium per well containing the tested materials in the indicated concentrations. In the case of HeLa, HEp-2, Vero, MCF7, MCF7/ADR, Hep G2, MDCK, LLC-MK<sub>2</sub>, and Caco-2 cell lines, the cultured cells were harvested from 75 % confluent tissue culture flasks with 0.2 % trypsin, 0.025 % versene solution and resuspended in the appropriate medium at a density of  $10^5$  cells/ml. Aliquots (100  $\mu\text{l}$ ) - in the case of Caco-2, 100  $\mu\text{l}$  and 50  $\mu\text{l}$  - were dispensed into 96-well micro plates, made up to 250  $\mu\text{l}$  with the appropriate medium and incubated for 24 h. Then the medium was gently discarded and replaced with 250  $\mu\text{l}$  fresh medium containing the tested compounds in the indicated concentrations. All kinds of cells were allowed to proliferate for 48 h.

The number of viable Sp2/0-Ag14, EL4, A20, Jurkat, and K562 cells was then counted microscopically with the trypan blue dye exclusion method.

The survival of HeLa, HEp-2, and Vero cells was measured by assessing endogenous alkaline phosphatase activity of cells [50]. The assay was validated before starting the experiments. Briefly, after incubation period the culture medium was removed from the well, the cells were rinsed with sterile PBS. Then 150  $\mu\text{g}$  of alkaline phosphatase substrate (4-nitrophenylphosphate, Sigma tablets No. 104-105) dissolved in 150  $\mu\text{l}$  fresh 10 % diethanolamine buffer (pH 9.8) was added to each well. During the addition and removal of solutions care was taken not to disturb the attached cells. Plates were incubated at 30 °C until the absorbance in the case of untreated cells reached a value of about 1. The reaction was stopped by adding 50  $\mu\text{l}$  of 3 M NaOH to each well. The absorbance was measured at 405 nm with the aid of a Dynatech ELISA reader. Peripheral wells of each plate were utilised for blank (N = 3) background determinations. Background values were subtracted from each reading.

The viability of Caco-2, MCF7, MCF7/ADR, Hep G2, MDCK, and LLC-MK<sub>2</sub> cells was assessed with the MTT colorimetric assay [51]. Because of disturbing effect of the tested compounds the modified assay was used [52]. In brief, after 48 h incubation the medium was removed from the wells and the cells were washed with sterile PBS. To the cells in each well was added 50  $\mu\text{l}$  of a 5 mg/ml sterile filtered solution of MTT in the applied medium. After incubating the plate for 4 h in 5 % CO<sub>2</sub> at 37 °C, the untransformed MTT was removed from the wells and the cells were washed with PBS. In all cases, the addition and removal of solutions were made carefully not to disturb the attached cells. Then 50  $\mu\text{l}$  isopropanol was added to all wells of the plate and thoroughly mixed in order to solubilize the formazan crystals. The quantity of formazan product formed was assessed by its absorbance at 550 nm on a Dynatech MR7000. Peripheral wells of each plate were utilised for blank (N = 3) background determinations. Background values were subtracted from each reading. Results were expressed in case of all cell lines as the percentage of the untreated control systems. All values are expressed as the mean $\pm$ SE.

### **Treatment Schedule and Dose**

The concentration of substances in the solution used for treatment of animals were: 0.1 M L(-)malic acid, 0.1 M L-phenylalanine, 0.1 M L-arginine, 0.1 M L-histidine, 0.1 M 2-deoxy-D-ribose, 0.002 M L-tyrosine, 0.1 M L-methionine, 0.05 M L-tryptophan, 0.05 M d-biotin, 0.1 M pyridoxine hydrochloride, 0.1 M L-ascorbic acid sodium salt, 0.005 M adenine, 0.001 M riboflavin, 0.146 M NaHCO<sub>3</sub>, and 0.004 M KHCO<sub>3</sub>. The compounds were dissolved in a buffer (8 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.1). The solution was prepared immediately before use and injected at 6.00, 9.00, and 12.00 a.m.; 3.00, 6.00, and 9.00 p.m. on each treatment day in a volume of 0.2 ml.

### **Determination of the Tumourigenicity of Sp2/0-Ag14 Mouse Myeloma Cells**

Tumourigenicity was assessed by i.p. injection of varying numbers of Sp2/0-Ag14 mouse myeloma cells into BALB/c mice (3 mice/ group). The mice were monitored daily for mortality [53].

### **Evaluation of Antitumour Activity in i.p. Tumour Model**

BALB/c mice (10 mice/group) were injected i.p. with  $5 \times 10^4$  Sp2/0-Ag14 mouse myeloma cells suspended in 0.2 ml RPMI 1640 on Day 0. The solution used for treatment was given i.p. daily according to the defined schedule from Day 1 after tumour inoculation for 10 days. Tumour-bearing control mice were given injections of 0.2 ml PBS. Mice were monitored daily for mortality. Efficacy of the solution used for treatment was expressed as the percentage increase in median survival time of treated over control tumour-bearing mice (T/C %) [54]. The log<sub>10</sub> cell kill was calculated from the formula  $(T-C) / 3.32 \times T_d$ , where T and C are the survival times for 50 % of the animals in days for the treated and the control groups, respectively; 3.32 is the number of cell doublings per log of cell growth; and  $T_d$  is the doubling time of Sp2/0-Ag14 mouse myeloma cells [55]. The *in vivo* tumour doubling time for Sp2/0-Ag14 cells was estimated from semilogarithmic plots of data of tumourigenicity experiment, where the number of cells in the inoculum was plotted on the ordinate in logarithmic scale versus the survival times in days for 50 % of the animals on a linear abscissa [55].

### **Determination of Cell Number in Ascitic Fluid**

BALB/c mice (5 mice/group) were injected i.p. with  $5 \times 10^4$  Sp2/0-Ag14 mouse myeloma cells suspended in 0.2 ml RPMI 1640. One day later the treatment was started. The solution used for treatment was given i.p. for 10 consecutive days according to the defined schedule. Tumour-bearing control mice were given injections of 0.2 ml PBS. After finishing the treatment, the mice were sacrificed by cervical dislocation, the skin was opened, and the ascitic fluid was sucked and stored separately. The peritoneal cavity was washed twice with 5 ml RPMI 1640 medium and the cell-containing fluid was collected. After centrifugation, the cells were resuspended in 5 ml RPMI 1640 medium and were counted.

### **Evaluation of Antitumour Activity in s.c. Tumour Model**

A cell suspension of  $5 \times 10^7$  HeLa cells/ml was prepared in Eagle's minimum essential medium and 0.1 ml of the cell suspension ( $5 \times 10^6$  cells) was implanted subcutaneously in the lower extremities of the BALB/c nude mice (5 mice/group). At the start of treatment all tumours had a volume of 30-60 mm<sup>3</sup>. The first day of treatment was indicated as day 0. The solution used for treatment was administered i.p. according to the defined schedule for 10 consecutive days. Tumour-bearing control mice were given injections of 0.2 ml PBS only. Digital callipers (Mitutoyo, Inc., Tokyo, Japan) were used to measure the length (L), width (W), and height (H) of each tumour or each lobe in multi-lobed tumours twice weekly and the tumour volumes were estimated by the formula of  $0.5 \times L \times W \times H$  because this way of determining the volume has proved to be the most accurate [56]. Because of the variation in size at the initiation of treatment, volumes were converted to the initial tumour volume. The relative tumour volume was expressed by the formula  $V_t/V_0$ , where  $V_t$  is the tumour volume on a given day of measurement, and  $V_0$  is the initial volume of the same tumour at the start of the treatment. The ratio of the mean relative volume of treated tumours over that of control tumours multiplied by 100, (T/C %), was calculated at each evaluation [57]. The criteria for effectiveness were the percentage of T/C value with 42 and less. Mean growth delay was measured based on the number of days required for mean relative tumour volume to reach nine fold of the initial volume [58].

### **Toxicity Testing**

Toxicity was monitored by weight loss and toxic death. A weight loss nadir of 20 % per mouse or greater or 20 % or more toxic death is considered an excessively toxic dosage of the given substances [59].

### **DNA Gel Electrophoresis**

The effect of the mixtures was assessed by adding the indicated concentrations of the components dissolved in the applied medium to cultures,  $16 \times 10^4$  Sp2/0-Ag14,  $4 \times 10^4$  K562,  $8 \times 10^4$  Vero cells per 1000  $\mu$ l medium. The composition of control mixture and active mixture are given in the legend to Fig. 10. The cells were allowed to proliferate for 24 h. Untreated cells were collected at the same time as treated samples. The number of viable cells was then counted microscopically with the trypan blue dye exclusion method. DNA fragmentation was monitored by gel electrophoresis as described previously [60]. Briefly, the untreated, control mixture or active mixture treated cells, were collected by centrifugation, washed in PBS, resuspended ( $5 \times 10^6$  cells) in 0.5 ml of 45 mM Tris-borate buffer-1 mM EDTA, pH 8.0, containing 0.25 % Nonidet P-40 and 0.1 % RNase A, incubated at 37 °C for 30 min and then treated with 1 mg/ml of proteinase K, and incubated for an additional 30 min at 37 °C. After incubation, 0.1 ml of loading buffer (0.25 % bromophenol blue, 30 % glycerol) was added and 40  $\mu$ l of the tube content were transferred to the gel. Electrophoresis was performed on 1.6 % agarose gel containing 0.5  $\mu$ g/ml ethidium bromide at 80 V for 1-2 h with a TAE running buffer. DNA was visualised under ultraviolet light and photographed.

### **Flow Cytometric Analysis**

Flow cytometric analysis was performed to identify apoptotic cells as described earlier [61]. Briefly, cells were fixed in 70 % ethanol overnight at 4 °C. Cells after fixation were incubated in PBS containing 50  $\mu$ g/ml RNase for 1 h, and stained with 65  $\mu$ g/ml propidium iodide for 1 h at 4 °C and then analysed by a FACSort flow cytometer.

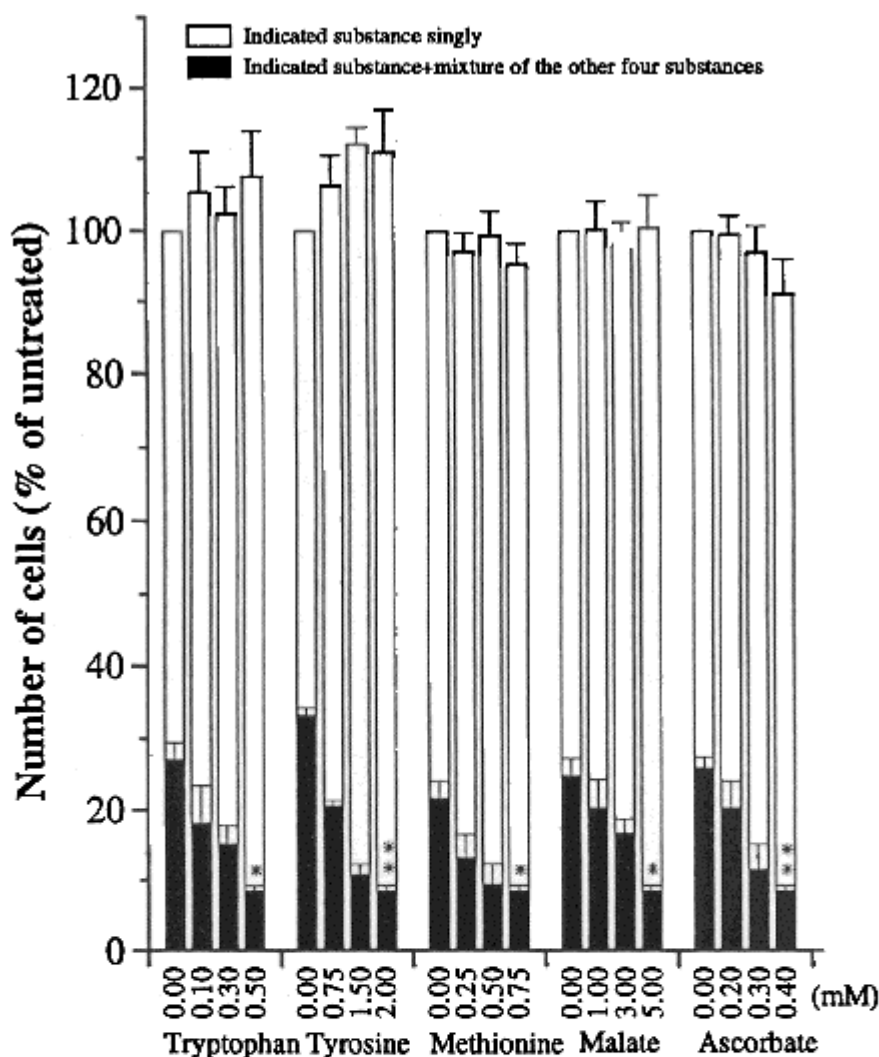
### **Statistical Analysis of Data**

The two-tailed Student's t test was used to determine the statistical significance of any changes observed.

## EXPERIMENTAL RESULTS

### Effects of L-Tryptophan, L-Tyrosine, L-Methionine, L(-)Malate, L-Ascorbate in Different Concentrations Singly and in Combination on the Survival of Sp2/0-Ag14 Mouse Myeloma Cells after a 48-h Incubation Period

It can be seen (Fig. 1) that using the above mentioned materials singly in the indicated concentrations (white columns), none of them showed tumour cell killing effect, only methionine and ascorbate decreased slightly but not significantly the proliferation of the cells in the highest 0.75 and 0.4 mM concentration. In the given concentrations tryptophan and tyrosine even increased the proliferation of cells compared to untreated cells. However, exposure of Sp2/0-Ag14 cells to combinations of four or five compounds caused a significant synergistic increase in tumour cell death (black columns). The effect of mixtures containing four compounds is shown by the first black column in each group because the concentration of the indicated fifth substances in these cases were chosen zero. The four-component mixtures in each group contained the four compounds in the concentrations given under the fourth column of the other four groups. For example in the tryptophan group the mixture having four components contained 2.0 mM tyrosine, 0.75 mM methionine, 5.0 mM malate, 0.4 mM ascorbate as a final concentration in a well. It appears (Fig. 1) that this four-component mixture has significant cytotoxic effect on tumour cells because the cell number was only 27.2 % compared to untreated cells. By increasing the amount of the fifth compounds the cell death further increased. For example, the additional tryptophan in 0.5 mM concentration decreased the cell number from 27.2±2.2 % of the untreated value to 8.5±0.6 % (black column). The same amount (0.5 mM) of tryptophan, when it was used without the other four substances, even slightly increased the proliferation of cells (white column). The differences between mixtures containing four components and mixtures containing five components and having the fifth components in the highest indicated concentration were significant (in the cases of malate, tryptophan, and methionine  $P<0.01$ , in the cases of ascorbate and tyrosine  $P<0.001$ ). The changes in different degrees of the white and black columns (in the cases of tryptophan and tyrosine even the opposite one) with the increasing amounts of the fifth materials prove the synergistic effect, and the black columns demonstrate the powerful tumour cell killing effect of the mixtures.

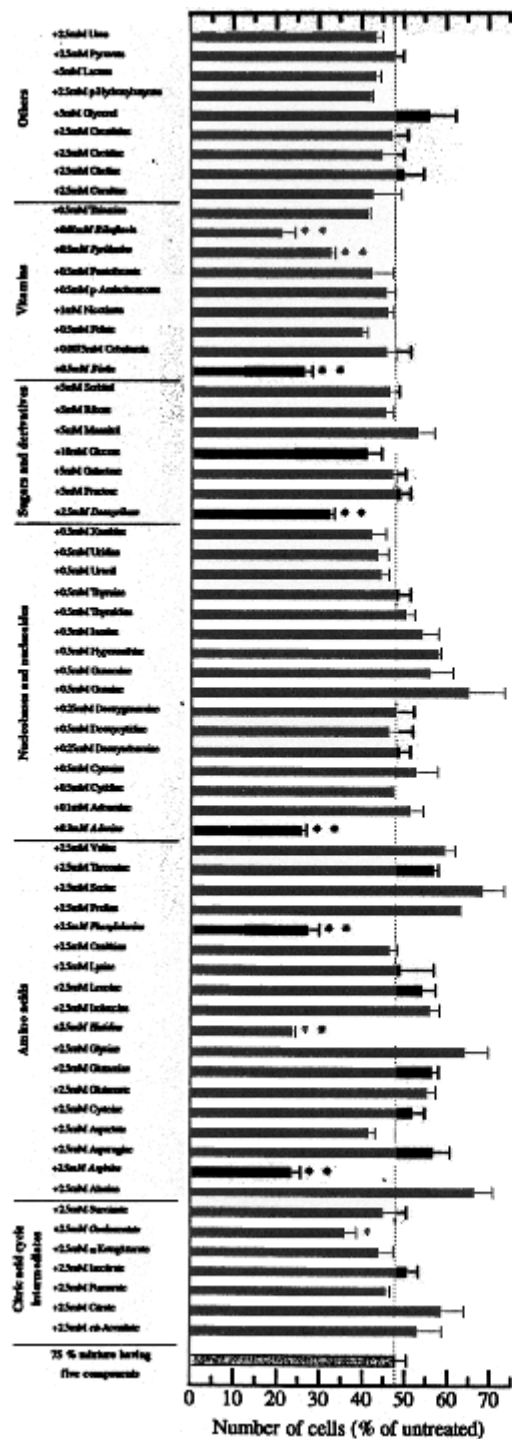


**FIGURE 1**

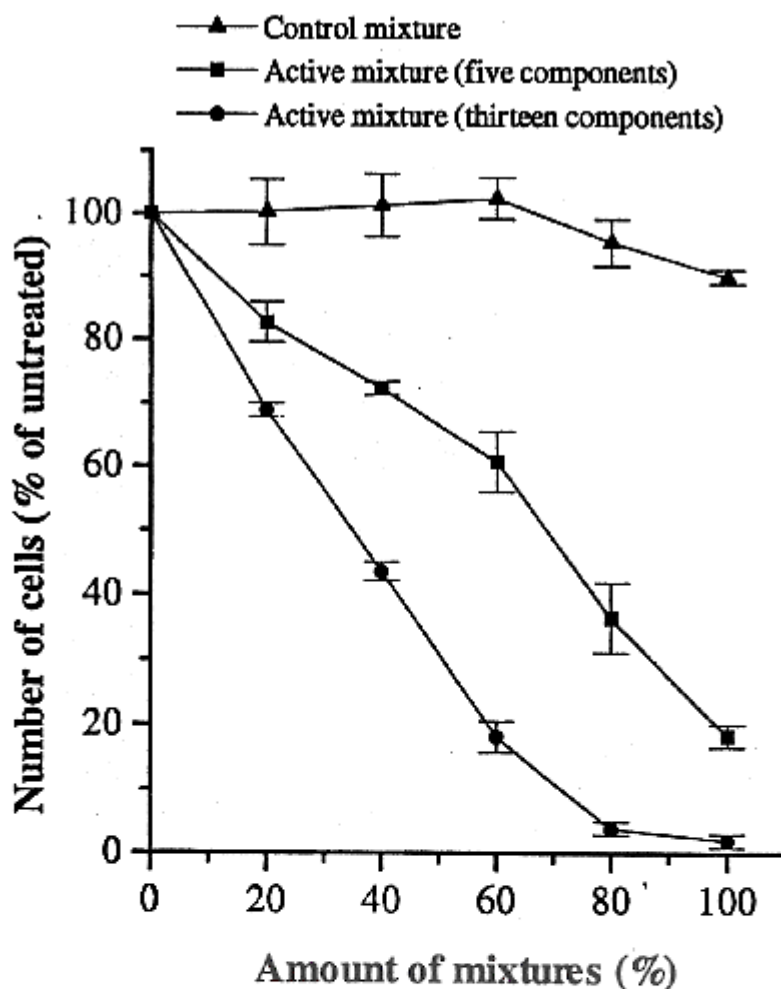
In vitro effect of L-tryptophan, L-tyrosine, L-methionine, L(-)-malic acid disodium salt and L-ascorbic acid sodium salt, singly and in combination, on the growth of Sp2/0-Ag14 mouse myeloma cells. The cells were treated with the indicated amounts of the substances singly (white columns) or in combination with the following mixtures (black columns): L-tryptophan with mixture of 2.0 mM L-tyrosine, 0.75 mM L-methionine, 5.0 mM L(-)-malic acid disodium salt and 0.4 mM L-ascorbic acid sodium salt; L-tyrosine with mixture of 0.5 mM L-tryptophan, 0.75 mM L-methionine, 5.0 mM L(-)-malic acid disodium salt and 0.4 mM L-ascorbic acid sodium salt; L-methionine with mixture of 0.5 mM L-tryptophan, 2.0 mM L-tyrosine, 5.0 mM L(-)-malic acid disodium salt and 0.4 mM L-ascorbic acid sodium salt; L(-)-malic acid disodium salt with mixture of 0.5 mM L-tryptophan, 2.0 mM L-tyrosine, 0.75 mM L-methionine and 0.4 mM L-ascorbic acid sodium salt; L-ascorbic acid sodium salt with mixture of 0.5 mM L-tryptophan, 2.0 mM L-tyrosine, 0.75 mM L-methionine and 5.0 mM L(-)-malic acid disodium salt. The concentrations are given as final concentrations in a well. When the amount of the indicated molecules is zero (first column of each group) the black column shows the effect of mixture containing four components and the white column shows the untreated cells. The cell cultures and cytotoxicity assay were made as described in "Materials and Methods." The results are expressed as percentage of untreated cells. The values are mean $\pm$ SE (*bars*) for three independent experiments. \* and \*\* significantly different from effect of mixture containing four components with  $P<0.01$  and  $P<0.001$ , respectively.

## **Effects of Various Compounds of the Circulatory System on the Effect of Five-Component Mixture on the Survival of Sp2/0-Ag14 Mouse Myeloma Cells**

To be able to detect the potentiating effect of the different compounds, the concentration of components of the five-component mixture was set to 75 % of their highest concentration used in the previous experiment. Thus, the mixture containing 0.375 mM tryptophan, 1.5 mM tyrosine, 0.56 mM methionine, 3.75 mM malate, and 0.3 mM ascorbate as a final concentration in a well decreased the cell number to  $47.8 \pm 2.8$  % compared to untreated cells. Of the 66 compounds examined, 9 compounds (adenine, L-arginine, L-phenylalanine, L-histidine, 2-deoxy-D-ribose, d-biotin, pyridoxine, riboflavin, and oxaloacetate) potentiated significantly ( $P < 0.001$ , for oxaloacetate  $P < 0.01$ ) the effect of the five-component mixture (Fig. 2). Using the above substances singly in the indicated concentration none of them had cell proliferation-decreasing effect, in fact, some of them even slightly increased the cell number compared to untreated cells (data not shown). Thus it can be stated that these compounds potentiated in a synergistic manner the effect of the five-component mixture in killing tumour cells.



**FIGURE 2**  
 Effect of the indicated amount of various molecules occurring in the circulatory system on Sp2/0-Ag14 mouse myeloma cells in combination with a 75 % mixture containing five components. The 75 % mixture contained 0.375 mM L-tryptophan, 1.5 mM L-tyrosine, 0.56 mM L-methionine, 3.75 mM L(-)malic acid disodium salt and 0.3 mM L-ascorbic acid sodium salt. The concentrations are given as final concentrations in a well. The molecules written italic potentiated the effect of this mixture significantly. The cell cultures and cytotoxicity assay were made as described in "Materials and Methods." The results are expressed as percentage of untreated cells. The values are mean±SE (bars) for three independent experiments. \* and \*\* significantly different from effect of 75 % mixture containing five components with  $P < 0.01$  and  $P < 0.001$ , respectively.



**FIGURE 3**

Comparison of the effect of different amounts of thirteen-component control mixture and active mixtures containing five or thirteen components on the growth of Sp2/0-Ag14 mouse myeloma cell line. The dilution of mixtures is expressed in percentage. The 100 % control mixture (filled triangles) contained 5 mM succinic acid disodium salt, 2.5 mM L-serine, 2.5 mM L-asparagine, 2.5 mM L-valine, 2 mM L-alanine, 0.75 mM glycine, 0.5 mM L-proline, 0.5 mM thiamine hydrochloride, 0.5 mM niacin, 0.4 mM folic acid sodium salt, 0.01 mM D-pantothenic acid hemicalcium salt, 0.2 mM hypoxanthine and 2.5 mM D(-)-ribose. The 100 % active mixture having five components (filled squares) contained 5 mM L(-)-malic acid disodium salt, 2 mM L-tyrosine, 0.75 mM L-methionine, 0.5 mM L-tryptophan and 0.4 mM L-ascorbic acid sodium salt. The 100 % active mixture having thirteen components (filled circles) contained the former five molecules plus 2.5 mM L-phenylalanine, 2.5 mM L-arginine hydrochloride, 2.5 mM L-histidine, 0.5 mM d-biotin, 0.5 mM pyridoxine hydrochloride, 0.01 mM riboflavin, 0.2 mM adenine hydrochloride and 2.5 mM 2-deoxy-D-ribose. The concentrations are given as final concentrations in a well. The cell cultures and cytotoxicity assay were made as described in "Materials and Methods." The results are expressed as percentage of untreated cells. The values are mean $\pm$ SE (bars) for three independent experiments.

### **Comparison of the Cytotoxic Effects of Active Mixtures Containing Five or Thirteen Components on the Survival of Sp2/0-Ag14 Mouse Myeloma Cells Compared to Control Mixture**

Treatment of the cells with mixture containing thirteen components (filled circles) caused significantly larger decrease of survival as a function of amount of mixture (Fig. 3) than mixture containing five substances (filled squares). The dilution of mixtures was expressed in percentage of a starting mixture called 100 % mixture. It means that all components of a mixture changed by the same proportion with dilution. The compositions of 100 % mixtures are indicated in Figure legend. In case of starting mixture (100 % mixture) containing five components the cell number was  $18.4 \pm 1.7$  % of the untreated cells. At the same time the mixture containing thirteen components could produce this result when the 60 % of the thirteen-component starting mixture was used. The control mixture (filled triangles) was not cytotoxic for Sp2/0-Ag14 cells at any amount. This control mixture contained thirteen compounds of similar characteristics (succinate, amino acids, vitamins, hypoxanthine, and ribose) as the thirteen-component active mixture at a concentration that ensured the same osmolarity as the thirteen-component active mixture. The components of control mixture were chosen from the compounds that were found in the previous experiment (Fig. 2) ineffective in potentiating the cell killing effect of the five-component mixture.

### **Comparison of the Effect of Thirteen-component Active Mixture and Control Mixture as a Function of Time on the Growth of Sp2/0-Ag14 Mouse Myeloma Cell Line Compared to Untreated Cells**

In this experiment the viable cells were counted microscopically with the trypan blue dye exclusion method after being cultured for 6, 12, 24, 36 or 48 hours. It can be seen (Fig. 4) that the number of untreated cells (filled squares) and of cells treated with the thirteen-component control mixture (filled triangles) increased exponentially. At the same time, the number of cells treated with thirteen-component active mixture (filled circles) decreased compared to starting value. In 48 hours the number of cells untreated and of the cells treated with 100 % control mixture increased from a starting number of  $129 \times 10^3$  to  $754 \times 10^3$  (584 % of starting) and to  $610 \times 10^3$  (473 % of starting), respectively. Over the same period, the number of cells treated with 100 % thirteen-component active mixture decreased from the starting value to  $30 \times 10^3$  (23.3 % of starting). This means that about 100,000 cells were killed by the active mixture. None of the components of active mixture had cytotoxic effect on cancer cells when they were used singly in the same amount as in active mixture (data not shown). This demonstrates that they increase the effect of each other synergistically. Osmotic effect, a non-specific consequence of the overload of substances,

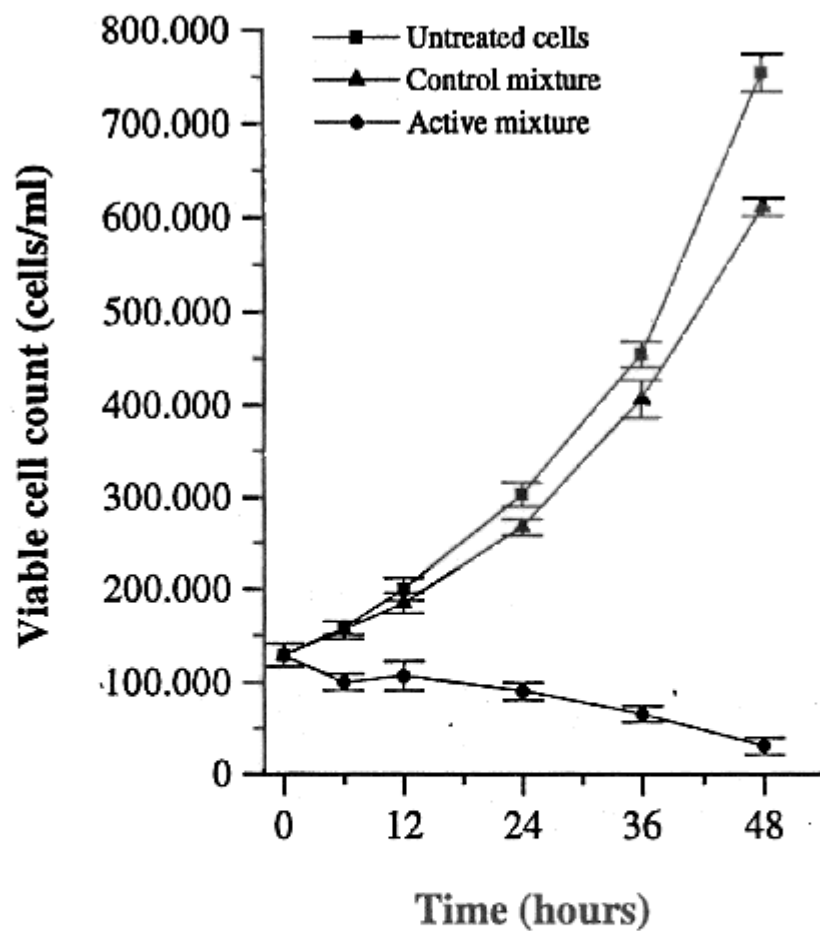
ammonium toxicity or amino acid imbalance, can be excluded as the causes of cell-killing effect because the control mixture was not cytotoxic for the cells.

### **Effects of Catalase on the Five- and Thirteen-component Active Mixture Mediated Inhibition of Sp2/0-Ag14 Mouse Myeloma Cell Growth**

The amount of catalase (Sigma, C-40) used was 2000 U/ml [62]. This amount did not decrease significantly the effect of either mixture (data not shown).

### **Effects of Various Counter Ions on the Thirteen-component Active Mixture Mediated Inhibition of Sp2/0-Ag14 Mouse Myeloma Cell Growth**

To find out whether counter ions have any role in the effects of the mixtures, experiments were carried out where  $K^+$  or  $Ca^{2+}$  were used instead of  $Na^+$  or sulfate instead of chloride. There were not any significant differences between the effects of mixtures using different counter ions. In fact, the results were essentially the same (data not shown).



**FIGURE 4**

Comparison of the effect of 100 % thirteen-component active mixture (filled circles) and control mixture (filled triangles) as a function of time on the growth of Sp2/0-Ag14 mouse myeloma cell line compared to untreated cells (filled squares). The composition of 100 % control mixture and 100 % thirteen-component active mixture are given in the legend of Fig. 3. The cell cultures and cytotoxicity assay were made as described in "Materials and Methods." The values are mean  $\pm$  SE (*bars*) for three independent experiments.

## **Effects of Compounds of the Circulatory System Found Ineffective on the Thirteen-component Active Mixture Mediated Inhibition of Sp2/0-Ag14 Mouse Myeloma Cell Growth**

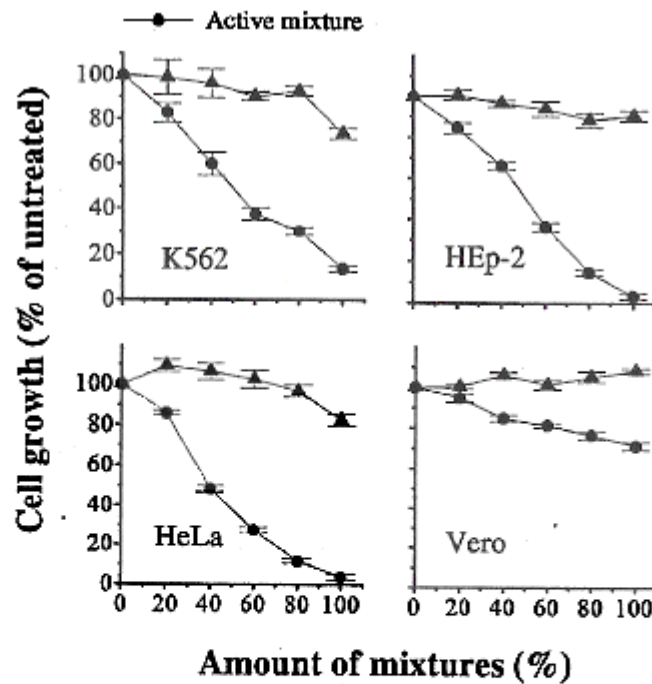
To find out whether the compounds of the circulatory system found ineffective in our experiments (Fig. 2) can influence the effect of substances found effective, we performed experiments where the cultures contained 20 % or 40 % thirteen-component active mixture together with an additional 60 % and 40 % control mixture, respectively. Thus, the osmolarity was equal with a 80 % mixture in both cases. The cell killing effect was exactly the same as in the case when only 20 % or 40 % thirteen-component active mixture was used (data not shown).

## **Comparison of the Effects of Control Mixture and Thirteen-component Active Mixture for Four Established Cell Lines**

It can be seen (Fig. 5) that the cytotoxic effect of the thirteen-component active mixture (filled circles) detected on all cell lines, except Vero normal cell line, was essentially directly proportional to the amount of mixture. The control mixture (filled triangles) was not cytotoxic for any cell lines in any amount, as was the thirteen-component active mixture for Vero normal cells. The proliferation of the Vero cells was only slightly decreased by the active mixture.

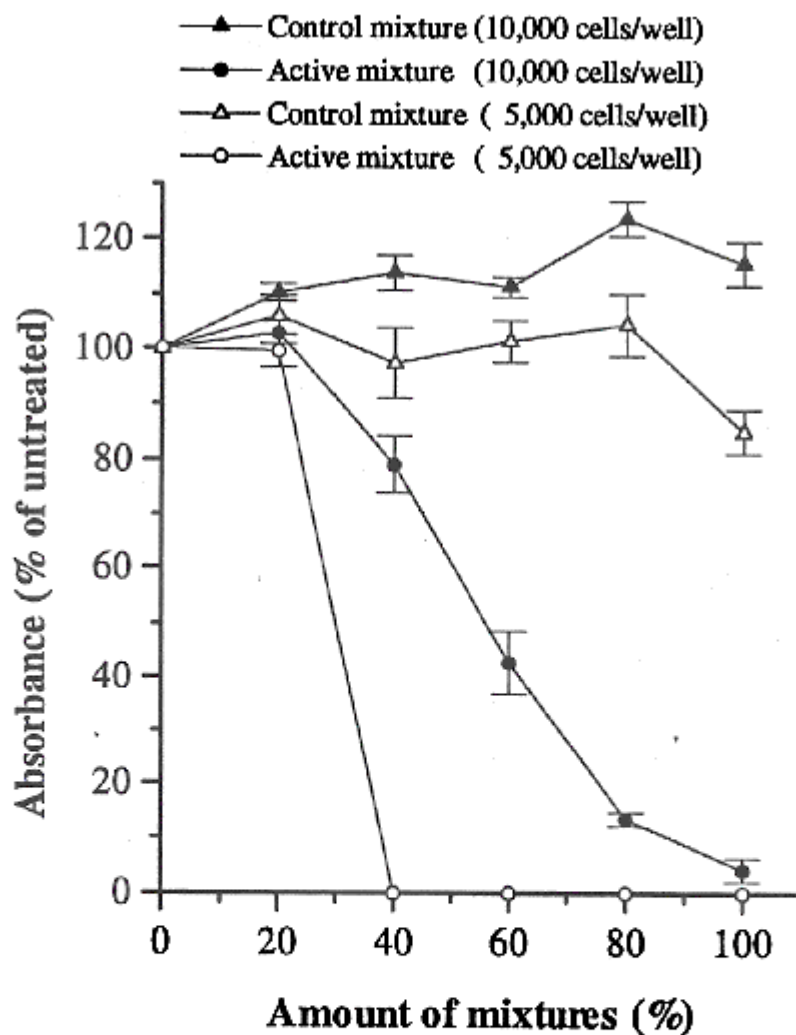
## **Comparison of the Effects of Control Mixture and Thirteen-component Active Mixture on Different Number of Caco-2 Cells**

It can be seen (Fig. 6) that the cytotoxic effect of the thirteen-component active mixture on  $5 \times 10^3$  Caco-2 cells/well (open circles) was significantly and markedly stronger than on  $10^4$  cells/well (filled circles). The control mixture (filled and open triangles) was not cytotoxic for any amount of cells.



**FIGURE 5**

Comparison of the effect of different amounts of thirteen-component active mixture (filled circles) and thirteen-component control mixture (filled triangles) on the growth of a normal (Vero) and three tumour cell lines (K562, HEp-2, HeLa). The dilution of mixtures is expressed in percentage. The composition of 100 % active mixture and 100 % control mixture are given in the legend of Fig. 3. The cell cultures and cytotoxicity assay were made as described in "Materials and Methods." The results are expressed as percentage of untreated systems. The values are mean  $\pm$  SE (bars) for in the case of K562 cells three and in the case of HeLa, HEp-2 and Vero cells five independent experiments.



**FIGURE 6**

Comparison of the effect of different amounts of thirteen-component active mixture (circles) and thirteen-component control mixture (triangles) on different number of Caco-2 cells. In the experiments  $10^4$  Caco-2 cells/well (filled symbols) and  $5 \times 10^3$  Caco-2 cells/well (open symbols) were used. The dilution of mixtures is expressed in percentage. The composition of 100 % active mixture and 100 % control mixture are given in the legend of Fig. 3. The cell cultures and cytotoxicity assay were made as described in "Materials and Methods." The results are expressed as percentage of untreated systems. The values are mean  $\pm$  SE (bars) for five independent experiments.

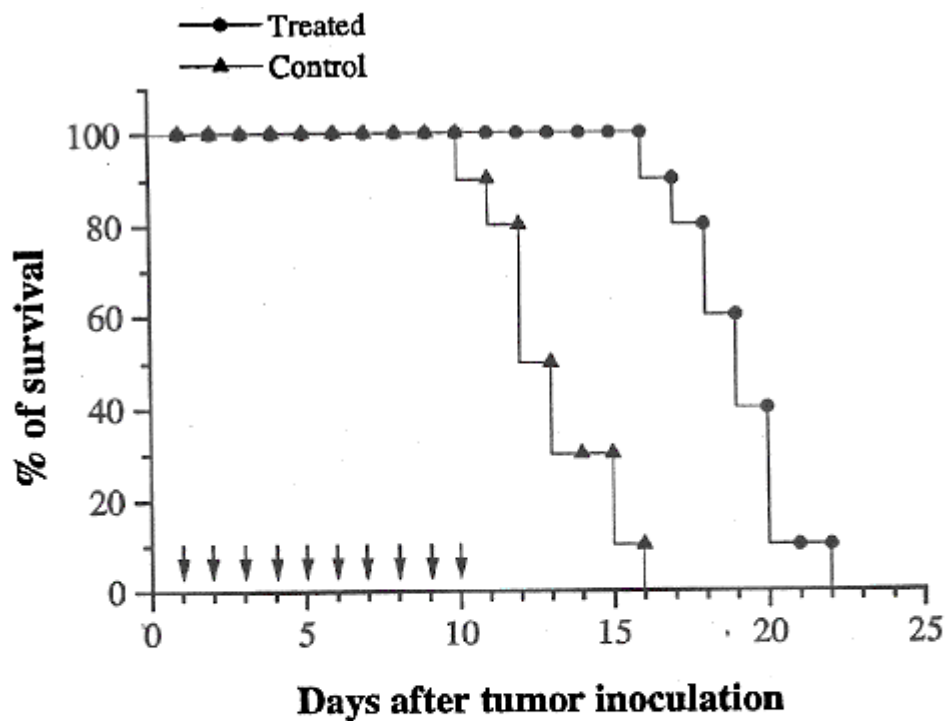
### **Tumourigenicity of Sp2/0-Ag14 Mouse Myeloma Cells**

A dose dependency was found between the number of cells injected and survival time, with survival times ranging from means of 11 to 22 days following injection of  $2.2 \times 10^5$  to  $1 \times 10^3$  cells, respectively (Fig. 7). Mice developed abdominal distention 3 to 4 days before death. At autopsy, 4 to 6 ml of ascitic fluid was present in the peritoneal cavity. Mice given injections of  $5 \times 10^3$  or less Sp2/0-Ag14 cell showed no evidence of i.p. tumour growth when sacrificed 100 days following injection of the cells. The *in vivo* tumour doubling time for Sp2/0-Ag14 cells was 1.0 day estimated from semilogarithmic plots (not shown) of data of Fig. 7 as described in "Materials and Methods".

### **Antitumour Efficacy of the Thirteen-component Active Mixture in i.p. Survival Model**

The treatment with solution of the thirteen-component active mixture given in "Materials and Methods" increased the survival time of the mice injected i.p. with Sp2/0-Ag14 cells (Fig. 8). The difference between mean survival time of control ( $12.9 \pm 0.6$  days) and treated ( $18.9 \pm 0.5$  days) group is highly significant ( $P < 0.001$ ). The T/C % calculated from the median survival time of control (13.5 days) and treated (20 days) group is 148.1 %. Comparison of the 50 % survival times (19 days) in the group of animals treated with the 50 % survival times (12 days) in control animals and the *in vivo* tumour doubling time (1.0 day) were used to estimate the  $\log_{10}$  cell kill *in vivo* as described in "Materials and Methods". The result (2.1) shows that the treatment with the mixture eliminated more than 2 logs (99 %) of Sp2/0-Ag14 mouse myeloma cells.





**FIGURE 8**

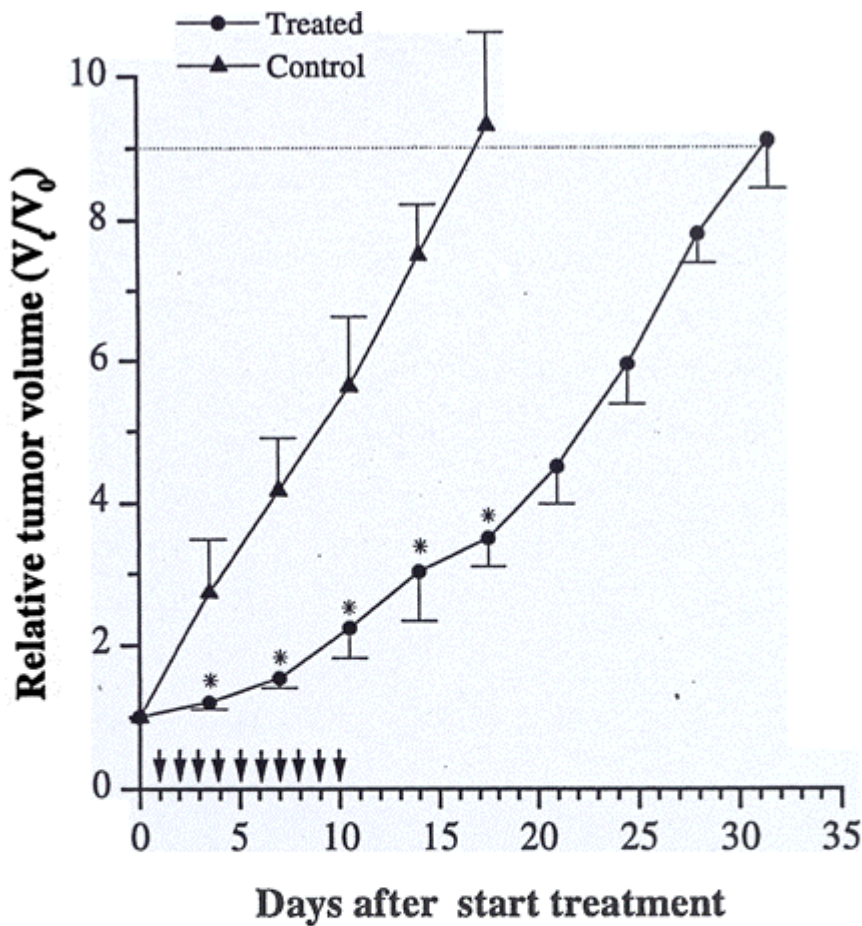
Effect of the thirteen-component active mixture on the survival of mice (filled circles) compared to mice treated by PBS (filled triangles). The composition of the mixture are given in "Materials and Methods." Female BALB/c mice (10 mice/group) were inoculated i.p. with  $5 \times 10^4$  cells of the Sp2/0-Ag14 mouse myeloma cell line on Day 0. The solution of the active mixture and PBS were given i.p. daily according to the defined schedule from Days 1 to 10. The mice were monitored daily for survival. *Arrows*, the days of treatment.

### **Effect of the Thirteen-component Active Mixture on the Number of Sp2/0-Ag14 Mouse Myeloma Cells in Ascitic Fluid**

To exclude the possibility that the increase of survival time of treated group was caused only by a roborating effect of the substances, we determined the number of tumour cells present in the ascitic fluid of treated and control mice (5 mice/group) after finishing treatment with solution of the thirteen-component active mixture given in "Materials and Methods". The significant ( $P < 0.001$ ) difference between mean cell number of control ( $9.68 \times 10^7$ ) and mean cell number of treated group ( $10.8 \times 10^5$ ) excludes the roborating effect as only reason of increase of survival time. The result also shows that the tumour cell kill of approximately 2 logs is practically the same as the calculated tumour cell kill value determined in the previous experiment.

### **Antitumour Efficacy of the Thirteen-component Active Mixture in s.c. Tumour Model**

The treatment with solution of the thirteen-component active mixture given in "Materials and Methods" decreased the growth of tumours in the BALB/c nude mice injected s.c. with HeLa cells (Fig. 9). The mean relative tumour volumes of the control (filled triangles) and treated (filled circles) groups differed significantly ( $P < 0.05$ ) in all cases. The T/C % was less than 42 % at each evaluation. The least value was 35.7 %. The mean growth delay was 16 days. The weight loss of control ( $-5.5 \pm 3.1$  %) and treated group ( $-7.6 \pm 3.2$  %) did not differ significantly.



**FIGURE 9**

Effect of the thirteen-component active mixture on the growth of HeLa s.c. tumour (filled circles) compared to the growth of tumour in control animals treated by PBS (filled triangles). The composition of the mixture are given in "Materials and Methods." The solution of the mixture and PBS were given i.p. daily according to the defined schedule from Days 1 to 10. The time to reach nine fold of starting tumour volume was arbitrarily chosen as the standard for determine of tumour growth delay. Plots, mean $\pm$ SE (bars); \*,  $P < 0.05$  by Student's *t* test; arrows, the days of treatment.

## **Investigation of the Potentiating Effects of Other Seventeen Compounds Occurring in the Circulatory System on the Five-Component Active Mixture Mediated Inhibition of Sp2/0-Ag14 Mouse Myeloma Cell Growth**

To investigate the potentiating effect of other seventeen compounds of the circulatory system on the effect of five-component active mixture we used the same method and the same mixture (described above) which was used when the 66 compounds of the circulatory system were tested (Fig. 2). The five-component mixture (as described in the legend to Fig. 2) contained 0.375 mM tryptophan, 1.5 mM tyrosine, 0.56 mM methionine, 3.75 mM malate, and 0.3 mM ascorbate as a final concentration in a well. Of the 17 compounds (5 mM taurine, 0.1 mM DL-6,8-thioctic acid sodium salt, 5 mM adonitol, 5 mM D(+)-mannose, 5 mM myo-inositol, 5 mM D(+)-glucosamine, 0.5 mM propionic acid sodium salt, 2.5 mM glutathione, 5 mM N-acetyl-D-glucosamine, 0.05 mM adenosine-5'-triphosphate disodium salt, 5 mM allantoin, 5 mM L(-)fucose, 1 mM orotic acid sodium salt, 5 mM hippuric acid sodium salt, 5 mM betaine, 5 mM levulinic acid, 0.05 mM guanosine-5'-triphosphate sodium salt) examined in this experiment, 3 compounds (orotic acid, hippuric acid, D(+)-mannose) were found to potentiate significantly ( $P < 0.001$ , for orotic acid sodium salt  $P < 0.01$ ) the effect of the five-component mixture (data not shown). Using the above substances individually at the indicated concentration, none of them had cell proliferation-decreasing effect (data not shown). Thus it can be stated that these compounds potentiated synergistically the effect of the five-component mixture in killing tumour cells.

## **Effect of Active Mixtures Containing Sixteen or Thirteen components on the Survival of Sp2/0-Ag14 Mouse Myeloma Cells Compared to Control Mixture**

It can be seen (Fig. 10) that the sixteen-component active mixture (filled circles) containing orotic acid sodium salt, hippuric acid sodium salt, and D(+)-mannose additionally to the thirteen-component active mixture has significantly higher toxic effect on the Sp2/0-Ag14 cell line than the thirteen-component active mixture (filled squares). However, the sixteen-component control mixture (filled triangles) was not cytotoxic at all for Sp2/0-Ag14 cells. The compositions of all the different mixtures used are indicated in the Figure legend.

## **Investigation of the Potentiating Effects of Various Ions of the Circulatory System on the Sixteen-Component Active Mixture Mediated Inhibition of Sp2/0-Ag14 Mouse Myeloma Cell Growth**

In order to detect the potentiating effect of different ions ( $\text{Fe}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cr}^{3+}$ ,  $\text{SeO}_3^{2-}$ ), the concentration of components of the sixteen-component active mixture was set to 40 % of their highest concentration used in the

previous experiment (given in the legend to Fig. 10). The 40 % sixteen-component active mixture decreased the cell number to  $41.6 \pm 2.1$  % compared to untreated cells. In the experiments the sulfate, chloride or sodium salt of the above ions were used ( $\text{FeCl}_3$ ,  $\text{CuSO}_4$ ,  $\text{ZnCl}_2$ ,  $\text{CrCl}_3$ ,  $\text{Na}_2\text{SeO}_3$ ), since it was demonstrated earlier that sulfate, chloride or sodium as counter ions did not have any influence on the effect of the active mixture. The final concentration of the salts in a well was set to 20  $\mu\text{M}$  taking their mean physiological concentration into consideration [63]. Of the ions examined, only  $\text{Cu}^{2+}$  potentiated significantly ( $P < 0.001$ ) the effect of the sixteen-component mixture (data not shown). The  $\text{Cu}^{2+}$  alone (i.e. without the active mixture) did not have any effect on the cell number. However, the 40 % sixteen-component mixture and the  $\text{Cu}^{2+}$  together decreased the cell number to  $12.1 \pm 1.3$  % compared to untreated cells. This effect could be prevented completely (data not shown) by catalase (Sigma, C-40; 2000 U/ml). On the other hand, the  $\text{Cu}^{2+}$  could not potentiate the effect of active mixture when the mixture did not contain ascorbate.

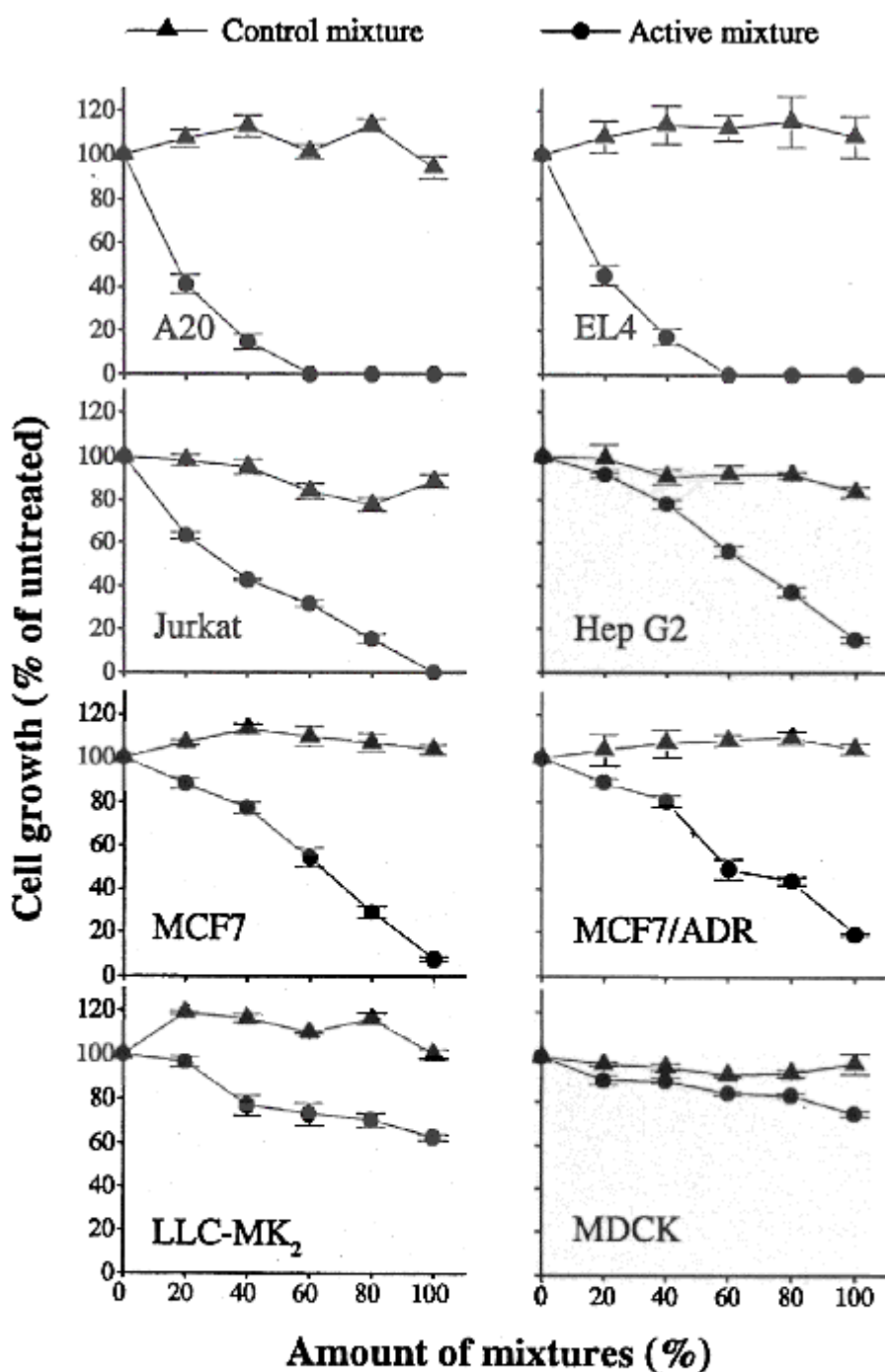
#### **Comparison of the Effect of Sixteen-component Active Mixture and Sixteen-component Control Mixture for Eight Established Cell Lines**

The sixteen-component active mixture (filled circles) had a significant cytotoxic effect on all cell lines except for the LLC-MK<sub>2</sub> and MCDK normal lines (Fig. 11). The degree of cell death was especially high in case of the A20 and EL4 lymphoma cell lines. These cell lines were so susceptible to active mixture that even the 60 % mixture could kill all the cells. The active mixture was also effective against both the MCF7 human breast adenocarcinoma and its adriamycin-resistant version, MCF7/ADR cell line. Proliferation of the LLC-MK<sub>2</sub> normal cells was only slightly decreased by the active mixture compared to untreated cells. In the case of the other normal cell line (MCDK), essential difference could not be observed between the effect of the active mixture and control mixture. The sixteen-component control mixture (filled triangles) was not cytotoxic for any cell lines up to any amount.

#### **Demonstration of the Selective Effects of Sixteen-Component Active Mixture on Various Tumour and Normal Cell Lines by Photographs using the MTT Colorimetric Assay**

The results of MTT colorimetric assay demonstrate (Fig. 12) the selective cytotoxic effect of the sixteen-component active mixture on Hep G2 and MCF7 tumour cells compared to LLC-MK<sub>2</sub> and MDCK normal cells. The results illustrated by the photographs of the microplates show that the survival of tumour cells strongly decreased as

the amount of the active mixture increased (the intensity of the purple colour decreases as the cells die because the purple formazan dye is produced only by living cells) but control mixture did not influence the number of cells (the intensity of the purple colour is essentially the same in the wells containing the cells treated by the control mixtures as in the wells containing the untreated cells). In contrast with tumour cells as shown in Fig. 12, the active mixture did not have a toxic effect on any normal cell lines. Only in the case of LLC-MK<sub>2</sub> cell line could be observed a slight difference between the colour intensity of the wells containing cells treated by 100 % active or 100 % control mixture. To avoid the “edge effect”, the peripheral wells of each plate contained only medium without cells.

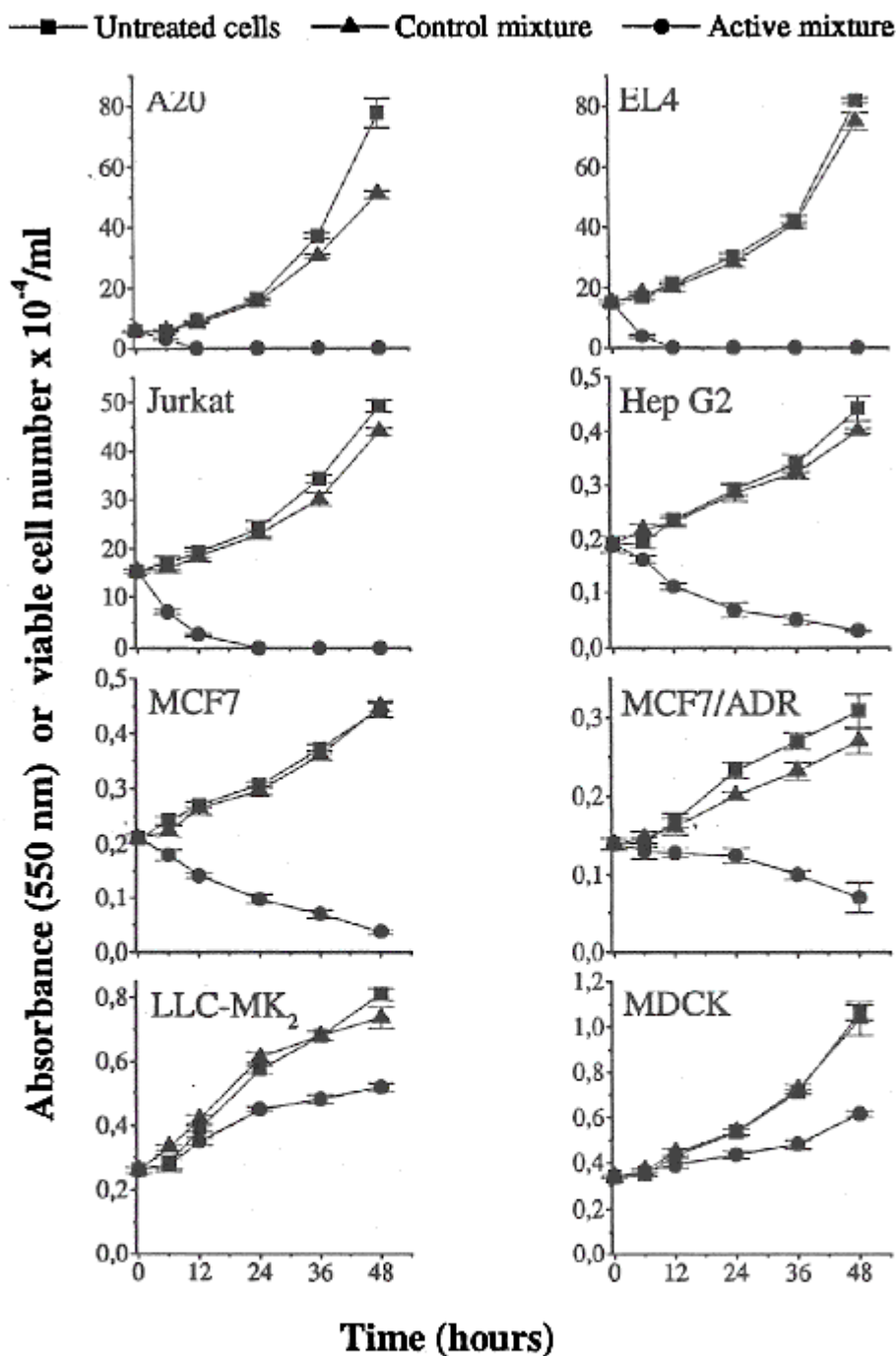


**FIGURE 11**

Comparison of the effect of different amounts of sixteen-component active mixture (filled circles) and sixteen-component control mixture (filled triangles) on the growth of various normal (LLC-MK<sub>2</sub>, MDCK) and tumour cell lines (A20, EL4, Jurkat, Hep G2, MCF7, MCF7/ADR). The dilution of mixtures is expressed as a percentage. The composition of 100% control mixture and 100% active mixture are given in the legend of Fig. 10. The cell cultures and cytotoxicity assay were made as described in "Materials and Methods." The results are expressed as percentage of untreated systems. The values are mean ± SE (*bars*) for A20, EL4 and Jurkat cells three and for Hep G2, MCF7, MCF7/ADR, LLC-MK<sub>2</sub>, and MDCK cells five independent experiments.

## **Comparison of the Effect of Sixteen-component Active Mixture and Control Mixture as a Function of Time on the Growth of Eight Established Cell Lines Compared to Untreated Cells**

In this experiment the viable cells were detected microscopically with the trypan blue dye exclusion method (A20, EL4, Jurkat) or by the MTT colorimetric assay (Hep G2, MCF7, MCF7/ADR, LLC-MK<sub>2</sub>, MDCK) depending on the cell types after being cultured for 6, 12, 24, 36 or 48 hours. It may be seen (Fig. 13) that the number of untreated cells (filled squares) and of cells treated with the control mixture (filled triangles) increased exponentially in the case of every cell line. The number of LLC-MK<sub>2</sub> and MDCK normal cells treated with the sixteen-component active mixture (filled circles) also increased, only the rate of proliferation was slightly lower than in the case of control mixture or untreated cells. This means that the active mixture did not have a toxic effect on normal cells at all. In contrast with normal cells, a large number of tumour cells were destroyed by the sixteen-component active mixture (filled circles) during the 48 hours incubation period and the number of all kinds of tumour cells decreased compared to starting value (Fig. 13). This experiment, like a previous one (Fig. 11) also demonstrated that the A20 and EL4 lymphoma cell lines were highly susceptible (more than the other cell lines) to the effect of the active mixture, since all the cells were killed after 12 hours of incubation.



**FIGURE 13**

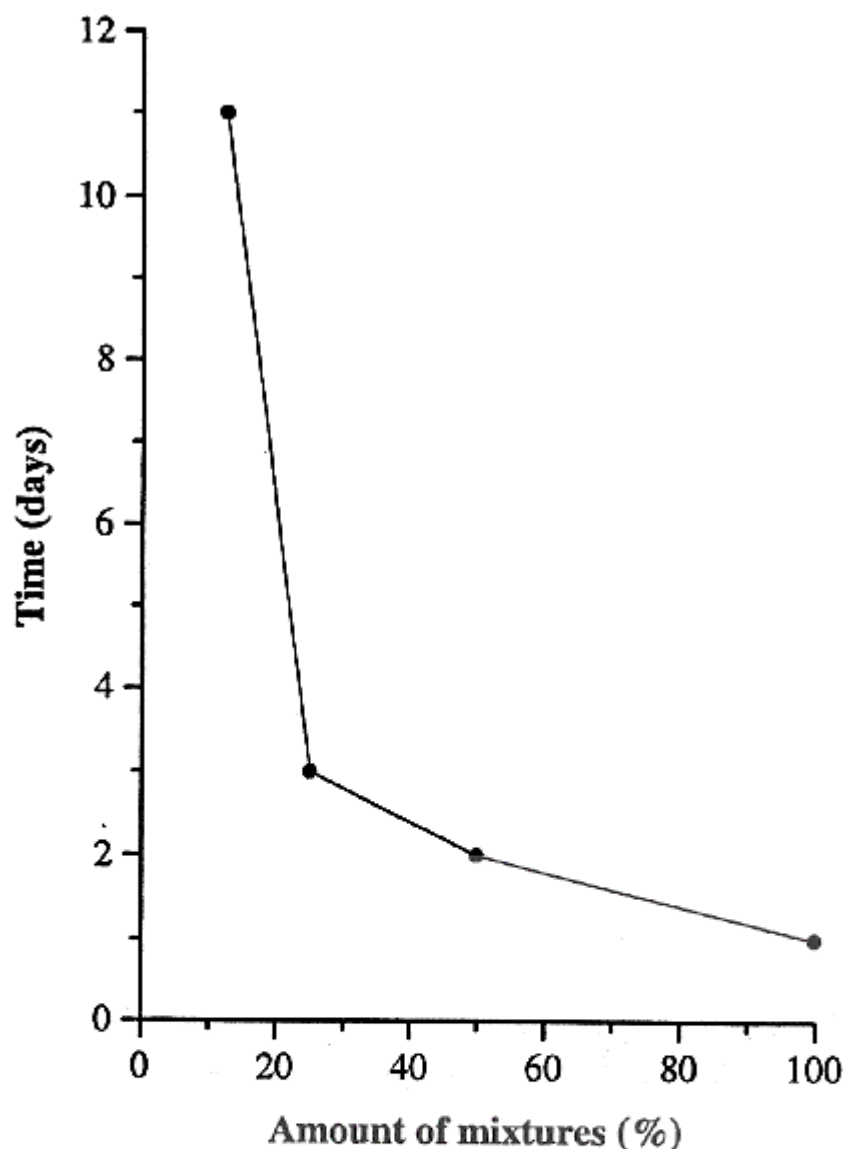
Comparison of the effect of 100 % sixteen-component active mixture (filled circles) and control mixture (filled triangles) as a function of time on the growth of various normal (LLC-MK<sub>2</sub>, MDCK) and tumour cell lines (A20, EL4, Jurkat, Hep G2, MCF7, MCF7/ADR) compared to untreated cells (filled squares). The composition of sixteen-component active mixture and control mixture are given in the legend of Fig. 10. The cell cultures and cytotoxicity assay were made as described in "Materials and Methods." In the case of A20, EL4 and Jurkat cells the number of viable cells  $\times 10^4$ /ml [mean  $\pm$  SE (bars), three independent experiments], in the case of Hep G2, MCF7, MCF7/ADR, LLC-MK<sub>2</sub> and MDCK cells the absorbance at 550 nm was plotted [mean  $\pm$  SE (bars), five independent experiments].

## **Comparison of the Effects of Active Mixtures Containing their Components in the Maximum or Minimum Concentration Occurring in the Blood on K562 and Sp2/0-Ag14 Cells Using Different Initial Cell Concentration**

The maximum and minimum concentration in the blood of the substances of active mixture can be found in the scientific literature [63-70]. In this experiment we investigated the effects of a maximal active mixture (the final concentration of its components in a well corresponded to their maximum concentration existing in the blood), a minimal active mixture (containing its components in the minimum concentration can be found in the blood) and a control mixture (the concentration of its components and thus the osmolarity of it corresponded to the maximal active mixture) on K562 and Sp2/0-Ag14 cells at different starting cell concentrations. The maximal active mixture was a model of an optimally operating defence system while the minimal active mixture was a model of a poorly operating defence system. Because some substances of the mixtures occur in the medium, we took the concentration of substances in the medium into account when the mixtures were prepared. The sum of the concentration of a given substance in the mixture and the concentration of the same compound in the medium corresponded to the concentration of the given substance in the serum. The composition of control mixture as well as maximal and minimal active mixture are given in the legend to Fig. 14. Since L-ascorbate was highly toxic *in vitro* even individually when it was applied as high concentration as its maximum concentration in the serum, therefore the concentration of ascorbate in the maximal active mixture was set lower than its maximum concentration in the serum. The 2-deoxy-D-ribose was omitted from the maximal active mixture because data about its serum concentration could not be found in the special literature. The cells were counted when the untreated cells proliferated to approximately  $5 \times 10^5$ . Obviously, as the initial cell concentration decreased the length of time needed for proliferation of the untreated cells to  $5 \times 10^5$  increased. The results were expressed as a percentage of untreated cells. As it appears (Fig. 14) the maximal active mixture (black columns) had a significantly higher effect on K562 cells at different initial cell concentration than the minimal active mixture (grey columns) and killed all the K562 cells under 125 cells/ml starting cell concentration. Although the minimal active mixture had an effect at any initial cell concentration, it was ineffective in destroying all the cells even at 60 cells/ml initial cell concentration. The control mixture (white columns) did not show this cell destructive effect at all and even in some cases it increased the proliferation of cells compared to untreated cells. The results were similar in the case of Sp2/0-Ag14 cells (data not shown). Adherent cells could not be used for these types of experiment because none of the cells, even the untreated ones could grow under about 2000 cells/ml initial cell concentration (data not shown).

## **Investigation of the Effect of Active Mixture on the Length of Time Needed for Death of All K562 Cells in Case of Increasing Mixture to Cell Ratio**

In this experiment, the relationship between the concentration of the active mixture (as a matter of fact, the concentration of its components) and the length of time needed for killing all the cells by the active mixture was investigated. Because the starting concentration of cells was constant and the concentration of mixture was increased, the mixture to cell ratio rose. The length of time needed for cell death was detected for 6.25, 12.5, 25, 50, and 100 % active mixtures. The concentration of components of the highest amount of active mixture (the 100 % active mixture) was set as high as possible so that we could avoid the osmotic effect (legend to Fig. 15). Therefore, we took in the case of each component of the active mixture the twenty-fold amount of their maximum serum concentration (can be found in legend to Fig. 14). In contrast with the preceding experiment the amount of components contained by the medium was not taken into consideration when the mixture was prepared because the components of the medium cannot be diluted. On the other hand, it was not of importance from the point of view of this experiment and it would have made the experiment unnecessarily complicated. Beside the 2-deoxy-D-ribose, the L-ascorbic acid sodium salt was also omitted from the mixture because it had, even alone, a high toxic effect on the given amount of cells at the higher concentrations applied and thus, it would have disturbed the experiment. The composition of the 100 % active mixture and 100 % control mixture are given in the legend to Fig. 15. To investigate the length of time of cell-death, it was an important condition that any amount of active mixture, except for the lowest (the 6.25 % mixture), should destroy all the cells. Because the death of all cells depends on the proportion of the concentration of active mixture and cells, on the basis of the previous experiment the concentration of cells was set to 300 cells/ml. Since the cells could not proliferate during the experiment, they even died under shorter and shorter time intervals as the amount of active mixture increased (12.5, 25, 50 or 100 % mixture), the result of the experiment could not be evaluated by the usual methods. Thus, the cell death was detected visually by invert microscope. The microplates were monitored every day at the same time and the day was taken as the time of death when all the cells died in every parallel well (six) in which the cells were treated by the same amount of active mixture. It can be seen (Fig. 15), that as the amount of the active mixture and thus the mixture to cell ratio increased, the length of time needed for death of the same number of cells decreased. Because the cells were not destroyed by the 6.25 % active mixture or by any amount of control mixture (as it was expected), the data concerning their effect could not be plotted in the above figure. These results of control mixture and 6.25 % active mixture also means that the cell death observed at the higher amounts of the active mixture was not an artefact but the consequence of the cell killing effect of the active mixture at higher concentrations.



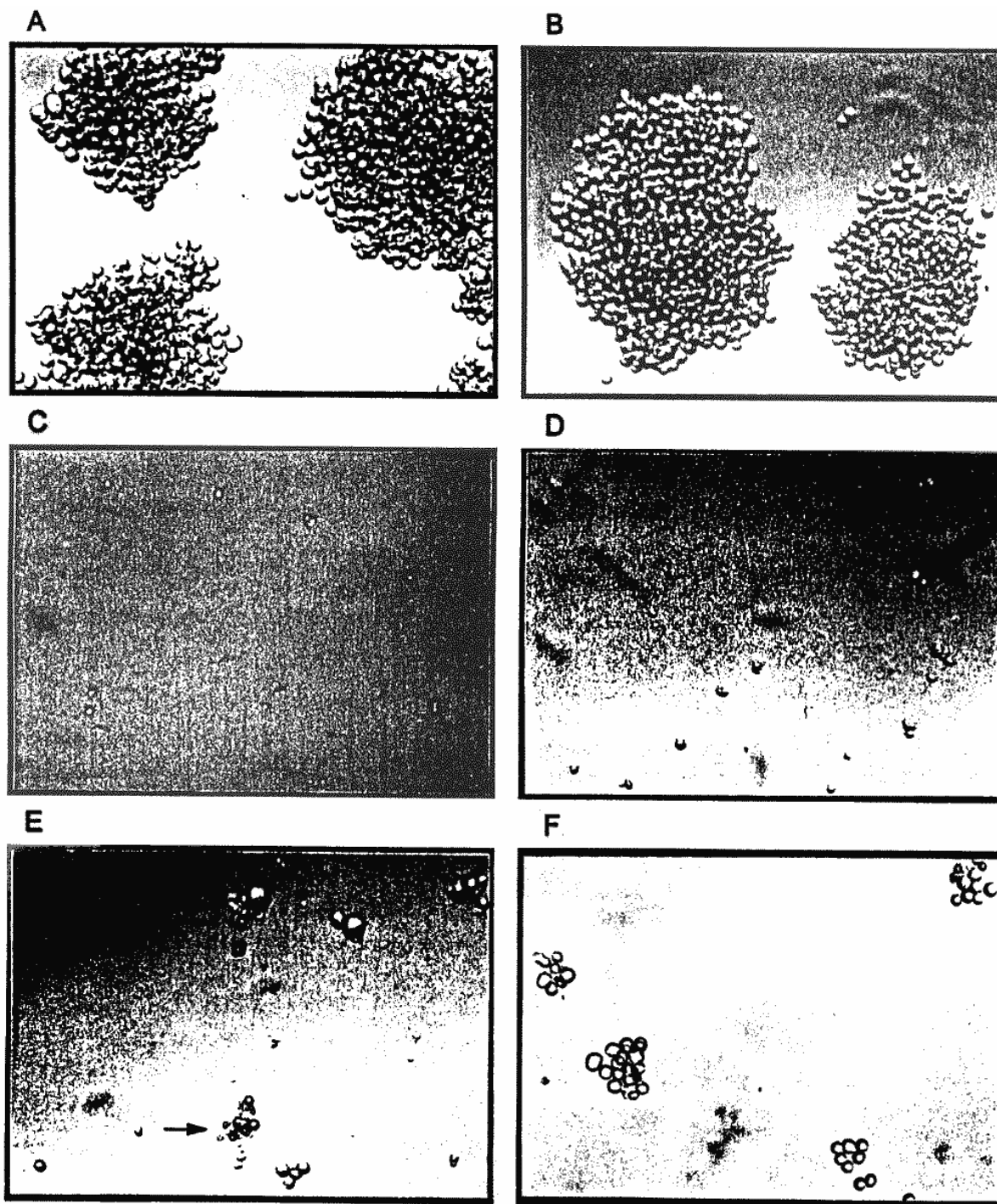
**FIGURE 15**

Investigation the effect of active mixture on the length of time needed for death of all K562 cells in the case of increasing proportion of the concentration of active mixture and tumour cells. The concentration of cells was 300 cells/ml. The 100 % active mixture contained 1.34 mM L(-)malic acid disodium salt, 4.84 mM L-phenylalanine, 3.48 mM L-arginine hydrochloride, 4.9 mM L-histidine, 2.76 mM L-tyrosine, 1.34 mM L-methionine, 2.94 mM L-tryptophan, 0.002 mM d-biotin, 3.9 mM pyridoxine hydrochloride, 0.022 mM adenine hydrochloride, 0.026 mM riboflavin, 2.44 mM D(+)-mannose, 0.67 mM hippuric acid sodium salt and 0.08 mM orotic acid sodium salt. The 100 % control mixture contained 1.34 mM succinic acid disodium salt, 4.84 mM L-serine, 3.48 mM L-asparagine, 4.9 mM L-valine, 2.76 mM L-alanine, 1.34 mM glycine, 2.94 mM L-proline, 0.002 mM thiamine hydrochloride, 3.9 mM niacin, 0.022 mM hypoxanthine, 0.026 mM D-pantothenic acid hemicalcium salt, 2.44 mM D-(+)-glucose, 0.67 mM betaine and 0.08 mM uracil. The concentrations are given as final concentrations in a well. The cell cultures were made as described in "Materials and Methods."

## **Visual Investigation of the Effect of Active Mixture on the Survival of K562 Cells in the Case of Decreasing Mixture to Cell Ratio**

In the current experiment we investigated visually by invert microscope what happens with the tumour cells when the cell number is constant and the cells are submitted to a diminishing amount of the active mixture (the mixture to cell ratio decreases), in other words, when the efficiency of defence mechanism declines. Since we used the same 100 % active and 100 % control mixtures than in the previous experiment (legend to Fig. 15), to be able to diminish the mixture to cell ratio, we had to set the cell concentration higher than in the previous experiment, as high as possible so that it could be killed only by the highest amounts of active mixture. Taking into consideration that the concentration of cells destroyed totally by the maximal active mixture in a previous experiment (Fig. 14) was about 100 cells/ml and that the 100 % active mixture in the current experiment is twenty-fold amount of the maximal active mixture used in the mentioned experiments (Fig. 14), the cell concentration was set to twenty-fold of 100 cells/ml, namely to 2000 cells/ml. Then, this amount of cells was exposed to the effect of 100 % control mixture or 100 %, 50 %, 25 % or 12.5 % active mixture. The cultures were photographed directly from the culture plates, using an inverted phase contrast microscope. The results are illustrated by the photographs taken after an eight day incubation period (Fig. 16).

It can be seen that the untreated cells (Fig. 16A) and the cells treated by the 100 % control mixture (Fig. 16B) proliferated to a high number of cells during the eight days and all of the cells were alive and well-conditioned at the end of the experiment. The bulk of cells shown in the photos arose from some mother cells. In contrast with that, all the cells treated with the 100 % active mixture were killed (Fig. 16C) without dividing even once. When the 50 % active mixture was applied (Fig. 16D), the cells also died, but some of them could divide once before their death and one of those seen in the photo could divide twice. Fig. 16E shows the cells when they were exposed to the 25 % active mixture. It can be seen in the photograph that at this mixture to cell ratio the cell death and the cell division compete with each other and the division and death of cells occurring simultaneously were approximately in equilibrium. When the amount of the active mixture was further decreased (to 12.5 %), proliferation overtook cell death (Fig. 16F). However, it is important to emphasise that some dying and dead cells can be seen in the photo and the cell number is significantly lower than the number of untreated (Fig. 16A) and control mixture treated cells (Fig. 16B).



**FIGURE 16**

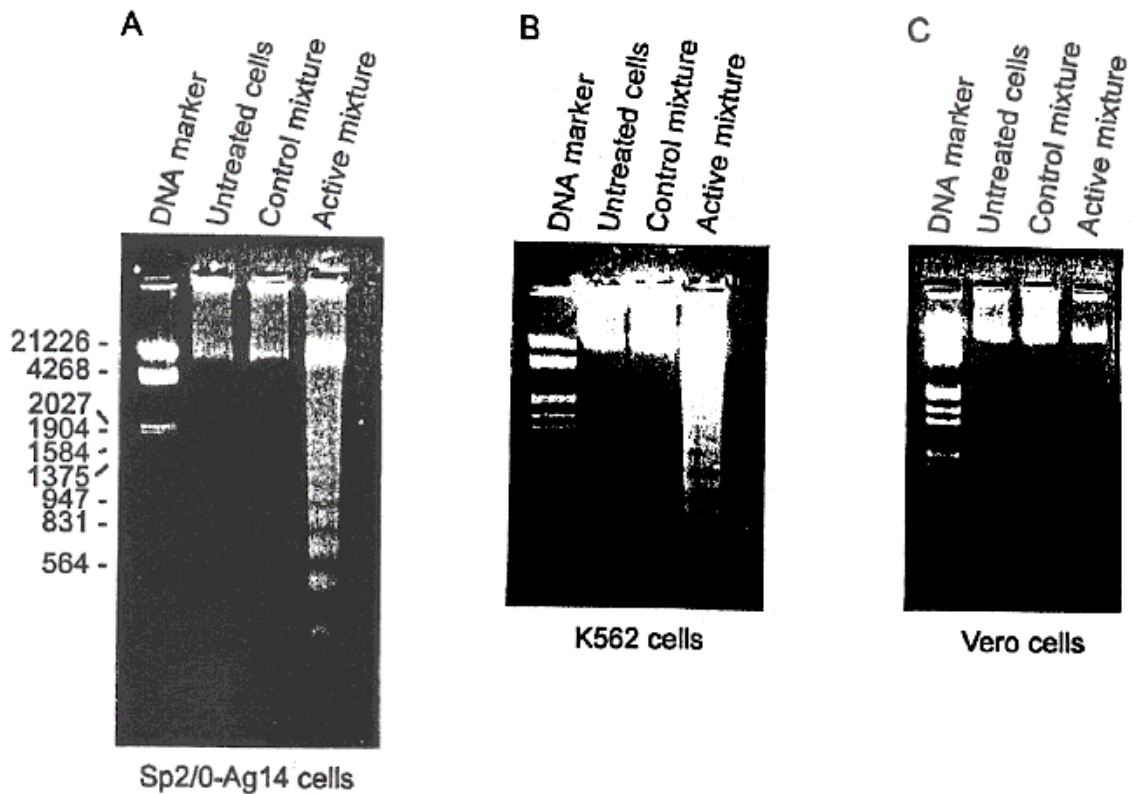
Visual comparison of the effect of active mixture and control mixture on the survival of K562 cells in the case of decreasing proportion of concentration of active mixture and tumour cells. The concentration of cells was 2000 cells/ml. The composition of the 100 % active mixture and 100 % control mixture are given in the legend of Fig. 15. Cells were incubated in the growth medium without (A) or with 100 % control mixture (B) or 100 % (C), 50 % (D), 25 % (E), and 12.5 % (F) active mixture for eight days. The cell cultures were made as described in "Materials and Methods." Arrows (E) show blebbing cells and apoptotic bodies.

### **Comparison of the Apoptosis Inducing Effect of the Sixteen-component Active Mixture and Control Mixture on Various Tumour and Normal Cell Lines Detected by Gel Electrophoresis**

The cells treated with the active mixture show fragmentation of DNA into endonucleosome-sized units characteristic of apoptotic cell death in the case of Sp2/0-Ag14 mouse myeloma (Fig. 17A) and K562 human erythroleukemia cells (Fig. 17B). In contrast, a ladder-like pattern of DNA fragmentation cannot be seen in the case of untreated cells and in the case of cells treated with the control mixture. No fragmentation is visible in the case of Vero normal cells (Fig. 17C) treated with active mixture under the same experimental conditions.

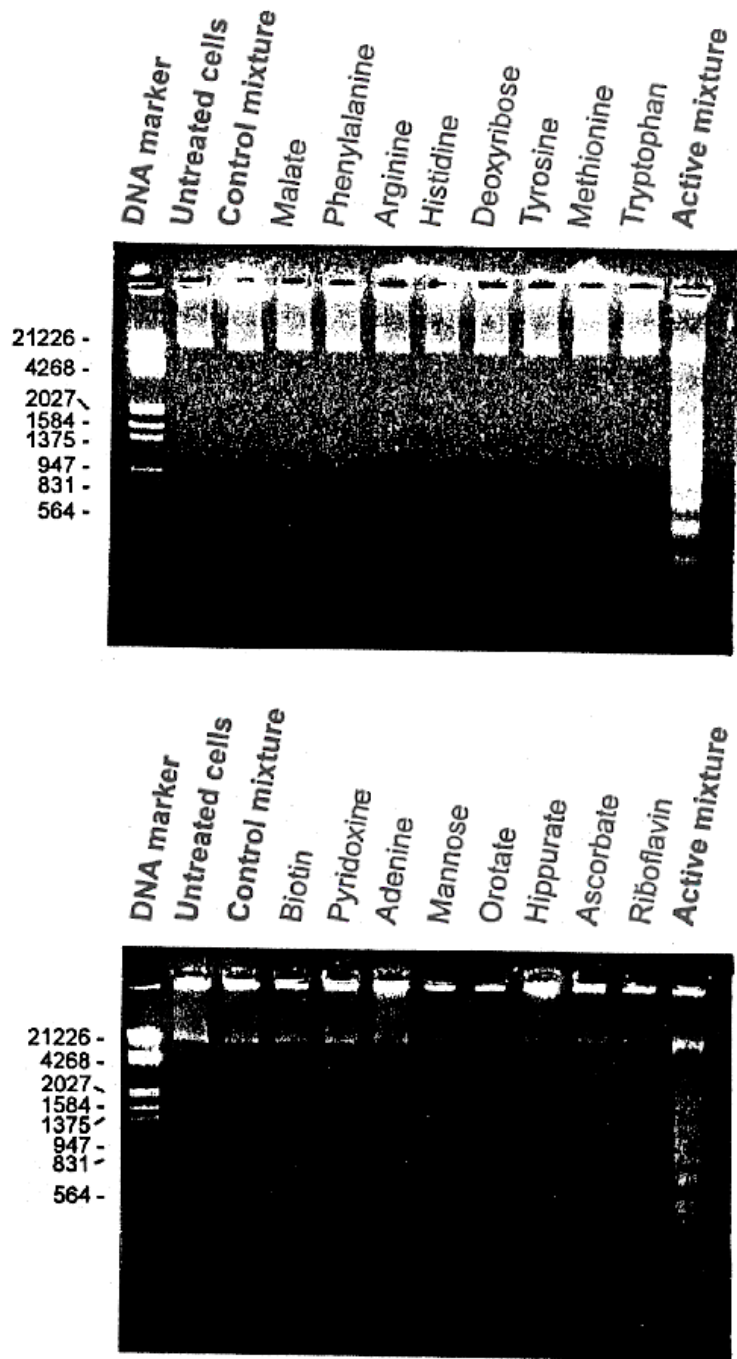
### **Comparison of the Apoptosis Inducing Effect of the Sixteen-component Active Mixture and its Components Singly Detected by Gel Electrophoresis**

As shown in Fig. 18, the components of the active mixture, when they were used singly in exactly the same concentration as in the active mixture, could not induce apoptosis of the tumour cells. The DNA ladder appeared only when the cells were exposed to the simultaneous effect of the substances.



**FIGURE 17**

Electrophoresis of DNA from Sp2/0-Ag14 mouse myeloma (A), K562 human erythroleukemia (B) and Vero African green monkey kidney normal (C) cells treated with control mixture or active mixture. DNA size marker: Lambda DNA EcoR I Hind III digest. The composition of the sixteen-component active mixture and control mixture are given in the legend of Fig. 10. The concentrations are given as final concentrations in a well. The cell cultures and DNA gel electrophoresis were made as described in "Materials and Methods".



**FIGURE 18**

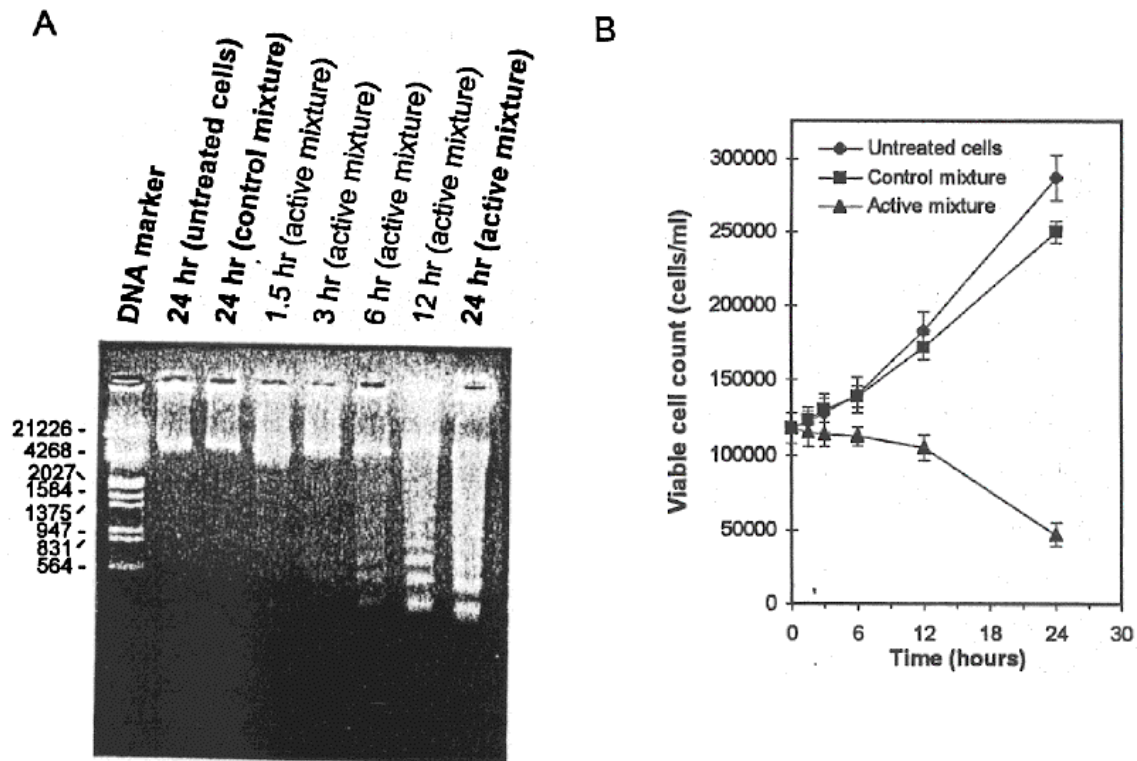
Agarose gel electrophoresis of DNA from Sp2/0-Ag14 cells after exposure for 24 h to active mixture, control mixture or to the components of active mixture alone. The composition of the sixteen-component active mixture and control mixture are given in the legend of Fig. 10. The concentration of the components, when they were used singly, was exactly the same as in the active mixture. Cells were maintained, treated and DNA fragmentation was assessed as described in "Materials and Methods".

### **Comparison of the Apoptosis Inducing Effect of Different Amount of the Sixteen-component Active Mixture Detected by Flow-cytometric Analysis and Gel Electrophoresis**

When cell cultures were incubated with different amounts of the active mixture, subsequent DNA flow-cytometric analysis revealed a number of cells with low DNA stainability, resulting in a sub-G<sub>1</sub> peak, designated as apoptotic cells (Fig. 19A). There is circumstantial evidence that this reduced DNA stainability may be the consequence of progressive loss of DNA from the cells, due to the activation of endogenous endonuclease, and subsequent leakage of the low-molecular-weight DNA products. The composition of the 100 % sixteen-component active mixture and 100 % sixteen-component control mixture are given in the legend to Fig. 10. The percentage of fluorescent events detected in the sub-G<sub>1</sub> region began to increase at the 80 % mixture amount (Fig. 19A). The DNA fragmentation into oligonucleosomal sized units detected by gel electrophoresis (Fig. 19B) fit in well with the result of the flow-cytometric analysis. Fluorescence in the sub-G<sub>1</sub> region, a ladder-like pattern of DNA fragmentation cannot be detected in the case of untreated cells and in the case of the control mixture (Fig. 19).

### **Comparison of the Effect of Sixteen-component Active Mixture and Control Mixture as a Function of Time on the Growth and Apoptosis of Sp2/0-Ag14 Mouse Myeloma Cell Line**

Internucleosomal DNA fragmentation was first detected after treatment for 1.5 h (the first time point examined) with the active mixture and became more prominent with longer treatment (Fig. 20A). There was no detectable DNA fragmentation in untreated, and control mixture treated cells after 24 h. The change of cell number as a function of time (Fig. 20B) detected by cell count supports these results.



**FIGURE 20**

Kinetics of DNA laddering (A) and growth (B) of Sp2/0-Ag14 mouse myeloma cells after exposure to active mixture, control mixture or to media alone. The composition of the sixteen-component active mixture and control mixture are given in the legend of Fig. 10. After the indicated times, cells were counted with the trypan blue dye exclusion method and then were lysed for agarose gel electrophoresis of DNA as described in "Materials and Methods". The values of the growth curves are mean  $\pm$  SE (bars) for three independent experiments.

## DISCUSSION

To our starting hypothesis, a Passive Antitumour Defense System exists in the living system besides the known immune mechanisms. The action of this defense system is the reason that only a few kinds of tumour develop when the activity of the known immune system is decreased (in AIDS or in other immune deficiency diseases) or suppressed (in organ allograft recipients). This defense system causes that tumours do not develop in the majority of population during their lifetime, although the majority of the clinically relevant tumours are not or only weakly immunogenic [2, 14]. We assumed that the effective agents of this defense mechanism are certain small substances occurring in the circulatory system. These molecules can enter both normal cells and tumour cells. However, their intake by normal cells is regulated, but by tumour cells it is unregulated and proportional to their availability [35-41]. We assumed that some of these substances together, by increasing the effect of each other synergistically, can kill the tumour cells if they can reach enough high concentration in the cells. The latter one, due to unregulated uptake of them by tumour cells, only depends on the availability. Thus, these substances can destroy the arising tumour cells in the living system, if the number of cells is not too high or the concentrations of the required substances are not too low. This happens in the majority of population during their lifetime. If the number of tumour cells arising simultaneously exceeds a critical value at which the divisions of the cells overcompensate for the killing of cells by the PADS and the cells are non-immunogenic (like the majority of clinically relevant tumours [2, 14]) then it is most likely that a tumour develops.

On the bases of literary data and theoretical considerations not detailed in this thesis, we selected L-tryptophan, L-tyrosine, L-methionine, L(-)-malate, and L-ascorbate as possible "killer" molecules. Examining the effect of them singly and in combination on Sp2/0-Ag14 mouse myeloma cells *in vitro* (Fig. 1) we found that they could really act in the supposed synergistic manner. To examine whether only these five compounds correspond to our assumption, or if some other substances occurring in the circulatory system can potentiate the effect of them as well, we tested 66 materials (Fig. 2). Nine of them, namely, L-arginine, L-phenylalanine, L-histidine, 2-deoxy-D-ribose, d-biotin, pyridoxine, adenine, riboflavin, and oxaloacetate were found effective. It also agrees with our assumption that according to literature these compounds, except for 2-deoxy-D-ribose, are accumulated by tumour cells [35-41]. Because the concentration of substances was chosen to be ineffective when they were used singly in the same concentration, the observed potentiating effects were in all cases the result of a synergistic interaction of the participating substances. The effect of oxaloacetate has only conceptual importance, since its concentration in the serum is very low compared to malate, and it is converted in the cells immediately to malate. Thus, it was not interesting regarding our starting hypothesis and we did not use it in our further experiments.

However its effect was interesting in a theoretical respect. In our theoretical considerations selecting malate as possible killer molecules we used the finding of Moreadith and Lehninger [71], that the extra- and intramitochondrial

malate has a different fate in tumour cells. Our results support their observation since oxaloacetate is the only intermediate of citric acid cycle that cannot readily cross the inner mitochondrial membrane and be converted immediately into malate in cytosol and since the other intermediates of citric acid cycle, which can readily enter mitochondria and can be converted into malate mainly intramitochondrially, were not found effective in our experiments (Fig. 2).

Comparing the effect of different amounts (expressed as percentage) of mixture containing the original five components selected on a theoretical bases and a mixture diluted in the same percentage containing the above five substances plus the eight experimentally selected compounds, the latter was found more effective than the former one (Fig. 3). Because certain brands or even lots of these substances can be contaminated and thus toxic, alone or in select combinations, we repeated the above experiments using different commercial products (obtained from Sigma, from Serva and from Reanal) and the result was the same in all cases (data not shown). We used in all other experiments cell culture tested biochemicals purchased from Sigma Chemical Co. (St. Louis, MO) which were the purest compounds available for these kinds of experiments. At the same time, we used the best quality biochemicals only for active mixture and the components of the control mixture were obtained from different sources and in different quality, as it was described in "Materials and Methods". In spite of these, the control mixture was not cytotoxic for any tumour cell lines at any amount. On the other hand, the thirteen-component active mixture was toxic both *in vitro* and *in vivo* only for different tumour cells and not for normal cells. On this basis, we think that the probability that contaminants have a significant role in the obtained effects is much less than the probability that the biochemicals themselves acted.

There are many publications [72-78] about the optimisation of composition of different tissue culture media. In opposition to us, they investigated the environment in which the different cells (malignant and normal) can growth ideally. They found that "all the amino acids are more or less inhibitory at 10-20 mM concentrations". They tested the toxicity of the substances singly and found that the reason of toxicity was "amino acid imbalance and ammonium toxicity". We determined the non-toxic amounts of all substances one by one by preliminary experiments and used these amounts in our experiments. Thus, the 100 % thirteen-component active mixtures in the experiments contained their components in such amounts in which they could not decrease the cell number compared to untreated cells when they were used singly. Naturally this was even more true for the 80 %, 60 %, etc., mixtures, in which the amounts of active materials were 20 %, 40 %, etc., less than the starting value. Thus, it can be stated that the cell killing effect was not caused by the individual toxicity of any components but it was strictly caused by the synergistic interaction of the given substances. On the other hand, in all experiments we used control mixtures that were composed of the same amounts of physiologically and chemically similar but - according to our previous experiments (Fig. 2) - ineffective compounds (succinate, amino acids, vitamins, hypoxanthine and ribose) in the

same dilution as the corresponding thirteen-component active mixture. Thus, the possibility that the measured effect in the experiments was a result of an osmotic effect or an aspecific overload of nutrients or an amino acid imbalance or ammonium toxicity could be excluded. Since the control mixtures also contained thirteen components it can also be excluded that the difference between mixtures containing five and thirteen compounds was caused by the increase of osmolarity. In addition, we found the thirteen-component active mixture effective not only in tissue culture but also *in vivo*.

We demonstrated (Fig. 4) that the thirteen-component active mixture destroyed the majority of Sp2/0-Ag14 mouse myeloma cells in 48 hours, whereas the control mixture only slightly influenced the proliferation of the same cells compared to untreated cells. The death of about 100,000 tumour cells proved that the synergistic interaction of the given substances did not only cause an inhibition of cell proliferation but it really killed the cells.

There was no significant difference at all between active mixtures containing the same thirteen compounds but different counter-ions (calcium or potassium instead of sodium and sulfate instead of chloride). Because the results with or without catalase were the same, it can also be excluded that the observed effect was caused by toxic hydrogen peroxide that can form by the action of light and oxygen on some substances [79-81]. The above results demonstrated that the measured cell killing effect is a fundamental feature of the mixture of the given substances. The effect of active mixtures did not change when it was complemented by different amounts of control mixture. This demonstrated again that the cell death was not caused by an imbalance. This finding also evidenced that the other compounds of the circulatory system could not antagonise the effect of active substances. Thus, the selected substances can act in physiological conditions, too.

Obviously, the various kinds of tumour cells differ from normal cells differently, and therefore it is presumable that the kind and amount of the substances effective against them also differ to a certain extent. However, a mixture containing many compounds is probably effective against many or all kinds of tumour cells. Naturally, the mixture having the most universal effect is the fluid of the circulatory system. Our further experiments render this speculation likely since the thirteen-component active mixture was found also significantly effective *in vitro* against K-562, HEP-2, HeLa (Fig. 5), and Caco-2 (Fig. 6) cell lines compared to the control mixture. At the same time the above mentioned mixture had no cytotoxic effect against the Vero normal cell line (Fig. 5); it only slightly decreased the proliferation of the cells. The effect of the thirteen-component active mixture on different number of Caco-2 cells (Fig. 6) corroborates in another respect the speculation that, when the proportion between the number of tumour cells and the amount of "killer" molecules is under the above mentioned critical value, then the cells are destroyed by the given molecules. Being the HEP-2 a "hardy cell line that resists temperature, nutritional, and environmental changes without loss of viability" (ATCC Catalogue of Cell Lines and Hybridomas, 1985) the obtained result proves again that the observed synergistic cell killing is not the result of a disturbing effect in the cell culture, but a fundamental

feature of the mixture of the given substances. To exclude that the measured effect was caused by the employed detection method, three different methods were used in our *in vitro* experiments to assay the cell viability. It is important to emphasise that in all experiments *in vitro* the concentration of the given substances was only about ten to thirty times larger than the physiological concentration in the serum of the same substances [63-70]. Considering the essentially direct proportion between the killed cells and the amount of mixture and dividing by thirty the number of tumour cells per millilitre used in the experiments, it can be stated that the mixture of selected substances can kill a certain number of tumour cells when the concentration of the molecules is a thirtieth of that used in the experiments, which is about the equivalent of their physiological concentration. This statement will also be evidenced by other experiments hereafter.

The aim of the *in vivo* experiments was to demonstrate that the substances of the circulatory system selected on the bases of starting hypothesis and found synergistically and selectively cytotoxic for tumour cells *in vitro* can also inhibit the growth of tumours *in vivo*. Considering the starting hypothesis, the most effective way of treatment would have been a continuous infusion, keeping the concentration of the selected substances in the circulatory system of the animals permanently on a fairly high level, to be abundant for tumour cells. For practical reasons we could use only a discontinuous, periodical treatment; thus, the effective level of substances in the serum could exist only for a short period. Although this treatment was not optimal to achieve a quantitative result and to demonstrate the total curative effectiveness of the thirteen-component active mixture, it was sufficient to get a qualitative view and to prove that the mixture of the substances selected by us could also act *in vivo* and could destroy a certain number of tumour cells in the living system. This treatment was equal to the task because the demonstration of the latter one was our only purpose. First, we examined and found that the mixture of the above mentioned substances significantly increased the survival time of mice injected i.p. with Sp2/0-Ag14 mouse myeloma cells (Fig. 8) by killing more than 2 logs (99 %) of the cells. This result is even more noteworthy considering that Sp2/0-Ag14 mouse myeloma is a highly aggressive and fast proliferating cell line with 1 day *in vivo* doubling time as it was revealed by tumourigenicity experiment (Fig. 7). The finding that the number of cells in the ascitic fluid of treated animals was significantly lower (about 2 logs cell kill) than in ascitic fluid of control animals excludes the possibility that the increase of survival time was caused by a simple roborating effect of the above substances. To show that the result was not due to a local effect (the i.p.-i.p. system) we experimented with an s.c. tumour model (HeLa human cervix epitheloid carcinoma). We found that the treatment significantly slowed down the tumour growth in treated animals compared to control ones (Fig. 9). It can be seen from the curve that the effectiveness of the treatment decreased with the increasing tumour volume. Considering the starting hypothesis, the probable explanation is that the proportion of the number of tumour cells and the amount of the substances reached by the discontinuous treatment was above a critical value during treatment. This is probably true also in the case of the other *in vivo* experiment made with mouse

myeloma cells. Thus the number of tumour cells arising by cell division was higher than the number of cells killed by the given molecules. The competition between cell division and cell death will be demonstrated in another experiment (Fig. 16). On the other hand, duration of the existence of substances in effective amounts ensured by one injected dose decreases as the number of tumour cells grows. This result fits well with the *in vitro* experiments (Fig. 6 and 14) that the effect of the mixture decreases as the number of tumour cells grows.

Body weight change is a generally used index to characterise the toxicity of a treatment. In our experiment the change in average body weight during treatment was not significant between the control and the treated group. Toxic death was not observed either during or after treatment, meaning that the substances in the given amount were not toxic. The reason for the small weight loss in both groups was probably the stress caused by frequent injections of the animals.

Testing other eighteen compounds of the circulatory system as described in the “Experimental Results”, we could select three additional substances (orotic acid, hippuric acid, D(+)-mannose) that could take part in the Passive Antitumour Defence System.

As it is also described in the “Experimental Results” section, only  $\text{Cu}^{2+}$  ion out of the different ions tested could potentiate the effect of the sixteen-component active mixture. The observation that the effect attained by  $\text{Cu}^{2+}$  could be prevented by catalase shows that the measured synergistic cytotoxic activity was due to  $\text{H}_2\text{O}_2$  formation and that this  $\text{H}_2\text{O}_2$  was produced outside the cells in the medium only. It follows from the latter that  $\text{Cu}^{2+}$  is not likely to play a role in the defence system under physiological conditions. It has been reported previously [82, 83] that the simultaneous presence of  $\text{Cu}^{2+}$  ions and ascorbate led to the formation of  $\text{H}_2\text{O}_2$ . Our findings can be explained by this observation because the potentiating effect of  $\text{Cu}^{2+}$  ions could be fully prevented by omitting the ascorbate from the active mixture. Using the ascorbate free active mixture, the catalase did not have a preventive effect. This result also shows that ascorbate is important for  $\text{H}_2\text{O}_2$  formation in the presence of  $\text{Cu}^{2+}$  ions. These findings makes it improbable that  $\text{Cu}^{2+}$  has a role in the PADS, therefore it was not used in our further experiments.

To demonstrate that the additionally selected three substances could have a role in the defence mechanism, the Sp2/0-Ag14 cell line was exposed to the effect of both of the sixteen- and thirteen-component active mixtures. It can be seen (Fig. 10) that submitting the cells to any amount of sixteen-component active mixture led to a significant decrease in cell survival compared to the cells treated with the same percentage of the thirteen-component active mixture.

The generality and selectivity of the effect of the sixteen-component active mixture were investigated on various cell lines (Fig. 11). Because the cell lines used in our previous experiments were not satisfactorily representative we used different tumour and normal cell lines in this experiments than previously. The sixteen-component active mixture showed significant cell-killing effect *in vitro* on A20, EL4, Jurkat, Hep G2, MCF7, and

MCF7/ADR tumour cell lines compared to the sixteen-component control mixture (Fig. 11). On the contrary, the sixteen component active mixture had no cytotoxic effect on the MDCK and the LLC-MK<sub>2</sub> normal cell lines (Fig. 11), it only slightly diminished the proliferation of the LLC-MK<sub>2</sub> cells. It is important to note that in the case of A20 and EL4 lymphoma cell lines the total cell death occurred at significantly lower concentration of the active mixture than in the case of the other cell lines. The remarkable effect of active mixture on both lymphoma cell lines compared to the other cells demonstrates that the lymphoma cells have a higher sensitivity to the cell-killing effect of the active mixture than the others. It is an important result because the active mixture could have therapeutic value to prevent lymphomas which frequently develop in the case of AIDS and in other immune deficiency diseases or in immunosuppressed patients. It is also worthy of note that the active mixture had cytotoxic effect on both MCF7 human breast adenocarcinoma cells and its adriamycin-resistant variant, MCF7/ADR cells. The importance of the latter result is emphasised by the facts reported earlier [48] that the MCF7/ADR cell line exhibited a multidrug-resistant phenotype and was cross-resistant to a wide range of antineoplastic agents including *Vinca* alkaloids, anthracyclines, and epipodophyllotoxins. These experiments corroborate the results of the previous ones (Fig. 5 and 6) and support our assumption that the substances selected by us experimentally as the members of the PADS have a general and selective effect on different tumour cell lines and that this effect is independent even from multidrug-resistance due to different mechanisms of action.



While the death of the tumour cells submitted to the active mixture may be observed very easily by invert microscope, the slight difference between the effect of the 100 % active mixture or 100 % control mixture on the normal cells can be perceived only to the skilled eye. This is well demonstrated by the photographs of the microplates (Fig. 12) after development of the MTT colorimetric assay. The significant difference between the effect of active mixture on the tumour and normal cells is the result of selectivity of the active mixture. The above observations bear importance upon our hypothesis of the PADS because the general toxic effect on tumour cells and the selectivity are a fundamental feature of a defence mechanism.

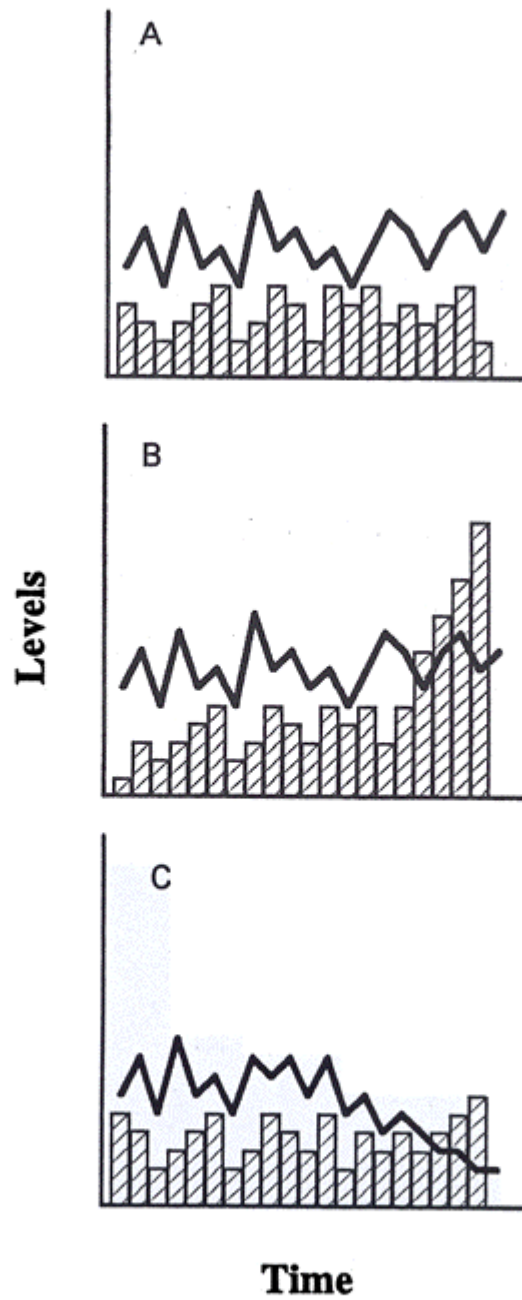
To interpret the slight proliferation decreasing effect of active mixture on LLC-MK<sub>2</sub> normal cells observed previously (Fig. 11 and 12), it may give rise to the next potentiality: if the LLC-MK<sub>2</sub> cells do not proliferate or proliferate very slowly, the slight difference between the effect of the active mixture or control mixture (Fig. 11 and 12) may be the result of cell death. To decide if the active mixture kills the normal cells or only decreases the proliferation of them, the change of cell number as a function of time with or without treatment by active mixture or control mixture was investigated. It was found (Fig. 13), that the normal cells either untreated or treated with control mixture had a fairly high proliferation rate similar to the tumour cells. The number of normal cells treated by 100 % active mixture also increased as a function of time (Fig. 13) and dying cells could not be observed microscopically in contrast to tumour cells treated by the same active mixture whose number decreased during the 48 hours incubation

period in consequence of cell death. The inverse change of cell number in the case of normal and tumour cells treated by the active mixture is unquestionable evidence for the selectivity of the sixteen-component active mixture.

In sum, considering the essentially direct proportion between the amount of mixture and the cell-killing effect in the *in vitro* experiments, the synergistic interaction of the substances, the number of killed cells at the given concentrations, the fact that the cell-killing effect is not antagonised by other substances of the circulatory system, the fact that the concentration of a given substance in the *in vitro* experiments was only about ten to thirty times larger than the physiological concentration in the serum of the same substance, the different (selective) effect of substances observed *in vitro* on normal and tumour cells, the non-toxic antitumour effects of the selected substances *in vivo*, it can be supposed that these substances existing together in the living system can really destroy a certain number of cancer cells in the body under physiological condition when their concentrations are in physiological range. This supports our hypothesis that in the living systems a Passive Antitumour Defence System exists and the compounds found by us play a role as effective agents in the operation of this defence system.

The next hypothetical figures illustrate (Fig. 21) the supposed operation of the Passive Antitumour Defence System. The amount of substances of the defence system continuously changes in the circulatory system depending on nutrition, age, life-style, etc. as shown by the lines. The bars symbolise the amount of arising tumour cells. If the number of arising cancer cells remains under a critical value then the concentration of substances of the defence system existing in the environment of the given cells is satisfactorily high in order to destroy all the arising cells (Fig. 21A). This happens in the majority of the population during their lifetime. If the number of tumour cells arising simultaneously exceeds a critical value because of some reason (e.g., strong carcinogenic effects, viruses, hereditary predisposition to cancer, etc.), then the tumour develops because above the critical value the divisions of the cells overcompensate for the killing of cells by the defence mechanism (Fig. 21B). The other way to develop a tumour is the decrease of the concentration of the above mentioned substances in the circulatory system in consequence of some reasons (e.g., malnutrition, disease, stress, etc.) and thus the impairment of the effectiveness of the PADS (Fig. 21C). Although in this case no more tumour cells arise than in the majority of the population, the number of cells can still reach the critical value because of the low concentration of the defence molecules. Obviously, the level of defence never decreases to zero since the majority of the substances taking part in the operation of the PADS have endogenous sources, only the defence cannot always operate optimally.

 Amount of arising tumor cells  
 Level of substances of defence system



**FIGURE 21**

Hypothetical figures to illustrate the supposed operation of the defence system. The changes of the amount of substances of the defence system are shown by the lines. The bars symbolise the amount of arising tumour cells.

To verify the above mechanism, the effect on tumour cells of active mixtures containing their components at concentrations corresponding to the maximum or minimum concentration of the given component in the serum was investigated (Fig. 14). As mentioned earlier, the maximum and minimum concentration in the serum of substances of the PADS can be found in the scientific literature [63-70]. When the substances of the active mixture were used in their maximum concentration existing in the blood (maximal active mixture: the model of the optimally operating defense system) and the tumour cells at different (decreasing) initial cell concentration were subjected to the effect of this mixture (Fig. 14), we could determine the critical cell number for this condition. This critical cell number was between 125 and 60 cells/ml (about 100 cells/ml). It can be seen (Fig. 14, black columns) that below this critical value all the cells were killed by the maximal active mixture. This corresponds to the condition that occurs in the majority of population during their lifetime shown in the hypothetical Fig. 21A. Although the maximal active mixture could not destroy all the tumour cells above the critical value (above 100 cells/ml) because the cell death was overcompensated by the cell division, it had some cytotoxic effect on the cells at any initial cell concentration compared to the control mixture. Obviously, this proliferation diminishing effect decreased as the initial cell concentration increased. This situation exists in the living system as it is shown in the hypothetical Fig. 21B when the cell number has got above the critical value for one reason or another (e.g., strong carcinogenic effects, viruses, hereditary predisposition to cancer, etc.) and thus the tumour develops. Since a human erythroleukemia cell line was used in our experiment, it is interesting to compare the result with an old-standing clinical observation. According to this observation, relapse does not occur in the case of haematological tumours if the cell number can be decreased by the usual treatment to  $10^6$ - $10^5$  because the defence mechanisms of the living system can destroy the remaining cells [84]. In our experiment (Fig. 14), the optimally operating defence mechanism (the maximal active mixture) could kill about 100 cells/ml that is 100,000 cells/l. Taking as an average about 5 litres of blood in a person, the total number of cells is about  $5 \times 10^5$  that corresponds to the above clinical observation. Because of the limited number of cells destroyed, the above clinical observation cannot be explained by the action of an activable defence mechanism like the known immune system it can only be explained by the operation of a relatively constant level, non-activable (passive) defence mechanism.

This statement is further supported by our previous tumourigenicity experiment (Fig. 7). Mice given injections of  $5 \times 10^3$  or less Sp2/0-Ag14 cells showed no evidence of i.p. tumour growth. Injecting twofold ( $1 \times 10^4$ ) or more amount of cells, tumours developed in all the rodents. Similar results were reported in other paper [53] about other cell line. This sharp limit between the cell number causing the development of tumour and the cell number showing no evidence of tumour growth is rather strange from the point of view of the known activable immune system. If the known immune system can kill  $5 \times 10^3$  or less cells, why cannot the same immune system kill  $1 \times 10^4$  cells? The above observations become explainable if the existence of a relatively constant level defence mechanism like the

PADS is accepted. Namely, in the case of  $5 \times 10^3$  or less cells the cell number is under the critical value and all the cells are destroyed by the PADS. In the case of  $1 \times 10^4$  or more cells the cell number is above the critical value and the cell division overcompensates for the amount of cell death. Thus, more cells arise than die and consequently the tumour develops.

It can be seen in Fig. 14 that the minimal active mixture (the model of the poorly operating defence system) also had an effect on the tumour cells at any initial cell concentrations. Obviously, its effect was lower than the effect of the maximal active mixture, what is more, the minimal active mixture could not kill all the cells even at the lowest initial cell concentration. That means, if the defence system cannot operate optimally then the tumour develops even at low concentration of arising tumour cells as it is illustrated in the hypothetical Fig. 21C. This happens when the amount of defence molecules decreases for some reasons (e.g., malnutrition, disease, stress, etc.). The finding that the control mixture had not cytotoxic effect on tumour cells at all (Fig. 14) demonstrates that the cell-killing effect (the defence) is a fundamental feature of the given substances selected experimentally from the compounds of the circulatory system. The adherent cells could not be used in similar experiments because they could not proliferate at the above mentioned low cell concentration even in the absence of any treatment. However, the results of experiments (Fig. 5, 6, 11, 12, and 13) performed with a higher cell number (40,000 cells/ml) and obviously with a higher amount of active mixture than in the present study make it probable that the substances of the defence mechanism can also kill a certain number of the adherent cells when their concentrations are the same as in blood.

The question may arise: whether the concentration or the total amount of given compounds in the circulatory system has higher importance from the point of view of the PADS. The experimental results show (Fig. 15) that the length of time needed for death of the same amount of cells decreases as the concentration of the active mixture increases. This means that the rate of cell death depends on the concentration of the active mixture. Obviously, the rate of the cell division is conditioned by the cell concentration. To our speculation the cell division and the cell death caused by the defence system compete with each other. The balance of them determines that the tumour may or may not develop. At the critical value the rate of division and the rate of death are equal. Below the critical value the rate of cell death is higher than the rate of division and so all the cells die (Fig. 14, below 125 cells/ml), but above the critical value the rate of cell division is higher than the rate of death and the tumour develops (Fig. 14, above 125 cell/ml and in the case of minimal active mixture). As it appears in Fig. 15, at 12.5 % of active mixture the length of time needed for cell death was 11 days. That is much longer than in the case of 25 % and higher amounts of mixture (3 days or less), what is more, the 6.25 % of active mixture could not kill all the cells. The reason for this is that the critical proportion (the critical value) of the cells applied at a constant amount and the active mixture used in various amounts is between the 6.25 % and 12.5 % of the active mixture. That is why the rate of cell death at 12.5 % of active mixture was only barely higher than the rate of cell division and the death of all cells needed 11 days. At

6.25 % of active mixture the rate of cell death was at such a low level that it was already overcompensated by the rate of cell division and accordingly, the 6.25 % of active mixture could not kill all the cells. These findings taken together with other results indicate the necessity to keep an optimal concentration of the above mentioned substances in the blood every time.

The importance of that is further corroborated by the experiment (Fig. 16) in which it was investigated visually (by photos) what happens with the tumour cells when the effectiveness of defence is shifted from the optimally operating to the poorly operating condition. It may be seen in the photos of Fig. 16 that as the amount of active mixture decreased, in other words, as the effectiveness of the defence system declined, the division of the K562 human erythroleukemia cells compared to the cell death became more and more dominant. While in the case of the 100 % active mixture all the cells were destroyed before they could divide only once (Fig. 16C), in the case of 50 % active mixture some cells could already divide once before their death (Fig. 16D). As the amount of the active mixture decreased further (to 25 %) the rate of cell division became commensurable with the rate of cell death (Fig. 16E). This is the point at which the mixture to cell ratio corresponds to the critical value and the cell division and cell death occurring simultaneously are in an equilibrium. It can be clearly seen in the photo that the cell death is accompanied by cell blebbing and formation of apoptotic bodies which has been discovered to be a late event in apoptosis and a marker of it. If in this situation in the living system the concentration of substances of the defence mechanism gets higher than the concentration existing at the critical value (e.g., due to nutrition or a preventive medicine containing the mentioned substances) then the equilibrium of death and division shifts to direction of death (to the direction of the previous state shown in the Fig. 16D) and all the tumour cells will die but if the concentration of the components of the defence system further decreases (e.g., in consequence of stress, illness, malnutrition) then the cell division will overcompensate the cell death and the tumour develops as can be seen in Fig. 16F. It is important to emphasise that in the last photo (Fig. 16F) some caduceus and dead cells can be seen and the number of cells is significantly lower than the number of cells untreated or treated by the control mixture (Fig. 16A and 16B). This means that the defence mechanism has a cell-destroying effect even after a tumour has developed and even if its effect is not enough to kill all the tumour cells. This statement is also supported by the observations that the maximal active mixture slowed down the proliferation of the cells compared to untreated cells above 125 cells/ml cell concentration (Fig. 14) and that in the *in vivo* experiments, although the concentration of the active mixture ensured by the treatment was not enough to kill all the tumour cells and cure the animals, it could still eliminate more than 99 % of the tumour cells increasing the survival time (Fig. 8) and slowed down significantly the growth of solid tumour (Fig. 9) in the treated animals.

The existence of the Passive Antitumour Defence System makes it possible to explain and understand the monoclonality of the majority of the tumours. This can be clearly demonstrated in the above photos. If the level of

defence high enough or the number of arising cell is low, in other words, the rate of cell death is much higher than the rate of cell division then all the cells die before they can divide only once. This situation occurs in Fig. 16C. As the number of the arising cancer cells (in other words, the rate of cell division) increases or the level of defence (in other words, the rate of cell death) declines, the probability rises that some of the cancer cells can divide once before they die, similarly as in Fig. 16D. The number of cells which can divide twice, three times, etc. decreases considerably. In the above mentioned photo (Fig. 16D), many cells can be seen which could divide once before death but only one cell can be seen that could divide twice. The more cancer cells arise (e.g., because of carcinogenic effect) or the lower the level of defence (e.g., because of malnutrition) the higher the probability that at least one of the cells can divide so many times that the number of its daughter cells reaches the critical value. Obviously, if the number of arising cells is very high due to determinants such as hereditary susceptibility then the probability increases that the number of daughter cells of more than one original cell can reach the critical value and the tumour will be polyclonal. These are in accordance with the observations that the majority of human tumours are of monoclonal origin excepting e.g., hereditary tumours [19, 85, 86]. The majority of tumours in animals also show monoclonality [86]. The clonal origin of the tumours cannot be easily explained by the effect of the known immune system. On one hand, many hereditary neoplasm's are polyclonal in patients with an intact immune system [86], on the other hand, the clonal origin of non-hereditary tumours is strongly suggested by various observations [86] in immunosuppressed patients. The phenomenon of monoclonality can only be elucidated by the existence of a critical cell number and a defence mechanism possessing a relatively constant level.

Because the process of cell death can occur by either apoptosis or by necrosis [87] we tried to identify, if the death induced by the substances of the PADS (by the sixteen-component active mixture) was of the apoptotic or necrotic type, although the cell blebbing and apoptotic bodies observed in an earlier experiment (Fig. 16E) made the apoptotic death probable.

Apoptosis and necrosis can usually be distinguished morphologically and biochemically [87]. Characteristics of apoptosis include cell shrinkage, chromatin condensation, apoptotic body formation, and DNA degradation. These properties differ significantly from characteristics of necrosis.

Cells with characteristic features of apoptosis were observed by light microscopy after 24 hours treatment of Sp2/0-Ag14, and K562 tumour cells by the active mixture. Drastic morphological changes occurred in the mentioned cells in comparison with Vero normal cells or tumour cells untreated or treated with control mixture (results are not shown).

The death of cells undergoing apoptosis is preceded by chromatin cleavage at the linker regions between nucleosomes by specific endonucleases, which results in a number of 180-200 base pair fragments and multiples of it [88]. In general, the DNA fragments can be demonstrated by agarose gel electrophoresis, wherein a characteristic

“ladder” develops. The agarose gel electrophoresis of DNA from cells treated with active mixture revealed the “ladder” pattern in the case of Sp2/0-Ag14 mouse myeloma (Fig. 17A) and K562 human erythroleukemia cells (Fig. 17B), indicating preferential DNA degradation at the internucleosomal linker DNA sections. In contrast, DNA fragmentation cannot be detected in the case of untreated cells and in the case of cells treated with the control mixture. Vero normal cells treated with active mixture under the same experimental conditions also failed to show evidence of endonucleolytic DNA degradation (Fig. 17C), although it was demonstrated earlier [89] that degradation of DNA in Vero cells giving rise to the typical ladder pattern on gel electrophoresis can be induced. These results are consistent with the previous findings demonstrating that the active mixture has cytotoxic effect on various cell lines except for normal cells. Thus, the result of the gel electrophoresis repeatedly demonstrates the selectivity of the active mixture. It is important to emphasise that K562 has been shown to be relatively resistant to a variety of apoptotic stimuli [90] (diphtheria toxin [91], etoposide [92], etc.) but the given substances of the circulatory system together could induce apoptosis of K562. On the other hand, other substances of the circulatory system (see control mixture) did not have an apoptosis inducing effect on the K562 cells.

As it was demonstrated above, the active mixture containing many compounds is effective against several kinds of tumour cells (Fig. 5, 6, 11, 12, and 13). From the cell lines investigated, the Sp2/0-Ag14 cell line was chosen previously (on account of practical reasons) to select the components of the active mixture (Fig. 1 and 2). That is why the Sp2/0-Ag14 cell line was also applied in the following experiments to investigate the characteristics of the apoptosis-inducing effect of the active mixture.

We demonstrated previously that the cell-killing effect of the active mixture was not caused by the individual toxicity of any components but was caused by the synergistic action of the given substances. Therefore, we investigated if the components of the active mixture can induce apoptosis singly or only together. The result of the experiment demonstrated that only the simultaneous effect of the substances could induce apoptosis of tumour cells (Fig. 18). Using the components individually in exactly the same concentration as in the active mixture, DNA fragmentation could not be detected (Fig. 18). This demonstrates the synergism in an early phase of the way leading to cell death, and proves together with the former results that the synergistic action is a fundamental feature of these substances.

Flow-cytometric measurement of DNA content of the Sp2/0-Ag14 cells after 24 h incubation with different amounts of the sixteen-component active mixture demonstrated the presence of cells with a fractional DNA content (sub-G1 peak), typical of apoptosis (Fig. 19A). There was concordance between the intensity of DNA fragmentation detected by gel electrophoresis (Fig. 19B) and the proportion of cells appearing apoptotic by flow-cytometric analysis (Fig. 19A) as the amount of the active mixture increased. On the bases of these experiments it could be

excluded that the apoptosis was induced by detection techniques or sample preparation procedure because the two different detection methods gave the same result.

Treating the Sp2/0-Ag14 cells with active mixture, the internucleosomal DNA fragmentation was apparent 1.5 h after incubation with the mixture (the first time point investigated) and continued to increase to 24 h, the end point of these experiments (Fig. 20A). DNA fragmentation could not be detected in untreated, and control mixture treated cells even after 24 h. Counting the cells, the results (Fig. 20B) fit in well with the results of gel electrophoresis (Fig. 20A).

According to our hypothesis, the arising tumour cells are extinguished by the simultaneous action of the mentioned substances of the circulatory system. These cells undergo apoptotic cell death as shown by the above results. In this context it is of interest that preneoplastic cells exhibited much higher apoptotic activity than the surrounding normal cells [88, 93] and single initiated cells should have a relatively high risk of elimination by apoptosis [93]. Both mathematical and biological analyses suggest that 80-90 % of the initiated cells induced by chemical carcinogens are eliminated by apoptosis [88, 93].

The existence of the PADS is supported not only by the above experiments but by many epidemiological and clinical observations, as well as other literary data which are detailed in the followings.

Evidently the defence against the development of a tumour takes place in two steps. In the first step the living system tries to prevent the formation of malignant cells. This defensive step includes the action of antioxidants, protective enzymes, repair mechanisms and the like. If this fails, the cancer cells come into existence. In the second step the living system tries to destroy the tumour cells arisen. In this second step the PADS and in the case of immunogenic tumours the known immune system has a role. Once the existence of the PADS has been accepted, various epidemiological observations can be explained. On the one hand, epidemiological studies accumulated evidence that consumption of different vegetables and fruits is associated with a decreased risk of cancer [94-96]. Of 170 epidemiological studies of cancer at all sites, 132 found a statistically significant protective effect associated with the highest intake of fruits and vegetables [94]. Individuals with low fruit and vegetable intakes (approximately 20-30 % of the population) have a cancer rate at least twice that of individuals with high intakes [94, 97]. However, it was also observed that the intake of total carotenoids, retinol and total vitamin A was weakly and inconsistently related to risk [98], and the protective effect of vegetables and fruits is made not only by ascorbic acid or carotenoids but other food constituents also play a role [96, 99, 100]. Taking into account that large quantities of pentose [63], malic acid [101, 102], ascorbic acid [38, 101], riboflavin [38, 103], pyridoxine [38, 103] as well as other water soluble vitamins and amino acids [102, 104] are found in vegetables and fruits and that the capacity of intestinal transport of all the amino acids, carbohydrates, and most water-soluble vitamins increases when there is a large food intake [105], it can be assumed that the substances of the PADS may be among the above-mentioned "other food

constituents". Thus, appropriate nutrition can positively affect the amount of these substances [106, 107] and the operation of the PADS. On the other hand, malnutrition can affect negatively [106, 108] the amount of substances of the PADS in the serum. However, the majority of the substances of the PADS selected by us have an endogenous source. Thus, malnutrition does not cause a substantial loss, but only a decrease in the effect of the PADS. This means that the PADS always operates, but at a lower level in cases of malnutrition than in normal conditions and the operation of it always depends on the quality of the nutrition. From the above the probability can be seen that the positive effect of proper nutrition is larger than the negative effect of malnutrition. Thus, the significant effect of malnutrition on the tumour incidence can probably be observed if prolonged malnutrition occurs in a large population. Fortunately, it was taken an observation fitting in well with our speculation that the risk of cancer related to poor nutrition in the poorly fed Moslem populations of Central Asia may be considerable, even without other detrimental effects [109].

The later stages of HIV-1 infection (AIDS) is frequently accompanied by malnutrition [110, 111]. It is not probable that the effect of this malnutrition on tumour incidence can be detected since dietary changes occur at the time of HIV-1 seropositivity diagnosis, with participants increasing their intake of vegetables and vitamin supplements [112]. At later stages, enteral feeding or, in the case of homosexual men who commonly have diarrhoea, parenteral nutrition is used in the management of patients [113, 114]. On the other hand, the duration of malnutrition in AIDS is rather shorter than in the case of e.g., a poorly-fed population.

Cancer is also often associated with malnutrition, manifested by weight loss, cachexia. This malnutrition is caused by tumour- and therapy-induced anorexia, nausea, vomiting and malabsorption, and exacerbated by the abnormal metabolism of nutrients and the competition of the tumour with the host for essential nutrients [39, 115, 116]. It has a low probability that the effect of this malnutrition on tumour incidence can be detected because of the relatively short duration of malnutrition, because of the total parenteral nutrition used often in cancer patients and because metachronous multiple tumour formation is influenced by many other factors (e.g., therapy).

Of course, not only nutrition and malnutrition can influence the amounts of the "defender" substances and thus, the effect of the PADS. For example, it has been observed by epidemiologists that alcohol increases the risk of cancer [109, 117], although the mechanisms by which alcohol induces cancer in humans are not clear [117]. At the same time, heavy alcohol abuse is associated with an inadequate intake of proteins and vitamins, and impairs absorption, utilisation, storage and excretion of nutrients [118], causes marked decreases the concentration in the plasma of each amino acids taking part in the defence mechanism [119] and enhances pyridoxine degradation [120]. The latter observations that the alcohol decreases the concentration of substances of the PADS in the circulatory system can give an explanation how the alcohol increases the risk of cancer.

The PADS must still have some influence even in the presence of a growing tumour because the tumour cells are always subjected to some effect of the "defender" molecules only in the case of a growing tumour the rate of cell division is higher than the rate of cell death. This speculation fits in well with opinion of others [88] that the growth of a tumour is regulated not only by the rate at which tumour cells divide, but more importantly by the rate at which they die. Our statement is supported by our experiments (Fig. 8, 9, 14, and 16F) and by the observations that the cell death rate is still high within non-necrotic tumour tissue [31] and that 70 % to 90 % of newly-produced tumour cells in humans die spontaneously by a mechanism that is yet poorly understood [19] and that the disparity between the observed tumour growth and the expected growth calculated from proliferation kinetics amounts to 70-90 % [88] and that in some cases spontaneous regression of tumours occurred even in AIDS patients [121, 122]. Furthermore, it was observed that in maximally immunodepressed recipients almost 80 % of the papillomas regressed, although cellular or humoral immune capacity in the animals could not be detected by conventional means [123]. It has been also reported that apoptosis may be an important pathway for cell loss even in untreated tumours [124] and that spontaneous regression may also be due to apoptosis, induced by unknown stimuli [88]. Taking into account that the majority of clinically relevant tumours are not immunogenic [2, 14], the above observations suggest that the PADS really have an effect on growing tumours by inducing the apoptosis of the tumour cells.

It is also probable that the PADS has a role in the defence against metastases. This is supported by the observations that the development of metastases is an inefficient process [32, 125] and a very small percentage (< 0.01 %) of circulating tumour cells initiate metastatic colonies [32] in spite of the above mentioned fact that the majority of clinically relevant tumours are not immunogenic [2, 14]. The observation that metastases did not necessarily develop even when large numbers of viable tumour cells regularly entered the blood in patients with peritoneovenous shunts [126, 127] further supports the role of the PADS in preventing metastases considering that in the above experiment host factors other than surveillance by the known immune system had to be responsible for local encouragement or suppression of metastatic growth because no cellular immune response was detected in response to micro metastases or isolated tumour cells [126, 127]. Mechanical factors such as blood turbulence or deformation of the cells in the microvasculature can also participate in the destruction of a part of released cancer cells [125, 128, 129], but, considering that more than  $10^4$  allogeneic, nonimmunogenic MCAIV tumour cells are required to transplant the tumour s.c. (when there are no considerable mechanical factors) into 50 % of 6 Gy whole-body irradiated athymic NCr/Sed nude mice [130], and considering that the cell dose required to transplant the tumour into 50 % of recipients could neither be increased by immunisation procedures nor decreased by six Gy whole-body irradiation prior to transplantation [130], it can be stated that this number of cells (more than  $10^4$ ) are being killed due to the PADS. Consequently, the PADS has a role to prevent the development of both tumour and metastasis. The difference is that in the latter case cancer cells originate from a growing primary tumour and a

detectable size metastatic tumour always releases enough cells to produce metastasis. It appears that the greater the number of cells released by a primary tumour, the greater the probability that the daughter cells of at least one of them in the given organ can reach the critical value. This means that the majority of metastasis incidence must also be of monoclonal origin. This is supported by literature [125, 131, 132]. Evidently, if the number of cells shed into the bloodstream is high enough, then many metastases develop. These originate from different cells but the majority of them are monoclonal [131]. Probably only primary tumours that reach a certain size can shed enough cells into the blood to bear down the PADS, to reach the critical value and to produce metastases. This is in accordance with the finding that the analysis of data from 2648 breast cancers treated between 1954 and 1972 at the Gustave Roussy Institute suggests the existence of each tumour at a given volume (threshold) at which the first remote metastasis is initiated [133]. Obviously, as the primary tumour further increases, the number of cells released into the circulatory system, the number of cells reached the proper organ and accordingly the number of metastases increases as well. On the other hand, a large tumour may also facilitate the formation of metastases since it can cause the decline of the PADS in consequence of the increased, unregulated uptake of the “defender” substances by the tumour. It is probable that this influence of the tumour ceases after removal of the tumour mass. This is supported by observations that the removal of the tumour restores to normal the abnormal profiles of plasma amino acids found during the tumour-bearing state [116], and micro metastases in breast cancer patients became undetectable for a while after surgery, both in treated and untreated patients [134].

It is well known that tumour cells released into the bloodstream are rapidly distributed to many organs, but they only grow to form metastases in certain sites [32, 125, 128, 132] because the microenvironmental conditions of the various organs (e.g., the concentrations of nutrients, organ-derived inhibitory factors [125, 128]) influence the survival of the tumour cells. To our speculation, certain PADS substances also have a role in this process. The amount and kind of PADS substances in the various organ environments probably differ, depending on the function and metabolism of the given organ. Cells released from various kinds of tumours may be sensitive to these substances in different ways. This assumption is supported by the findings that some organs rapidly and effectively diminish the number of live tumour cells [135]. These *in vitro* experiments clearly demonstrated that the inhibitory effects are dose-dependent, and due to the presence of soluble, dialyzable, non-immunologic agents of small molecular weight diffusing out of organs. The effects of the given organs on the given tumour cells *in vitro* were in clear agreement with the *in vivo* observations [135]. It can be found in other reports as well that diffusible inhibitors of tumour growth may be released by certain tissues [125, 136]. Obviously, our assumption does not exclude the role of other factors such as motility, invasiveness, and cell adhesion, and does not contradict the "seed-and-soil" hypothesis of Paget. Rather, it completes it.

It is well known that immune function declines with advancing age [137]. Looking at the incidence of cancer in AIDS and in other immune-deficient patients, it might be expected that only some kinds of tumour increase in incidence with advancing age similarly as in AIDS and in other immune-deficient patients. Contrary to expectation, the incidence of most types of cancer increase [137, 138]. This contradiction shows that a different and more general defence mechanism than the declined known immune system protects the living system against tumour development and that this defence mechanism also declines with advancing age. All of these are in consonance with the observations that the molecules of the PADS also decline with advancing age and this decline is prolonged. Namely, the prevalence of digestive disorders is increased in the elderly [139]. Intestinal absorption of nutrients [139, 140], including amino acids [141] shows a decrease with ageing. Ingestion of an amino acid load from protein hydrolysate resulted in serum amino acid concentration a lower rise in older human subjects than in young adults [140]. Even the fasting serum level of all amino acids occurring in the PADS was significantly lower in elderly people as compared to young subjects [142]. Malnutrition is relatively frequent among elderly ambulatory patients [143] and the elderly have more likely than young adults low or deficient levels of ascorbic acid [139, 140, 144], riboflavin, and pyridoxine [139, 144].

Finally, it is a further important evidence for the existence of PADS that it was reported earlier about many substances taking part in the operation of PADS, namely, about ascorbic acid [145, 146], arginine [147], 2-deoxy-D-ribose [148], mannose [149], orotic acid [150], pyridoxine [151], riboflavin [152], tyrosine [153], etc. that they had antitumour effect. It is important to emphasize that the above substances were used singly in the mentioned experiments. Taking into account that the same substances could increase synergistically the effect of each other as it was demonstrated by us, the above observations also support our hypothesis and corroborate our results.

The exact mechanism of action of the substances participating in the PADS and the exact mechanism of apoptosis caused by the mixture of the above substances needs further investigation, which are in progress in our laboratory.

### **The Possible Practical Applications**

The existence of the PADS and the knowledge of substances taking part in it offer the possibility of practical employment. Considering the hypothesis and the results, this can be executed by keeping the concentration of the selected substances in the circulatory system permanently on a fairly high level, to be enough to destroy the tumour cells. The possibilities of employment depending on dose are: for prophylaxis in the case of old people, different

diseases, stress situations, malabsorption, alcoholism, smoking and in the case of populations which have a larger susceptibility to tumour because of hereditary features or for people with unusual lifestyles or modified diets; for prevention of tumour development in the case of AIDS patients and organ transplant recipients; for prevention of metastasis; for adjuvant, combined or direct treatment of patients having tumour. As it obviously follows from the theory, the treatment must be permanent in all cases. On the other hand the method can change from tablets to infusion, depending on the aim of the treatment, the probable number of tumour cells and thus the needed dose. This kind of treatment has remarkable advantages. First, the molecules of the PADS are natural compounds. It is therefore probable that the tumour cells do not produce against them damage-recognition proteins and P-glycoproteins and thus it is also probable that resistance does not develop against them. The latter statement was demonstrated by us because the mixture of the “defender” molecules could also destroy the MCF7/ADR adriamycin-resistant cells (Fig. 11 and 13). Furthermore, the effect of these substances is general and selective and they are without considerable adverse effects at the required and applicable dosage.

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## THE NEW RESULTS OF THIS DISSERTATION

1. We selected five molecules (L-tryptophan, L-tyrosine, L-methionine, L(-)malate, and L-ascorbate) as possible participators of the hypothetical defense system on the bases of literary data. Examining the effect of them singly and in combination on Sp2/0-Ag14 mouse myeloma cells *in vitro* we found that the mixture of them is really toxic for tumour cells and they increase the effect of each other synergistically (Fig. 1).

2. Then we selected experimentally nine additional substances that could potentiate synergistically the effect of the former five. Of the 66 compounds examined, 9 compounds (adenine, L-arginine, L-phenylalanine, L-histidine, 2-deoxy-D-ribose, d-biotin, pyridoxine, riboflavin, and oxaloacetate) potentiated significantly ( $P < 0.001$ , for oxaloacetate  $P < 0.01$ ) the effect of the five-component mixture (Fig. 2).

3. It was found that treatment of the cells with different amount of mixture containing thirteen (the first five substances plus the next eight, without oxaloacetate) components caused significantly larger effect to decrease cell proliferation than mixture containing the five substances. The control mixture was not cytotoxic for Sp2/0-Ag14 cells at any amount (Fig. 3).

*In this and in all the other experiments we used control mixtures. In this experiment the control mixture contained thirteen compounds of similar characteristics (succinate, hypoxanthine, ribose, amino acids, etc.) as the thirteen-component active mixture at a concentration that ensured the same osmolarity as the thirteen-component active mixture. The components of control mixture were chosen from that part of 66 compounds that were found in the previous experiment ineffective in potentiating the cell killing effect of the five component mixture. Thus, the possibility that the measured effect in the experiments was a result of an osmotic effect or an aspecific overload of nutrients or an amino acid imbalance or ammonium toxicity could be excluded.*

4. We investigated the effect of the thirteen-component active mixture and control mixture as a function of time on the growth of Sp2/0-Ag14 mouse myeloma cell line compared to untreated cells (Fig. 4). The number of untreated cells and cells treated with the control mixture increased exponentially in 48 hours. At the same time, the number of cells treated with thirteen-component active mixture decreased compared to starting value. The death of about 100,000 tumour cells proved that the synergistic interaction of the given substances did not only cause an inhibition of cell proliferation but it really killed the cells.

5. There was no significant difference at all between active mixtures containing the same thirteen compounds but different counter-ions (calcium or potassium instead of sodium and sulphate instead of chloride).
6. The effect of active mixtures did not change when it was complemented by different amounts of control mixture. This demonstrated besides others that the cell death was not caused by an imbalance. This finding also evidenced that the other compounds of the circulatory system could not antagonise the effect of active substances.
7. The thirteen-component active mixture was found also significantly effective *in vitro* against K-562, HEp-2, HeLa (Fig. 5), and Caco-2 (Fig. 6) tumour cell lines compared to the control mixture. The same active mixture had no cytotoxic effect against the Vero normal cell line (Fig. 5).
8. We examined and found that the mixture of the thirteen components significantly increased the survival time of mice injected i.p. with Sp2/0-Ag14 mouse myeloma cells by killing more than 2 logs (99 %) of the cells (Fig. 8). The difference between mean survival time of control ( $12.9 \pm 0.6$  days) and treated ( $18.9 \pm 0.5$  days) group is highly significant ( $P < 0.001$ ). The T/C % calculated from the median survival time of control (13.5 days) and treated (20 days) group is 148.1 %.
9. The finding that the number of cells in the ascitic fluid of treated animals was significantly ( $P < 0.001$ ) lower ( $10.8 \times 10^5$ ) than in ascitic fluid of control animals ( $9.68 \times 10^7$ ) excludes the possibility that the increase of survival time was caused by a simple roborating effect of the above substances.
10. The treatment with mixture of the thirteen components decreased the growth of tumours in the BALB/c nude mice injected s.c. with HeLa cells (Fig. 9). The mean relative tumour volumes of the control and treated groups differed significantly ( $P < 0.05$ ) in all cases. The T/C % was less than 42 % at each evaluation. The least value was 35.7 %. The mean growth delay was 14 days.
11. The change in average body weight during above treatment was not significant between the control and the treated group and toxic death was not observed either during or after treatment, meaning that the substances in the given amount were not toxic.
12. Testing other seventeen compounds of the circulatory system we could select experimentally three additional substances (orotic acid, hippuric acid, D(+)-mannose) for the active mixture.

13. We demonstrated that the sixteen-component active mixture containing orotic acid sodium salt, hippuric acid sodium salt, and D(+)-mannose additionally to the thirteen-component active mixture has significantly higher toxic effect on the Sp2/0-Ag14 cell line than the thirteen-component active mixture (Fig. 10).

14. Investigating the effect of different ions ( $\text{Fe}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cr}^{3+}$ ,  $\text{SeO}_3^{2-}$ ), it was found that only  $\text{Cu}^{2+}$  potentiated significantly ( $P < 0.001$ ) the effect of the sixteen-component mixture. This effect could be prevented completely by catalase (Sigma, C-40; 2000 U/ml). On the other hand, the  $\text{Cu}^{2+}$  could not potentiate the effect of active mixture when the mixture did not contain ascorbate. This findings made improbable that  $\text{Cu}^{2+}$  could play a role under physiological conditions and could take part in the PADS, therefore it was not used in our further experiments.

15. The sixteen-component active mixture showed significant cell-killing effect *in vitro* on A20, EL4, Jurkat, Hep G2, MCF7, and MCF7/ADR tumour cell lines compared to the sixteen-component control mixture (Fig. 11). On the contrary, the sixteen component active mixture had no cytotoxic effect on the MDCK and the LLC-MK<sub>2</sub> normal cell lines. The degree of cell death was especially high in case of the A20 and EL4 lymphoma cells. It is an important result because the active mixture could have therapeutic value to prevent lymphomas which develop frequently in the case of AIDS, in other immune deficiency diseases or in immunosuppressed patients. It is also worthy of note that the active mixture had cytotoxic effect on both MCF7 human breast adenocarcinoma cells and its adriamycin-resistant variant, MCF7/ADR cells. The sixteen-component control mixture was not cytotoxic for any cell lines in any amount.

16. To decide if the active mixture kill the normal cells or only decrease the proliferation of them, the change of cell number as a function of time with or without treatment by active mixture or control mixture was investigated. It was found (Fig. 13), that the normal cells either untreated or treated with control mixture had a fairly high proliferation rate similarly to the tumour cells. The number of normal cells treated by 100 % active mixture also increased as a function of time and dying cells could not be observed microscopically in contrast to tumour cells treated by the same active mixture whose number decreased during the 48 hours incubation period in consequence of cell death. The inverse change of cell number in the case of normal and tumour cells treated by the active mixture is an unquestionable evidence for the selectivity of the sixteen-component active mixture. This experiment also demonstrated that the A20 and EL4 lymphoma cell lines were highly susceptible (more than the other cell lines) to the effect of the active mixture, since all the cells were killed after 12 hours of incubation.

17. The maximal and minimal concentration of the substances of active mixture in the blood can be found in the scientific literature. The active mixture, when the final concentration of its components corresponded to their maximum concentration existing in the blood, killed all the K562 cells under 125 cells/ml initial cell concentration whereas the untreated cells proliferated to  $5 \times 10^5$  (Fig. 14). The minimal active mixture was ineffective to destroy the cells even at 60 cells/ml initial cell concentration. The control mixture did not show cell killing effect at all. The results were similar in the case of Sp2/0-Ag14 cells. The maximal active mixture was a model of a fairly operating and the minimal active mixture of a poorly operating defence system. It results from these experiments that the selected substances may act in physiological conditions, too. These experiments also demonstrates the importance of the optimal amount of the above substances in the blood.

18. We demonstrated (Fig. 15), that the length of time needed for death of the same amount of cells decreases as the concentration of the active mixture increases. This means that the rate of cell death depends on the concentration of the active mixture.

19. It was investigated visually (by photos) using the active mixture as a model of defence system, what happens with the tumour cells when the effectiveness of defence is shifted from the well operating to the poorly operating condition. It can be seen in the photos of Fig. 16 that as the amount of active mixture decreased, in other words, as the effectiveness of the defence system declined, the division of the K562 human erythroleukemia cells compared to the cell death became more and more dominant. However, the photos also demonstrate that the defence mechanism has a cell-killing effect even after a tumour developed and even if its effect is not enough to kill all the tumour cells.

20. The cells treated with the sixteen-component active mixture showed fragmentation of DNA into endonucleosome-sized units characteristic of apoptotic cell death in the case of Sp2/0-Ag14 mouse myeloma (Fig. 17A) and K562 human erythroleukemia cells (Fig. 17B). In contrast, a ladder-like pattern of DNA fragmentation could not be seen in the case of untreated cells and in the case of cells treated with the control mixture. No fragmentation was visible in the case of Vero normal cells (Fig. 17C) treated with active mixture under the same experimental conditions, although it was demonstrated earlier that degradation of DNA in Vero cells giving rise to the typical ladder pattern on gel electrophoresis can be induced. It is important to emphasise that K562 has been shown to be relatively resistant to a variety of apoptotic stimuli but the given substances of the circulatory system together could induce apoptosis of K562. On the other hand, other substances of the circulatory system (see control mixture) had not effect even together.

21. As shown in Fig. 18, the components of active mixture, when they were used singly in exactly the same concentration as in the active mixture, could not induce apoptosis of the tumour cells. The DNA ladder appeared only when the cells were exposed to the simultaneous effect of the substances. This demonstrates the synergism in an early phase of the way leading to cell death, and proves together with the former results that the synergistic action is a fundamental feature of these substances.

22. When cell cultures were incubated with different dilution of the active mixture, subsequent DNA flow-cytometric analysis revealed a number of cells with low DNA stainability, resulting in a sub-G<sub>1</sub> peak, designated as apoptotic cells (Fig. 19A). The DNA fragmentation into oligonucleosomal sized units detected by gel electrophoresis (Fig. 19B) fit in well with the result of the flow-cytometric analysis. Fluorescence in the sub-G<sub>1</sub> region and a ladder-like pattern of DNA fragmentation cannot be detected in the case of untreated cells and in the case of the control mixture (Fig. 19). On the basis of the above results, it could be excluded that the apoptosis was induced by detection techniques or sample preparation procedure because the two different detection methods gave the same result.

23. Internucleosomal DNA fragmentation was first detected after treatment for 1.5 h (the first time point examined) with the active mixture and became more prominent with longer treatment (Fig. 20). There was no detectable DNA fragmentation in untreated, and control mixture treated cells after 24 h.

## **PUBLICATIONS BEING THE BASIS OF THIS DISSERTATION:**

### **Papers**

1. Kulcsár Gy. (1995) Inhibition of the growth of a murine and various human tumor cell lines in culture and in mice by mixture of certain substances of the circulatory system. *Cancer Biotherapy*, 10: 157-176.
2. Kulcsár Gy. (1995) Kísérletes bizonyítékok a passzív tumorelleses védelmi mechanizmus létezése mellett. I. *Egészségtudomány*, 3-4: 334.
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7. Kulcsár Gy. (1998) Synergistic potentiating effect of d(+)-mannose, orotic and hippuric acid on selective toxicity of mixture of thirteen substances of the circulatory system for various tumour cell lines in culture. *The European Journal of Cancer*, submitted
8. Kulcsár Gy. (1998) Further experimental evidence for the existence of a passive antitumor defense system formed by the synergistic action of certain small substances of the circulatory system. *Experimental Cell Research*, submitted

## Patents

1. Kulcsár Gy. (1994) Pharmaceutical Compositions for Prevention and Treatment of Cancerous Disease and Process for Their Preparation. *International Application Published under the Patent Cooperation Treaty (PCT)*. International Application Number: PCT/HU94/00049.
2. Kulcsár Gy. (1995) Pharmaceutical Compositions for Prevention and Treatment of Cancerous Disease and Process for Their Preparation. *Application for European Patent (Belgium, France, Italy)*. Application Number: 95901556.1.
3. Kulcsár Gy. (1995) Pharmaceutical Compositions for Prevention and Treatment of Cancerous Disease and Process for Their Preparation. *Application for National Patent (Austria, Appl. No.: A-9008/94; Australia, Appl. No.: 10749/95; Canada, Appl. No.: 2 151 826; Switzerland and Liechtenstein, Appl. No.: 02054/95-9; China, Appl. No.: 94190904.2; Czech Republic, Appl. No.: PV-1773/95; Germany, Appl. No.: 44 98 692; Spain, Appl. No.: 9550024; Finland, Appl. No.: 953369; Japan, Appl. No.: 7-513708; Republic of Korea, Appl. No.: 95-702773; Netherlands, Appl. No.: 9420013; Poland, Appl. No.: P 309 600; Russian Federation, Appl. No.: 95116361; Sweden, Appl. No.: 9502474-1; USA, Appl. No.: 08/481 352) on the bases of PCT/HU94/00049.*
4. Kulcsár Gy. (1996) Arzneimittelkompositionen zum Vorbeugen und Hellen von Krebserkrankungen und Verfahren zu Ihrer Herstellung. *Erfindungspatent für die Schweiz und Liechtenstein*, Patentnummer: CH 686 867 A5.
5. Kulcsár Gy. (1997) Tumoros megbetegedések megelőzésére és kezelésére használható gyógyászati kompozíciók és eljárás azok előállítására. *Magyar szabadalom*, Lajstromszám: 213 677.
6. Kulcsár Gy. (1997) Pharmaceutical compositions for prevention and treatment of cancerous disease and process for their preparation. *Australian patent*, Patent number: 682.735.
7. Kulcsár Gy. (1997) Composiciones farmacéuticas para la prevención y el tratamiento de enfermedades cancerosas y procedimiento para su preparación. *Española patente*, Numero de patente: P 9550024.

## Podium presentations

1. Kulcsár Gy. Evidence for the Existence of the Passive Antitumor Defense System. *First Eurasia Congress of Medicine*, Gyor, 1994.

2. Kulcsár Gy. Kísérletes bizonyítékok a passzív tumorellenes védelmi mechanizmus létezése mellett. I. *Magyar Higiénikusok Társasága VI. Nemzeti Kongresszusa*, Pécs, 1995.
3. Kulcsár Gy. Passzív tumorellenes védelmi mechanizmus: egy új megközelítés. *IV. Sejt- és Fejlődésbiológiai Napok*, Visegrád, 1996.
4. Kulcsár Gy. Passzív tumorellenes védelmi mechanizmus: egy új elmélet és kísérletes bizonyítékai. *XXVII. Membrán-transzport konferencia*, Sümeg, 1997.
5. Kulcsár Gy. A passzív tumorellenes védelmi mechanizmus és a tumorellenes prevenció lehetősége. *MÁOTE VII. Országos Jubileumi Kongresszusa*, Balatonfüred, 1997.
6. Kulcsár Gy. The Passive Antitumor Defence System and the Culevit Food Supplement Developed on the Bases of It. *Conference of the Medical Committee of the Hungarian Fitness League and the Scientific Committee on Sport*, Budapest, 1997.
7. Kulcsár Gy. A szervezetünkben működő , ez idáig ismeretlen tumorellenes védelmi mechanizmus létezésének kísérletes bizonyítékai és az ebből adódó gyakorlati lehetőségek. *Pécsi Fitoterápiás Napok*, Pécs, 1998.

### **Poster presentations**

1. Kulcsár Gy. Kísérletes bizonyítékok a passzív tumorellenes védelmi mechanizmus létezése mellett. II. *Magyar Onkológusok Társaságának XXI. Nemzetközi Kongresszusa*, Pécs, 1995.
2. Kulcsár Gy. Az apoptózis szerepe a passzív tumorellenes védelmi mechanizmusban. *XXVI. Membrán-transzport konferencia*, Sümeg, 1996.
3. Ormos A., Kulcsár Gy. A szabadgyökök szerepének vizsgálata a passzív tumorellenes védelmi mechanizmus által indukált apoptózisban. *XXVII. Membrán-transzport konferencia*, Sümeg, 1997.

## **Working place presentations**

1. Kulcsár Gy. Kísérletes és irodalmi bizonyítékok a passzív tumorellenes védelmi mechanizmus létezéséről.

*POTE Tudományos Ülés, Pécs, 1995.*

2. Kulcsár Gy. A passzív tumorellenes védelmi mechanizmus által indukált apoptózis (programozott sejthalál)

vizsgálata. *POTE Tudományos Ülés, Pécs, 1996.*

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