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

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It is well documented that despite global abnormalities of the immune system in AIDS and other immune deficiency diseases or in immunosuppressed patients, the incidence of only a few kinds of tumors increases, and that the degree of immunosuppression seems not to be a critical factor in the development of even these tumors. The fact that tumors do not develop in the majority of population during their lifetime, despite the ineffectiveness of the known immune system against the majority of tumors, can only be explained by hypothesizing that the living system has an additional defense mechanism against tumors. On the bases of literary data, it can be assumed that the effective agents of this defense mechanism are certain substances of the circulatory system. We proved this hypothesis by being able to select thirteen substances of the circulatory system from 71 compounds tested, using the synergistic tumor cell-killing effect as criteria. The mixture containing the thirteen substances (L-hyptophan, L-tyrosine, L-methionine, L(-)malate, L-ascorbate, L-arginine, L-phenylalanine, L-histidine, 2-deoxy-D-ribose, d-biotin, pyridoxine, adenine and riboflavin) had a cytotoxic effect against Sp2/0-Ag14 mouse and K562, HEp-2, HeLa and Caco-2 human tumor cell lines in well-controlled conditions, but it was not cytotoxic against Vero normal cell line. The mixture of the above substances increased significantly the survival time of mice (T/C% 148.1) injected i.p. with Sp2/0-Ag14 mouse myeloma cells by killing more than 2 logs (99%) of the cells. Approximately the same 2 logs cell kill was found counting the Sp2/0-Ag14 cells in the ascitic fluid of control and treated animals after finishing treatment. The above mixture slowed down the growth of HeLa solid tumor significantly (T/C%, the least value 35.7). The weight loss of control and treated group during treatment did not differ significantly.

INTRODUCTION

In another paper (Kulcsár Gy. Theoretical and Literary Evidences for the Existence of the Passive Antitumor Defense System. Manuscript in preparation.) we expounded a hypothesis.
about a Passive Anti\textit{tumor} Defense System (PADS) which is supported by many epidemiological and clinical observations, as well as other literary data. Briefly, the well-known observations that full-blown AIDS is associated with substantial loss of virtually all cellular and humoral immune responses\textsuperscript{3,6} are unquestionably evidenced by rejection-free renal graft survival in a patient with AIDS despite the significant and prolonged withdrawal of the usual immunosuppressive agents\textsuperscript{7}. In spite of all these and contrary to expectation, the incidence of only a few kinds of tumor increases in patients with AIDS (mainly Kaposi's sarcoma and non-Hodgkin's lymphoma)\textsuperscript{8,6} and even in the development of tumors in question the degree of immunosuppression seems not to be a critical factor\textsuperscript{10-14}. To take also into account the similar observations made in other immune deficiency diseases\textsuperscript{15,16} and immunosuppressed patients\textsuperscript{16-20}, it can be stated that the known immune system is not or not the only mechanism that prevents the development of tumors. This fits in well with findings that the majority of clinically relevant tumors are not or are only weakly immunogenic\textsuperscript{2,19}.

There are two possibilities to explain that tumors do not develop in the majority of population during their lifetime despite the ineffectiveness of the known immune system against the majority of tumors. The first possibility is that there is no defense mechanism against non-immunogenic tumors; that the rise of a cancer cell is only a rare singular event but a tumor may develop from all cells arising. This assumption includes the high vitality of these cancer cells under any conditions. However, this contradicts the observations that the cell death rate is still high within non-necrotic tumor tissue\textsuperscript{21} and that 70\% to 90\% of newly produced tumor cells in humans die spontaneously by a mechanism that is yet poorly understood\textsuperscript{22} and only a very small percentage (<0.01\%) of circulating tumor cells can initiate metastatic colonies\textsuperscript{23}. The other possibility is that cancer cell formation is quite common but the majority of cells are not able to multiply to produce a tumor because they die shortly after they arise. The reasons for this may either be entirely random effects or the action of a \textit{systematic} defense system. The former ones can be excluded because were cell death an absolutely accidental event, the simultaneous development of a number of primary tumors in organs should be a relatively frequent occurrence. However, the development of even double synchronous primary tumors is rather \textit{rare}\textsuperscript{23,24}. Consequently, the death of non-immunogenic cancer cells can only be explained by the existence and effect of a hitherto \textit{unknown} defense mechanism.

It is obvious that the components of an antitumor defense system must be in the circulatory system. On the basis of literary data, it can be assumed that the effective agents of this defense mechanism come from small molecules occurring in the circulatory system (amino acids, monosacharides, nucleobases, vitamins, membrane permeable intermediates of the cell metabolism, etc.). Certain features of these compounds make them suitable for this role. They can reach and enter all cells because they are components of the circulatory system and their presence is fundamental for normal cell functions. Their intake by normal cells is regulated. However, it is well documented that their intake by tumor cells is increased, unregulated and proportional to their \textit{availability}\textsuperscript{25,31}. Thus, the tumor cells have a reproductive advantage over their normal neighbors in the competition for these essential \textit{substrates}\textsuperscript{25,26}. According to our speculation, this feature of tumor cells, i.e. that they can accumulate unregulatedly certain substances of the circulatory system, provide an advantage for them over normal cells only when there are many tumor cells and they have to compete with normal cells for these substances. The same feature, i.e. the unregulated uptake of these substances, may be highly disadvantageous, even fatal when there is no need for competition between cells because there are plenty of the substances in question. It is well known that tumor cells \textit{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 tumor cells at all. This precludes the possibility that a single molecule can kill the
tumor cells selectively. However, certain molecules together increasing the effect of each other synergistically can kill the cells if these "killer" molecules can reach an enough high concentration in them. Because of uncontrolled uptake of certain substances, this kind of cell death is probably a common fate of tumor cells if there is sufficient amount of the given molecules in the environment of the cells. It can be assumed that the uncontrolled uptake and high concentration of the substances in question in the cells, their atypical metabolism and synergistic effect, together with the huge abnormal deviations of almost all cell functions (transport systems, membrane permeability, enzyme amounts, enzyme activity, enzyme regulation, different metabolic pathways, isozyme composition, various control factors, etc.) in tumor cells\textsuperscript{36-34} can increase the anomalies in the cancer cells to such a degree that they can no longer survive. To our hypothesis this happens with arising tumor cells in the majority of the population during their lifetime if the number of the arising cells 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 tumor cells arising can exceed a critical value at which the divisions of the cells compensate for the killing of cells by PADS and if the cells are non-immunogenic it is most likely that a tumor develops.

To test this hypothesis, we selected five substances (tryptophan, tyrosine, methionine, malate, ascorbate) of the circulatory system as possible participants of the hypothetical defense system on the basis of literary data and theoretical considerations not detailed in this paper. We found that the mixture of them is really toxic for tumor cells and they increase the effect of each other synergistically. Then we selected experimentally eight additional substances that could potentiate synergistically the effect of the former five and examined the effect of a mixture containing the above mentioned five plus eight materials on different tumor and a normal cell line. We also investigated the in vivo effect of the above mentioned substances and demonstrated that it could inhibit the growth of Sp2/0-Ag14 mouse myeloma and HeLa human cervix epithelioid carcinoma in mice. These findings support our hypothesis and it can be stated that these substances occurring together in the living system can destroy a certain number of cancer cells in the body even if their concentrations are in physiological range.

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, sodium bicarbonate (all of them were cell culture tested biochemicals) were purchased from Sigma Chemical Co. (St. Louis, MO). 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 (No. D-3916), Medium 199 (No. M-5017), RPMI-1640, trypan blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), adenosine, D(+)-galactose, folic acid, hypoxanthine, D-pantothenic acid hemicalcium salt, p-aminobenzoic acid, L-carnitine, DL-isocitric acid, cis-aconitic acid, pyruvic acid, uridine, cobalamin, 4-nitrophenylphosphate (Sigma tablets No. 104-105) and catalase (No. C-40) were purchased from Sigma Chemical Co. (St. Louis, MO). 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).

Mice

Six- to eight-week-old female BALB/c mice and BALB/c (nu/nu) mice were obtained from
Biotechnological Facility, 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.

**Tumor Cells and Culture**

The Sp2/0-Ag14 mouse myeloma cell line was kindly provided by Dr. P. Ntmeth (Biotechnological Facility, University Medical School of Pécs). The K562 human erythroleukemia, the HeLa human cervix epithelioid carcinoma, the HEp-2 human larynx epidermoid carcinoma and the Vero African green monkey kidney cell lines were generously provided by Prof. Dr. J. Szekeres (Department of Microbiology, University Medical School of Pécs). The Caco-2 human colon adenocarcinoma cell line was kindly provided by Dr. Gy. Szics (Department of Virology & Laboratory, ÁNTSZ, County Institute of National Public Health Service, Pécs).

The Sp2/0-Ag14 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. HeLa 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% CO₂ 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 it was added to culture once every 24 h³⁵ 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% CO₂ 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 and K562 lines, the logarithmically growing cells were harvested from the medium and resuspended to a final concentration of 4x10⁵ cells of Sp2/0-Ag14 and 2x10⁶ of K562 in 250 µl appropriate medium per well containing the tested materials in the indicated concentrations. In the case of HeLa, HEp-2, Vero 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 10⁵ cells/ml. Aliquots (100 µl) in the case of Caco-2, 100 µl and 50 µl - were dispensed into 96-well micro plates, made up to 250 µl with the appropriate medium and incubated for 24 h. Then the medium was gently discarded and replaced with 250 µl 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 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 cells³⁶. 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 (phosphate-buffered saline: 140 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.1). Then 150 µg of alkaline phosphatase substrate (4-nitrophenyolphosphate, Sigma tablets No. 104-105) dissolved in 150 µl 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 μl 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 utilized for blank (N = 3) background determinations. Background values were subtracted from each reading.

The viability of Caco-2 cells was assessed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Because of disturbing effect of the tested compounds the modified assay was used. 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 μl of a 5 mg/ml sterile filtered solution of MTT in the applied medium. After incubating the plate for 4 h in 5% CO₂ 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 μl 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 utilized 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±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-lysine, 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 NaHCO₃ and 0.004 M KHCO₃. The compounds were dissolved in a buffer (8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 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 Tumorigenicity of Sp2/0-Ag14 Mouse Myeloma Cells

Tumorigenicity 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.

Evaluation of Antitumor Activity in i.p. Tumor Model

BALB/c mice (10 mice/group) were injected i.p. with 5 x 10⁴ 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 tumor inoculation for 10 days. Tumor-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 tumor-bearing mice (T/C%). The log, cell kill was calculated from the formula (T - C) 13.32 X T, 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, is the doubling time of Sp2/0-Ag14 mouse myeloma cells. The in vivo tumor doubling time for Sp2/0-Ag14 cells was estimated from semilogarithmic plots of data of tumorigenicity 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.

Determination of Cell Number in Ascitic Fluid

BALB/c mice (5 mice/group) were injected i.p. with 5 x 10⁴ 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. Tumor-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 Antitumor Activity in s.c. Tumor 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 tumors had a volume of 30-60 mm$^3$. 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. Tumor-bearing control mice were given injections of 0.2 ml PBS only. Digital calipers (Mitutoyo, Inc., Tokyo, Japan) were used to measure the length (L), width (W), and height (H) of each tumor or each lobe in multi-lobed tumors twice weekly and the tumor volumes were estimated by the formula of $\frac{0.5 \times L \times W \times H}{3}$ because this way of determining the volume has proved to be the most accurate. Because of the variation in size at the initiation of treatment, volumes were convened to the initial tumor volume. The relative tumor volume was expressed by the formula $V/V_0$, where $V$ is the tumor volume on a given day of measurement, and $V_0$ is the initial volume of the same tumor at the start of the treatment. The ratio of the mean relative volume of treated tumors over that of control tumors multiplied by 100, $(T/C\%)$, was calculated at each evaluation. 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 tumor volume to reach nine fold of the initial

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.

Statistical Analysis of Data

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

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 tumor 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 tumor 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
were 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, 0.50 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." All results are expressed as percentage of untreated cells. The values are means ± 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.

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, 0.50 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." All results are expressed as percentage of untreated cells. The values are means ± 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.

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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±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 tumor 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 bold) 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.
Comparison of the Cytotoxic Effects of 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±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 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 129x10^3 to 754x10^3 (584% of starting) and to 610x10^3 (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 30x10^3 (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\textsuperscript{6}. 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\textsuperscript{+} or Ca\textsuperscript{2+} were used instead of Na\textsuperscript{+} 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).

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 osmolality 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, 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 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 (filled and open circles) on $5 \times 10^3$ Caco-2 cells/well was significantly and markedly stronger than on $10^4$ cells/well. The control mixture (filled and open triangles) was not cytotoxic for any amount of cells.

Tumorigenicity 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 $1 \times 10^5$ 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. tumor growth when sacrificed 100 days following injection of the cells. The in vivo tumor doubling time for Sp2/0-Ag14 cells was 1.0 day estimated from semilogarithmic plots (not shown) of data of Fig. 6 as described in "Materials and Methods".

Antitumor 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±0.6 days) and treated (18.9±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 heated with the 50% survival times (12 days) in control animals and the in vivo
tumor doubling time (1.0 day) were used to estimate the tumor 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.

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 tumor 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 x 10⁵) and mean cell number of treated group (10.8 x 10⁵) excludes the roborating effect as only reason of increase of survival time. The result also shows that the tumor cell kill of approximately 2 logs is practically the same as the calculated tumor cell kill value determined in the previous experiment.

Antitumor Efficacy of the Thirteen-component Active Mixture in s.c. Tumor Model

The treatment with solution of the thirteen-component active mixture given in "Materials and Methods" decreased the growth of tumors in the BALB/c nude mice injected s.c. with HeLa cells (Fig. 9). The mean relative tumor volumes of the control (filled circles) and treated (filled squares) 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. The weight loss of control (-5.5±3.1%) and treated group (-7.6±3.2%) did not differ significantly.

DISCUSSION

To our starting hypothesis, a Passive Antitumor Defense System (PADS) 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 tumor 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 tumors do not develop in the majority of population during their lifetime, although the majority of the clinically relevant tumors are not or only weakly immunogenic.\(^\text{19}\) We assumed that the effective agents of this defense mechanism are certain small molecules occurring in the circulatory system. These molecules can enter both normal cells and tumor cells. However, their intake by normal cells is regulated, but by tumor cells it is unregulated and proportional to their availability.\(^\text{25-31}\) We assumed that some of these substances together, by increasing the effect of each other synergistically, can kill the tumor cells if they can reach enough high concentration in the cells. The latter one, due to unregulated uptake of them by tumor cells, only depends on the availability. Thus, these substances can destroy the arising tumor 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. If the number of tumor cells arising simultaneously exceeds a critical value at which the divisions of the cells compensate for the killing of cells by PADS and the cells are non-immunogenic than it is most likely that a tumor develops.

On the bases of literary data and theoretical considerations not detailed in this paper, 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 tumor cells.\(^\text{25}\) Because the concentration of substances was chosen to be ineffective when they were used singly in the

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**Figure 8.** Effect of the thirteen-component active mixture on the survival of mice. The composition of the mixture is given in "Materials and Methods." Female BALB/c mice (10 mice/group) were inoculated with \(5 \times 10^4\) cells of the \(Sp2/0\)-Ag14 mouse myeloma cell line on Day 0. The solution of the mixture and PBS were given i.p. daily according to the defined schedule from Days 1 to 10. Arrow, the days of treatment.

**Figure 9.** Effect of the thirteen-component active mixture on the growth of HeLa s.c. tumor. The composition of the mixture is 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. Arrow, the days of treatment.
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 Lehninger⁴, that the extra- and intramitochondrial malate has a different fate in tumor 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 tumor 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 tumor 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 publications⁴⁸-⁵⁴ about the optimization of composition of different tissue culture media. In opposition to us, they investigated the environment in which the different cells (malignant and normal) can grow 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 nontoxic 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 osmolality. 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 tumor 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. 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 antagonize the effect of active substances. Thus, the selected substances can act in physiological conditions, too.

Obviously, the various kinds of tumor 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 tumor cells. 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 tumor 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 to assay the cell viability. It is obvious that cell lines used in our experiments are not satisfactorily representative but our purpose was only to demonstrate that the mixture of the selected substances is effective against more than one tumor cell line and it is not toxic for normal cells. The latter one, considering also the non-toxic in vivo effect of the mixture, is evidenced. It is important to emphasize that in all experiments in vitro the concentration of the given substances was only about ten to one hundred times larger than the physiological concentration in the serum of the same substance. Considering the essentially direct proportion between the killed cells and the amount of mixture and dividing by a hundred the number of tumor cells per milliliter used in the experiments, it can be stated that the mixture of selected substances can kill a certain number of tumor cells when the concentration of the molecules is one hundredth of that used in the experiments, which is about the equivalent of their physiological concentration.

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 tumor cells in vitro can
also inhibit the growth of tumors 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 tumor 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 mixture of the substances selected by us could act in vivo and could destroy a certain number of tumor 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 tumorigenicity 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 mborating 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. tumor model (HeLa human cervix epithelioid carcinoma). We found that the treatment significantly slowed down the tumor 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 tumor volume. Considering the starting hypothesis, the probable explanation is that the proportion of the number of tumor 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 tumor cells arising by cell division was higher than the number of cells killed by the given molecules. On the other hand, duration of the existence of substances in effective amounts ensured by one injected dose decreases as the number of tumor cells grows. This result fits well with the in vitro experiment (Fig. 6) that the effect of the mixture decreases as the number of tumor cells grows.

Body weight change is a generally used index to characterize 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. d conclusion, 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 antagonized by other substances of the circulatory system, the fact that the concentration of a given substance in the in vitro experiments was about ten to one hundred times larger than the physiological concentration in the serum of the same substance, the different effect of substances observed in vitro on normal and tumor cells, the non-toxic antitumor effects of the selected substances in vivo, it can be stated 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 Antitumor Defense System exists and the compounds found by us participate in this defense system. Obviously, the function of these molecules is generally not the protection of the organism. They play such a protective role only when
tumor cells arise and exist. This protective role is very similar to the protective role of substances (i.e., fatty acids, porphyrins, lactic acid, etc.) which are bactericidal to certain pathogenic microorganisms, only the killing of tumor 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 microorganisms. The existence of PADS is also supported by many epidemiological and clinical observations, as well as other literary data as detailed in the mentioned paper (Kulcsár Gy. Theoretical and Literary Evidences for the Existence of the Passive Antitumor Defense System. Manuscript in preparation). For example, it was observed 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. Of course poor nutrition can negatively influence the known immune system too, but the majority of tumors not being immunogenic, this observation may be convincing evidence for the existence of PADS. The spontaneous regression of tumors occurring in AIDS patients also makes the existence of PADS probable. It was observed that more than 10⁴ allogeneic, nonimmunogenic McAlV tumor cells required to transplant the tumor s.c. (when there are no considerable mechanical factors) into 50% of 6 Gy whole-body irradiated athymic NCr/Sed nude mice. This finding suggests that up to 10⁴ McAlV cells were killed by the PADS. It is probable that in the given system the about 10⁴ cells are the critical value. The existence of PADS is also corroborated by the findings on over 50 separate naturally occurring murine mammary tumors that some organs in vivo rapidly and effectively diminish the number of live tumor cells and 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 existence of PADS and the knowledge of the participator molecules 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 abundant for tumor cells. The possibilities of employment depending on dose are: for prophylaxis in the case of old people, different diseases, stress situations, malabsorption, alcoholism and in the case of populations which have a larger susceptibility to tumor because of hereditary features or for people with unusual lifestyles or modified diets; for prevention of tumor development in the case of AIDS patients and organ transplant recipients; for prevention of metastasis; for adjuvant, combined or direct treatment of patients having tumor. 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 tumor cells and thus the needed dose. This kind of treatment has remarkable advantages. First, the molecules of PADS are natural compounds. It is therefore probable that the tumor 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. Furthermore, they are without considerable adverse effects at the required and applicable dosage.

On the basis of preliminary observations it is probable that the substances found effective by us cause apoptosis, but the exact mechanism of their action and the application of treatments needs further investigation, which is in progress in our laboratory.

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