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The effect of lysine deprivation on leukemic blood

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Summary. Studies have shown that specific amino acids are required for optimal growth of leukemic versus normal cells, and it is believed that the depletion of selected amino acids can abrogate tumor growth. We have developed a technique for studying the effect of amino acid deprivation on leukemic cell proliferation. The technique is based on the controlled enzymatic removal of the amino acid from leukemic blood and the subsequent measurement of cell proliferative capacity. The specific system being studied is the removal of lysine from blood using immobilized L-lysine α-oxidase.

A reactor has been designed that consists of L-lysine α-oxidase and catalase co-immobilized within the void space of the porous region of asymmetric hollow fiber (ultrafiltration) membranes. Blood from leukemic sheep is currently being treated *in vitro* with this reactor. By varying treatment time, the amount of enzyme immobilized, and the blood flow rate, the amount of lysine removed from the blood can be varied and controlled. Preliminary data indicate that 80% depletion of lysine from leukemic blood is enough to cause a significant (25%) decrease in total white cell count as well as a decrease in the proliferative capacity of the leukemic cells.

Keywords: Amino acids – L-lysine oxidation – Immobilized enzymes – Membranes – Leukemia

Introduction

Leukemia is a disease of unknown cause characterized by rapid and abnormal proliferation of leukocytes in the blood-forming organs (bone marrow, spleen, lymph nodes) and the presence of immature leukocytes in the peripheral circulation. To be effective against leukemia, a treatment must exert some differential cytotoxic effect upon the malignant cell. Qualitative biochemical differences between leukemic cells and normal cells which would permit the design of drugs

having specific cytotoxicity for leukemic cells are not known. Consequently, present chemotherapy must rely on small quantitative differences between the sensitivities of leukemic cells and normal cells that are the target of the drug's toxicity.

Exogenous amino acids have been shown to be primary nutritional requirements for certain types of leukemic cells. This requirement is the result of either a failure of the cell to synthesize the amino acid in an amount needed for the maintenance of normal metabolism (Dimitrov et al., 1971) or an increased demand for certain amino acids necessary for protein synthesis. Since various studies have shown that specific amino acids are required for the optimal growth of malignant versus normal cells (Dimitrov et al., 1971; Iyer, 1959), it is believed that the depletion of selected amino acids can abrogate leukemic cell growth by preferentially affecting the leukemic cells. These amino acids include asparagine, glutamine, serine, cysteine, arginine, and lysine (Holcenberg, 1981; Tivey, 1954). In particular, lysine and serine have been shown to be consumed to the greatest extent (Tivey, 1954).

Dietary restriction of amino acids has proven to be an unsuccessful method of therapy largely due to the overall detrimental effects on patient health (Sugimura et al., 1959). Recently, several enzymes that degrade essential amino acids have been shown to depress tumor growth. Of the antitumor enzymes studied, only L-asparaginase has been accepted clinically for the treatment of acute lymphatic leukemia (ALL). In this treatment, asparaginase is combined with vincristine (an alkaloid) and prednisone (a steroid) in order to induce remission in patients with ALL.

Although this combination therapy has been successful in inducing remission, several problems exist which decrease the overall effectiveness of L-asparaginase therapy. Large amounts of enzyme are required to maintain adequate therapeutic levels in the blood. Repeated administration of enzymes often leads to the development of an immune response which causes undesirable side-effects and also limits the long-term effectiveness of the therapy. Furthermore, L-asparaginase has a spectrum of severe toxicity resulting from a) its ability to cause the cessation of protein biosynthesis in tissues dependent on exogenous asparagine and b) the uncontrolled, unmanaged total depletion of asparagine from the blood of patients which is a typical result of enzyme injection treatments (Holland and Ohnuma, 1981; Hanefeld and Riehm, 1980). The disadvantages of present methods of enzymatic cancer therapy suggest the use of immobilized enzyme treatments. Since the enzyme is immobilized, it maintains its catalytic activity for longer periods, it is protected from attack by the patient's immune system, and the enzymes are reusable.

The specific system being studied in this work is the removal of lysine from blood using immobilized L-lysine α-oxidase. This enzyme catalyzes the α-oxidative deamination of L-lysine as follows:

$$\begin{array}{l}\text{L-lysine} + O_2 + H_2O \rightarrow \alpha\text{-keto-}\varepsilon\text{-aminocaproate} + NH_3 + H_2O_2 \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad \downarrow\uparrow \pm H_2O \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad \Delta^1\text{-piperidine-2-carboxylate}\end{array} \tag{1}$$

Table 1. Michaelis constant of selected antitumor enzymes

Enzyme	Michaelis constant K_m(mM)
Phenylalanine ammonia-lyase	.25
Methionine γ-lyase	78–90
Tyrosine phenol lyase	.28
Threonine deaminase	8.0
Lysine α-oxidase	.04
L-Asparaginase	.02

L-lysine α-oxidase has been shown to completely inhibit the growth of mouse leukemic cells *in vitro* (Kusakabe et al.,1980). When injected into the blood of mice suffering from leukemia, average lifespan was increased 34 to 48% over the control animals. Other researchers (Faguet, 1986) have shown L-lysine α-oxidase to be effective in killing human leukemic T-cells *in vivo*. Furthermore, of the antitumor enzymes that have been investigated, only L-asparaginase has a lower Michaelis constant, K_m (see Table 1). A low K_m indicates a strong enzyme/substrate affinity and is therefore desirable. The favorable physiological and enzymatic properties of L-lysine α-oxidase make it an excellent choice for immobilization in hollow fiber reactors.

We have developed an immobilized enzyme hollow fiber reactor prototype for the removal of amino acids from blood. The reactor operates according to the following scheme that we have previously described (Reiken and Briedis, 1990): hollow fiber membranes consist of an ultrathin inner membrane surrounded by a porous "sponge-like" annular section which is approximately 80 to 90% void (Fig. 1). The ultrathin inner membrane is impermeable to macromolecular species, such as enzymes, but not to species of molecular weight below the nominal cutoff of the membrane (e.g. amino acids). Enzyme is flushed into and trapped in the spongy layer, and blood is fed at low pressures through the fiber lumen. The ultrathin membrane allows the passage of the amino acid from the blood into the spongy layer, where it is consumed by the enzymatic reaction, and the reaction products diffuse back into the lumen.

Our immobilized enzyme reactor consists of a single hollow fiber encased in a protective glass shell (Fig. 2). The single fiber reactor (SFR) is a unique small-scale reactor which is an ideal, economical tool for kinetics studies and development work since it requires small amounts of blood and biochemicals for experimentation. In addition, SFR data provide results that are scaleable to larger (hollow fiber cartridges) systems and are valid for the design of clinical-scale reactors.

In addition to overcoming the problems associated with enzyme injection therapy, the immobilized enzyme reactor is able to vary and control levels of lysine removal by choice of the amount of enzyme immobilized and the treatment time. This enables the experimenter to study the effect of different levels of lysine deprivation on the proliferative capacity of leukemic cells. Furthermore, the ability to control amino acid levels is clinically important because

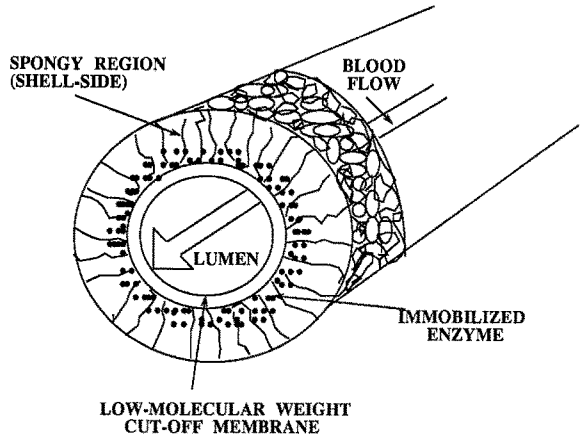

Fig. 1. Schematic of a hollow fiber. The fiber consists of three regions; the fiber lumen, the fiber membrane, and the spongy region. The membrane separates the lumen from the spongy region in which the enzyme is immobilized

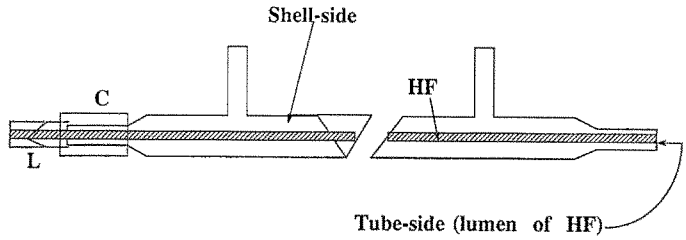

Fig. 2. Single fiber reactor (SFR)—Shell material is borosilicate glass, 21.5 cm overall length, 0.8 cm O.D.. Fittings illustrated on the left were applied to both ends of the reactor: *L* male and female Luer lock fittings; *C* Tygon tube; *HF* Hollow ultrafiltration-fiber. The hollow fiber was retained by a plug of epoxy potting resin (Dow Chemical) between the Luer lock fittings and the hollow fiber

amino acids are also necessary for the healthy growth of normal cells. Because the procedure converts an essential amino acid, the treatment should not be complicated by the body's synthesis of the very substrate that the reactor is removing.

Materials and methods

Materials

All ultrafiltration (UF) fibers were donated by Romicon, Inc. (Woburn, MA). The L-lysine α-oxidase was donated by Yamasa Shoyu Co., LTD (Tokyo, Japan). L-lysine, α-ketoglutarate, NADH, saccharopine dehydrogenase, catalase, and all the chemicals for flow cytometry analysis were purchased from Sigma Chemical Co. (St. Louis, MO).

Analyses

Lysine concentrations were determined by the saccharopine dehydrogenase method described by Nakatani et al. (1972). Blood analyses were performed by the Veterinary Clinic's pathology laboratory at Michigan State University. Cell viability was measured by uptake of trypan blue dye, and blood cell counts were determined using the Technichon H1 blood analyzer (Metpath, Inc., Des Plaines, IL). The Technicon H1 is a discrete mode bench top hematology analyzer designed to perform a complete blood count and a full white cell differential count.

Flow cytometry

Leukemic cells progress through the normal phases of cell division ($G_1 \rightarrow S \rightarrow G_2 \rightarrow M$) (Alberts et al., 1983). In the G_1 phase, protein and RNA are actively synthesized, but DNA is not. Following the G_1 phase, the chromosomes are replicated (DNA and protein synthesis) in the S phase. The significance of the G_2 phase is not fully understood at present. After the completion of the G_2 phase, cell division starts (M phase). Sometimes cells enter a resting state designated as the G_0 phase. Cells in the different stages of the cell cycle have different, but predictable amounts of DNA. Because of this, the DNA labelling technique of flow cytometry can be used to determine the exact number of cells in each phase of the cell cycle (G_0/G_1, S, and G_2/M), and the proliferative capacity of the lymphocytes (defined as the fraction of cells in the S plus G_2/M phases) can be calculated.

Flow cytometry was used to determine the proliferative capacity of treated and control lymphocytes. This technique was performed by the pathology laboratory of St. Lawrence Hospital (Lansing, Michigan). The analysis is based on the following principals: fluorochromes are available that stoichiometrically bind to DNA, and, as discussed earlier, cells in different stages of the cell cycle have different, but predictable amounts of DNA. Normal cells are euploid and have a known amount of DNA, whereas malignant cells are frequently aneuploid (have an abnormal number of chromosomes).

The first step in utilizing flow cytometry is to separate the white cells from the whole blood. This is accomplished by a sedimentation technique routinely used at St. Lawrence Hospital. After the lymphocytes are isolated, they are stained with a fluorescent label. The labelled cells are then pumped under slight pressure into the Becton-Dickinson FAC-SCAN flow cytometer. The cells enter a detection zone where they are illuminated by a precisely focussed laser beam. This technique measures physical and chemical characteristics of the cells as they pass single file in a fluid stream through the focussed laser beam. The energy of the laser excites the fluorescent stain attached to the cells. The fluorescence intensity is a direct measure of the amount of nuclear DNA. The data are acquired and analyzed by a computer program developed at St. Lawrence Hospital. Fluorescence intensity and light scattering signals can be evaluated simultaneously. Information on relative cell size, relative granularity, and flourescence intensity can be recorded for a large number of cells in a short time.

Source of leukemic blood

Bovine leukemia virus (BLV) is biochemically and biologically similar to human T-cell leukemia virus (HTLV I), an oncogenic retrovirus of humans implicated in the etiology of adult T cell leukemia (Gross, 1983). Both viruses cause rapidly fatal leukemia and lymphoma. Because of these similarities between BLV and HTLVs, interest has arisen concerning the role of BLV as a model virus for studying human leukemia and lymphomas caused by retroviruses.

A self-propogating flock of sheep chronically infected with BLV was used as the source of leukemic blood for these experiments. Only 100–200 ml were needed per experiment (up to one liter of blood may be withdrawn without endangering the animal). The blood was heparinized and maintained viable by storing with nutritive (RPMI 1640) medium. It should be noted that RPMI 1640 is lysine-free.

Evaluation of lysine removal from blood

Several experiments were conducted to evaluate SFR performance with the blood of BLV-infected sheep. Blood was removed from the sheep and treated with the anticoagulant heparin. The blood was then divided into two samples. One sample was continuously recirculated through the immobilized enzyme reactor (Fig. 3) for about four hours; blood aliquots were taken from the treated blood at regular intervals for analysis. By varying the flow rate, amount of enzyme immobilized, and the time of treatment, we could control and vary the amount of lysine removed from the blood. The other sample served as a control and was maintained at the same temperature over the same time period as the treated blood. The blood samples were analyzed 24 hours after the completion of the treatment to allow sufficient exposure of the lymphocytes to reduced lysine levels in the blood. Analyses of both the treated and control blood samples included cell blood counts, proliferative capacity, and lysine concentration.

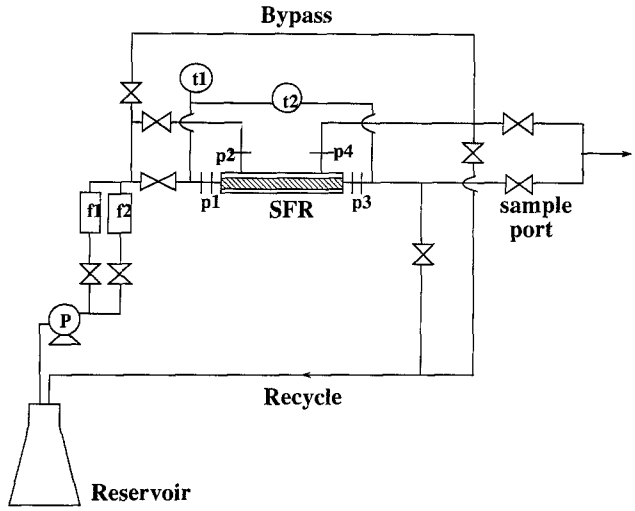

Fig. 3. Single fiber reactor system (*SFR*) – *Res* reservoir flask; *P* pump; *f1*, *f2* flowmeters; *p1* lumen-side inlet port; *p2* shell-side inlet port; *p3* lumen outlet port; *p4* shell-side outlet port; *t1* 15 psig pressure transducer; *t2* 5 psi differential pressure transducer; ⧖ tubing clamps for stopping flow

Results

Fig. 4 shows the effect of lysine deprivation on the total amount of white cells in the leukemic blood. It is seen that as the amount of lysine removed from the blood increases, the total number of white cells in the blood decreases until approximately 70% of the lysine has been removed. At this point, the lysine deprivation effect levels off. Fig. 5 shows similar results for the proliferative capacity (defined as the percentage of lymphocytes in the actively proliferating phases of the cell cycle) of the cells.

These results are important for two reasons. First, the reduction of both the total white cell count and the proliferative capacity of the lymphocytes represents the first step in the induction of remission of leukemia. Remission is defined to occur when the blood cell counts and proliferative fraction are within specified ranges for normal sheep.

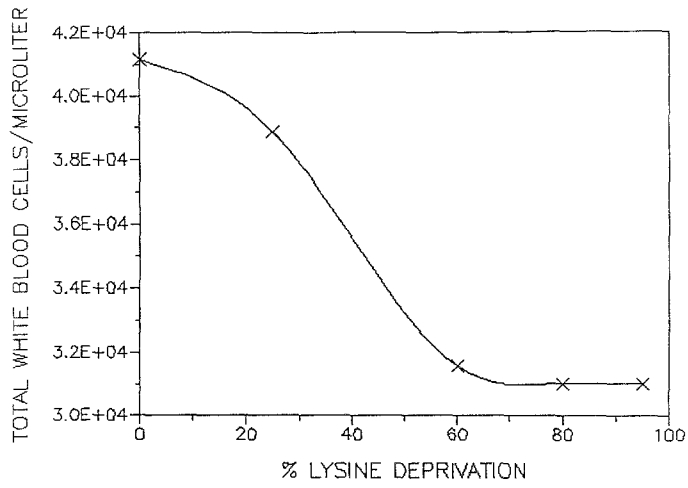

Fig. 4. The effect of lysine deprivation on total white cell count. Exposure time is 24 hrs

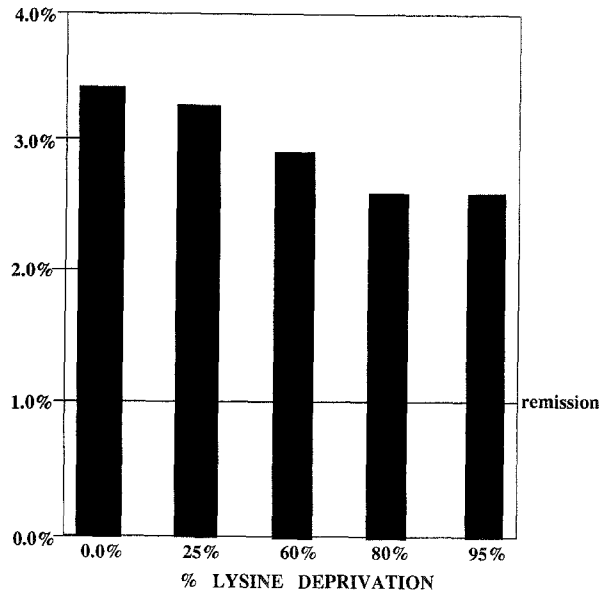

Fig. 5. The effect of lysine deprivation on cell proliferative capacity; percentage of proliferating lymphocytes versus percentage lysine removed. Exposure time is 24 hours

Although the cell counts and proliferative capacity of the treated blood are still above the norm (normal white cell count in sheep is approximately 4,000 cells/μl with less than 1% in the proliferative stages of the cell cycle), these results represent the effect of lysine deprivation after only one treatment and a 24 hr exposure period. A normal treatment regimen would keep lysine levels low for approximately one month. Therefore, the 25% decrease in total white cell count and the significant decrease in proliferative capacity represent an important step towards the induction of remission. In addition, these experiments indicate that the effects of lysine deprivation level off before all the lysine has been removed from the blood. This is important clinically because exogenous amino acids are

important for the metabolism of normal cells and tissues. These results further show that the depletion of lysine can be controlled in such a manner as to have a significant effect in inducing remission of this disease, but still leave enough exogenous amino acid in the bloodstream for the normal function of healthy cells and organs.

Discussion

Remission induction therapy is the first step in the clinical treatment of leukemia, and enzyme therapy is an important part of this procedure. The currently popular enzyme, L-asparaginase, however, causes severe side-effects in some patients. Its administration consumes large amounts of enzyme and offers little control over the levels of amino acid depletion in patient blood. Enzyme immobilization, as opposed to its injection into the blood, prevents many harmful side-effects of direct blood contact with the foreign protein. The immobilized enzyme reactor is therefore viewed as a potentially superior treatment to L-asparaginase injection therapy by offering good control of amino acid depletion, avoidance of undesirable side-effects from injection, and about 100-fold less enzyme needed for treatment. Furthermore, precedent exists for the extracorporeal treatment of leukemic blood (Schiffer et al., 1966).

The preliminary data presented in this paper indicate the potential use of the immobilized enzyme reactor in the treatment of leukemia. In addition to the *in vitro* data collected as described above, live leukemic sheep will be used for the *in vivo* evaluation of the enzymatic reactor. These experiments will yield important information on the effect of lysine deprivation on both blood cells and bone marrow. This information will be used to develop and refine a mathematical model that will be used to generate a computer simulation of the effects of the immobilized enzyme reactor treatment on leukemic lymphocyte survival rates. The rationale for developing a mathematical model for computer simulations is that, as long as the model realistically depicts physical phenomena, it can serve as a predictive tool for optimizing treatment and interpreting experimental data. A model also will quantify the effects of amino acid deprivation on leukemic cell proliferation kinetics to further our understanding of the development of leukemia. As mentioned above, additional data are necessary in order to calculate model parameters. Once these model parameters are validated by their ability to predict experimental data, computer simulations may be used as a "decision-making" tool for choosing therapy protocols which balance leukemic cell death with normal cell survival.

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