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A Simple Method for Long-Term Drug Infusion in Mice: Evaluation of Guanazole as a Model[1] (38488)

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(Introduced by E. Mihich)

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In order to exploit fully the relatively narrow differences existing between tumor and host tissues, the need for selection of optimum drug dosage and schedules has been acutely felt in the chemotherapy of cancer. For a number of anticancer agents with reversible inhibitory actions, chemotherapeutic activity is dependent upon both the intensity and the duration of drug levels at target sites (1). In treatment with such agents as arabinosylcytosine or guanazole (GZ), which have short plasma half-lives, it is therefore necessary to maintain desired drug levels for desired time periods by continuous iv infusion or by multiple injections. For the *in vivo* treatment of experimental tumors in mice, multiple dosing has been widely used to approximate the sustained blood levels achievable by iv infusion. A technique for drug infusions in unrestricted swiss mice reported by Plager (2) involves the use of a specially tooled stainless steel needle as cannula necessitating complete immobilization of the tail during cannulation as well as during infusion. Bennett and Dave (3) have recently described a procedure for infusing restricted rats by a direct cannulation of the tail vein with a piece of polyethylene tubing. Thus it seemed feasible to couple the advantages of both methods (2, 3) for the development of a technique simple enough for infusing mice, in particular, DBA/2J mice, which are used in *in vivo* cancer chemotherapy studies.

GZ (3,5-diamino, 1,2,4-triazole, NSC 1895) a clinically effective antileukemic agent (4, 5), was chosen to test the usefulness of this experimental method. GZ was selected because it has significant activity against mouse leukemia L1210 only when administered in multiple doses (5, 7), because it has a very short half-life, namely, about 30 min in mice (8, 9), 68 min in rat (10) and 5–7 hr in man (9, 11) and because it is excreted unchanged in these species, thus eliminating response variables due to differences in drug metabolism.

Materials and Methods. Chemicals. Guanazole (GZ) was originally supplied by the National Cancer Institute, Chemical Trials Evaluation Branch and was found to be 98% pure by thin layer chromatography (10). (U-^{14}C)-Uridine, 509 mCi/mmole, was obtained from the Amersham/Searle Corp. Dimethyl POPOP and PPO, the counting fluors, were obtained from Packard Instruments Co. Beckman Biosolv-3 (BBS-3) was obtained from Beckman Instruments Co. Other chemicals were of enzyme or analytical grade and were obtained from Sigma Chemicals Co. or Fisher.

Intravenous Infusion Procedures. DBA/2J female mice (16–18 g) obtained from Jackson Laboratories, Bar Harbor, Maine, were used for this study. For cannulation, the mouse was temporarily restrained by placing it in a piston-type Lucite chamber (chamber: 15 cm × 2.5 cm i.d., piston rod: 11 cm × 1.5 cm). For convenience, a snap-on type door, prepared from 20 gauge sheet metal, was used in place of the hinged plastic door described by Plager (2). The tail was cleaned and wiped with ethanol. A surgical suture needle (Ethicon, Type B #682) was inserted to a distance of 1–2 mm into a dorsal or lateral tail vein and then was removed. This puncture facilitated subsequent cannulation with a saline-filled polyethylene tubing (i.d. 0.011″ × o.d. 0.024″, Intramedic PE 10, Clay Adams Co.) which was directly introduced to a distance of about 1.5–3 cm into the vein. The other end of the tubing was connected to a Luer lock syringe (Eisle Co.) by means of a hypodermic needle (27 gauge, Yale Co.). Proper positioning in the vein was

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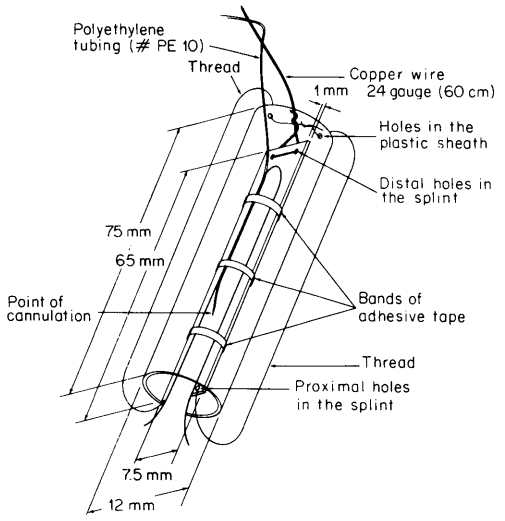

FIG. 1. A diagrammatic representation of the assembly for cannulation of the tail vein of a mouse.

verified by the observation that back-flow of blood into the polyethylene tubing occurred in the absence of applied pressure and also by lack of edema formation following infusion of 0.1–0.2 ml of saline. The tubing was held in place by spraying this area with adhesive dressing (Aeroplast Corp., product ≠112-C). In addition, a splint (65 × 7.5 mm), cut from a plastic ruler (1 mm thick), was used for supporting and immobilizing the tail during infusion (Fig. 1). A pair of holes, bored with a hot wire, were made at each end of this splint and 20 cm of thread was passed through the set of holes positioned proximally to the mouse. A 60 cm loop of copper wire (24 gauge) was passed through the distal set of holes. The tail, along with the connected polyethylene tubing, was then attached to the supporting splint by wrapping with 3 strips of adhesive tape. In order to protect the cannula, a cylindrical sheath (75 mm × 12 mm o.d.), made by cutting off the bottom of a 90 mm plastic test tube (Falcon Co., Oxnard, CA) was then slipped over the whole assembly bearing the cannulated tail vein. A pair of holes were also made in the distal end of this sheath. Each end of the piece of thread, which had been looped through the pair of proximal holes in the splint, was passed through the corresponding hole in the plastic sheath and then the ends were knotted together (Fig. 1). This held the cylindrical sheath in place over the tail, providing protection for the infusion system. The free ends of the copper wire were wound around a metal ring on a stand located about 40 cm above the cage. As the animal moved freely in the cage (30 × 12 cm, 15 cm deep), the wire suspended the tail at a minimum 30° angle with the floor of the cage and also prevented kinking of the infusion tubing.

The syringes, coupled to the cannulating tubing, were mounted on a Harvard infusion pump (Harvard Apparatus Co., Millis, MA), allowing changes in pump rates and in substances infused, without interruption of the animal activities. Food was provided ad libidum in the form of pellets placed in the bottom of cages. Based upon the approximate average daily oral consumption by mice, adequate water was provided by the iv administration.

Effect of GZ on the incorporation of radiolabeled nucleosides. Groups of mice were infused, as described above, with saline or with saline containing GZ (30 mg/ml) for 47 hr at a flow rate of 0.3 ml/hr. Immediately following termination of infusion, the cannulating tube was cut 2–3 cm from the point of its insertion and ^{14}C-uridine mixed with unlabeled uridine to give 2.5 μCi/0.4 μmole in 0.2 ml vol was injected into each mouse with a 27 gauge needle inserted into the cannulating tube. The tube was then sealed with a hot clamp in order to prevent loss of the precursor. An aliquot (5 μl) of this radiolabeled nucleoside mixture was counted in order to determine the actual radioactivity present. Fifteen minutes after the injection of precursors, mice were killed by cervical dislocation. Brain, heart, kidneys, thymus and about a 200 mg section of liver were rapidly removed, frozen in liquid nitrogen, and homogenized in 1–5 ml (ice-cold) 10% trichloracetic acid (TCA) by 10 strokes with a Ten-Broeck hand homogenizer. Bone marrow cells were recovered from both femurs by rinsing the marrow cavities with 1 ml saline per femur.

The homogenates were centrifuged at 1000 *g* for 10 min at 4°C. The TCA-soluble supernatant fraction containing nucleosides, nucleotides as well as GZ, was assayed for total radioactivity content and for the GZ present as described below.

The TCA-insoluble pellet, containing nucleic acids and protein, was made free from acid-soluble radioactivity by washing with three 5 ml aliquots of ice-cold TCA. DNA and RNA were separated from each other as described by Schneider (12). This procedure involved alkaline hydrolysis of RNA in 1 ml of 1 *N* NaOH (37°, 1 hr) followed by neutralization with 1 *N* HCl, and extraction of RNA hydrolysate with ice-cold TCA. DNA was extracted from the residue with hot TCA.

Aliquots of RNA and DNA extracts were used for counting radioactivity and also for colorimetric estimation. RNA content was determined by the orcinol reaction (12). DNA was quantitated by a routinely used adaptation of the method of Burton (13), where 0.6 m*M* paraldehyde was substituted for 1.8 m*M* concentration of acetaldehyde in a colorimetric reaction using diphenylamine. Substitution of acetaldehyde by paraldehyde was found extremely useful because of the ease of handling paraldehyde. The sensitivity of the original procedure was, however, unaltered under this condition. Recently, other modifications have also been suggested in the Burton's procedure in addition to the one described above (14). GZ was colorimetrically determined by a procedure involving diazotization and coupling with diphenylamine (10).

Measurement of radioactivity. Radioactivity was determined by counting up to 1 ml aliquots of the aqueous samples in 10 ml of a scintillation cocktail composed of toluene containing 0.6% w/v PPO, 0.06% w/v dimethyl POPOP, and 15% v/v BBS-3. The counts for quench using a Wang 700 model calculator (15, 16).

Results. In spite of the difficulty in visualizing the veins in these brown mice, it was indeed found relatively easy to cannulate the tail vein. The correct insertion of the tubing was always verified by the back-flow of blood, in the absence of any external hydrostatic pressure, at the beginning and at the end of the infusion. With practice, it was possible for one person to successfully cannulate 90–95% of the animals in less than 5 min per mouse.

GZ infusion resulted in a primary inhibition of incorporation of ^{14}C-uridine into DNA of various tissues (Table I). Maximal inhibition occurred in spleen. Thymus and bone marrow were other hemopoietic tissues in which the uridine incorporation into DNA was also considerably inhibited by the drug. The order of inhibition was spleen > thymus > bone marrow > heart, brain. No

TABLE I. LEVELS OF GZ IN DBA/2J MICE FOLLOWING IV INFUSION OF THE DRUG AND ITS EFFECTS ON THE INCORPORATION OF URIDINE INTO NUCLEIC ACIDS OF VARIOUS TISSUES.[a]

Tissues	Guanazole μg/g tissue	Percent uridine incorporation into	
		DNA	RNA
Spleen	407.6 ± 7.6	16.4 ± 0.5	43.1 ± 5.1
Thymus	398.1 ± 11.0	33.6 ± 2.8	84.3 ± 4.2
Bone marrow	—	58.4 ± 9.7	91.0 ± 5.4
Heart	233.3 ± 6.5	74.5 ± 1.1	82.3 ± 4.0
Brain	145.1 ± 5.9	81.4 ± 0.4	104.3 ± 9.1
Kidney	248.4 ± 6.7	103.8 ± 13.7	102.1 ± 8.1
Liver	215.9 ± 12.7	177.0 ± 7.1	131.8 ± 8.0

[a] Each number is an average of two experimental values ± 0.5 Range. GZ (30 mg/ml) was infused for 47 hr at the rate of 0.3 ml/hr. ^{14}C-Uridine (2.5 μCi/0.4 μmole) was injected (0.2 ml) 15 min before sacrifice and removal of tissues. GZ in tissues was determined from the TCA-soluble extracts as described in the text and expressed as μg per g wet weight of tissues. Tissues from saline-treated mice were used as blanks. Specific incorporations of uridine into DNA and RNA were determined from the TCA-insoluble pellet and the results are expressed as percent of the corresponding saline-treated controls. The average saline-treated control values for uridine incorporation into DNA (dpm/mg DNA) of spleen, thymus, bone marrow, heart, brain, kidney and liver were 26,653; 1,274; 35,141; 6,829; 1,115; 6,198 and 12,198 respectively and those into RNA (dpm/mg RNA) were 11,119; 9,113; 4,396; 21,394; 8,993; 10,106 and 11,119 respectively. The individual control values ranged between 90 and 110% of their mean.

inhibition occurred in kidney. Increased incorporation occurred in liver. It should be noted that under the conditions of the experiments, no detectable change occurred in the total DNA content per g of various tissues (data not shown). In comparison with the uridine incorporation into DNA, that into RNA was less affected or not at all (Table I). Levels of GZ in different tissues were measured 15 min following termination of infusion. Therefore, these values represent approximately 70% of their steady state levels based on the assumptions that plasma half-life of GZ is 30 min (8, 9) and that the drug is equilibrated instantaneously between various compartments. The drug levels in spleen and thymus were the highest followed by those in the other tissues. These levels are in agreement with the observed inhibition of uridine incorpsration into DNA (Table I). For technical reasons, it was not possible to determine the drug content in the marrow.

Discussion. The present method is a considerable improvement over the techniques described for swiss mice by Plager (2) and for rats by Bennett and Dave (3). The most important contribution of this method is the direct cannulation of the tail vein by polyethylene tubing as was done for immobilized rats (3). The elimination of the stainless steel needle as the cannula (2) not only prevented the accidental damage to the vein, but greatly simplified the technique. Furthermore, in cannulation with polyethylene tubing, it was no longer necessary to completely immobilize the tail. This greatly simplified the splint design and method for its attachment to the tail. These technical simplifications allowed a considerable saving of time. Also, it was possible to insert the polyethylene cannula 1–3 cm into the vein in contrast to only a few millimeters with a rigid needle. This resulted in a more secure cannulation. Use of plastic sheath instead of glass and the use of copper wire reduced, respectively, the strain on the mouse and on the cannulating apparatus. No complication was encountered during the 2-day infusion. At the arbitrary termination of infusion, every animal showed backflow of blood into the cannula and lack of edema.

Using the technique, it was observed that inhibition of uridine incorporation occurred primarily into DNA as a result of GZ treatment. This is consistent with the reported *in vitro* inhibition of ribonucleoside diphosphate reductase by GZ (6). It is noteworthy that the measured concentration of GZ in spleen (0.4 m*M*) at about 70% of the estimated steady state value is in the same order of magnitude as that reported for 50% inhibition of reductase *in vitro* (6). More data on the sensitivity of reductase as well as on pool sizes of various deoxynucleosides and -tides, would be necessary before any satisfactory explanation for the increased incorporation into liver DNA could be given. With the exception of liver, a correlation seems to exist between the level of GZ in the tissue and the degree of inhibition of incorporation into DNA. This suggests similarity in the sensitivities of ribonucleoside diphosphate reductases to GZ in different tissues. The data also indicates that rapidly proliferating normal tissues like spleen, thymus and bone marrow, are most sensitive to inhibition by GZ. This is consistent with the known immunosuppressive and myelosuppressive actions of GZ in animals (7) and man (4, 5).

In conclusion, the newly developed technique for infusion in mice is significantly better than past techniques. Because of its simplicity, it has the potential of being routinely used for the evaluation of pharmacologic agents with a short plasma half-life under the conditions of desired steady state levels. Since most animal tumor models *in vivo* are maintained in mice, this technique will be of particular value for evaluating anticancer agents.

Summary. A simplified technique for iv infusion in unrestricted DBA/2J inbred mice has been described. The method, which involves direct cannulation of the tail vein with polyethylene tubing, is suitable for routine use. Guanazole, an antileukemic agent with a short plamsa half-life, was evaluated as a model compound. After administration for 47 hr at the rate of 0.3 ml/hr, guanazole (30 mg/ml) caused a marked inhibition of incorporation of ^{14}C-uridine, administered 15 min before sacrifice, primarily into DNA of spleen, thymus and bone marrow in decreasing order. Inhibition of incorporation into RNA was less marked but followed a similar

pattern. The effects on the incorporation of uridine in nucleic acids of kidney, heart and brain were minimal. Increased incorporations into RNA and DNA occurred in liver. The data for the hemopoietic and lymphoid organs, namely, spleen, thymus and marrow, are consistent with the reported immunosuppressive and myelosuppressive effects of the drug and also with the inhibition of ribonucleoside diphosphate reducaste by guanazole.

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