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5-Azacytidine: Microbiological Assay in Mouse Blood

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A 6-chloropurine-resistant strain of *Escherichia coli* has been used to develop a microbiological assay for the estimation of 5-azacytidine distribution in mouse tissues.

5-Azacyidine (NSC 102816), a new antitumor agent, has been reported to strongly inhibit lymphoid leukemia in mice (5, 6), and clinical studies are now in progress. The successful development of a potentially useful chemotherapeutic agent is dependent in part on the ability to measure tissue distribution and concentration of the new agent. The reported antimicrobial activity of 5-azacytidine (2, 7) led us to develop a logarithmic-ratio microbiological assay which has been used to measure the distribution and concentration of 5-azacytidine in the tissues of mice which have been injected with nonlethal doses of this new pyrimidine analogue.

Previously described methods (3) were used to select an appropriate microorganism for the assay of 5-azacytidine. From more than 100 species of bacteria tested for sensitivity to this compound, a strain derived from *E. coli* ATCC 9637 as resistant to 6-chloropurine (designated *E. coli*/6-chloropurine) was selected as the assay organism. The culture is maintained on agar slants of a simple glucose-salts medium supplemented with 0.5 mg of 6-chloropurine (6-CPU)/ml. For the preparation of seeded agar plates (90 by 15 mm, no. 1029, Falcon Plastics Inc., Los Angeles, Calif.), stationary cultures of *E. coli*/6-CPU were grown for 16 to 18 hr at 37 C in the glucose-salts medium. Cells from these cultures were collected and washed by centrifugation in saline (0.85% NaCl),

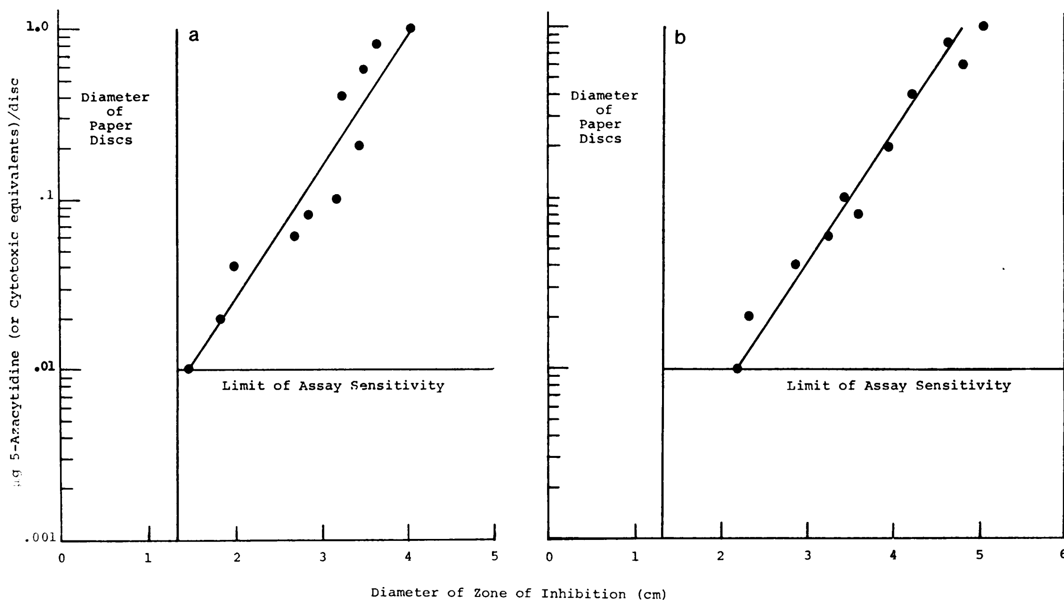

FIG. 1. *Standard assay curves (constructed by method of least squares) for the logarithmic-ratio microbiological assay of 5-azacytidine in the blood of mice. Assay microorganism: Escherichia coli ATCC 9637/6-chloropurine. (a) Blood standard curve (index of precision, 0.0597). (b) Saline standard curve (index of precision, 0.0489).*

resuspended in saline, and adjusted to 20% light transmittance (660 nm) in a Spectronic 20 Colorimeter (Bausch & Lomb, Inc., Rochester, N.Y.). The suspension was diluted 1:200 in saline, and 10 ml of this suspension was added to 1 liter of cooled (50 to 55 C) glucose-salts medium. A 5-ml amount of the inoculated agar was added to the previously described plates containing 8 ml of congealed sterile agar. *E. coli*/6-CPU is a stable mutant and the addition of 6-CPU to the assay plates is not required.

A stock solution of 5-azacytidine was prepared in sterile saline and was appropriately diluted so that 0.08 ml of the various dilutions was added to filter-paper discs (1.27 cm in diameter). The following concentrations were obtained (micrograms of 5-azacytidine/disc): 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, and 3.0. An additional 5-azacytidine stock solution was prepared in which the drug was dissolved in mouse blood and diluted with saline. Filter-paper discs (1.27 cm in diameter, no. 740-E, Schleicher and Schuell Co., Keene, N.H.) were impregnated with 0.08 ml of the respective solutions containing graded concentrations of the drug. These discs were placed on the surface of each seeded agar plate and pressed down securely with flamed forceps. All plates and drug concentrations per disc were prepared in triplicate. Each individual plate contained a maximum of three discs. Two discs contained, individually, either experimental samples or standard curve solutions of different concentrations. A third control disc, containing an empirically selected concentration (3.0 μg of 5-azacytidine/disc) was added to each plate, which allowed for correction of plate-to-plate variation in zone sizes. All plates were incubated at 37 C for 18 to 22 hr simultaneously with plates which contained discs impregnated with blood from animals which received 5-azacytidine. The resulting zones of inhibition on the triplicate standard plates were measured and corrected as follows. If the mean diameter of all the control disc zones is greater than that of an individual control disc zone, the difference is added to all of the zones on that plate. Conversely, if the average diameter of the control disc zones is less than that of an individual control disc, the difference is subtracted from all the zones on that plate. The mean diameter of these corrected zones is determined for each drug concentration and entered on semilogarithmic paper—the diameter of zones on the ordinate and concentrations of drug/disc on the abscissa. A straight line of best fit is drawn through the points thus obtained. This line constitutes a standard curve (*see* Fig. 1). From the zones which developed around the discs that received blood samples from the treated animals,

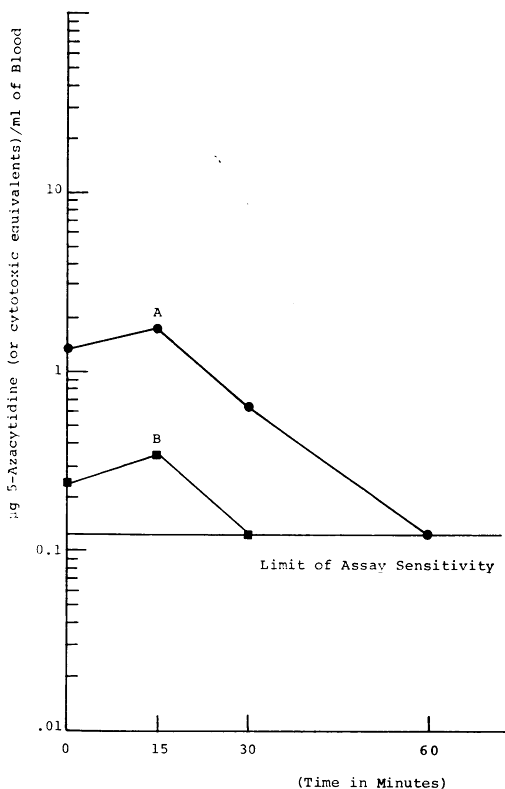

FIG. 2. *Concentrations of 5-azacytidine detected in the blood of mice by microbiological assay. Each point represents the mean drug level of five mice. Assay organism: Escherichia coli ATCC 9637/6-chloropurine. (A) Animals were injected with a single* LD_{10} *intraperitoneal dose (9.5 mg/kg) of drug. (B) Animals were injected with a single 0.5* LD_{10} *intraperitoneal dose (4.75 mg/kg) of drug.*

drug concentrations (micrograms/disc) were read directly from the abscissa of the standard curve. Drug concentrations (micrograms per milliliter) in the blood of treated mice were calculated by multiplying the microgram/disc values by the appropriate dilution factor. Standard curves were prepared each time blood samples were assayed for drug levels. Since the diameter of the paper discs was 1.27 cm, no zone was measurable that had a diameter less than that of the filter-paper discs.

Microbiological standard inhibition assay curves obtained for NSC 102816 are presented in Fig. 1. The indices of precision (λ) (1) of the respective curves are 0.0597 and 0.0489.

In all experiments, BDF mice (mixed sexes, 18 to 22) were used. Drug was administered in two doses (LD_{10} and 0.5 LD_{10}) in 0.2 ml of 0.85% NaCl. The method used for securing blood samples from treated animals was cardiac puncture of sacrificed mice.

Figure 2 summarizes the levels of drug detected in the blood of sacrificed mice which were given 9.5- and 4.75-mg/kg doses (LD_{10} and 0.5 LD_{10}, respectively) of 5-azacytidine. Maximal drug concentrations were obtained prior to 15 min after drug administration, but rapidly diminished; all measurable drug was gone by 1 hr after injection of an LD_{10} dose and by 30 min after injection of an 0.5 LD_{10} dose. No drug was detected in the liver, lungs, brain, spleen, or kidneys of the mice at any time after administration of the drug. Failure to detect 5-azacytidine in the solid tissues of mice may be due to the possibility that 5-azacytidine, like 6-azacytidine (4), is deaminated.

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LITERATURE CITED

1. Bliss, C. I. 1952. The statistics of bioassay, p. 472. Academic Press Inc., New York.
2. Doskocil, J., and F. Sorm. 1967. The action of 5-azacytidine on bacteria infected with bacteriophage T_4. Biochim. Biophys. Acta 145:780–791.
3. Hunt, D. E., and R. F. Pittillo. 1968. A microbiological assay for estimating concentrations of camptothecin lactone in mouse tissues. Appl. Microbiol. 16:867–870.
4. Skoda, J. 1963. Mechanism of action and application of azapyrimidines, p. 197–219. *In* Davidson, J., and Cohn, W. (ed.), Progress in nucleic acid research, vol. 2. Academic Press Inc., New York.
5. Sorm, F., A. Piskala, A. Cihak, and J. Vesely. 1964. 5-Azacytidine, a new, highly effective cancerostatic. Experientia 20:202–203.
6. Sorm, F., and J. Vesely. 1964. The activity of a new antimetabolite, 5-azacytidine, against lymphoid leukemia in AK mice. Neoplasma 11:123–130.
7. Zadrazil, S., V. Fucik, P. Bartl, Z. Sormova, and F. Sorm. 1965. The structure of DNA from *Escherichia coli* cultured in the presence of 5-azacytidine. Biochim. Biophys. Acta 108:701–703.