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STUDIES ON 6-AZAURIDINE AND 6-AZACYTIDINE—I.
TOXICITY STUDIES OF 6-AZAURIDINE AND 6-AZACYTIDINE IN MICE

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Abstract—The acute toxicity of 6-azauridine and 6-azacytidine after intraperitoneal injection in mice is very low. After repeated daily administration, however, the following toxic manifestations developed: leukopenia, hemorrhagic diarrhea, and finally death of the experimental animals. Mortality was related to size of daily dose, as well as to the duration of drug administration, and a linear relationship was found between the logarithm of the daily dose and the logarithm of the corresponding mean lethal time (LT_{50}).

Morphological findings revealed marked changes in the structure of the spleen and the lymph nodes. At a very low level of dosage (50 mg/kg daily) irritation of lymphopoiesis was seen in both organs, whereas after higher doses progressive depressions of lymphopoiesis, together with a proliferation of reticulum cells, were found. This proliferation often was accompanied by the occurrence of nuclear anomalies in the proliferated reticulum cells. Marked proliferation of the endothelial lining of blood capillaries also was observed in the lymph nodes. The cells and tissues of the small intestine, as well as of the kidney and liver; were affected only with increased dosage of both compounds; under these circumstances, necrobiotic changes of the intestinal epithelium and fatty degeneration in the liver, as well as in the cortical tubuli of the kidney, were observed.

THE antimetabolites of pyrimidine metabolism prepared within the last years[1–3]—6-azauracil, 6-azauridine and 6-azacytidine—are known for their inhibitory activity on the growth of some animal tumours.[4–9] While 6-azauracil had to be discarded from human therapy because of neurotoxicity,[10] both 6-azauridine and 6-azacytidine have been investigated in therapy of human hemoblastosis with positive results.[11, 12]

Additional pharmacological investigations of 6-azauridine and 6-azacytidine, together with more detailed morphological studies in relation to toxicity, have been performed in our laboratory. Presented herewith are the results of the studies undertaken by our group with a view to the detection of possible correlations between morphological and functional features of both drugs.

METHODS

Acute toxicity

Acute toxicity studies were performed in mice of both sexes of the Konárovice strain weighing 16–20 g, kept at a constant temperature and on the same diet. The drugs were administered intraperitoneally in five doses, each dose given to ten animals.

The mean lethal doses (LD_{50}) were calculated by the procedure of Litchfield and Wilcoxon.[13]

Subacute toxicity and blood cell counts

Subacute toxicity studies were performed on groups of mice of the same weight containing 10–20 animals. The compounds, dissolved in physiological salt solution, were administered intraperitoneally in daily doses ranging from 0·5 to 5·0 g/kg. A group of control animals was injected with the same amount of the saline solution. In daily doses of 1·0 to 5·0 g/kg the administration of 6-azauridine and 6-azacytidine was maintained until at least 50 per cent of the animals died. The number of dead animals was recorded daily. From these data the corresponding mean lethal times (LT_{50}), expressed in days from the beginning of the administration of the drugs, were calculated using the graphical probit procedure of Litchfield.[14] The surviving animals were killed and the tissues from various organs removed for histological examination. With lower daily doses, 50 mg/kg of 6-azauridine given subcutaneously or 250 to 500 mg/kg of either compound injected intraperitoneally, only the mortalities on the 20th and 33rd day after the beginning of dosage were determined. The white blood cells were counted in all groups, including controls, at the beginning and at the end of drug treatment.

Morphological effects

The lymphatic nodes from maxillary, cervical, axillary (both superficial and deep), mediastinal, renal and pelvic groups, as well as the spleen, kidney and liver, were investigated histologically. A piece of small intestine also was taken for microscopic examination from animals with hemorrhagic diarrhea. The material was fixed in Baker's neutral formol and in Clark's fluid. After the fixation with formol, the frozen sections were cut for the demonstration of lipids with the aid of Oil Red O according to the method of Lillie. The material fixed in Clark's fluid was passed through *n*-propanol and lanolin-paraffin and then embedded into paraffin. Sections were stained with hematoxylin-eosin, with hemotoxylin-picrofuchsine (according to the method of van Gieson), and with Goldner's modification of Masson's trichrome method.[15] The reticular framework was impregnated in the manner described by Gordon-Sweet. Nucleic acids were demonstrated by the Feulgen-reaction, by Turchini's method, by Chrom-Gallocyanine, and by Toluidine blue staining. Extraction controls were treated with either 10% perchloric acid, according to the method of Erickson, or with Streptokinase, according to that of Jackson and Dessau. The air-dried blood-smears were fixed with absolute methanol, then stained, using Pappenheim's panoptic method. On the smears, the peroxydase reaction (according to Satō), the PAS-reaction, and the demonstration of ribonucleoprotein-induced basophilia (with the aid of Toluidine Blue) were performed.[16] If not quoted otherwise, all techniques were performed in the manner described by Pearse[17] and Schudel.[18]

Solutions

Solutions in saline of each of the compounds were employed and all solutions were freshly prepared before each experiment.

RESULTS

Acute toxicity

The extremely low acute toxicities of 6-azauridine, as well as of 6-azacytidine, are indicated by Table 1, which shows the corresponding LD_{50}-values for each of these compounds after a single intraperitoneal injection in mice.

TABLE 1. ACUTE TOXICITIES OF 6-AZAURIDINE AND 6-AZACYTIDINE AFTER INTRAPERITONEAL INJECTION IN MICE

Compound	LD_{50} (g/kg)	Limits of confidence ($p = 0{\cdot}95$)
6-Azauridine	11·25	9·3–13·6
6-Azacytidine	14·0	11·1–17·6

Subacute toxicity and blood cell counts

With repeated daily administration of these compounds, death results even from relatively low dosages, and a linear relationship between dosage and mortality was found within the 1·0 to 5·0 mg/kg dose range, if the logarithms of the LT_{50}-values were plotted against the logarithms of corresponding single daily doses. As can be seen from Fig. 1, the LT_{50}-values for 6-azauridine and 6-azacytidine do not differ

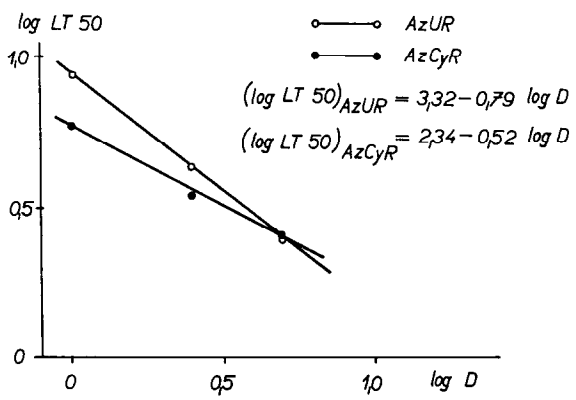

FIG. 1. The relationship between the logarithm of daily dose D (abscissa) and the logarithm of the mean lethal time (LT_{50}) (ordinate) for 6-azauridine (AzUR) and 6-azacytidine (AzCyR).

greatly, although in lower doses the LT_{50}-values for 6-azacytidine, as compared to those of 6-azauridine, are slightly smaller. Diarrhea, very often hemorrhagic, was a salient sign in the preterminal stages of intoxication with either compound. Deaths of experimental animals also were observed as a result of the prolonged administration of daily doses of 250–500 mg/kg of either compound (Table 2). Since in this dose range the treatment was carried on only within a limited time-interval, during which mortality did not exceed 50 per cent of the experimental animals, the calculation of mean lethal times was not performed. After daily doses of 50 mg/kg of 6-azauridine were injected subcutaneously, deaths did not occur within 33 days of drug administration.

TABLE 2. MORTALITY OF MICE CAUSED BY DAILY ADMINISTRATION OF 6-AZAURIDINE AND 6-AZACYTIDINE

Compound	Daily dose (mg/kg)	No. of animals	Mortality on 20th day (%)	Mortality on 33rd day (%)	Route of administration
6-Azauridine	50	20	0	0	Subcutaneously
	250	20	35	35	intraperitoneally
	500	20	47	not determined	intraperitoneally
6-Azacytidine	250	20	35	45	intraperitoneally
	500	20	53	not determined	intraperitoneally

White blood cell counts were carefully compared with control values from animals injected with saline. Changes after daily doses of 250–500 mg/kg of either compound did not differ essentially from those of control animal values. Only the higher daily dose range (1·0 to 5·0 g/kg) caused a substantial decrease of the lymphocyte counts, as compared to those of control animals. In addition, 6-azacytidine produced a statistically significant reduction in the number of neutrophiles; however, this effect was not dependent on dose.

Morphological effects

Spleen. After the subcutaneous administration of 50 mg of 6-azauridine per kg daily for 33 days, the spleens in experimental animals were enlarged. On section, the follicles were discernible, the pulp was of deep violet colour and had a moderately firm consistence. Microscopically, in comparison with control animals, a moderately increased number of mature lymphocytes and a smaller number of lymphoblasts were found in the Malpighian bodies. The reticulum cells in the Malpighian bodies were increased in number, but the amount of the reticular fibres was not enhanced. After the intraperitoneal administration of 500 mg of 6-azauridine per kg daily for 17 days, however, the size of the spleen was diminished. Their capsules were faint and wrinkled, with a smooth and bright surface. On section (Fig. 2), the Malpighian bodies were diminished in size and their borders were not sharply delineated. The pulp was deeply violet, of a moderately firm consistence. Microscopically, a decreased number of lymphocytes and lymphoblasts was found in the Malpighian bodies, the germinal centers were enlarged, with an increased number of reticulum cells. The nuclei of several reticulum cells showed some anomalies of form and chromatin configuration, but the number of mitoses was not increased; the red pulp was congested. After the daily intraperitoneal administration of 6-azauridine, 1 g/kg, for 7 and 13 days, respectively, or in daily doses of 2·5 g/kg for 13 days, the findings were similar. The same was true for daily doses of 6-azacytidine given intraperitoneally either for 32 days, 25 mg/kg, or for 7 days, 2·5 g/kg, or for 3 or 4 days, 5 g/kg.

After the daily intraperitoneal administration of 250 mg of 6-azauridine per kg for 32 days, all changes in the spleen described in the previous group were present, but some patterns were more intensive (Fig. 3); these concerned mainly the reticulum cells. Anomalies of form and configuration of chromatin were more numerous and

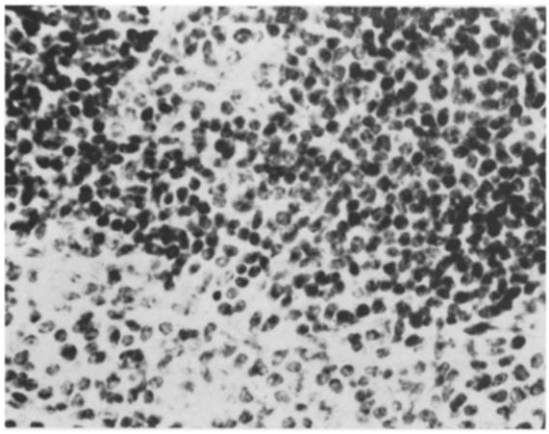

FIG. 2. Malpighian body of the mouse spleen after 17 daily intraperitoneal doses of 6-azauridine, 500 mg/kg. A diminished number of lymphocytes and lymphoblasts, as well as an increased number of reticulum cells, are indicated. Haematoxylin-eosin. (× 238).

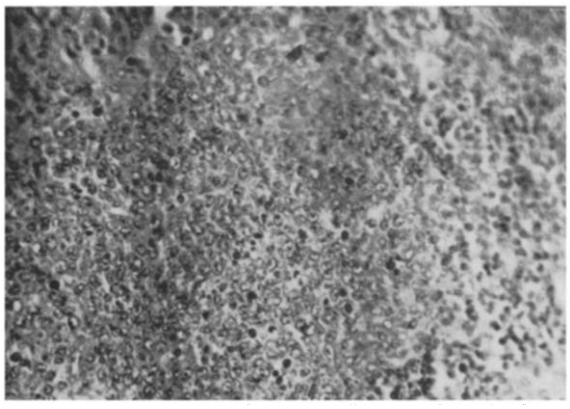

FIG. 3. Malpighian body of a mouse spleen after 32 daily intraperitoneal doses of 6-azauridine 250 mg/kg. A progressive decrease in the number of lymphocytes and lymphoblasts, is observed, while the number of reticulum cells is increased. Haematoxylin-eosin. (× 168).

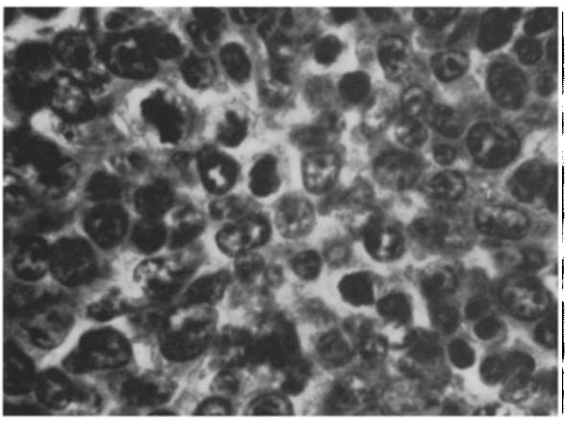

FIG. 4. Detail from Fig. 3. Anomalous nuclei in the reticulum cells, with some atypical mitoses, are shown. Masson-Goldner. (× 840).

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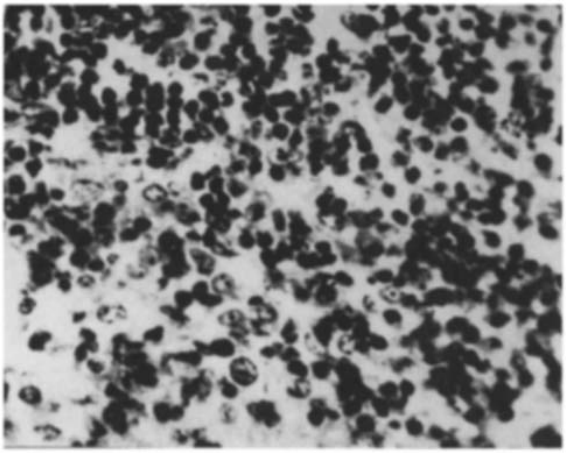

FIG. 5. Cortical nodule of a lymphatic node of a mouse given 17 daily intraperitoneal doses of 6-azauridine, 500 mg/kg. There is a diminished number of lymphoblasts and lymphocytes, as well as an increased number of reticulum cells. Haematoxylin-eosin (×238).

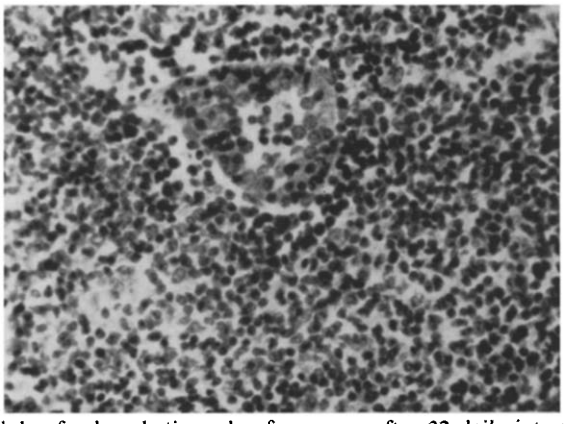

FIG. 6. Cortical nodule of a lymphatic node of a mouse after 32 daily intraperitoneal doses of 6-azauridine 250 mg/kg. In comparison with Number 5, there is a further decrease in the number of lymphocytes and lymphoblasts, while the number of reticulum cells is increased. A proliferation of endothelial lining of a venous arm of capillary loop, with a narrowing of the lumen, is shown. Masson-Goldner (×168).

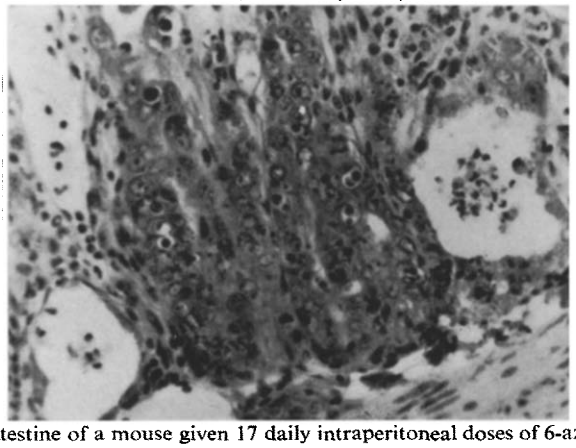

FIG. 7. The small intestine of a mouse given 17 daily intraperitoneal doses of 6-azauridine 500 mg/kg. A proliferation of enterocytes in the basal parts of Lieberkühn's crypts can be seen, as well as an increased penetration of lymphocytes into the mucous membrane. Masson-Goldner (×168).

were accompanied by an increased number of mitoses—often atypical (Fig. 4). The same result was obtained after the administration of 500 mg of 6-azacytidine per kg daily for 17 days, or of 1·0 g/kg for 8 days.

Lymph nodes. After the subcutaneous administration of 50 mg of 6-azauridine per kg for 33 days, some groups of lymph nodes were slightly enlarged, without other striking changes. Histologically, the number of lymphocytes was increased in the cortical nodules. In the germinal centers an increased number of reticulum cells was found; the nuclei of those cells showed only few anomalies.

After the daily intraperitoneal administration of 500 mg of 6-azauridine per kg for 17 days, the lymph nodes were slightly diminished in size. Microscopically (Fig. 5), in the cortical nodules, a decreased number of lymphocytes and lymphoblasts was found. The germinal centers were enlarged, with an increased amount of reticular cells. Several hyperchromic nuclei of these cells were anomalous in shape. A heightening of endothelial cells was observed in some small venous branches of blood capillaries at the border between cortex and medulla of the lymph nodes. These changes were found only in a part of the lumen lining, but the lumina of such capillaries were markedly narrowed. The changes of this type also were found after the daily intraperitoneal administration of 6-azauridine, 1·0 g/kg, for 7 and 13 days, or 2·5 g/kg for 7 or 13 days, respectively, or 6-azacytidine, 250 mg/kg, for 32 days, or 2·5 g/kg for 7 days, or 5·0 g/kg for 3 and 4 days, respectively.

After the daily intraperitoneal administration of 250 mg of 6-azauridine for 32 days, the lymph nodes of a firm consistence were diminished distinctly in size, particularly those of the axillary groups. Microscopically (Fig. 6), a loss of borders of the cortical nodules, with a marked decrease in number of lymphocytes and lymphoblasts was found. In the enlarged germinal centers a proliferation of reticulum cells with an increased number of nuclear anomalies and mitoses, which often were atypical, was found. In a majority of the experimental animals, a proliferation of the endothelial lining of blood capillaries was observed, either in the whole circumference or in the form of cushion-like thickening. The lumina of capillaries were markedly narrowed and sometimes they disappeared completely. The changes of this type also were found after the daily intraperitoneal administration of 500 mg of 6-azacytidine per kg for 17 days or of 1 g/kg for 8 days.

Kidney and liver

The extent of the changes in the kidney, caused by the repeated administration of 6-azauridine and 6-azacytidine, were dose-dependent. Only after the daily administration of high doses (1·0 g/kg or more) was a diffuse steatosis of the cortical tubuli observed in the kidney. A similar situation was found in the liver, in which these doses led to an irregular fatty degeneration of liver cells in form of small droplets; the changes were present particularly in the central areas of the liver lobules.

Small intestine

After the administration of 250 mg of 6-azauridine per kg daily for 32 days, or of 500 mg/kg for 17 days, vacuolar degenerations with necrobiosis and desquamation of epithelium at the tops of the intestinal villi were found (Fig. 7). In the basal parts of Lieberkühn's crypts, marked proliferation of enterocytes was seen, with numerous mitoses; the latter often were atypical. An increased penetration of lymphocytes

into mucous membranes also was observed. In the lamina propria, a scanty polymorphonuclear cellulization and a small congestion of blood vessels were found.

The histochemical reactions showed no marked changes in the distribution of deoxyribonucleoproteins, ribonucleoproteins, lipids and polysaccharides in the cells investigated.

DISCUSSION

Our experiments show that from the point of view of their acute toxicity, 6-azauridine, as well as 6-azacytidine, are much less toxic than 6-azauracil. Clinical trials, however, revealed that for successful therapy of neoplastic diseases the dosage of both 6-azauridine and 6-azacytidine should be kept relatively high.[11, 12] For this reason, the development of signs of toxicity during the long-continued administration of these compounds in animals without tumors deserves attention, in order to gain information concerning tolerance when the usual therapeutic dose range is surpassed. In this respect our results show that, under the conditions of chronic administration in mice, definite signs of toxic affects may appear, and these coincide roughly with those described in dogs by Handschumacher *et al.*[12] In our experiments they were characterized by leukopenia, diarrhea, blood in the stools and finally by death of the experimental animals.

The development of toxicity also was indicated by the morphological picture observed in various organs. The morphological changes in the structure of hemopoietic organs, i.e. lymph nodes and spleen, can be classified according to their severity into three groups. The changes in the first group, seen after the lowest dose of 6-azauridine, afford evidence for a stimulation of the leukopoietic system, as they consist in an increased number of lymphoblasts and mature lymphocytes, as well as in a moderate proliferation of the reticulum cells; these effects are not lethal. Lethal effects emerged with a progressive depression of lymphopoiesis that is marked in the second, or even more in the third, group of our histological findings, in which proliferation of reticulum cells, as well as of the endothelial lining of venous capillaries in the lymph nodes, was accompanied by decreased numbers of lymphoblasts and mature lymphocytes. In the third group the irritation of reticulum cells was accentuated by the occurrence of atypical nuclei and mitoses. In animals suffering from hemorrhagic diarrhea, vacuolization or even necrobiosis of the epithelium at the top of intestinal villi could be observed, together with a proliferation of enterocytes in the deeper layers of Lieberkühn's crypts. Higher doses also produced fatty degeneration in the liver and in the epithelial cells of the renal cortical tubuli.

The severity of toxic signs, however, varied not only in relation to the amount of drug injected daily, but also with the duration of administration of drug. As far as mortality is concerned, a definite relationship relating daily dose to the achievement of a certain effect, i.e. death of 50 per cent of the animals, was observed at least within a limited range of doses. Depression of lymphopoiesis sometimes appeared to be more marked with lower doses and prolonged treatment than with very high doses, in which case death occurred prior to the possibility of development of intensive morphological changes.

It may be concluded, therefore, that from the morphological point of view the repeated administration of 6-azauridine or 6-azacytidine produces changes primarily in those organs and tissues in which mitotic activity is observed, i.e. in cells possessing

basopholic cytoplasm and nucleoli containing ribonucleoproteins, both indicators of an active production of proteins. In the kidney, however, in which the microscopic alterations in cortical tubuli has not the character of a nuclear damage, the changes of structure might be related to the massive elimination of the compounds by means of an active tubular mechanism, as described for 6-azauridine by Volle *et al.*[19] in the hen.

The character of the morphological changes produced by prolonged treatment with 6-azauridine and 6-azacytidine suggests a similarity to those observed after ionizing radiations[16–19] or alkylating agents,[20, 21] in spite of the different sites of action at the biochemical level. Moreover, the nuclear and mitotic atypias of reticulum cells, observed with higher doses of the compounds, resemble strikingly the initial phases of neoplastic proliferation of reticulum. In this connection, it is important to note that after the use of these drugs for their chemosterilant effect, tumor-like cells were found by Landa and Řežábová[22] in the ovaries of *Musca domestica*.

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