Biochemical and Morphologic Changes in a Mast-Cell Neoplasm during Treatment with Cyclophosphamide*

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SUMMARY

Treatment of mice bearing the P-815 ascites mast-cell neoplasm produced biochemical and morphologic alterations in the cell population. The metachromasia of cells increased concomitantly with a rise in serotonin content, while the histamine content of cells remained unchanged. The urinary excretion of 5-hydroxyindoleacetic acid was enhanced, and serotonin was excreted in large amounts in the urine. The excretion of tryptophan metabolites along the kynurenine pathway decreased. The significance of these observations and the usefulness of functional animal tumor systems are discussed. A method for the simultaneous assay of serotonin and histamine content of this neoplasm is described.

Cyclophosphamide (CTX) has been found to be highly effective against a variety of well established animal tumors (7–10, 18). Recent studies in this laboratory showed that CTX was the most effective of several agents tested against the P-815 mast-cell ascites tumor (11). In the ascitic form this tumor grows rapidly and kills at about the 10th day. The ascites is not bloody, and the tumor cell population is morphologically and biochemically stable from one transplant generation to the next. The cells contain metachromatic granules and measurable quantities of 5-hydroxytryptamine (5-HT), histamine, and heparin (3–6, 13, 16). The investigations reported here deal with the biochemical and morphologic alterations produced in the tumor cell population during treatment with CTX. A simple method for the simultaneous determination of 5-HT and histamine in the tumor cells is described.

MATERIALS AND METHODS

Tumor system and animals.—The mast-cell tumor, P-815, in the ascitic form was obtained from Dr. Michael Potter and was maintained in our laboratory by weekly transfer into (BALB/c X DBA/2) F1 (CDBA) mice of a 1:10 dilution of ascites in Locke solution. A subline with higher levels of 5-HT developed in this laboratory was also used (12).

Preparation of drug.—Solutions of crystalline CTX1 were prepared freshly before administration by dissolving CTX in physiological saline in such concentration that the derived dose per kg. animal body weight was contained in 10 ml. of solution (i.e., 0.01 ml/gm body weight).

Histologic preparation.—Fresh, thinly spread, air-dried smears of ascites were stained with Wright’s stain as used routinely. Metachromasia was demonstrated by staining with toluidine blue according to the method of Padawer (13). The periodic acid-Schiff (PAS) reaction was also used. Stains were performed on all samples obtained for daily biochemical assays.

Assay of cell content of 5-HT and histamine.—The intracellular content of 5-HT and histamine was measured by modifications of previously described methods (15, 19). (a) Aliquots of ascitic fluid, 0.1–0.5 ml., were used for each determination, and the volume of packed cells was determined in capillary microhematocrit tubes. (b) Cells were sonicated in 5 ml. of 0.4 N perchloric acid

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after three washes in ice-cold Locke solution, and the sonicate diluted to 8 ml. with normal saline and centrifuged for 30 minutes at 2500 r.p.m. (c) For 5-HT, 1 ml. of the supernate was added to 0.3 ml. 12 N HCl in a cuvette and the fluorescence read in a spectrophotofluorometer (activation wave-length, 310 mm.; fluorescence wave-length, 550 mm.) against a standard and blank prepared with 1 ml. of normal saline. (d) For histamine, to a test tube containing 2 ml. of 0.1 N HCl, 0.4 ml. of 1 N NaOH, and 0.025 ml. of the supernate was added 0.1 ml. of orthophthalaldehyde solution (1 per cent in absolute methyl alcohol). After 4 minutes 0.2 ml. of 3 N HCl was added, and the fluorescence at 450 mm. was measured (activation wave-length, 370 mm.) against a blank, and a standard prepared in similar fashion, with saline used in place of the supernate. (e) Results were expressed in μg/ml packed cells.

The high concentration of histamine in these tumor cells made it possible to develop directly the fluorophore with orthophthalaldehyde solution without the extraction procedure outlined by Shore et al. (15). It was possible to obtain reproducible results at concentrations of over 100 μg/ml packed cells, when verified by the extraction procedure. Values for 5-HT were no different from those obtained when ZnSO4 and NaOH, as described by Weissbach et al. (19), was used for protein precipitation.

For 5-tIT, 1 ml. of the supernate was added to 0.3 ml. 12 N HCl in a cuvette and the fluorescence read in a spectrophotofluorometer (activation wave-length, 310 mm.; fluorescence wave-length, 550 mm.) against a standard and blank prepared with 1 ml. of normal saline. (d) For histamine, to a test tube containing 2 ml. of 0.1 N HCl, 0.4 ml. of 1 N NaOH, and 0.025 ml. of the supernate was added 0.1 ml. of orthophthalaldehyde solution (1 per cent in absolute methyl alcohol). After 4 minutes 0.2 ml. of 3 N HCl was added, and the fluorescence at 450 mm. was measured (activation wave-length, 370 mm.) against a blank, and a standard prepared in similar fashion, with saline used in place of the supernate. (e) Results were expressed in μg/ml packed cells.

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Assays of ascites from two animals in treated and untreated groups were performed daily. Repeated aspirations in any one animal occurred at no less than 7-day intervals.

**Assay of tryptophan metabolites in urine.**—UrinEs from six animals were collected daily for 24-hour periods in individual glass metabolic cages. Three were used for assay of 5-hydroxyindoleacetic acid and three for paper chromatography. Quantitative assay of the urinary excretion of 5-hydroxyindoleacetic acid was carried out by the method of Udenfriend et al. (17).

**Paper chromatographic separation and identification of tryptophan metabolites in urine** were performed by a modification of the method described by Dalglish (1, 2). Flash evaporation at 38° C. and 15 mm. Hg pressure was used instead of steam distillation to concentrate the eluate and eliminate the phenol. Compounds were identified by their position on the paper and by fluorescence and absorbance spectra after elution.

**RESULTS**

**Effect of CTX on survival of mice with the P-815 mast-cell ascites tumor.**—The survival time of mice with the mast-cell tumor was substantially prolonged by oral administration of CTX. This is illustrated in Chart 1, in which the median survival times of mice given single and repeated (every 7 days) doses of CTX have been plotted.
covering a dose range of 133–675 mg/kg. In these experiments, treatment was initiated 7 days after tumor implantation, at which time the tumor was well established, and considerable ascites had formed. In repeated experiments the range in survival time of untreated mice varied from 9 to 14 days, with medians of 9–11 days. As shown in the figure, CTX was effective over a wide range of doses, and a greater therapeutic effect was obtained with repeated administration than by a single treatment. Weekly oral administration of 300 mg/kg CTX increased the median survival to 36 days (287 per cent longer than controls), and a single dose of 450 mg/kg increased survival by 156 per cent over that of untreated mice.

Indications of therapeutic effectiveness of CTX were evident within 24–48 hours after administration of a single dose. The general appearance of the animals improved, and the body weight, which had increased sharply just prior to treatment owing to formation of ascitic fluid, returned to pretumor inoculation levels. There was a decrease in the number of tumor cells per unit volume of ascitic fluid as well as the total volume of ascites. Maximum improvement was noted within 4–9 days after a single treatment. Subsequently, body weight increased concomitantly with increasing ascites volume and rising tumor cytocrit. On repeated administration, CTX produced a pattern of response similar to that described above, although the regrowth of tumor was less pronounced.

Morphology of the tumor cell population.—The morphologic change in the tumor cell population after a single dose of CTX is indicated in Chart 2. The change in the granular content of the tumor cells was demonstrated best by the toluidine blue stain. In the untreated tumor there was a small rise in the number of tumor cells containing metachromatic granules as animals neared death. In contrast, in the treated tumor, 15–20 per cent of cells contained metachromatic granules within 4 hours after treatment, and metachromasia was observed in 60–80 per cent of cells by the 4th posttreatment day. The number of granules per cell increased, and the granules appeared larger. These granules also stained PAS-positive. As a new cell population emerged and the ascites reaccumulated, the number of cells with these granules decreased. Retrieval of animals resulted in repetition of these morphologic changes. Examples of toluidine blue-stained smears of ascites before and after treatment are shown in Figures 1–4.

Biochemical changes.—Concomitant with the tumor regression and the increased cellular metachromasia there was a marked rise in the 5-HT content of the remaining tumor cells. This occurred in two strains of tumor mast cells containing different levels of 5-HT and was in the order of a threefold rise in both strains. Associated with the increased 5-HT cell content there was an enhanced excretion of urinary 5-hydroxyindoleacetic acid. The histamine content of these tumor cells did not change significantly. Chart 3 summarizes the patterns of these metabolites with and without treatment in a typical experiment. With retreatment this pattern of response was also repeated.

Chart 4 shows schematic diagrams of tryptophan metabolites, separated by paper chromatography, in urine of tumor-free mice, urine of mice bearing the mast-cell tumor, and in urine of tumor-bearing, CTX-treated mice. In normal mouse urine indole, indoleacetic acid, and kynurenine were noted on all papers in moderate amounts, while xanthurenic acid, kynurenic acid, and o-

[Charts 2, 3, and 4 are not transcribed here but are included in the full text.]
aminohippuric acid did not appear on all papers and usually were present in small quantities, as judged by intensity of fluorescence and stain and the size of the spot. In the urine of animals bearing the mast-cell tumor the amount of kynurenic, xanthurenic, and o-aminohippuric acids and of kynurenine increased, and 5-hydroxykynurenine was noted. In addition, very large quantities of 5-hydroxyindoleacetic acid were noted, and 5-hydroxytryptophan appeared. After treatment the amounts of kynurenine decreased and kynurenic, xanthurenic, and o-aminohippuric acids and 3-hydroxykynurenine were not detected in the urines of most animals even after elution of suspect areas on the paper and fluorometric ex-

DISCUSSION

Ideally, animal tumor systems used for the study of potential antitumor agents should predict effects on analogous human tumors and provide opportunities to clarify mechanisms of action, proper dose schedules, and biological effects of the agent studied. Tumor growth rate and prolonged survival have been the commonly used parameters in animal tumor systems to assay the effect of treatment. Biochemical and morphologic changes in tumor cells may give more precise data in following the effects of treatment. The P-815 mast-

**CHART 4.**—Schematic diagrams of paper chromatograms of urinary tryptophan metabolites.

A. Tumor-free CDBA ♀ mice.
B. CDBA ♀ mice with 7-day-old P-815 ascites mast-cell tumor.
C. CDBA ♀ mice bearing the mast-cell tumor 4 days after treatment with CTX (250 mg/kg p.o.).

Light stipple indicates small quantities of metabolite not appearing on all papers.
Medium stipple indicates those metabolites noted on all papers.
Black spots indicate very large quantities of metabolite.

amination. Large amounts of 5-hydroxyindole-
acetic acid were still noted. In addition a large, slightly fluorescent spot which stained an intense violet-blue with Ehrlich's reagent, having an Rf1 (organic phase) of .40 and Rf2 (aqueous phase) of .37, was noted in all urines of treated animals. Activation and fluorescent spectral peaks at 310 mm. and 550 mm., respectively, proved this compound to be 5-HT.

As the ascites reaccumulated, the patterns obtained were similar to those of pretreatment tumor-bearing animals.

Twenty normal animals were given the same dose of CTX and biochemical studies carried out in a similar fashion. Since these animals did not have ascites, histamine and 5-HT determinations were carried out on homogenates of whole mice. There was no change in total-body content of these metabolites, and the urinary excretion of 5-hydroxyindoleacetic acid and paper chromato-

cell tumor was selected for study because the cells contain metachromatic granules and measurable quantities of 5-HT, histamine, and heparin; in addition, the urine of tumor-bearing mice can be examined quantitatively for excretion of 5-hydroxyindoleacetic acid and by paper chromatographic separation for many tryptophan metabolites. This tumor might be considered comparable to urticaria pigmentosa, solid mastocytoma, and mast-cell leukemia in man, and, because of the high cell content of 5-HT, possibly malignant carcinoid.

Studies showed that CTX was the most effective of a large number of agents tested in prolonging the survival of mice with well established mast-cell ascites tumors, and this drug was selected for the present study.

The results indicated an association of biochemical and morphologic changes with antitumor activity.
The untreated tumor had few cells with granules that stained metachromatically. This was noted by Padawer in his description of the staining technic (13). The increased metachromasia and positive PAS-staining reaction after treatment indicated increasing cell content of mucopolysaccharide or mucoprotein. The associated rise in 5-HT content while histamine remained unchanged was of interest, since previously reported studies indicated a closer binding of heparin to 5-HT than to histamine (4). The rise in 5-HT and mucopolysaccharide indicated some continuing cytoplasmic-metabolic differentiation and production in remaining cells. Another explanation for the different cell content of 5-IIT and histamine may be found in recent work indicating biochemical heterogeneity in this tumor cell population in terms of 5-IIT and histamine (12). It is possible that CTX may have damaged predominantly one cell type.

The enhanced excretion of 5-hydroxyindoleacetic acid in the urine of treated tumor-bearing animals was probably secondary to the release of 5-IIT from damaged cells. The appearance of 5-IIT in the urine of treated animals also indicated the release of large quantities of this metabolite. The changes in urinary excretory patterns of tryptophan metabolites of the tumor-bearing mice indicated increased production along the major pathways. After treatment, the decreased excretion of metabolites along the pathway to niacin was striking and suggested a block along this pathway. Since metabolites from kynurenine and beyond were decreased there could be two possible sites: the conversion of tryptophan to formylkynurenine by tryptophan pyrrolosine or the subsequent hydrolysis to formic acid and kynurenine by the enzyme formylase.

Alternatively, it is possible that, as the tumor cells were destroyed by treatment, precursor substances for the indole pathways were reincorporated into the fewer and fewer remaining cells, while those necessary to kynurenine metabolism were irretrievably lost.

Examination of the changes after treatment noted in this study suggested that the antitumor action of CTX was mediated primarily against cell division, allowing some continued cytoplasmic function as indicated by increasing mucopolysaccharide and 5-HT content.

Similar studies with a relatively ineffective agent, 5-fluorouracil, showed no significant biochemical or morphologic change during treatment. Duazaomyein,\(^2\) which ranked second to CTX in prolonging survival, produced similar morphologic and biochemical changes of shorter duration and lesser degree. Of further note is the fact that in CTX-treated animals biochemical and morphologic changes were present and definite before conclusions regarding effectiveness could be made on the basis of prolonged survival. With additional studies it may be possible to predict activity of agents against this tumor on the basis of morphologic and biochemical changes and to gain additional information as to the mechanism of action of agents.

Studies aimed at clarifying the basic mechanisms of these changes in the tumor cell population and the usefulness of this tumor in predicting antitumor activity of agents in patients with malignant carcinoid or mastocytosis are under way.

This tumor may offer a good system for the study of potential antitumor agents with the biochemical and morphologic alterations used as additional parameters of effectiveness.

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\(^2\) Supplied through the courtesy of Chas. Pfizer & Co., N.Y.


Fig. 1.—Ascites mast-cell tumor, untreated. Few cells contain the metachromatic granules. Toluidine blue, X1200.

Fig. 2.—Same, 4 days after treatment with CTX. Increased number of cells with metachromatic granules. Toluidine blue, X1200.

Fig. 3.—Same, 8 days after treatment with CTX. Marked increase in metachromasia. Toluidine blue, X1200.

Fig. 4.—Same, 15 days after treatment. Appearance of new cell population without metachromatic granules. Toluidine blue, X1200.
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