Factors Affecting the Site and Degree of Localization of Tetracycline in Sarcoma 37 Tumors*

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SUMMARY

A study has been made of the localization of tetracycline (TTC) in transplantable tumors of the mouse (S-37) by utilizing the radioactivity and fluorescence of tritiated TTC as measures of concentration. Evidence is presented that the drug is sequestered within the tumor; that the avidity of the tumor for TTC increases with increasing age of the tumor; and that the actual site of affinity is the necrotic or prenecrotic area of the tumor, as detected by gross and histologic investigations.

Fluorescence of the tumor appears definitely related to tumor age. No tumors less than 10 days old fluoresced, whereas 100 per cent of tumors 13 days or more old were fluorescent.

When tritiated TTC was injected into mice bearing 9-day-old S-37 tumors, the concentration of TTC in the necrotic areas was demonstrated within 2 days after injection. The TTC concentration in the nonfluorescent viable areas fell precipitously within 2 to 6 days, whereas the TTC concentration in the necrotic fluorescent areas decreased much more gradually, demonstrating that TTC is retained more avidly by the necrotic zones.

Histologic studies indicate that the S-37 tumor cell does not display fluorescence until it begins to show cytologic evidence of necrosis. The typical zones which fluoresced under ultraviolet light manifested obvious necrosis, but had not undergone the most advanced changes of fragmentation and loss of structure.

An attempt is made to hypothesize the mechanism of localization by relating the well-known propensity of the tetracyclines for binding with a variety of biologic materials to the phenomena occurring during the necrosis of tissues.

Rall et al. (26) first described the localization and persistence of a yellow fluorescence in bone (32) and in a number of different animal tumors following tetracycline administration. The localization of fluorescence in a variety of human tumors has been confirmed (21, 25, 33) and has been used as a clinical cytologic test for gastric carcinoma (4, 12, 15) and as an aid at the operating table (20, 31).

Since tetracycline drugs themselves have been shown to be without therapeutic efficacy in a large spectrum of experimental tumors (11), it has been suggested that the drug could be coupled to other agents, resulting in their migration to and concentration in tumors. McLeay et al. (21) have suggested the use of a boron-coupled tetracycline which could be bombarded in situ with a neutron beam.

There is considerable difference of opinion regarding the site of localization, within the tumor, of the fluorophor. Fluorescence has been localized by various investigators in inflammatory tissue (9), in the macrophages (33), and in the mitochondria of normal liver and kidney cells (6).

The present studies were undertaken to determine the selectivity of TTC concentration in tumor tissue and the relationship between fluorescence and TTC concentration as measured by tritiated TTC, which differs from the parent molecule only by the substitution of tritium for hydrogen in the 7-position. The experimental conditions under which maximal tumor fluorescence and maximal TTC concentration might be attained were also investigated. Histologic studies were performed to determine the site of localization of the TTC within the tumor.

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MATERIALS AND METHODS

Sarcoma 37 tumors were transplanted subcutaneously into the intrascapular area of BAF-J mice 5–6 weeks of age, of both sexes, obtained from the Roscoe B. Jackson Memorial Laboratories. There were 97 animals in this group (Group A). In one series (Group B) Ehrlich solid tumor was transplanted intramuscularly into the right thigh of C-57 strain mice of the same age. The animals were given injections either intramuscularly, intraperitoneally, or subcutaneously of tritiated TTC in doses varying from 82 to 371 µc. (Each injection contained 6–16 mg. of TTC, depending upon the specific activity.) The TTC was dissolved immediately before use in isotonic saline containing ascorbic acid in an ascorbate-to-TTC ratio of 3:2 by weight.

Groups of animals were sacrificed 5–19 days after tumor implantation, the duration from implantation to sacrifice being designated as “tumor age.” The tritiated TTC was injected at periods varying from 1 to 8 days prior to sacrifice of the animals. The interval in days between injection of the TTC and sacrifice is designated as “interval.”

After sacrifice the organs were examined immediately for fluorescence with an ultraviolet light source of 3660 Å. The organs were then weighed on a torsion balance and frozen immediately.

In order to compare the concentration of TTC in the necrotic areas of the tumor with that in the non-necrotic areas (Table 3), Sarcoma 37 tumors were implanted simultaneously into BAF-J mice, and 9 days later each mouse received 8 mg. of tritiated TTC intraperitoneally. Animals were sacrificed 1, 2, 5, and 6 days later, and the tumors were inspected under ultraviolet illumination. Samples of necrotic and viable areas were then removed, weighed, and frozen. Necrotic areas were easily distinguished by their mushy consistency and yellowish discoloration. The viable tumor tissue was firm and glistening white.

Tritium assay.—The frozen tissue was dried in a dialysis bag, with an infrared lamp as a heating source. The bag containing the tissue was then placed in a platinum basket and ignited in a 4-l. flask in an oxygen atmosphere (14). The products of combustion, CO₂ and water, were then condensed by setting the flask in a bath of dry ice and acetone and were collected in 25 cc. of polyether-611 mixture containing phosphors (14). This mixture (20 cc.) was then placed in a vial and counted in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Company). An internal standard was used for each individual sample and the results were expressed as disintegrations/min/mg of tissue.

Histology and fluorescence.—Two different methods and two different groups of animals were used.

Animals carrying S-37 tumor 8 or 12 days old were prepared. Half of each group was given injections of radioactive TTC 5 days prior to the resection of the tumor, and the other half was killed without receiving injections. The fluorescence was observed grossly, with an ultraviolet source of 3660 Å, and the outlines of the fluorescent spots were marked by infiltrating India ink around their margin.

Representative slices of tumor tissue were fixed in formalin, imbedded in paraffin, and stained with hematoxylin and eosin.

Since the fluorescence disappeared with fixation, tumors were quickly frozen in dry ice and sectioned immediately with the cryostat microtome. The slides were observed unstained, under the ultraviolet microscope. With a diamond objective, some fluorescent and some nonfluorescent areas were demarcated. The slides were then stained with hematoxylin and eosin, and the marked fields were observed for their morphologic features. Some sections were stained for DNA by the Feulgen method. Photomicrographs of identical fields, both stained and under ultraviolet light, were taken.

Alizarin red S.—A group of ten BAF-J mice in which S-37 tumors had been implanted 9 days previously were given I.P. injections of 90 mg. of Alizarin red S. The mice were sacrificed 36 hr. later, and the localization of the dark red staining was noted by gross observation of the tissues.

RESULTS

Fluorescence and concentration of TTC.—Although fluorescence was observed in many organs, the present report is confined to liver, heart, bone, and tumor (Table 1). Three different patterns of organ fluorescence were noted:

a) The liver and heart presented a diffuse, homogeneous, yellow fluorescence on the cut surface. This fluorescence appeared within the first 24 hr. after the injection of tetracycline and usually was not present to a significant degree after 48 hr.

b) Bone fluorescence was also homogeneous, appeared within 24 hr., and persisted for the duration of the experiment (8 days).

c) Tumor fluorescence was not homogeneous but was distributed in well-demarcated areas differing in shape and size and located both on the surface and within the tumor.

Fluorescence was related to tumor age. Forty-seven per cent of the 10-day-old tumors and 100 per cent of those 13 days or older were fluorescent. None of the tumors of less than 10 days of age showed the characteristic localized fluorescence.

The mean weight for the group of fluorescent tumors was 2.87 gm. and for the nonfluorescent 1.34 gm., reflecting the relationship of fluorescence to tumor age.

The concentration of TTC in various tissues relative to dose or interval varied markedly between groups of animals and among animals of the same group. Dose was shown statistically to be a nonsignificant variable. There was no constant relationship between the amount of TTC in the tumor and the interval or tumor age. However, the ratio of the concentrations of TTC between two tissues such as the heart and liver (H/L) or tumor and liver (T/L) remained relatively constant after the first day among the animals in a given group, and between groups, regardless of interval. Consequently, in Table 1 the concentrations of TTC are recorded for the individual tissues.
Concentrations of various tissues as ratios: T = tumor; L = liver; H = heart.

Certainly established by analyzing the entire tumor be of counts in the nonfluorescent tumors would be diminished tion in count for the fluorescent tumors. The dispersion anticipated, probably because of the considerable varia

hr., all tissues appear to be saturated with TTC indis removed 24 hr. after TTC injection. During the first 24 become established only after the first day and remain criminately. Gradients in concentration between tissues are compared with the ratios of the animals bearing nonfluorescent tumors (5—8 days old), the mean ratios of the fluorescent group are significantly greater than those of the nonfluorescent group (P < 0.005) (Table 2).

cause of the variable amount of necrosis within the tumors; but when the ratios of TTC concentration in the tumor to that in the liver (T/L) or to that in the heart (T/H) of the animals bearing fluorescent tumors (10—15 days old) are compared with the ratios of the animals bearing nonfluorescent tumors (5—8 days old), the mean ratios of the fluorescent group are significantly greater than those of the nonfluorescent group (P < 0.005) (Table 2). The ratio of the concentration of tetracycline in two parenchymal tissues such as heart and liver (H/L) as shown by a correlation matrix was not significantly modified by any of the explanatory variables (age, interval, dose, route) and was essentially the same in the groups bearing fluorescent and nonfluorescent tumors (Table 2). This demonstrates that the significant difference which exists between T/H for the fluorescent group and T/H for the nonfluorescent, and between T/L for the fluorescent and T/L for the nonfluorescent group,

For tumors less than 10 days old, the T/H concentration ratios between groups varied from 0.70 to 0.81, regardless of interval (after the first day); the T/L ratios varied from 0.27 to 0.38; and the H/L ratios from 0.29 to 0.50. In mice bearing tumors for 10 days or more, the concentration ratios between tissues manifested greater varia

showed route to be a nonsignificant variable. (tumor, liver, heart) and as ratios among tumor, heart, and liver (T/H, T/L, and H/L).

Although no direct association between concentration and fluorescence could be established in a group of tumors with numerous variables with respect to age, interval, and TTC dosage, a statistical study was undertaken to determine whether such a relationship could be established in a group of tumors in which the variables might be taken into account or netted out. The results of the least squares prediction equation indicate that with other variables netted out, the presence of fluorescence in a tumor is associated with an increment of about 700 disintegrations/min/mg in radioactivity, an expression of increased TTC concentration. The value of the correlation of fluorescence and concentration (+0.35), although significant, is not as high as might be anticipated, probably because of the considerable variation in count for the fluorescent tumors. The dispersion of counts in the nonfluorescent tumors would be diminished by omitting from the calculations the counts of tumors removed 24 hr. after TTC injection. During the first 24 hr., all tissues appear to be saturated with TTC indiscriminately. Gradients in concentration between tissues become established only after the first day and remain relatively stable thereafter (Table 1).

The retention of TTC in necrotic tissue cannot be definitely established by analyzing the entire tumor be-
attributed to the presence or absence of fluorescence in the tumor, is related to such fluorescence and not to any differences between the groups.

The Ehrlich solid tumor, which grows at a considerably slower rate than the S-37 tumor, was implanted intramuscularly in a group of C57 mice which were subsequently treated by injection with TTC on day 14 and sacrificed on day 19. All tumors showed gross fluorescence. The values for T/H, T/L, and H/L (Table 1) were comparable to those observed in the tissues of animals bearing fluorescent S-37 tumors.

Fluorescence of the tumor in these experiments appears definitely to be related to the age of the tumor. The correlation between fluorescence and tumor age was highly significant: +0.78 (significant correlation: +0.25).

Histology.—Because fluorescence, on gross inspection of the tumors, appeared to be distributed in small, discrete areas, a histologic study was made in an attempt to relate the localization of fluorescence to alterations in cytologic structure. TTC alone did not influence the histologic appearance of the tumors, and no cytologic difference was noted between tumors from the TTC-treated and the control animals.

As expected (29), all 12-day tumors showed a fairly large number of necrotic and hemorrhagic zones. Only the 12-day old tumors of animals treated with TTC showed any yellow fluorescence grossly; the 8-day-old tumors did not.

Under microscopic observation, the India ink particles injected into areas of gross fluorescence were located in a transition zone between well-preserved tumor cells and damaged cells. The intact cells showed a basophilic and homogeneous cytoplasm, and a variety of uniformly hyperchromatic nuclear shapes. Approaching the India ink spots from the viable areas, the cells began to show signs of necrosis. The nuclei became smaller and palely staining, some showing pyknosis, and the cytoplasm became eosinophilic. Odd-shaped particles about the size of a nucleus were apparent which were basophilic and Feulgen-positive, indicating the presence of DNA. Toward the center of the marked area, cell necrosis appeared more advanced.

Under the fluorescence microscope, the sections showed a faint general greenish-blue autofluorescence of the cytoplasm (Fig. 1). This contrasted with a spotty, bright-yellow to gold fluorescence throughout the cytoplasm of tumor cells in the same general area where fluorescence had been seen grossly (Figs. 2, 3, 4). No fluorescence was seen in the interstitial spaces, and the nuclei produced a negative image (Fig. 3).

The slides stained with hematoxylin and eosin demonstrated that the nonfluorescent areas were generally constituted by well-preserved tumor cells; that in zones where the necrosis was very advanced (karyolysis present and cell morphology indistinct), no fluorescence was seen; and that the fluorescent spots all corresponded to groups of tumor cells in more or less advanced states of degeneration (Fig. 2). The cytoplasm of these cells was always eosinophilic and most of the nuclei were small and hyperchromatic. No macrophages were seen in the fluorescent areas.

These histologic studies indicate that the S-37 tumor cell does not display fluorescence until it begins to show cytologic evidence of necrosis.

The data in Table 3 substantiate the conclusion suggested by the previous data; namely, that TTC is retained more avidly in the necrotic areas. Mice bearing S-37 tumors were treated by injection with TTC 9 days after tumor implantation. On the first day thereafter there was an equally high concentration of TTC in the grossly necrotic fluorescent areas and in the viable areas. On subsequent days, the TTC concentration in the viable areas fell precipitously, whereas the TTC in the necrotic areas decreased much more gradually, so that after the first day the concentration of the TTC in the necrotic areas was always considerably higher than in the viable areas (Table 3).

**DISCUSSION**

In view of the results of a previous investigation on a variety of human tumors (33), and on the basis of the present report, which deals with transplantable animal tumors, it seems fairly unequivocal that TTC, as distinguished by its characteristic fluorescence, is retained in greater quantities in those areas of the tumor which are dead or dying. In each of these studies this phenomenon was demonstrated histologically. Our quantitative studies on the concentration of isotopically-labeled TTC in the necrotic areas of the tumor suggest that the localization of fluorescence in necrotic tissue is a real manifestation of a differential distribution of TTC within the tumor and not an artifact due to selective quenching of the fluorescence. This suggests a degree of sequestration of the TTC subsequent to its entry into the necrotic or “neerotizing” areas, either by complexing with some...
The critical determinant of fluorescence is the degree of necrosis in the tumor. The relationship of fluorescence to the age of the tumor probably reflects the fact that age correlates well with the degree of necrosis in S-37 tumors (28). Fluorescence does not appear until the tumor cell begins to show cytologic evidence of necrosis.

The fluorescent tumor may contain relatively more TTC than the nonfluorescent tumor or other tissues because of the deposition of TTC in the necrotic zones of the tumor in addition to the intracellular and extracellular TTC already present. This could explain the increased T/L and T/H ratios for animals bearing fluorescent tumors. TTC concentration alone, however, is probably not the only determinant of fluorescence. Local tissue factors may also play a role. TTC does not fluoresce in the absence of metallic cations (5). Its fluorescence is quenched by most histologic fixatives and by exposure to light (25), and is pH-dependent.

The measurement of the activity of tritiated TTC in tissues, however, appears to be an accurate reflection of the actual concentration of the unaltered drug, since it is not metabolized to any degree (13, 14).

The histologic localization of TTC in S-37 tumors may differ from that in human tumors. Although Vassar et al. (33) found TTC fluorescence almost invariably associated with macrophages in the stroma and debris of the human tumor, macrophages were almost entirely absent from all sections of our S-37 tumors. This appears to disqualify the phagocytic cell as the instigating force in the concentration of TTC in these areas, in spite of the known affinity of the reticuloendothelial system for TTC (2). Whereas Vassar et al. disclaimed intracellular fluorescence in human tumor cells, we encountered definite intracytoplasmic fluorescence in S-37 tumors.

The relationship of cell death to fluorescence deserves further elaboration. The typical zones which fluoresced under ultraviolet light in our histologic study manifested obvious necrosis but had not undergone the most advanced changes of fragmentation and loss of structure. Those cells in the transition zone between the obviously viable and the obviously necrotic tumor were already dead but were not yet morphologically necrotic (fragmented). It was this area which presented the greatest intensity of fluorescence, and it was these cells which demonstrated intracellular localization of the fluorescence.

The morphologic changes which have come to be associated with the death of cells probably appear long after the precise moment of irreversible depression of the life-maintaining processes. Majno et al. (18) noted that the histologic diagnosis of cellular necrosis by standard methods of examination could not be established earlier than the eighth hour after total ischemia in the rat liver at 37°C. Cellular death, defined as "the time when depression of metabolic processes becomes irreversible," occurs after a maximum of 1 hr. of total anoxia (3). One of the earliest signs of cell injury is the loss of selective membrane permeability which might lead to a passive assimilation of free or protein-bound TTC.

It would seem appropriate to attempt to relate the chemical and physical changes which characterize the death and necrosis of cells and tissues to the known propensities of the tetracyclines to form chelates and complexes. The ability of the tetracyclines to form chelates with metal cations has been amply demonstrated (1, 22). In all probability, it is chelation with calcium which results in the incorporation of TTC into newly-formed bone (24), metastatic calcifications (8), and areas of pancreatic fat necrosis (18, 19).

The relationship of calcium to necrosis of tumors is of particular interest in view of the evidence presented here which relates tumor necrosis to TTC content. Shear, reviewing the earlier literature covering human and experimental tumors, concluded that the non-necrotic areas of degenerating tumors contained relatively little calcium whereas the necrotic areas contained excessive amounts of calcium (28). A later study revealed a linear relationship between increasing necrosis and increasing calcium content in epidermal tumors of mice, whereas the calcium content of the completely viable tumor was quite depressed relative to the tissue of origin (30). Thus, a body of circumstantial evidence lends some credence to the hypothesis that the predilection of TTC for devitalized tumor tissue exists by virtue of its ability to chelate calcium.

When we administered Alizarin red S, an acid dye which chelates calcium, systemically to mice bearing S-37 tumors, the dye was visible grossly, after 36 hr., only in the necrotic areas of the tumors and in the bones. Since closely related dyes may localize either in the necrotic (trypan red) or in the viable (trypan blue) areas of tumors (7), this localization of Alizarin red S cannot necessarily be attributed to its calcium-chelating propensity.

A number of other mechanisms have been proposed to explain TTC binding in tumor or devitalized tissue. These include TTC linkages with peptides (17), proteins (as protein-bound complexes) (34), or depolymerized mucopolysaccharides and chondroitin sulfate (9).

To the extensive spectrum of tissue components which may bind TTC must be added the serum lipoproteins, especially the β fraction (16). This affinity is so pronounced (in the presence of calcium ions) that Lacko et al. (16) proposed it as a method for quantitative precipitation of β-lipoproteins. We have demonstrated in our laboratory that TTC is bound to the β-lipoproteins of liver mitochondria in an analogous fashion (3, 23).

Higuchi and Bolton (10) found that DNA formed complexes with TTC more effectively than many other organic compounds tested. Although several observers have attributed an insignificant role to the nucleus in the intracellular localization of TTC (6), they used fluorescence alone as the quantitative parameter. Similarly, the nuclei of TTC-containing tumors do not appear to fluoresce by histologic examination, although radioautographic studies suggest that a significant amount of TTC is localized in the nucleus (2).

Since, in addition to TTC, several chemical species of grossly unrelated structure, such as hematoporphyrins (27) and dyes (trypan red), appear to localize preferentially in necrotic tumor tissue, one might speculate that there is, perhaps, a single basic physiologic defect or physico-
chemical interaction underlying the behavior of all of them. There is probably an element of increased capillary permeability involved in the less viable areas of tumors (7), predisposing to leakage of various native and foreign materials into these areas. However, there undoubtedly exists some sort of mechanism for binding or sequestering these substances from the general circulation and extracellular fluid. What this mechanism might be has been conjectured on the basis of the known pathophysiology of tissue necrosis and the physicochemical properties of the substances which demonstrate this peculiar affinity for nonviable tumor tissue.

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REFERENCES

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Cancer Res 1964;24:1845-1853.

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