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IODODEOXYURIDINE ADMINISTERED TO MICE IS DE-IODINATED AND INCORPORATED INTO DNA PRIMARILY AS THYMIDYLATE

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SUMMARY: Iododeoxyuridylic acid, a structural analog of thymidylic acid, is extensively de-iodinated in vivo by the enzyme thymidylate synthetase. Substantial amounts of the deoxyuridylic acid formed by this process are subsequently methylated and incorporated into DNA as thymidine. As a result, when mice are given tritiated iododeoxyuridine, most of the tritium incorporated into their DNA is present in thymidine rather than in iododeoxyuridine. Some, but not nearly as much, tritium from tritiated bromodeoxyuridine is also incorporated into DNA thymidine.

In the course of a study of mouse cell kinetics in which cell migration and death was determined by means of labeled IdUrd (5-iodo-2'-deoxyuridine) we noticed that IdUrd labeled with ^{125}I behaved differently than IdUrd labeled with ^{3}H. Mice given IdUrd labeled with tritium at carbon atom #6 of the pyrimidine retained the label significantly longer than mice given IdUrd labeled with ^{125}I. This was unexpected since IdUrd is specifically incorporated into cellular DNA and therefore is lost from tissues only upon cell death or migration (1). At first we attributed the more rapid loss of ^{125}I to the greater radiotoxicity of ^{125}I relative to ^{3}H (2). However, when ^{125}I and ^{3}H labeled IdUrd were mixed and then injected into mice the same behavior was observed, ^{125}I was again lost much more rapidly than ^{3}H. Under the conditions of this experiment each proliferating cell would incorporate both labels, and cell death due to ^{125}I would release equivalent amounts of each label. Further experiments have led us

Abbreviations Used: BrdUrd, 5-bromo-2'-deoxyuridine; IdUrd, 5-iodo-2'-deoxyuridine; FdUrd, 5-fluoro-2'-deoxyuridine; BrdUMP, IdUMP and FdUMP represent the 5' phosphates of BrdUrd, IdUrd and FdUrd.

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to the conclusion that this effect is due to de-iodination of an IdUrd metabolite to deoxyuridylic acid which is then converted to dTMP and ultimately incorporated in substantial amounts into DNA. As a result the ^{3}H label from IdUrd appears in the IdUrd and thymidine residues of DNA whereas the ^{125}I label appears in the IdUrd residues of DNA only.

METHODS

Chromatography of nucleoside digest of DNA. Nucleoside digests were chromatographed on a 0.9 x 53.5 cm Sephadex G10 column as described by Braun (3) or on a 0.9 x 22.6 cm carboxymethylcellulose column as described by Aoyagi et al. (4).

Preparation of nucleoside digest of DNA. Organs were homogenized in equal parts 8% ammonium acetate and 88% phenol. The nucleic acid extract was dialyzed to remove phenol, and heated at 37° in 0.3N NaOH for 18 hr to hydrolyze RNA. DNA was precipitated with ethanol, dried under vacuum, and re-suspended in .01M $MgSO_4$, .01M tris-tris HCL pH 7.9, 0.5 mg/ml pancreatic DNase at 25° for 4 hours. The pH was raised to 9.4 with ammonium hydroxide and 0.2 mg/ml venom diesterase was added. The solution was stirred at 25° for 4 hours, 0.1 mg/ml bacterial alkaline phosphatase was added and the solution stirred one hour.

Measurement of radioactivity. ^{125}I activity was measured by means of γ-ray spectrometry with a well type thallium activated sodium iodide crystal. Tritium activity was measured by liquid scintillation. When samples contained both ^{125}I and ^{3}H, it was necessary to subtract out the contribution of ^{125}I to the liquid scintillation measurement in order to determine ^{3}H activity. This was done by determining the ratio of counts by liquid scintillation to counts by γ-ray spectrometry for a sample containing ^{125}I only. The counts in liquid scintillation due to ^{125}I in any sample could then be calculated by multiplying this ratio by the ^{125}I activity determined for the sample by γ-ray spectrometry. All counts were corrected for background and liquid scintillation counts were corrected for quench by means of internal standards.

RESULTS

Mice were injected with a mixture of ^{3}H and ^{125}I labeled IdUrd. DNA was isolated from their intestines, enzymatically digested to nucleosides and chromatographed on Sephadex G10. The ^{125}I and ^{3}H content of each fraction is shown in the lower panel of Figure 1. Nearly all ^{125}I migrated as a single component with a peak at fraction #30. It was identified as IdUrd since it appeared in the same position as an IdUrd sample run separately on the same column. Tritium appeared as three well resolved components with peaks at fractions 12, 17 and 30. They represented 13%, 54% and 33% of the total tritium and were originally identified as deoxycytidine, thymidine and IdUrd respectively on the

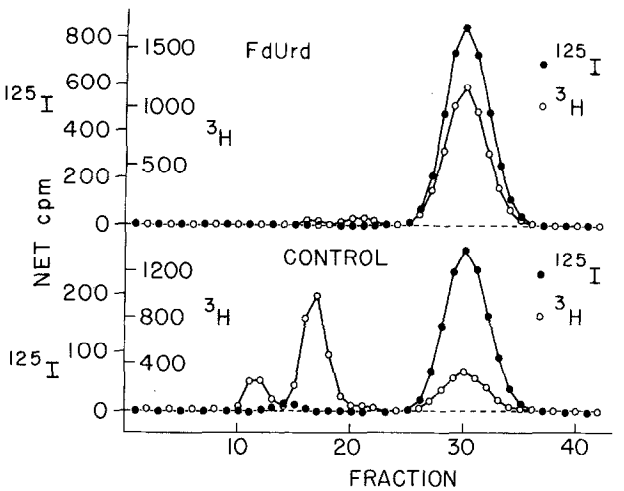

Figure 1. Chromatography on Sephadex G10 of a nucleoside digest of DNA from mice given a mixture of ^{3}H and ^{125}I labeled IdUrd. Mice were given 15 nanomoles FdUrd (upper panel) or saline (lower panel) followed 10 minutes later by a mixture containing 3 μCi each of ^{3}H and ^{125}I labeled IdUrd. One hour later the intestines were removed, DNA was isolated and digested to nucleosides. Open circles represent net cpm due to ^{3}H in each fraction and closed circles represent ^{125}I.

basis of their ultraviolet profile and position in the chromatogram. However oligonucleotides were found to elute at the same position as deoxycytidine. Therefore, if DNA digestion were incomplete, some or all of the first component might represent tritium from IdUrd and thymidine in the oligonucleotides. This was checked by chromatographing on carboxymethylcellulose a nucleoside digest of DNA from mice given (^{3}H)IdUrd. The tritium content and ultraviolet absorbance of each fraction is shown in Figure 2. No tritium was observed in the deoxycytidine (peak at fraction #27), deoxyadenosine (peak at fraction #23) or deoxyguanosine components (peak at fraction #19). The presence of as little as 0.2% of the total eluted tritium in any of these components could have been detected. All tritium appears to elute from this column as a single component only partially in coincidence with thymidine (peak at fraction #15). In fact it represents 2 poorly resolved components - thymidine and IdUrd (peak at fraction #17).

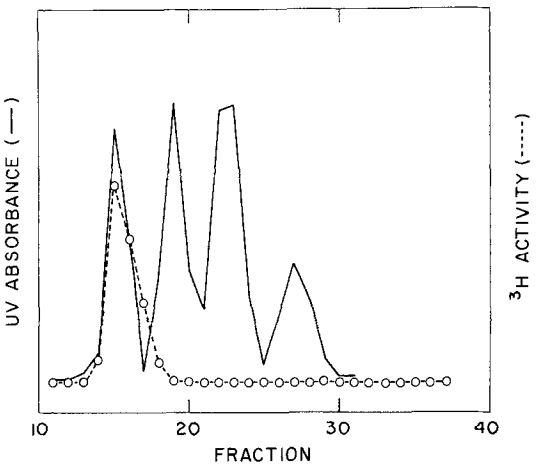

Figure 2. Chromatography on carboxymethylcellulose of a nucleoside digest of DNA from mice given 5 μCi ^{3}H labeled IdUrd. One hour after injection of [^{3}H]IdUrd, into mice, their intestines were removed, DNA was isolated and digested to nucleosides. The solid line represents ultraviolet absorption, the open circles connected by dotted lines represent ^{3}H activity.

The upper panel of Figure 1, which represents a nucleoside digest of DNA from mice pretreated with FdUrd, indicates that FdUrd prevented tritium from appearing in thymidine. All ^{125}I and over 98% of the ^{3}H in this digest appear as IdUrd.

Similar results were obtained with BrdUrd (bromodeoxyuridine). We chromatographed a nucleoside digest of mouse intestine DNA prepared in the same way as the digest shown in the lower panel of Figure 1 except that mice were given 3 μCi tritiated BrdUrd instead of IdUrd. We found that 92% of the tritium appeared as BrdUrd and 8% as thymidine.

The ratio of ^{3}H to ^{125}I in DNA after administration of ^{3}H and ^{125}I labeled IdUrd is affected both by FdUrd and the interval between labeling and removal of tissue. This is shown in Table 1. The results are expressed as the ratio of ^{3}H to ^{125}I in isolated DNA divided by the ratio of ^{3}H to ^{125}I labeled IdUrd in the injection mixture. In the absence of discrimination against incorporation of either isotope into DNA, the isotope ratio in DNA should equal the isotope ratio of IdUrd in the injection mixture. This was nearly the case for DNA from FdUrd treated mice (first column). Significant discrimination against ^{125}I

Table 1. The Ratio of ^{3}H to ^{125}I or ^{14}C in DNA

	$^{3}H/^{125}I$*				$^{3}H/^{14}C$*
Tissue	†FdUrd	Control	Control	Control	Control
	1 hr	1 hr	1 day	8 day	1 hr
Bone marrow††	.90	1.91	-	4.15	1.00
Intestine	.91	2.65	2.89	6.75	.98
Spleen	.94	2.31	2.81	3.62	.98
Thymus	1.01	2.34	2.38	5.48	.99

Mice (10 per group) were injected intravenously with a mixture containing either 3 μCi each of IdUrd labeled with ^{125}I plus IdUrd labeled with ^{3}H at carbon atom #6 of the pyrimidine or with 1 μCi each of thymidine labeled with ^{14}C on carbon atom #2 plus thymidine labeled with ^{3}H on the methyl group. Organs were removed at the times indicated, DNA was isolated and its isotope content measured.

*The isotope ratio in DNA divided by the isotope ratio in IdUrd or thymidine in the injection mixtures which had been chromatographed on Sephadex G10 to remove impurities. In the absence of discrimination the ratios should equal 1.

†These animals received 15 nanomoles FdUrd 10 minutes before injection with labeled IdUrd.

††From femurs.

incorporation was seen, however, in all tissues from the one hour control group (second column), and it increased with time (third and fourth columns).

DISCUSSION

The principal biological effect of FdUrd is the irreversible inactivation of thymidylate synthetase mediated by its metabolite, FdUMP. Wataya and Santi (5) have demonstrated that purified preparations of this enzyme can, in the presence of dithiothreitol, dehalogenate both IdUMP and BrdUMP. Our results suggest that the dehalogenation of IdUMP by thymidylate synthetase is not an in vitro artifact, that it occurs in mice, that it is extensive, and that substantial amounts of the dUMP formed are subsequently converted to dTMP by the same enzyme and incorporated into DNA.

Nearly all the results of this study can be explained on this basis. The original observation that ^{125}I disappears more rapidly than ^{3}H from the tissues

of mice given ^{125}I and ^{3}H labeled IdUrd is a consequence of the large amount of ^{3}H present in DNA thymidine. Tritiated DNA thymidine disappears from tissues more slowly than the ^{125}I label from DNA IdUrd (6). This accounts for the increase in the $^{3}H/^{125}I$ ratio seen with time (Table 1). DNA from the one hour control group contains over twice as much ^{3}H as ^{125}I because ^{3}H enters DNA as thymidine and IdUrd, whereas ^{125}I enters only as IdUrd.

Our results also suggest an additional explanation for the six-fold greater efficiency of thymidine incorporation into DNA compared to IdUrd. This has been attributed to discrimination against IdUrd phosphorylation by thymidine kinases (7) and to the more rapid degradation of IdUrd by the liver (1). We found that prior injection of FdUrd triples the incorporation of ^{125}I labeled IdUrd into intestine and spleen DNA but does not affect the incorporation of ^{3}H from ^{3}H labeled IdUrd. Evidently FdUrd increases IdUrd incorporation by inactivating thymidylate synthetase as well as by decreasing thymidine pool size as proposed by Hughes et al (1). Thymidylate synthetase has no effect on dTMP which corresponds to its effect on IdUMP. This was shown by an experiment in which mice were given a mixture containing 1 μCi each of thymidine labeled with ^{14}C at carbon atom #2 and thymidine labeled with ^{3}H on the methyl group. The data in column 5 of Table 1 show that the isotope ratio in DNA isolated from these mice was the same as in the thymidine mixture injected. Therefore it seems that thymidine is more efficiently incorporated into DNA than is IdUrd principally because dTMP is inert with respect to thymidylate synthetase.

There are two observations for which additional explanations are required. The data in column 1 of Table 1 suggest that in the presence of FdUrd, some discrimination against tritium occurs in the bone marrow, intestine and spleen, but not thymus. Although this effect is small, it appears to be real. It can not be explained by impurities in the injection mixture, differences in quenching or selective loss of ^{3}H during alkaline hydrolysis of RNA. The injection mixture was purified by chromatography on Sephadex G10. Quenching was checked by internal standards. Less than 2% of either ^{3}H or ^{125}I became acid

soluble after alkaline hydrolysis of labeled DNA (18 hr, 37° in 0.3 N NaOH). Furthermore the isotope ratio in the acid soluble fraction was nearly the same as that of DNA. Perhaps discrimination against tritium results from enzyme catalyzed exchange of tritium on carbon atom #6 of IdUrd. The other puzzling observation is the large discrepancy between the apparent rate of dehalogenation of IdUrd relative to BrdUrd found in vitro and in vivo. We found that nearly 20 times as much IdUrd is dehalogenated and incorporated into DNA as thymidine whereas Wataya and Santi (5) found that, in vitro, BrdUrd is dehalogenated somewhat faster than IdUrd.

The effect of thymidylate synthetase on IdUMP in vivo has a number of interesting implications. Clearly, tritiated IdUrd is not as suitable for cell kinetics as ^{125}I labeled IdUrd, nor is it a reliable means of determining the amount of IdUrd incorporated into DNA unless FdUrd is present to prevent its incorporation as thymidine. Also, some of the biological effects of IdUrd may result from the significant amounts of intracellular deoxyuridylic acid formed. Deoxyuridylic acid can be phosphorylated and incorporated into DNA. It is subsequently removed (8) but its temporary presence or the repair processes involved in its removal might account for part of the mutagenic or viral induction effects of IdUrd.

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