

## DOUBLE LABELING WITH [<sup>3</sup>H]THYMIDINE AND [<sup>125</sup>I]IODODEOXYURIDINE AS A METHOD FOR DETERMINING THE FATE OF INJECTED DNA AND CELLS IN VIVO

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The present experiments illustrate the application of a new and convenient method for measuring the breakdown and reincorporation of nucleosides from exogenous DNA. Many experiments have been reported indicating that exogenous DNA preparations can be taken up by mammalian cells both in vitro and in vivo (see references 1-3). However, this material is subject to hydrolysis by enzymes in the blood stream and tissues (cf. references 2 and 4). If the DNA has been labeled with, for example, [<sup>3</sup>H]thymidine (TdR), much of the labeled TdR resulting from DNA hydrolysis would be reutilized by the tissues for the synthesis of new DNA (2, 4, 5). Consequently, the presence of [<sup>3</sup>H]TdR in tissue DNA would not be distinguished between breakdown products and intact polynucleotide chains that had been incorporated from the exogenous DNA.

In the present study, we have utilized a double label with [<sup>3</sup>H]TdR and the thymidine analogue [<sup>125</sup>I]iododeoxyuridine (IUdR). As long as the polynucleotide chains remain intact, the ratio of <sup>3</sup>H/<sup>125</sup>I label in the DNA fraction should be unaltered. However, if the polynucleotide chains are hydrolyzed to nucleosides, the labeled TdR should be reutilized more efficiently than the labeled IUdR (6). The discrimination between these precursors does not occur at the cellular membrane but during the intracellular utilization

of the deoxyribonucleosides.<sup>1</sup> Thus, if the exogenous DNA is taken up by pinocytosis or phagocytosis (cf. reference 7), and if the DNA is hydrolyzed and the breakdown products are released, it is to be expected that salvage of the nucleosides even within the phagocytizing cell would be accompanied by a discrimination in favor of TdR. Consequently, the <sup>3</sup>H/<sup>125</sup>I quotient in the DNA fraction of the tissues should increase considerably. The present experiments demonstrate the applicability of this technique.

### MATERIALS AND METHODS

Doubly labeled preparations of DNA were obtained from female NMRI albino mice, approximately 2 mo old and weighing approximately 27 g, that were placed on drinking water containing 0.1% sodium iodide, commencing 2 days before injection to minimize reutilization of radioactive iodide liberated during the catabolism of IUdR (8). Groups of 12-20 mice were injected intraperitoneally with 5  $\mu$ Ci [*methyl*-<sup>3</sup>H]TdR (Amersham/Searle Corp., Arlington Heights, Ill.; sp act 22 Ci/mmol) plus 10  $\mu$ Ci [<sup>125</sup>I]IUdR (Amersham/Searle; sp act 2-4 Ci/mmol) per mouse. The injections were repeated five times at intervals of 1 h, and the animals were killed 2 h after the last injection. Nucleoprotein was purified from the

<sup>1</sup> Myers, D. K., and L. E. Feinendegen. Incorporation of thymidine and iododeoxyuridine into the DNA of mouse tissue. *Canad. J. Physiol. Pharmacol.* In press.

washed small intestine of these animals by the method of Maruschige and Bonner (9) up to the stage of centrifugation through sucrose solution. Intact nuclei were prepared from the spleen by homogenizing in saline at 0°C and were washed but not further purified. To prepare intact thymus cells, of which approximately 10% would be labeled (10), the thymus glands were minced finely in 0.9% NaCl at 0°C, the resulting suspension was filtered through several layers of surgical gauze and then centrifuged for 10 min at low speed at 0°C, and the cells were resuspended in 0.9% NaCl at 0°C before injection. More than 95% of the radioactivity in these preparations was associated with the DNA.

Experiments were also done with a line of human kidney T cells (HKT cells) that had been grown in tissue culture for 26 h at 37°C in Eagle's basal medium with Earle's salts plus 10% (vol/vol) newborn calf serum, 0.2  $\mu$ Ci [ $^{125}$ I]IUdR/ml, 1  $\mu$ Ci [methyl- $^3$ H]TdR/ml,  $10^{-6}$  M IUdR,  $10^{-6}$  M TdR, and  $10^{-5}$  M deoxycytidine. These cells were suspended by trypsinization followed by repeated washing with fresh medium and saline and were finally brought into suspension in 0.9% NaCl solution. The three HKT preparations used for injection contained an average of  $1.4 \times 10^4$  dpm  $^3$ H/ml and  $1.7 \times 10^6$  cells/ml of cell suspension, 98% or more of their radioactivity being in the DNA fraction.

The doubly labeled DNA preparations were injected intravenously (0.2 ml), intraperitoneally (0.5 ml), or subcutaneously (0.5 ml) into groups of 3–5 female NMR1 mice, approximately 8 wk of age, that had been placed on drinking water containing 0.1% NaI for 2 days before the injection (6, 9). Unless otherwise stated, the recipient animals were killed 24 h after injection. The organs were taken out and placed immediately in ice-cold 0.5 M perchloric acid. The bone marrow from one femur was also removed in some experiments. The tissues were homogenized, centrifuged at 0°C, and the acid-soluble

extract was removed. After the precipitate was washed twice with cold perchloric acid, lipid was extracted with ethanol and RNA with 0.5 M perchloric acid at 4°C for 22 h. In most tissues the lipid and RNA fractions contain very little radioactivity. The residue was washed again with cold perchloric acid, and DNA was extracted with 0.5 M perchloric acid at 80°C for 1 h.

$^{125}$ I in the DNA extracts was measured in a Packard Autogamma counter (Packard Instrument Co., Inc., Downers Grove, Ill.); liquid scintillation fluid was then added, and  $^3$ H in the same samples was subsequently measured in a Packard liquid scintillation counter with an external standard for estimation of quenching.<sup>1</sup>  $^3$ H activity was calculated by means of standard quench curves. Recoveries were expressed as a percentage of the total amount of each isotope injected into the whole animal.

## RESULTS

Table I summarizes the data obtained 1 day after injection of doubly labeled nucleoprotein or nuclei. Both preparations yielded similar results. The recoveries of labeled IUdR were 4- to 10-fold smaller than those of TdR. Moreover, the  $^3$ H/ $^{125}$ I ratios were consistently higher in duodenum and mesenteric lymph node, for example, than in thymus and bone marrow. These ratios are very similar to those obtained after simultaneous injection of the two nucleosides in free form (6 and footnote 1), indicating that most of the incorporation of label from the exogenous nucleoprotein or nuclei involved hydrolysis of these preparations to the nucleoside level and subsequent reutilization of these products. The results with nuclei were not altered when these suspensions were heated at

TABLE I  
Recovery of Labeled TdR and IUdR from the DNA of Six Mouse Tissues 1 day after Intraperitoneal Injection of Doubly Labeled Nucleoprotein or Nuclei

Tissue	Intestinal nucleoprotein			Spleen nuclei		
	[ $^3$ H]TdR	[ $^{125}$ I]IUdR	$^3$ H/ $^{125}$ I	[ $^3$ H]TdR	[ $^{125}$ I]IUdR	$^3$ H/ $^{125}$ I
Bone marrow (one femur)	0.04 $\pm$ 0.02*	0.01 $\pm$ 0.007*	4	—	—	—
Mesenteric lymph node	0.15 $\pm$ 0.02	0.02 $\pm$ 0.01	8	—	—	—
Thymus	0.35 $\pm$ 0.1	0.07 $\pm$ 0.03	5	0.38 $\pm$ 0.1	0.08 $\pm$ 0.03	5
Spleen	1.7 $\pm$ 0.7	0.19 $\pm$ 0.1	9	1.8 $\pm$ 0.6	0.3 $\pm$ 0.1	6
Duodenum	2.6 $\pm$ 0.6	0.25 $\pm$ 0.1	10	3.4 $\pm$ 0.9	0.4 $\pm$ 0.1	9
Liver	0.54 $\pm$ 0.3	0.11 $\pm$ 0.1	5	0.40 $\pm$ 0.1	0.06 $\pm$ 0.03	7

\* The recoveries of [ $^3$ H]TdR and of [ $^{125}$ I]IUdR are expressed as percent of the injected activities; the data represent the mean  $\pm$  SD of the individual measurements on each animal. 13 animals were used for the marrow and lymph node and 24 for the other four tissues after injection of nucleoprotein; 22 animals were used for intraperitoneal injection of nuclei.

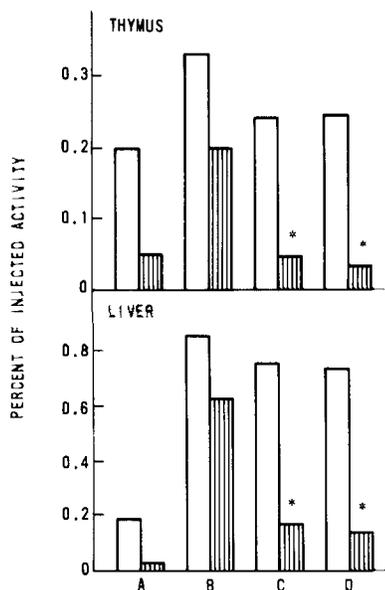


FIGURE 1 Recovery of labeled TdR (open bars) and IUDR (shaded bars) from the DNA fraction of thymus and liver 1 day after injection of doubly labeled thymus cells. Each bar represents an average from the data obtained with 10 mice; the standard deviations ranged from 28 to 85% of the mean values. The four groups of mice are designated on the abscissa as follows: (A) 4  $\mu$ Ci [ $^3$ H]TdR plus 2  $\mu$ Ci [ $^{125}$ I]IUDR injected intravenously for comparative purposes, (B) normal thymus cells labeled with [ $^3$ H]TdR and [ $^{125}$ I]IUDR injected intraperitoneally, (C) the same thymus cells as in B were heated at 60°C for 20 min before intraperitoneal injection, (D) the same thymus cells as in B were exposed to 10,000-R  $\gamma$ -radiation at 0°C before intraperitoneal injection. Values in C and D that were significantly different from the corresponding control in group B are indicated with an asterisk.

60°C for 20 min or exposed to 10,000-R  $\gamma$ -radiation before intraperitoneal injection.

The amounts of [ $^3$ H]TdR and [ $^{125}$ I]IUDR recovered from the DNA fraction of the tissues were similar whether the nucleoprotein and nuclei were injected intravenously, subcutaneously, or intraperitoneally. Similar ratios, but lower recoveries, were also observed after injection of doubly labeled nucleoprotein into mice that were exposed to 800-R whole-body X-radiation either 1 or 24 h before injection.

When intact mouse thymus cells were injected into normal mice, the [ $^3$ H]TdR/[ $^{125}$ I]IUDR quotient in the DNA fraction of the tissues 1 day later was 1.5–2 times higher than that in the original cell suspension (Fig. 1). A comparison of these quo-

tients with those obtained after injection of nucleoprotein, nuclei (Table 1), or free TdR plus IUDR (Fig. 1) suggests that 60–90% of the exogenous DNA was still intact in the tissues 1 day after intravenous injection of the thymus cells. If the cells were killed either by heating at 60°C for 20 min or by exposure to 10,000-R  $\gamma$ -radiation before injection, the recovery of [ $^{125}$ I]IUDR in the DNA fraction was greatly reduced (Fig. 1) and the  $^3$ H/ $^{125}$ I quotients approached those observed with free TdR plus IUDR.

In contrast to the mouse thymus cells, the DNA

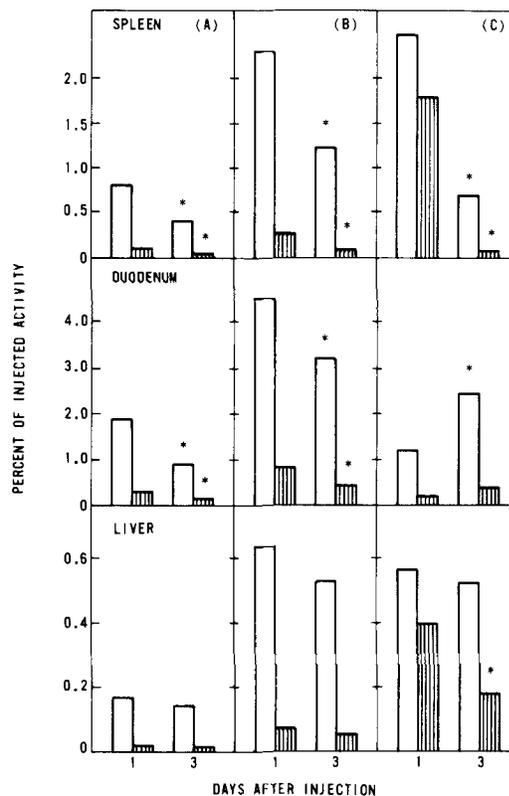


FIGURE 2 Recovery of labeled TdR (open bars) and IUDR (shaded bars) from the DNA fraction of spleen, duodenum, and liver 1 day and 3 days after injection of doubly labeled HKT cells. Each bar represents an average from the data obtained with five mice; the standard deviations ranged from 24 to 50% of the mean values. The three groups of mice are designated from left to right as follows: (A) 4  $\mu$ Ci [ $^3$ H]TdR plus 2  $\mu$ Ci [ $^{125}$ I]IUDR injected intravenously for comparative purposes, (B) HKT cells labeled with [ $^3$ H]TdR and [ $^{125}$ I]IUDR injected intravenously, (C) the same HKT cells as in B injected intraperitoneally. Values at 3 days that were significantly different from the corresponding values at 1 day are indicated with an asterisk.

of HKT cells did not usually appear to survive for 1 day after injection into normal mice. In duodenum (Fig. 2), lymph node, bone marrow, and thymus, the recovery of [ $^3\text{H}$ ]TdR was about sixfold greater than that of [ $^{125}\text{I}$ ]IUdR in the DNA fraction of the tissues. These results parallel those obtained after injection of nucleoprotein, nuclei (Table I), or free TdR plus IUdR (Figs. 1 and 2). In all of the above cases, the  $^3\text{H}/^{125}\text{I}$  quotients were not appreciably altered when the HKT cells were exposed to 60°C for 20 min or to 10,000-R  $\gamma$ -radiation before injection.

Exceptions were noted with spleen and liver after intraperitoneal, but not after intravenous, injection of normal HKT cells. The [ $^3\text{H}$ ]TdR/[ $^{125}\text{I}$ ]IUdR quotient remained close to that of the injected cells in these two instances (Fig. 2). Presumably the HKT cells, which normally adhere to a solid surface during growth, were still intact and had attached themselves to the surface of the liver and spleen, though not of the duodenum or mesenteric lymph node in the same peritoneal cavity. However, most of the doubly labeled DNA of the HKT cells associated with the spleen and liver did not survive for 3 days after intraperitoneal injection into normal mice. By this time, the  $^3\text{H}/^{125}\text{I}$  quotients were approaching those found after intravenous injection of HKT cells or of free TdR plus IUdR (Fig. 2).

## DISCUSSION

The present experiments demonstrate the validity of double labeling with [ $^3\text{H}$ ]TdR and [ $^{125}\text{I}$ ]IUdR as a method for following the survival of exogenous DNA in the animal. The reutilization of [ $^3\text{H}$ ]TdR from labeled exogenous nucleoprotein, nuclei (Table I), or intact cells (Figs. 1 and 2) is a very efficient process in the mouse, and even more efficient than the uptake of injected free TdR (Figs. 1 and 2). By combining the TdR label with that of IUdR, which is less efficiently reutilized (6), one can distinguish clearly between intact exogenous polynucleotide chains in the tissues and *de novo* synthesis from breakdown products.

The double-labeling procedure is most useful for measuring the survival of the major portion of exogenous DNA. If, for example, we assume an average  $^3\text{H}/^{125}\text{I}$  quotient of 6 for *de novo* DNA synthesis from free nucleosides in the mouse (6), then 20% breakdown and reutilization of the exogenous DNA in the tissues would yield a net  $^3\text{H}/^{125}\text{I}$  quotient of 2, which is readily distinguished experimentally from the value of 6 for 0%

survival of polynucleotide chains from the exogenous DNA. However, 20% survival of the exogenous DNA with 80% breakdown and reutilization would yield a net quotient of 5 which is not readily distinguished from the value of 6. Thus, the present experiments do not indicate, for example, whether or not small amounts of intact polynucleotide chains from the exogenous DNA might be transferred into tissue cells and whether or not small numbers of the doubly labeled exogenous cells might survive in the animal. For this purpose other experimental techniques are required.

An advantage of the double-labeling procedure is that the fate of polynucleotide chains can be followed even when the doubly labeled DNA preparations or cells do not themselves necessarily remain intact. Thus, this method provides a useful and convenient adjunct to other techniques that can be used to follow the fate of exogenous DNA.

## SUMMARY

Mice were injected intravenously and intraperitoneally with preparations of intestinal nucleoprotein, spleen nuclei, mouse thymus cells, or human kidney T cells whose DNA had been labeled with both [ $^3\text{H}$ ]thymidine (TdR) and [ $^{125}\text{I}$ ]iododeoxyuridine (IUdR). Since free TdR is reutilized more efficiently than free IUdR produced by enzymic hydrolysis of the exogenous DNA, the ratio of [ $^3\text{H}$ ]TdR/[ $^{125}\text{I}$ ]IUdR in the DNA fraction of the tissues of the recipient mice provides a measure of the amount of intact exogenous DNA in the tissue. In most instances, the doubly labeled exogenous DNA was almost completely hydrolyzed within 1 day after injection, but survival of the DNA from whole cells could be demonstrated in some cases.

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