Radioactive Triiodothyronine As a Tumor Cell Label for Kinetic Studies

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Summary
Triiodothyronine tagged with 131I has been used as a label for Walker 256 tumor cells. This hormone attaches rapidly to the surface of tumor cells, and after 60 min less than 5% of the radioactivity was removed by each washing. Radioactive iodine administered as triiodothyronine remained in the hormone during experiments several hr in duration. A considerable portion of the hormone penetrated into cells within 1 hr as indicated by radioautographs made by tritiated triiodothyronine.

The use of this technique for labeling tumor cells permits studies of the kinetics of embolic tumor cell passage through the pulmonary blood vessels of rats.

Introduction
Malignant tumors commonly spread by development of metastatic deposits in the lungs. Most of these metastases are thought to be caused by the lodgment and growth of embolic tumor cells carried in the blood from the primary tumor to the lungs. Understanding of the mechanism by which tumor cells are trapped and grow or fail to grow in the lungs is limited. Studies of the kinetics of emboli passage through the pulmonary vasculature have obvious importance for better understanding of the process of metastasis.

In pursuing kinetic studies of tumor cell passage through the lungs, it has become desirable to develop a long lasting method for tagging tumor cells. If an appropriate tag for tumor cells can be developed, it may become possible to follow the arrest of tumor cells in pulmonary vasculature and their release into the blood stream in experiments of long duration. Earlier efforts to develop such a tag have not been entirely satisfactory for various reasons. Tagging with radioactive 32P, used by Ambrus and his co-workers (1), is complicated by the fact that phosphorus rapidly enters into the metabolism of the cells and the surrounding medium. Raker and Skinner (7) employed 65K as a cell label in previous experiments. This label is useful for experiments of short duration, but because potassium exchanges rapidly with the surrounding medium, this tagging of the tumor cells is not stable enough to permit experiments of long duration. Even in experimental studies of short duration an inconvenient correction factor for leakage of tag from cells must be introduced.

In a search for a more suitable radioactive tag for tumor cells, the use of a naturally occurring hormone seemed worth investigation. Triiodothyronine was selected because of its general application in cellular metabolism (3, 4) and because a convenient label with 131I was available.

Materials and Methods

The tumor cells used in this study were Walker 256 cells carried in the ascites form in Sprague-Dawley white rats.2 Injection of 1000–2000 cells was sufficient to transplant the tumor consistently. Cells were harvested after 5–7 days growth. They were washed and resuspended in 10–20 ml of Ringer's solution, and radioactive triiodothyronine3 was added to the mixture. Total triiodothyronine varied from 0.1 to 3.5 µg/50,000 cells. Specific activity varied from 5.75 µc/0.1 µg to 5.75 µc/3.5 µg. The mixture was incubated at 37°C for varying periods of time up to 4 hr. Subsequent washings were carried out with Ringer's solution. Appropriate 1.0-ml aliquots of cell suspensions and wash fluids were counted in a scintillation well counter.

Attempts were made to locate the radioactivity attached to tumor cells, both to determine whether it was still in the form of triiodothyronine and to locate radioactivity within or on the surface of the cells. Tumor cells tagged with radioactive triiodothyronine in the usual fashion for 3 hr were mechanically fragmented as described by Stanbury (8). Repeated paper chromatographic studies of all of the fractions of the cells were carried out and analyzed utilizing a butanol-acetic acid system and standard solutions of iodide and organic iodine compounds for comparison with the cell fractions. The location of radioactivity in the chromatograms was determined.

After fragmentation of tagged tumor cells, standard ultracentrifugation techniques were used to separate cell fractions. The radioactivity of all cell fractions was measured.

Unlabeled tumor cells were fragmented and separated into

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2 The Walker 256 tumor cells were originally obtained by courtesy of Mr. I. Wodinsky of A. D. Little Co., Cambridge, Mass.

3 Triomet-131 (lithryronine-131) commercially available from Abbott Laboratories was used. In this preparation l-triiodothyronine is contained in 50% propylene glycol. Of the 131I present in the preparation, 98% is estimated to be in the form of l-triiodothyronine and 2% in the form of inorganic iodide.

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Triiodothyronine-\textsuperscript{131}I As Tumor Cell Label

their fractions by ultracentrifugation. The fractions were then exposed to radioactive triiodothyronine to determine their capacity to be labeled by free hormone.

Triiodothyronine tagged with \textsuperscript{131}I was used to label tumor cells, and radioautographs were prepared as described by Vickery (9).

Tumor cells labeled with triiodothyronine-\textsuperscript{131}I were injected into the abdomen of Sprague-Dawley rats to establish the viability of cells treated in this fashion. The usual transplantation doses of 1000–2000 cells were used.

In order better to localize the site of the hormone tagged to tumor cells, tritiated triiodothyronine was prepared by heating triiodothyronine in the presence of tritiated water and acetic anhydride.\footnote{The final preparation was carried out by the New England Nuclear Co. after the method was suggested by Dr. Robert Loftfield and pilot preparations in our laboratory had been successful.} Five mg of triiodothyronine were dissolved in 0.2 ml of acetic acid-acetic anhydride (1:1) containing 10 c of tritiated water and 10 mg of prereduced platinum catalyst. The reaction mixture was heated at 100°C overnight with magnetic stirring. Volatiles were removed in vacuo. The catalyst was removed by filtration, 1 ml of 6 \textit{n} hydrochloric acid being used. The solution was heated at 100°C for 1 hr and the solvent was then removed in vacuo. The remaining triitated triiodothyronine was dissolved in 2 ml of 1 \textit{n} hydrochloric acid. Total activity of the preparation was 1.2 mc. Specific activity was 0.24 mc/mg. Radiochromatographic tracings of the triitated hormone were prepared and compared with the chromatographic distribution of standard iodide and organic iodine compounds.

Tritiated triiodothyronine prepared in this way was used to tag tumor cells for 3 hr in the usual fashion. Radioautographs were prepared from these tumor cells embedded and sectioned in order to show the distribution of radioactivity within and on the cells.

Results and Discussion

Triiodothyronine absorption by RBC is commonly used as an indirect measure of thyroid function (6). The hormone concentrates preferentially in certain solid tumors and leukocytes (2, 5). The mechanism and site of attachment of triiodothyronine to erythrocytes and tumor cells is unknown. Triiodothyronine attaches itself rapidly to glass and polyethylene surfaces. The use of silicone effectively prevents this surface action. This evidence suggests that the initial attachment of triiodothyronine to tumor cells may involve a surface phenomenon of comparable nature.

As shown in Chart 1, triiodothyronine tagged with \textsuperscript{131}I was rapidly taken up by tumor cells so that a maximum of 75\% of available hormone was tagged to cells within approximately 3 hrof incubation at 37°C in Ringer's solution.

The binding of hormone to cells became progressively stronger with the passage of time, and after 30 min the binding of triiodothyronine to tumor cells was quite stable. Chart 2 demonstrates that after 30 min less than 10\% of the radioactivity tagged to tumor cells was removed by 1 washing with Ringer's solution and that successive washings removed progressively less and less of the radioactivity tagged to the cells. Prolonging the exposure time beyond 60 min did not increase the strength of labeling affinity. These facts may indicate either a time-related physicochemical binding process or that triiodothyronine first is bound loosely to cell surfaces and later enters the cell to participate in intracellular processes.

By varying the specific activity of the triiodothyronine to which cells were exposed, no demonstrable threshold for the uptake of the hormone by tumor cells was found. Amounts of nonradioactive triiodothyronine in excess of those usually used failed to saturate the cells; the uptake of labeled hormone was not blocked.
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TABLE 1
RADIOCHROMATOGRAPHIC DISTRIBUTION OF 131I IN FRACTIONS OF TUMOR CELLS LABELED WITH THIODOOTHYRONINE-131I

<table>
<thead>
<tr>
<th>RADIOCHROMATOGRAPHIC SECTIONS</th>
<th>RADIOACTIVITY (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole homogenate</td>
</tr>
<tr>
<td></td>
<td>Nuclear fraction</td>
</tr>
<tr>
<td>Origin</td>
<td>14</td>
</tr>
<tr>
<td>Unknown No. 1</td>
<td>29</td>
</tr>
<tr>
<td>Iodide</td>
<td>41</td>
</tr>
<tr>
<td>Unknown No. 2</td>
<td>34</td>
</tr>
<tr>
<td>MIT</td>
<td>20</td>
</tr>
<tr>
<td>DIT</td>
<td>57</td>
</tr>
<tr>
<td>T3-T4</td>
<td>27</td>
</tr>
<tr>
<td>Solvent front</td>
<td>35</td>
</tr>
</tbody>
</table>

*MIT, monoiodotyrosine; DIT, diiodotyrosine; T3, triiodothyronine; T4, thyroxine.

TABLE 2
CHANGE IN DISTRIBUTION OF 131I WITH INCREASED Fragmentation of Cells Labeled with Thiiodothronine-131I

<table>
<thead>
<tr>
<th>STATE OF CELL FRAGMENTATION</th>
<th>131I (% of cpm of original homogenate)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole homogenate</td>
</tr>
<tr>
<td></td>
<td>Nuclear fraction</td>
</tr>
<tr>
<td>Nuclei intact</td>
<td>100</td>
</tr>
<tr>
<td>Nuclei broken</td>
<td>100</td>
</tr>
</tbody>
</table>

The treatment of tumor cells by amounts of 131I triiodothyronine used in this study did not impair viability. Doses of labeled cells required for successful transplantation to the abdomen of a new host were no larger than the numbers of unlabeled cells usually required.

Studies of the distribution of 131I attached to tumor cells showed that the triiodothyronine molecule remained largely intact while on or in the cells for intervals up to several hr. Paper chromatographic separation showed that in every instance the vast majority of the radioactivity persisted in the triiodothyronine-thyroxin fraction, although there were scattered traces of radioactivity in the inorganic iodide, monoiodotyrosine, and diiodotyrosine fractions. Table 1 shows a sample distribution of radioactivity in cell fractions as determined by radiochromatographic sections.

Considerable variation was noted in the distribution of 131I among the various ultracentrifugation fractions, depending upon the effectiveness with which the labeled cells were broken up. If the nuclear fraction showed microscopic integrity of nuclear membranes, the largest concentration of radioactivity remained in this fraction. If fragmentation was carried to the point where the nuclei were broken and no recognizable nuclei could be seen by microscopic examination, the mitochondrial fraction then contained 40-55% of the radioactivity and increased amounts of radioactivity appeared in the microsomal fraction and in the soluble fraction. Table 2 shows the change in distribution of radioactivity in ultracentrifuged cell fractions with increasing fragmentation. Comparable distributions of radioactivity were obtained by fragmenting the cells mechanically and exposing the fractions to 131I-labeled triiodothyronine. These findings suggest that triiodothyronine becomes physically attached to or within the cells and that it redistributes itself among cell fragments and particles when the cells are disrupted.

The radioautographs of tumor cells labeled with 131I-triiodothyronine demonstrated clearly the presence of radioactivity associated with the cells; but the resolution afforded by these studies utilizing 131I was not sufficient to distinguish between location of the hormone on the cell surface or within the cells. The preparation of tritiated triiodothyronine was undertaken in order to obtain radioautographs of better resolution. The radiochromatographic tracing of Chart 3 demonstrates that tritium in this preparation was actually incorporated into the triiodothyronine molecule.

The radioautographs of tumor cells labeled with tritiated triiodothyronine are shown in Fig. 1. They show random distribution of radioactivity over the sectioned planes of labeled cells rather than limitation to the region of the cell membrane. This finding is evidence that tritiated triiodothyronine did not merely remain attached to the cell membrane indefinitely; after sufficient exposure time some of the hormone had entered the intact cells.

Conclusions
These data are interpreted to mean that the mechanism of attachment of triiodothyronine to cells is not simple; the initial
attachment may be a coating of the cell surfaces, but after 60 min the hormone has entered the cells. Though the hormone penetrates the cell membrane and can be removed from intact cells only with difficulty, disruption of the cells permits redistribution of the hormone among fragments.

Radioactive iodine-tagged triiodothyronine is a promising means of labeling tumor cells for kinetic experiments. With cells tagged by this hormone, it is possible to study the kinetics of the passage of tumor emboli through the vasculature of the lung in experiments lasting as long as 30 min. A technic for isolation of the recipient animal's heart and lungs is utilized (7). The influences of different environmental conditions and agents upon the effectiveness of the lung for trapping tumor emboli are under investigation.

Acknowledgments

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References

FIG. 1.—Radioautograph of paraffin section of ascites tumor cells labeled with tritiated triiodothyronine. Cells exposed to hormone 120 min. Radioautograph exposure time, 27 days. × 1600.
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