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RNA Metabolism in Sponge Granuloma: Decreased [^{14}C]RNA Synthesis Due to Enzymatic Conversion of Precursor Nucleosides (37779)

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Granulation tissue may be produced in the rabbit by subcutaneous implantation of polyvinyl sponge squares. During the first 2 weeks after implantation, a proliferative fibroblastic reaction occurs in the sponge capsule. This connective tissue grows into the sponge during the next 4 weeks. During the first 2 weeks, the sponge contains only fluid and inflammatory cells, and it is not useful for study of granulation tissue metabolism (1). We have used the connective tissue capsule surrounding the sponge for studies of RNA synthesis *in vitro*. When this tissue is incubated in the presence of radioactive nucleosides, these markers are incorporated into CTAB[2]-precipitable RNA. We have found that the addition of rabbit sponge inflammatory fluid or blood to the incubation mixture results in a marked decrease of incorporation of uridine and cytidine.

The studies presented describe these effects on nucleoside incorporation rates. The probable mechanisms of these effects are also discussed. Failure to detect these effects would have led to erroneous interpretation of the data.

Materials and Methods. [^{14}C]adenosine (55 mCi/mmole) and [^{14}C]uridine (55 mCi/mmole) were products of the New England Nuclear Company. [^{14}C]Cytidine (42 mCi/mmole) and [^{14}C]uracil (55 mCi/mmole) were purchased from Schwartz Bio Research Company. Pronase (nuclease free) was obtained from Cal Biochem.

New Zealand albino rabbits were kept in separate cages and maintained on an *ad libitum* diet of water and Purina rabbit chow checkers.

Two unimplanted rabbits were killed by air embolism, and they served as a source of unstimulated connective tissue. This was obtained by dissection from the dorsal subcutaneous fascial cleft (zero day tissue).

Five rabbits were each implanted with 10 polyvinyl sponge squares (2 × 2 × 0.5 cm) in the dorsal subcutaneous region. These rabbits were sacrificed by air embolism 4, 5, 9, 19, and 22 days after implantation, and the capsular granulation tissue removed and used as 0.2- to 0.6-g aliquots. This tissue was added immediately to tubes (8 × 45 mm) containing 1 ml of solution D[3] (2, 3), 0.1 ml of glucose (10 mg/ml), and 0.1 ml (1 μCi) of [^{14}C]nucleoside.

These tubes were covered with a double layer of gauze and placed in a Dubnoff incubator in a 37° water bath. The atmosphere was maintained at 95% O_2 and 5% CO_2 by continuous flow, and the tubes were gently shaken at a rate of 120/min. Tissue aliquots were devitalized at zero time by heating to 60° for 2 min. Incubated killed

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[2] Cetyltrimethylammonium bromide.

[3] Solution D when diluted to 1.2 ml as described contains in meq/liter: 133 Na, 106 Cl, 4.4 Ca, 4.8 H_2PO_4, 2.39 Mg, 3.2 citrate, 27.6 HCO_3, 4.8 K. This mixture maintains a pH of 7.4 in a 5% CO_2 atmosphere.

tissue and tissue kept at 4° in air were used as additional controls. Each experimental and control group consisted of eight tissue aliquots. Experimental groups were incubated after replacement of 0.5 ml of solution D with an equal volume of sponge fluid, subfractions of this fluid, plasma, blood, or hemolysate. All work was done with sterile technique.

Inflammatory fluid was obtained from sponges by manual compression and blood by cardiac puncture. Sponge fluid was fractionated by centrifugation at 4° in an International centrifuge at 800*g* for 30 min. Supernatant sponge fluid was ultrafiltered using a UM-10 filter (Aminico), and the filtrate collected. Each blood sample was immediately mixed with heparin to a final concentration of 40 U/ml and was centrifuged as above. The plasma was removed, and used to replace solution D.

Incubation times of 10–180 min were used in different experiments. At the end of the incubation interval, each incubation fluid was cultured. Each tissue aliquot was blotted and frozen in liquid N_2. These tissues were then pulverized and the frozen powder suspended in 22.5 ml of 1 *M* NaCl, 0.02 *M* Tris buffer, pH 7.4, containing 5 mg of pronase and 5.6 mg of $CaCl_2$. This mixture was heated to 60° for 20 min, cooled, and an additional 5 mg of pronase was then added. This solution was reincubated at 37° for 1 hr and filtered through a GF/C glass fiber filter (Whatman). Each filtrate was diluted 1:4 with water, and an aliquot used for quantitative precipitation of RNA with cetyltrimethylammonium bromide on a GF/C filter (4). These filters were solubilized in 1 ml of NCS (Nuclear Chicago) by heating to 50° for 2 hr. After addition of a BBS-3 Omnifluor mixture, these samples were counted in a Packard Tri-Carb liquid scintillation spectrometer. [^{14}C]Toluene was used as an internal standard. DNA was determined on the pronase digest by the method of Ceriotti (5).

Incubation fluid was deproteinized by addition of an equal volume of 10% trichloroacetic acid (TCA). The TCA was removed from the supernatant fluid by three serial extractions with diethyl ether. Ascending chromatography on thin-layer cellulose (tlc) or Whatmann 3MM paper (P) was performed with these samples. The following chromatographic systems were used for analysis of incubation fluids:

1. [^{14}C]Uridine-containing samples—isobutyric acid:water:concd NH_4OH (66:33:1) (P).

2. [^{14}C]Cytidine-containing samples—*n*-butanol:water:formic acid (77:13:10) (P). [^{14}C]Adenosine-containing samples were deproteinized in 0.8 *M* perchloric acid and absorbed on activated Norit, eluted, lyophilized, and chromatographed in *three different systems* for separation and identification of [^{14}C]adenosine derivatives. The systems used were:

1. Isobutyric acid:water:concd NH_4OH (66:33:1) (P).

2. *N*-butanol:acetic acid:water (50:25:25) (tlc).

3. Water, pH adjusted to 10 with NH_4OH (tlc).

Standard compounds were run in parallel with unknown radioactive samples. The areas in the radioactive channels corresponding to the R_f value of the standards were cut out and counted as above. Results were expressed as the percentage of the total counts chromatographed per channel recovered in the areas under the peaks of each channel.

Results. The incorporation rate of [^{14}C] uridine into the connective tissue RNA of zero day tissue was studied in a variety of incubation fluids *in vitro* (Table I). The incubation fluid was also analyzed by chromatographic methods and the extent of conversion of uridine to uracil measured. Neither plasma nor inflammatory fluid ultrafiltrate had any significant effect on incorporation rate relative to the control group, and only 10% of the extracellular uridine was converted to uracil. However, addition of 0.5 ml of blood, inflammatory fluid, or hemolysate to the incubation fluid resulted in marked inhibition of uridine incorporation coincident with 80–95% conversion of uridine to uracil. Numerous control experiments have demonstrated that uracil incorporation into RNA in this system is negligible (less than 1% of the uridine rate).

TABLE I. Effect of Inflammatory Fluid and Blood on [^{14}C]Uridine Incorporation into [^{14}C]RNA and Conversion to [^{14}C]Uracil in Areolar[a] Tissue Incubated *in Vitro*.

Incubation fluid	Connective tissue [^{14}C]RNA synthesis rate (dpm/μg DNA) M	SD	[^{14}C]RNA synthesis rate (% Control)	% Conversion of [^{14}C]uridine to [^{14}C]uracil in incubation fluid at 3 hr
Buffer control	491	220	100%	8
22-Day inf. fluid (ultrafiltrate)	451	170	92%	10
Plasma	367	138	75%	10
22-Day inf. fluid[a]	30	8	6%	95
Whole blood	128	25	26%	80
Hemolysate	28	7	5%	95

[a]Areolar tissue is zero-day tissue. Each value is the mean of eight determinations.

Uridine-converting activity was destroyed completely by heating inflammatory fluid at 75° for 5 min. These data suggest the presence of an extracellular enzyme of molecular weight greater than 10,000. Such enzyme activity has been found in inflammatory fluid supernatants and in lysates of rabbit red cells and leukocytes.

Granulation tissue removed from sponge capsules at Days 5 and 19 was incubated in the presence of [^{14}C]cytidine (Table II). When the tissue was incubated in the presence of inflammatory fluid, there was a 40–45% decrease in the rate of incorporation of cytidine into RNA. Chromatographic analysis of incubation fluids revealed the presence of radioactive uridine, uracil, and cytidine after a 3-hr incubation. In the presence of inflammatory fluid, conversion to uracil was 76–81%. The inhibition of cytidine incorporation into RNA was less marked than the effect seen with uridine.

When [^{14}C]adenosine was incubated in the presence of granulation tissue, it was found that extensive conversion of this nucleoside to inosine or hypoxanthine occurred (Table III). The conversion to inosine occurred in 15 sec at 4°, but extensive conversion to hypoxanthine required incubation at 37°.

Several alterations of incubation conditions were made to minimize uridine to uracil conversion (Table IV). Use of a short incubation time of 40 min without added inflammatory fluid resulted in a 32% conversion. Washing of the tissue, use of a low phosphate concen-

TABLE II. Effect of inflammatory fluid on [^{14}C]Cytidine Incorporation into [^{14}C]RNA and Conversion to [^{14}C]Uracil in Granulation Tissue Incubated *in Vitro*.

Gran. tissue age (day)	Incubation fluid	Connective tissue [^{14}C]RNA synthesis rate (dpm/μg DNA) M	SD	[^{14}C]RNA synthesis rate (% control)	% Conversion of [^{14}C]cytidine to [^{14}C]uracil in incubation fluid at 3 hr
5	Buffer control	904	244		27
5	Inflammatory fluid	521	191	55	76
19	Buffer control	766	228		20
19	Inflammatory fluid	460	183	60	81

TABLE III. Conversion of Incubation Fluid [^{14}C]Adenosine to [^{14}C]Inosine and [^{14}C]Hypoxanthine by Granulation Tissue.

Gran. tissue (age)	Incubation conditions		Distribution of total cpm in incubation fluid at end of incubation period		
			[^{14}C]aden.	[^{14}C]inos.	[^{14}C]hypoxanth.
	Time (Min.)	Temp.	(%)	(%)	(%)
No tissue	5	4	95		
0 Day	0.25	4	1	83	15
4 Day	5	4	1	88	8
4 Day	40	37	1	16	77
Washed 4 day	40	37	1	17	70
4 Day	40	37	1	14	79

tration in the medium, or addition of a protein-denaturing reagent resulted in lower rates of uracil production. The increased uridine incorporation noted after washing was due to more rapid transport and will be the subject of a future report. Washing had no effect on [^{14}C]adenosine degradation by tissue.

Discussion. Decreased incorporation of [^{14}C]uridine into connective tissue RNA *in vitro* has been found in the presence of inflammatory fluid, whole blood, or a hemolysate. This decreased RNA synthesis has been associated with a high rate of conversion of uridine to uracil in the incubation fluid. Other studies have shown that uracil is not converted to RNA *in vitro* and that the uridine-degrading activity is heat labile, has a molecular weight of greater than 10,000, and requires phosphate. Such activity is present in rabbit red cells and leukocytes and inflammatory fluid. Inflammatory fluid activity probably results from cell lysis or secretion since both types of cells are present in these fluids. The activity described closely resembles that of a uridine phosphorylase. Granulation tissue has an extracellular fluid space of 75% of the total wet weight of the tissue. Since this space is directly continuous with the sponge fluid space, aliquots of tissue will also contain uridine phosphorylase activity. Quantitative studies of RNA synthesis in this tissue can be affected by the presence of this activity. It can be removed by repeated washing with incubation fluid.

Conversion of cytidine to uracil proceeds by deamination to uridine and then conversion to uracil. The evidence for this is the presence of the three radioactive substances named on chromatograms of cytidine-containing extracellular fluids. The sum of the counts

TABLE IV. Effects of Experimental Conditions on [^{14}C]Uridine Incorporation into [^{14}C]RNA and Conversion to [^{14}C]Uracil by Granulation Tissue Incubated *in Vitro*.

Incubation fluid	Tissue RNA synthesis rate (dpm/μg DNA)		% Conversion [^{14}C]uridine to [^{14}C]uracil in incubation fluid at 40 min
	M	SD	
Buffer control	88	31	32
Buffer, low phosphate	82	17	5
Washed tissue[a] Soln. D	202	99	6
DEP[b] shock	30	10	4

[a]Washing of tissue was for three 10-min intervals in solution D (electrolyte bicarbonate buffer).

[b](DEP) diethylpyrocarbonate (200 μl per tube) shock was for 3 min followed by replacement with fresh solution D. DEP alkylates proteins and denatures them.

of the three compounds mentioned account for 95% of the total applied counts.

To minimize precursor destruction when studying granulation tissue RNA synthesis with radioactive nucleosides, we have found the following procedures to be useful:

(a) Utilization of cytidine instead of other nucleosides since it is more rapidly transported and incorporated and more slowly degraded by extracellular enzymes.

(b) Washing of the tissue with large volumes of incubation medium to remove enzyme activity.

(c) Limitation of incubation time to less than 40 min.

(d) Use of incubation fluid without phosphate.

(e) Preincubation of tissue with uridine or cytidine for 1 hr to label intracellular pools before addition of inflammatory fluid. This allows observation of the effect of this fluid on synthesis rates. No satisfacory method for prevention of adenosine conversion to inosine has been found.

Failure to consider these effects would have resulted in misinterpretation of the data obtained. These effects have not been described before in work with granulation tissue. The small number of available studies on granulation tissue RNA synthesis are concerned with other subjects or involve the use of ^{32}P labeling which is not affected by the enzymes described above (6–12). Similar enzymatic effects on radioactive nucleosides resulting in precursor destruction have been noted in other systems. Such activities have been described in ground squirrel spleen cells, normal and leukemic human leukocytes and in mycoplasma-infected cell cultures (13–16). These enzymes have also been responsible for decreased synthesis of radioactive RNA from labeled nucleosides (14), and for decreased cell growth in cultures (15).

Summary. The incorporation of radioactive nucleosides into rabbit granulation tissue RNA has been studied *in vitro*. Polyvinyl sponge implantation was used to obtain tissue and inflammatory fluid. Decreased nucleoside incorporation occurred in tissue incubated with inflammatory fluid or blood. These fluids contained extracellular enzymes that degraded radioactive uridine and cytidine to uracil which is not incorporated. Since granulation tissue also consists of a large amount of enzyme-containing inflammatory fluid in its extracellular space, quantitative studies of RNA synthesis in this tissue may be affected. Utilization of incubation times of less than 40 min, washing of tissue, and use of a low phosphate concentration in the incubation fluid will minimize these effects. Failure to detect these effects would have led to erroneous interpretation of the incorporation rate data obtained.

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