An Evaluation of Differential Methylation of Ribonucleic Acid in Cystic Fibrosis

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Extract

No differences were found in the methylation of total nucleic acids, total RNA, DNA, tRNA, or rRNA from lymphocytes or fibroblasts obtained from normal subjects, obligate heterozygotes, or individuals with cystic fibrosis. Similar results were obtained using four methods for isolation of the nucleic acids.

Speculation

The reasons for the failure of two laboratories to discern the reported variations in methylation of RNA in the normal, carrier, or cystic fibrosis state may be obscure differences in culture conditions or conditions of extraction.

Introduction

Many unsuccessful attempts have been made to determine the underlying biochemical defect in cystic fibrosis (CF). Most recently, Rennert *et al.* [6, 7] reported that patients with the disease and their heterozygote parents methylate the tRNA of lymphocytes and skin fibroblasts to a lesser extent than do normal individuals. This is potentially a very important finding and we attempted to confirm it using the methods outlined by Rennert [6, 7, and personal communications] and others. We were unable to find any difference in the extent of methylation of either RNA or DNA in lymphocytes or skin fibroblasts of normal (C/C), heterozygote (C/c) or CF (c/c) individuals. A similar conclusion has been reached by Klagsburn and Farrell [5] based on examination of the skin fibroblasts of a smaller population.

Materials and Methods

Probandes were taken from CF families under study. All had elevated sweat chlorides and typical CF symptomatology. Skin biopsies and heparinized venous blood samples were obtained from probands, their parents, a few siblings and presumed normal controls (C/-, 0.95 probability of C/C). Leukocytes were separated from the blood by gravity sedimentation and the lymphocytes cultured in Gibco 1A medium for two days. They were then transferred to medium containing 4 μ Ci of 14 C-methyl L-methionine (14 C-Met, 46.3 mCi/mole). Clumping of the cells made it difficult to relate 14 C uptake to cell number. Therefore 10 μ Ci of 5- 3 H-uridine (3 H-U, 10.5 Ci/mole) was added to label RNA, and results are reported as the ratio of methylated (14 C) to total (3 H) RNA. After 24 hours in labeled medium, the cells were collected and tRNA (and DNA) was separated as described by Rennert [6, 7] (Method 1). Fibroblasts were obtained from skin with the aid of trypsin and were sown (2×10^6 cells) in 75 cm² Falcon flasks in Eagle's MEM [10] or F-10, each supplemented with 15% fetal calf serum, 30 mM HEPES [11], pH 7.2, 1.1% sodium bicarbonate, glutamine, penicillin and streptomycin (100 u/ml). Cells were grown for several days until they were confluent and fresh medium containing 2 μ Ci each of 14 C-Met and 3 H-U were added. In some experiments the labels were reversed: 3 H-Met (1.2 Ci/mole) and 14 C-U (160 mCi/mole) were used. Incubation was continued for 20-24 hr., the monolayer washed three times *in situ* with normal saline, and the nucleic acids extracted.

Nucleic acids were isolated as described by Rennert [6, 7] for the experiments with lymphocytes (Method 1). In the fibroblast experiments, nucleic acids were isolated by the method of Sein *et al.* [9] followed by separation of the DNA and tRNA by the Drasher [1] modification of the method of Schmidt and Thannhauser [8] (Method 2). In a few experiments total nucleic acids were precipitated from crude cell extracts with cetyltrimethyl ammonium bromide [2] (Method 3). In 2 experiments the nucleic acids were fractionated on sucrose gradients containing sodium dodecyl sulfate [3] (Method 4). The details are described in the legend to Fig. 1. Nucleic acids were solubilized in NCS [12] solubilizer and counted in POPPOP-PP0 toluene in a Packard liquid scintillation counter. Data were corrected for channel crossover and the ratio of the disintegrations per minute (dpm) of the isotopes calculated by computer.

Results

The cultured lymphocytes from the members of 8 families were examined by Method 1 (Table 1). In only 2 instances (Families 5 and 6) did the ratio of methylated to total RNA appear significantly lower in the family members than in the controls and in most instances it was not possible to distinguish among the samples.

The fibroblasts from 6 families were examined with 14 C-methionine and 3 H-uridine and two with the reverse label. Nucleic acids were extracted by Method 2. It was not possible to distinguish among the genotypes in any instance. An example of the results is given in Table 2.

In another experiment the methylation of the total nucleic acids (obtained by Method 3) of one obligate heterozygote, one CF patient, one sibling and 9 normals were compared. No differences were apparent (data not shown).

The RNA obtained from fibroblasts of a CF patient, an obligate heterozygote, and a normal heavily labeled with 3 H-Met was separated on a sucrose density gradient (Method 4) in two experiments. The results obtained in one experiment are shown in Fig. 1. In neither case were there differences in the methylation of either rRNA or tRNA.

Discussion

The results presented here indicate that nucleic acids in general and tRNA in particular obtained from lymphocytes or skin fibroblasts are as fully methylated in CF as they are in normal cells. These findings contrast with those of Rennert and coworkers [6, 7] but are similar to those of Klagsburn and Farrell [5]. The latter used fibroblasts in their work and suggested that the differences from Rennert's results might owe to the different growth conditions used by each group. This possible explanation is not a likely one, for our experiments like Rennert's were performed on confluent cells, and similar (negative) results were obtained with lymphocytes as well as fibroblasts. Moreover similar results were obtained whether total nucleic acids, DNA, rRNA or tRNA (i.e., those species known to be methylated in mammalian cells) were examined. The reasons for the differences in the results obtained in Rennert's and the other 2 laboratories remain obscure. However, despite careful control, it is a truism that conditions for the cultures of tissues differ among laboratories in subtle ways and some such factor could conceivably account for the differences noted. Differences in extraction procedure may result in divergent partition of the nucleic acids in the organic and aqueous phases, a variable that would probably not affect the results obtained with Method 4. Until such conditions are elucidated, extent of methylation cannot be recommended for distinguishing the various genetic states of CF.

References and Notes

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- Minimum essential medium (Eagle), Auto-Pow, Flow Laboratories, Rockville, Maryland.
- N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, Calbiochem, La Jolla, California.
- Amersham/Searle, Arlington Heights, Illinois.
- Supported in part by PHS Research Grant AM12328-04, The Indiana Elks, and the J. W. Riley Memorial Association.
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- Accepted for publication November 14, 1973.

Fig. 1. Density gradient separation of RNA. The cells from four (C/-) or five (C/c and C/C) 75 cm² falcon flasks were labeled for 16 hr. with 56 μ Ci 3 H-Met (1.3 Ci/mole) and 1.43 μ Ci 14 C-U (498 mCi/mole) in 5 ml of F-10 medium:15% fetal calf serum containing 1×10^{-6} M adenosine [4]. The cells were washed, scraped from the bottles and lysed in hypotonic buffer. The cytoplasm was separated after treatment in a teflon-in-glass homogenizer, made 1% in SDS and layered on 26 ml of a 15-30% (w/w) gradient of sucrose in SDS buffer (0.5% SDS, 0.1 M NaCl, 0.01 M Tris-HCl, pH 7.5, and 0.001 M EDTA). The gradients were centrifuged for 16 hr. at 20 $^\circ$ in Spinco rotor SW25.1 at 23,000 rpm. The tubes were fractionated from the bottom (to the left), 1 ml fractions collected, the A₂₆₀ determined and trichloroacetic acid insoluble material collected on GF/A filters. These were washed, dried, solubilized with NCS solubilizer and counted in dimethyl POPPOP-PP0. The A₂₆₀ pattern corresponded to those shown for radioactivity. —, 3 H-Met label; Δ — Δ , 14 C-U label.

Table I. Methylation of tRNA of lymphocytes¹

Family	$^{14}\text{C}/^3\text{H} \times 10^5$			
	Affected c/c	Mother C/c	Father C/c	Control C/-
1.	684	365	401	746
2.	57	45	43	---
3.	---	73	126	91
4.	93	255	93	102
5.	375	573	381	1400
6.	985	712	867	1596
7.	115	360	733	561
8.	721	937	756	---

¹The tRNA was extracted by the method described by Rennert [6, 7]. The Control (unrelated to the family) was examined at the same time as the family.

Table II. Methylation of tRNA and DNA of fibroblasts¹

Label	Diagnosis	Combined NA	tRNA	DNA
$^3\text{H-Met}/^{14}\text{C-U}$	Mother(C/c)	.337	.338	.277
	Father(C/c)	.319	.394	.243
	Normal(C/-)	.371	.383	.264
	CF (c/c)	.296	.380	.260
$^{14}\text{C-Met}/^3\text{H-U}$	Mother(C/c)	.015	.016	.059
	Normal(C/-)	.015	.014	.049
	CF (c/c)	.015	.014	.049
	CF (c/c)	.022	.021	--

¹The nucleic acids were extracted and separated by Method 2.

18-0112-1-8

Corrigendum

Glucose Phosphate Isomerase Deficiency with Congenital Nonspherocytic Hemolytic Anemia: A New Variant
(Type Nordhorn) I. Clinical and Genetic Studies

By W. Shröter *et al.*

Pediatric Research, Vol. 8, No. 1

p. 18 and *Contents*—Dr. Arnold's name should have appeared as "H. Arnold" instead of "A. Arnold."