

ALTERED NUCLEIC ACID METABOLISM IN HUMAN CELL CULTURES INFECTED WITH MYCOPLASMA*

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Many human diploid cell strains cultured in one of these laboratories† have in the course of their serial propagation developed profound and permanent changes in the pattern of nucleic acid synthesis.¹ These changes included (a) the appearance of a new species of RNA, apparently synthesized in the cytoplasm, with a guanine plus cytosine content of 33 per cent and a sedimentation constant of 14–20S; (b) decreased C¹⁴-uridine incorporation into nuclear 35–45S ribosomal precursor RNA, to the degree that it could no longer be demonstrated by either autoradiography or sucrose density centrifugation; and (c) a decreased ribosomal RNA content per cell. The incorporation of C¹⁴-uridine into this 14–20S RNA was not decreased in confluent and nongrowing cultures of “altered” cells, as contrasted with the 90–95 per cent decrease in the incorporation of C¹⁴-uridine into normal ribosomal precursor RNA.²

More recent experiments³ have shown that the newly synthesized RNA consists of at least two distinct species (14S and 20S), separable either by density gradient centrifugation or gel electrophoresis, and in contrast to mammalian ribosomal RNA has a high turnover rate. With similar techniques, analogous changes have been found in the pattern of DNA synthesis: radioactive thymidine is incorporated by altered cultures into a “light” DNA species, apparently synthesized in the cytoplasm, and also with a high turnover rate. The cytoplasmic synthesis of the abnormal RNA, perhaps on the new DNA species as a template, was inhibited by actinomycin D.

Paradoxically, these profound alterations in the pattern of nucleic acid synthesis did not usually affect the gross appearance, growth rate, or life expectancy of the cultures; and no significant chromosome alterations could be detected. The causative role of mycoplasma in effecting these changes was previously considered possible, but unlikely.¹ The results here reported, based on more extensive isolation tests in a number of laboratories, as well as deliberate inoculation experiments, indicate that these altered patterns of nucleic acid synthesis in cultured human cells did indeed stem from infection with mycoplasma and that a single species (*M. hyorhina*) was involved.

Materials and Methods.—The cell strains used were all human diploid fibroblasts; their source and methods for propagation have been listed elsewhere.⁴ Although penicillin, streptomycin, and aureomycin were routinely added at 50, 50, and 10 µg/ml, respectively, only penicillin was used in inoculation experiments with mycoplasma cultures. Methods for nucleic acid characterization, including density gradient centrifugation, base composition analysis, and autoradiography, have also been previously described.¹ Methods for the cultivation and enumeration of mycoplasma were substantially those used by Hayflick⁵ and by Thomas and McGregor.⁶ Broth cultures for inoculation and agar plates for colony formation were incubated at 37°C in a CO₂ incubator. Except where indicated,

the numbers given in the tables represent "cell-associated" mycoplasmas, i.e., colony-forming organisms in a trypsinized suspension of cultured cells. Colonies were counted after 3-4 days' incubation, but were not considered negative until after 2 weeks' incubation. The serologically distinct mycoplasma species were identified by suitable growth-inhibition tests with dried antiserum-impregnated paper disks.⁷

Results.—Correlation between presence of mycoplasma and synthesis of 14-20S RNA: Earlier studies¹ showed no clear correlation between the presence of mycoplasma and synthesis of 14-20S RNA. Some cell strains with a normal pattern of RNA synthesis proved contaminated, whereas other strains with the characteristic "altered" patterns of RNA synthesis were not. Continuing studies, however, showed that infected cultures with a normal pattern of RNA synthesis developed the characteristic changes in later subcultures (Table 1, footnote a). Conversely, the apparent absence of mycoplasma in strains with an altered pattern of RNA synthesis proved due to wide variations in the ability of different laboratories to culture mycoplasma. Thus, when replicate cultures of "altered" cells were simultaneously submitted for mycoplasma isolation to three laboratories, the first detected mycoplasma in all of seven samples, the second found six cultures positive, and the third laboratory detected mycoplasma in only three. Such lack of agreement has been noted repeatedly during these investigations. However, in two of the cooperating laboratories (D and E in Table 1) there was consistently a high correlation between contamination with mycoplasma and the "altered" pattern of RNA synthesis, indicating a causal relationship (Table 1). In nine different "altered" strains of human fibroblasts, all isolates were identified as *M. hyorhinis*; all were resistant to a combination of 50 $\mu\text{g/ml}$ streptomycin and 10 $\mu\text{g/ml}$ aureomycin (cf. ref. 9); and attempts to "cure" cultures with Kanamycin, Tylosin, or high concentrations of aureomycin were unsuccessful.

Appearance of 14-20S RNA in cell cultures after deliberate inoculation with mycoplasma: To confirm the causal relationship between mycoplasma infection and the observed changes in nucleic acid synthesis, cultures showing normal C¹⁴-uridine incorporation into ribosomal precursor RNA were inoculated with nine

TABLE 1. Correlation between altered pattern of RNA synthesis in cultured human cells and mycoplasma contamination.

Pattern of RNA synthesis	Results of Mycoplasma Tests on Cell Cultures		Laboratory
	Positive cultures	Negative cultures	
Normal	0	1	A
	1	2	B
	0	0	C
	2 ^a	2	D
	1 ^a	31 ^b	E
Abnormal	4	6	A
	4	8	B
	0	2	C
	22 ^c	1	D
	15	0	E

^a Altered pattern of RNA synthesis appeared in subsequent passages.

^b Including three attempts to cultivate mycoplasma from heteroploid cell lines.

^c Including 11 isolations from "altered" heteroploid cell lines.

TABLE 2. *The pattern of RNA synthesis in cultured human cells^a infected with mycoplasma.*

Mycoplasma species inoculated	Size of inoculum ^b	Results in Successive Subcultures		
		Subculture number ^c	No. of cell-associated mycoplasma ^d	Pattern of RNA synthesis ^e
<i>hyorhinitis</i> ^f	1×10^9	0	3×10^7	Abnormal
		0	4×10^7	Normal
		1	2×10^7	Abnormal ^g
	8×10^8	2	2×10^7	Abnormal
		0	1×10^6	Normal
		1	6×10^7	Normal
	4×10^8	2	2×10^7	Abnormal
		0	6×10^3	Abnormal
		0	4×10^7	Mixture
<i>pulmonis</i>	2×10^8	1	3×10^7	Mixture
		2	4×10^7	Abnormal
		3	—	Abnormal
		0	6×10^6	Normal
<i>neurolyticum</i>	8×10^9	1	2×10^7	Abnormal
		2	3×10^6	Abnormal
		0	6×10^6	Abnormal
<i>orale I</i>	8×10^9	0	6×10^4	Normal
		2	5×10^1	Normal
	8×10^5	0	2×10^6	Abnormal
<i>arthritidis</i>	1×10^{10}	1	2×10^4	Normal
		2	—	Normal
		0	7×10^7	Abnormal
<i>fermentans</i>	6×10^8	1	2×10^4	Normal
		2	0	Normal
		0	4×10^7	Abnormal
<i>pneumoniae</i>	4×10^7	1	0	Normal ^h
		2	—	Normal
		0	7×10^5	Normal
<i>hominis I</i>	2×10^{10}	1	8×10^6	Normal
		2	2×10^6	Normal ^h
		0	2×10^6	Normal
<i>orale II</i>	4×10^5	1	1×10^6	Normal ^h
		2	—	Normal
		0	3×10^6	Normal
<i>salivarium</i>	5×10^9	1	1×10^3	Normal
		2	0	Normal

^a Illustrative experiments with six different human diploid strains. With a given mycoplasma species, all gave qualitatively similar results, and they are not distinguished in the table.

^b Total number of colony-forming units added to 8–10 ml of culture fluid.

^c For "subculture 0," most cultures were tested 24 hr after inoculation, five after 4 days, two after 5 days, and one after 7 days; for "subculture 1," the cultures were subdivided and retested for mycoplasma 6–10 days after inoculation; similarly, "subculture 2" was subdivided a second time and tested 14–17 days after the original inoculation, and "subculture 3," 21 days after inoculation.

^d Total number of cultivable cell-associated mycoplasmas (approximately 10^6 diploid cells per flask). At "subculture 0," 24 hr after inoculation, most of the mycoplasmas were in the supernatant medium; in following subcultures, up to 80% were cell-associated.

^e Pattern of radioactive peaks in sucrose density gradients of sodium dodecyl sulfate extracts prepared from cells labeled for 20 min at 37° with C^{14} -uridine at 0.2–0.5 μ c/ml. Normal = 35–45S ribosomal precursor (nuclear); abnormal = 14–20S RNA ("cytoplasmic"); mixture = two discrete peaks of C^{14} -uridine labeled RNA, corresponding to normal ribosomal precursor and abnormal cytoplasmic RNA.

^f The inoculum of 8×10^8 organisms was from a stock strain of *M. hyorhinitis*; the others were originally isolated from contaminated cell cultures.

^g Synthesis of abnormal 14–20S RNA associated with continuing multiplication of mycoplasma in high titer.

^h Normal pattern of RNA synthesis despite high titer of cell-associated mycoplasma. Mycoplasmas often were no longer demonstrable in following subculture (spontaneous "cure"?).

species of mycoplasma. *M. hyorhina* regularly produced an ongoing infection, in which organisms continued to propagate in subculture in high titer (Table 2); and in ten experiments with five different cell strains, there was a concomitant alteration in the pattern of RNA synthesis, similar to that observed in "naturally" infected cultures (cytoplasmic synthesis of new species of RNA sedimenting at 14–20S, with a guanine plus cytosine content of 34.7%).

Similar results were obtained with *M. pulmonis* and *M. neurolyticum*. Five other mycoplasma species (*M. arthritidis*, *M. fermentans*, *M. hominis*, *M. orale I* and *II*, and *M. pneumoniae*) usually did not permanently alter the pattern of RNA synthesis. With large inocula there was often a temporary appearance of 14–20S RNA in infected cells. In subculture, however, as the number of viable cell-associated mycoplasmas fell off, sometimes disappearing abruptly, C¹⁴-uridine was again incorporated into normal nuclear ribosomal precursor. In several experiments, the abnormal RNA could unaccountably not be demonstrated despite the presence of viable cell-associated mycoplasma in large numbers (footnote *h*, Table 2).

The simplest explanation for the appearance of abnormal, cytoplasm-synthesized RNA in mycoplasma-infected cells is that these nucleic acid species are those of mycoplasma itself; and results obtained on density gradient centrifugation of uridine-labeled mycoplasma RNA suggest that this is in fact the case.³

Ribosomal RNA synthesis in infected cultures: It had previously been shown in these infected cultures that labeled uridine was not demonstrably incorporated into ribosomal precursor RNA, that incorporation into 28S ribosomal RNA was decreased by 80–85 per cent, and that the absolute amount of ribosomal RNA was reduced by 50 per cent.¹ Nevertheless, the growth rate and viability of such cultures were not noticeably affected.

Some of these paradoxical results were resolved by the finding that the addition of unlabeled uridine to the medium increased the incorporation of precursor uridine into ribosomal 28S RNA (Table 3). Failure to detect such incorporation in mycoplasma-infected cells with tracer amounts of labeled uridine could be due to several factors. There may be changes in cell permeability (cf. ref. 13), suppression of critical enzymes systems within the host cell,¹³ or the metabolism¹⁴ or preferential utilization of exogenous uridine by extracellular mycoplasma in the medium or adherent to the surface of the cell.¹⁵

In agreement with previous findings with naturally infected cells, deliberate inoculation with mycoplasma caused a 30–50 per cent reduction in the absolute amount of 28S RNA per cell. That decrease was not due to increased nuclease activity, since 24-hour infection with mycoplasma had no effect on the amount of labeled ribosomal RNA recovered from prelabeled cells (421,000 cpm from infected cells; 413,000 cpm from control cells).

Discussion.—The present demonstration that mycoplasma infection in cultured human cells is followed by profound changes in the pattern of nucleic acid metabolism is consistent with several previous studies. Randall *et al.*¹⁰ observed an increased lability of host cell DNA in mouse cells following mycoplasma infection. Nardone and co-workers¹¹ concluded from autoradiographic data that incorporation of uridine and thymidine in such infected cultures is cytoplasmic,

TABLE 3. *Effect of added unlabeled uridine on the pattern of uridine-H³ incorporation into RNA of mycoplasma-infected cultures.^a*

Cell strain and number	Added unlabeled uridine (mM)	Final specific activity of H ³ -uridine ($\mu\text{c}/\mu\text{mole}$)	Incorporation into Cellular RNA				
			"28S" RNA ^b Cpm	"28S" RNA ^b $\mu\mu\text{Moles}^d$	"18S" RNA ^c Cpm	"18S" RNA ^c $\mu\mu\text{Moles}^d$	Ratio "28S"/"18S"
(A) Detroit	0	20,000	1,390 ^e	0.0695	370,000	18.5	0.00376
510	0.01	500	760	1.52	38,700	77.4	0.0197
(2 × 10 ⁶)	0.10	50	1,008	20.2	4,650	93.0	0.217
(B) E-699	0	20,000	14,200 ^e	0.710	417,000	20.9	0.0341
(4 × 10 ⁶)	0.01	500	5,130	10.3	43,900	87.8	0.117
	0.10	50	2,480	49.7	5,470	109.	0.452

^a Replicate cultures, contaminated with mycoplasma and synthesizing "abnormal" 14-20S RNA, were incubated for 6 hr in growth medium containing tritiated uridine (specific activity 20 curies/mole) at 5 $\mu\text{c}/\text{ml}$ (0.00025 mM), and unlabeled uridine at the concentrations indicated in the table. Whole cell lysates prepared with SDS were sedimented in sucrose density gradients so as to separate ribosomal "28S" RNA from the mixed peak of ribosomal "18S" RNA, and mycoplasma "14-20S" RNA. The tabular data represent the total TCA-precipitable radioactivity in the various peaks.

^b Ribosomal "28S" RNA.

^c Radioactivity peak sedimenting in the region of ribosomal "18S" RNA and abnormal "14-20S" RNA.

^d Calculated assuming 10⁶ cpm = 1 μc .

^e No discernible peak.

rather than nuclear, and Hakala *et al.*¹² have described enhanced deoxynucleoside cleavage in mycoplasma-contaminated cells.

The serious complications introduced by mycoplasma contamination in cells or cell extracts used for studies on nucleic acid metabolism are self-evident. Continuous monitoring of cell cultures is clearly indicated, either by mycoplasma cultivation or by determination of the type or locus of nucleic acid synthesis in pulse-labeled cells.¹ The factors responsible for the inconsistent results obtained by some laboratories in testing for mycoplasma are not clear. There seems little question that different species of mycoplasma differ in their ease of cultivation, and new methods have been suggested to increase the reliability of procedures (cf. ref. 8). In the more recent of the present experiments (Table 1, laboratory E), shipment time was minimized, agar plates were freshly prepared, and both cell-associated and free mycoplasma in the culture fluid were assayed (at least 80% of the organisms were cell-associated). Thirty-seven "altered" cultures have so far been tested in this manner, and mycoplasma has been successfully cultivated from all.

The etiology of mycoplasma infection in cultured cells has been examined by many workers. Among the suggestions has been the presence of mycoplasma (a) in the environment (dust, air), (b) in the nasopharynx of those handling cultures, or (c) in the materials used for cell propagation (media, serum, trypsin, etc.). At least in the present series of experiments, the validity of (b) and (c) is questionable. Over the past two years, every mycoplasma strain isolated from a wide variety of cultures in one of these cooperating laboratories† has proved to be *M. hyorhinae*, and all were resistant to penicillin, streptomycin, and aureomycin at the concentrations used in cell culture. Although some of the workers harbored mycoplasma in the nasopharynx, these isolates were serologically identified as *M. salivarium* and *M. orale I*. Recent experiments further minimize the role of the

materials used in cell propagation as the contaminating source and indicate that mycoplasma are usually introduced into cell cultures by a technical lapse in manipulation. In this, the use of antibiotics may paradoxically be a contributory factor. Ten strains of human diploid cells were subcultured weekly in antibiotic-free media for three to eight months, in the same laboratory in which cultures containing antibiotics regularly became mycoplasma-infected after an average of six weeks.¹ Over this period, occasional cultures became bacterially contaminated, but no culture without such frank bacterial contamination was mycoplasma-positive (cf. also ref. 15), and all continued to show a normal pattern of nucleic acid synthesis, with no cytoplasmic synthesis of 14–20S RNA. Apparently, when technical blunders result in simultaneous introduction of both bacteria and mycoplasma, cultures in antibiotic-free media (or if the bacterial contaminant is antibiotic-resistant) become obviously contaminated and are then discarded. In media with antibiotics, however, antibiotic-sensitive bacteria would be eliminated, whereas antibiotic-resistant mycoplasma survive. The maintenance of cultures in antibiotic-free media thus materially reduces unrecognized contamination with mycoplasma (cf. refs. 13 and 14).

The present experiments have been carried out with human diploid fibroblasts. Preliminary experiments with a wide spectrum of heteroploid cells—either derived from cancers, transformed *in vitro* by viruses, or arising “spontaneously” in the course of serial cultivation—and studies also with cells deriving from other animal species indicate a qualitatively similar relationship between contamination with mycoplasma and an altered pattern of nucleic acid metabolism.

Summary.—The permanently altered pattern of nucleic acid metabolism frequently observed in serially cultivated human cells has been shown to be due to contamination with *M. hyorhinis* and may be produced by deliberate inoculation of normal cultures with a variety of mycoplasma species. The apparently decreased incorporation of tracer radioactive uridine into ribosomal RNA in the mycoplasma-infected cultures is probably due to the failure of the exogenous uridine to reach the nuclear site of RNA synthesis.

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