In Vitro Safety Assessment of Double-Stranded Polynucleotides Poly I:C and Mu-9 (35754)

CLARENCE L. BAUGH, ALFRED A. TYTELL, AND MAURICE R. HILLEMAN

Division of Virus and Cell Biology Research, Merck Institute for Therapeutic Research, West Point, Pennsylvania 19486

The induction of interferon and resistance to viral infections in cell cultures and animals by double-stranded polynucleotides, including polyriboinosinic–polyribocytidylic acid complex (Poly I:C, rIn:rCn), was first reported from our laboratories in 1967 (1–4) and has since been confirmed by other workers (5–8). The further objectives of our group have centered on the application of interferon inducers such as poly I:C in human and animal medicine. As part of preclinical safety assessment, a study was made of the effect of poly I:C and double-stranded RNA from MU-9 coliphage (Mu-9 ds RNA) on the rate of growth, morphology, karyotype, and possible transformation of a human diploid cell strain in culture. A previous report summarized the preliminary findings (9). The present report presents the detailed results of the tests.

Materials and Methods. Polynucleotides. Poly I:C was prepared using individual homopolymers (poly rI and poly rC) obtained from the Miles Laboratories, Elkhart, Indiana. The ribopolynucleotides were complexed as described earlier (2). Mu-9 ds RNA was prepared by Drs. B. Lago and J. Birnbaum, Process Research Laboratories of Merck & Co., Rahway, N. J., employing *E. coli* infected with an amber protein coat mutant (Mu-9) of MS-2 coliphage and methods described previously (4).

Cell culture. Strain HFL-1B of diploid human lung cells established by this laboratory (10) was employed. This cell strain carries the male chromosome, is diploid, has a finite lifespan, and does not form tumors when inoculated into the cheek pouch of cortisone-conditioned hamsters. This cell strain, unlike strain WI-38, is susceptible to induction of resistance to viral infection by double-stranded ribonucleic acids (10). HFL-1B strain cultures usually fail to grow well beyond passage 17 and are regarded as "senescent" at this passage level.

The HFL-1B cells were propagated in Eagle's basal medium (EBME) containing 10% fetal calf serum and penicillin and streptomycin. Routine testing proved the absence of pleuropneumonia-like organisms. The parent stocks of HFL-1B cells were stored frozen at second passage *in vitro* and were used at subsequent passage levels in the experiments to measure the effect of added polynucleotides. Cultures of identical passage history but without added drug were used for control purposes.

Karyotype analysis. The cells were cultivated in the presence or absence of added polynucleotide and were analyzed for karyotype according to procedures outlined in the Minutes of the Sixth Meeting of the Committee on Cell Culture, 1969 (11).

Hamster cheek pouch analysis. Random-bred Syrian hamsters obtained from Lakeview Hamster Colony, Newfield, New Jersey, were used. Animals, weighing 60 g each, were conditioned by twice weekly injections of 2.5 mg each of cortisone. Cells treated with poly I:C or held as untreated controls were injected into the cheek pouch at the 1×10^5 level; cells treated with Mu-9 ds RNA and controls were injected at the 1×10^6 cell level. Positive controls consisted of HEp-2 human tumor cells injected in similar fashion. Observations were made twice weekly for formation and regression of nodules.

Growth studies. Poly I:C or Mu-9 ds RNA were incorporated into the growth medium in the passages and concentrations reported in the text. This medium was replaced at 3

TABLE I. Lack of Effect of Poly I:C and Mu-9 ds RNA on the Proliferation of HFL-1B Cells in Culture.[a]

Poly I:C present [cell culture passage(s)]	Polynucleotide		Cell protein (μg/culture) according to day of incubation		
	Kind	Dose (μg/ml)	3	7	10
8	Poly I:C	100[b]	124	116	64
		25	125	165	53
		5	124	131	53
		0 (Control)	127	143	69
15	Poly I:C	100	82	148	155
		25	90	146	156
		5	88	156	141
		0 (Control)	85	152	156
12	Poly I:C	5	97	88	183
		0 (Control)	94	98	145
16	Poly I:C	5	69	124	142
		0 (Control)	86	123	183
5–12	Poly I:C	5	104	98	145
		0 (Control)	111	84	163
5–16	Poly I:C	5	98	142	179
		0 (Control)	92	132	181
6	MU-9 ds RNA	100	99[c]	129[c]	165[c]
		25	109	124	193
		5	97	130	182
		0	93	140	168

[a] The medium was EBME with 10% fetal calf serum.
[b] Average values, 6 tubes/test.
[c] Days 4, 8, and 11.

and 7 days for studies with poly I:C and at 4 and 6 days for studies with Mu-9 ds RNA. Replicate cell cultures without drug served as controls and were treated in the same manner. Cell growth was measured in terms of cell protein according to Oyama and Eagle (12).

Results. Effect of polynucleotide on cell growth. Poly I:C or Mu-9 ds RNA was added to HFL-1B diploid human cell cultures in the dose and numbers of passages shown in Table I. Cell growth was measured in terms of micrograms of washed cell protein per roller tube culture. There were no marked differences in values obtained in cultures with drug compared with untreated controls, indicating no apparent effect on cell proliferation within the time and dose parameters measured.

Effect of polynucleotide on appearance of senescence. HFL-1B cells were passed serially in the presence of 5 μg/ml of poly I:C starting with passage no. 5. Untreated replicate controls were examined at the same time. The findings shown in Table II revealed that there was no effect of poly I:C on cell growth through the sixteenth passage and that the presence of poly I:C did not prevent the appearance of senescence which occurred at the seventeenth serial passage. Mu-9 ds RNA also failed to extend the lifespan of HFL-1B cells in similar experiments not recorded here.

Effect of polynucleotide on the karyotype of HFL-1B cells. The cells were transferred serially in the presence or absence of poly I:C or Mu-9 ds RNA and the karyotype was examined at the passage levels shown in Table III. There was no alteration in karyotype by polynucleotide with respect to tetra-

TABLE II. Development of Senescence in HFL-1B Cells Cultivated Serially with Poly I:C.

Cell culture passage no. examined	Cultures	Cell protein (μg/culture) according to day of incubation		
		3	7	10
8	Test (poly I:C)[a]	98	128	77
	Control	98	126	66
12	Test (poly I:C)	110	102	145
	Control	103	112	158
16	Test (poly I:C)	80	121	168
	Control	93	118	171
17	Test (poly I:C)	10	26	33[b]
	Control	8	31	26[b]

[a] Five μg/ml concentration.

[b] Cell sheets did not become confluent and cells were not recoverable on subculture.

ploidy, chromosome breaks, or alteration in chromosome number.

Failure of polynucleotide to cause neoplastic transformation of HFL-1B cells. Poly I:C or MU-9 ds RNA was added to cells during propagation at the passage levels indicated in Table IV. The cells were tested at the passage levels shown for ability to produce persistent or growing nodules when inoculated into the cheek pouch of hamsters. The findings showed that the HFL-1B did not produce persistent nodules, when propagated either in the presence or absence of polynucleotide. Littermates of these same hamsters did develop persistent nodules when inoculated with neoplastic HEp II cells for control purpose.

Discussion. Double-stranded RNA, such as poly I:C, offer promise for broad-spectrum short-term protection against certain viral diseases of man and animals, especially those for which vaccination does not provide a practicable approach. The continuing accumulation of refined and meaningful safety assessment data is essential to the expansion of clinical trials of these materials.

Previous *in vivo* tests for acute toxicity were carried out (13) in dogs, mice, rats, and

TABLE III. Karyotypic Analysis of HFL-1B Cells Cultivated in the Presence of Poly I:C or Mu-9 ds RNA.

Kind	Polynucleotide[a]		Karyotype analysis; % cells with:				
	Drug added (passage level)	No. of further passages[b]	Tetraploidy	Chromosome breaks	Chromosome no. <46	46	>46
Poly I:C	5	4 WD	2.5	3	22	78	0
	5	4 WO	2.9	8	15	78	6
	5	8 WD	3.3	8	12	78	10
	5	8 WO	3.3	1	18	77	5
	5	12 WD	1.6	4	12	80	7
	5	12 WO	1.8	4	10	79	8
Mu-9 ds RNA	4	9 WD	2.0	4	16	80	4
	4	9 WO	2.9	5	16	80	4
	4	12 WD	2.3	5	17	79	4
	4	12 WO	3.4	2	14	83	3

[a] 5 μg/ml concentration.

[b] WD, with drug; WO, without drug.

TABLE IV. Test for Neoplastic Transformation of HFL-1B Cells by Added Polynucleotide Measured in the Hamster Cheek Pouch Assay.

	Polynucleotide test with HFL-1B cells				Control tests with HEp II cells
Kind of drug[a]	Drug present (cell passage levels)	Cell passage level tested in hamster cheek pouch	Group[b]	Hamsters with persistent nodules (total no.)	Hamsters with persistent nodules (total no.)
Poly I:C	5– 7	7	WD	0/6	
			WO	0/6	5/6
	5–11	11	WD	0/6	
			WO	0/6	5/5
	5–15	15	WD	0/6	
			WO	0/6	4/6
MU-9 ds RNA	4–13	13	WD	0/6	
			WO	0/6	3/5
	4–16	16	WD	0/3	
			WO	0/4	4/5

[a] Five μg/ml concentration.
[b] WD, with drug; WO, without drug.

monkeys. Poly I:C was shown to cause marked toxic effects, especially in dogs given the drug by the intravenous route, and these appeared to have a vascular, hepatic, or hematologic basis. Administration of the RNA subcutaneously produced a far less effect and nasal administration was essentially innocuous. The administration of poly I:C to 20 human subjects (14) by the intravenous route was remarkably free of adverse effect, even when large doses (up to 4 mg/kg) were given repeatedly. The only consistent clinical manifestation of poly I:C was a febrile response and there was no evidence of disturbance of liver, kidney, or bone marrow functions. There were no effects on the clotting mechanism. Demonstrable interferon induction was noted in 16 of the subjects.

The findings in the present studies extend the knowledge of safety of both poly I:C and Mu-9 ds RNA from the standpoint of *in vitro* effect on cell replication and on the chromosomes. It is evident that there was no limitation by drug of growth of the HFL-1B cells in culture. Further, presence of the RNA did not prevent the appearance of senescence and deterioration of diploid cells having a finite lifespan. There was no observable effect of the polynucleotides on the nuclear chromosomes and the treated cells did not show evidence of neoplastic transformation when tested in the cheek pouch of cortisone-treated hamsters.

Summary. The double-stranded RNA's poly I:C (rIn:rCn) and Mu-9 ds RNA, which are potent inducers of interferon, did not affect the rate of growth, did not prevent the development of senescence, and did not cause detectable alteration of the karyotype of human diploid fibroblast cell line HFL-1B when present in single or in serial passage. Additionally, the cells propagated in the presence of the RNA's did not become neoplastic as determined by assay in the cheek pouch of cortisone-treated hamsters.

The authors are indebted to Dorothea Estok for excellent technical assistance in these studies.

1. Lampson, G. P., Tytell, A. A., Field, A. K., Nemes, M. M., and Hilleman, M. R., Proc. Nat. Acad. Sci. U.S.A. 58, 782 (1967).

2. Field, A. K., Tytell, A. A., Lampson, G. P., and Hilleman, M. R., Proc. Nat. Acad. Sci. U.S.A. 58, 1004 (1967).

3. Tytell, A. A., Lampson, G. P., Field, A. K., and Hilleman, M. R., Proc. Nat. Acad. Sci. U.S.A. 58, 1719 (1967).

4. Field, A. K., Lampson, G. P., Tytell, A. A., Nemes, M. M., and Hilleman, M. R., Proc. Nat. Acad. Sci. U.S.A. 58, 2102, (1967).

5. Colby, C., and Chamberlin, M. J., Proc. Nat. Acad. Sci. U.S.A. **63**, 160 (1969).

6. Falcoff, E., and Perez-Bercoff, R., Biochim. Biophys. Acta **174**, 108 (1969).

7. Vilcek, J., Ng, M. H., Friedman-Kien, A. E., and Krawciw, T., J. Virol. **2**, 648 (1968).

8. DeClerq, E., and Merigan, T. C., Nature (London) **222**, 1148 (1969).

9. Hilleman, M. R., Arch. Intern. Med. **126**, 109 (1970).

10. Field, A. K., Tytell, A. A., Lampson, G. P., and Hilleman, M. R., Proc. Nat. Acad. Sci. U.S.A. **61**, 340 (1968).

11. International Association of Microbiological Societies, Cell Culture Committee of the Permanent Section on Microbiological Standardization, Minutes of the Sixth Meeting, New York (1969).

12. Oyama, V. I., and Eagle, H., Proc. Soc. Exp. Biol. Med. **91**, 305 (1956).

13. Philips, F. S., Fleisher, M., Hamilton, L. D., Schwartz, M. K., and Sternberg, S. S., Int. Symp. Mol. Biol., 4th New York (1970), in press.

14. Field, A. K., Young, C. W., Krakoff, I. H., Tytell, A. A., Lampson, G. P., Nemes, M. M., and Hilleman, M. R., Proc. Soc. Exp. Biol. Med. **136**, 1180 (1971).

Received Jan. 14, 1971. P.S.E.B.M., 1971, Vol. 137.