Influence of Synthetic (Poly I:C) and Viral Double-Stranded Ribonucleic Acids on Adenovirus 12 Oncogenesis in Hamsters* (34028)

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Previous reports (1–6) from this laboratory recorded that several double-stranded ribonucleic acids from various sources were efficient inducers of interferon and resistance to ordinary lytic viral infections *in vitro* and *in vivo*. Important among them was the complex of polyriboinosinic (Poly I or rI) and polyribocytidylic (Poly C or rC) acids, (2, 5, 6), commonly referred to as rI:rC or Poly I:C. These studies have been extended to measure the influence of Poly I:C and of other interferon inducers on virus-induced neoplasia in animals given the substances in prophylactic and/or therapeutic regimens. Adenovirus type 12 was selected to represent a highly oncogenic DNA virus which is characterized by a short tumor latent period and by a high incidence of "virus-free" tumors in newborn hamsters. The present report summarizes the findings in tests to measure the effect of Poly I:C, of Poly I, and of Poly C alone, of double-stranded MU9 coliphage RNA, and of endotoxin and pyran on the oncogenesis of adenovirus 12 in newborn hamsters.

Materials and Methods. Hamsters. Pregnant random-bred golden Syrian hamsters were obtained from the closed breeding unit of the Lakeview Hamster Colony, Newfield, New Jersey. *Adenovirus* type 12, strain Huie, which was isolated in these laboratories and passed seven times in primary cell cultures of human embryonic kidney (HEK) was used. The infectivity titer of the virus was $10^{-8.7}$ $TCID_{50}$/0.1 ml when titrated in HEK. *Polyriboinosinic* (Poly I) and *polyribocytidylic* (Poly C) acids were purchased from Miles Laboratories, Elkhart, Indiana. The individual homopolymer solutions and Poly I:C (rI:rC) complex were prepared as described previously (2) and were furnished by Drs. A. K. Field and A. A. Tytell of these laboratories. The *double-stranded replicative-form RNA of MU9 mutant of MS2 coliphage (MU9-RNA)* was prepared as described in an earlier report (4) and was furnished by Dr. A. A. Tytell and Mr. G. P. Lampson of these laboratories. *Pyran copolymer (divinyl ether maleic anhydride)* (NSC-46015) was obtained from the Drug Development Branch, Chemotherapy, Cancer Control National Service Center, National Cancer Institute, National Institutes of Health, Bethesda, Maryland. The polymer was rehydrated in phosphate-buffered saline solution (PBS) (0.006 *M* sodium phosphate, 0.15 *M* NaCl), pH 7.0, and stored at 4° until used. *Endotoxin.* Lipopolysaccharide W (*E. coli* 0127:B8) obtained from Difco Laboratories, Detroit, Michigan, was rehydrated in PBS (100 μg/ml), sterilized by filtration with an ultrafine sintered glass filter, stored at 4° for no longer than 7 days, and diluted to the desired concentration, immediately prior to use. *Experimental design.* The hamsters were mixed at random 14–18 hr after birth and were divided into the experimental groups. At "0" time, 0.2 ml of undiluted adenovirus 12 was inoculated subcutaneously into the scapular region. Drug or PBS was given intraperitoneally in 0.1-ml volume in single or repeat doses as indicated in the text. The animals were palpated twice weekly for 6 weeks for subcutaneous tumors and once a week thereafter. Internal tumors were detected at autopsy after death of the animals. The location of tumors was recorded and their identity was established by gross and histopathologic examination.

* This study was conducted under Contract PH43-64-55 within the Special Virus Leukemia Program of the National Cancer Institute, NIH, USPHS.

TABLE I. Distribution of Adenovirus Type 12 Tumors in Newborn Hamsters According to Site and Multiplicity.

Site or multiplicity of tumors in placebo and untreated controls	Number (%) of tumor-bearing hamsters with indicated tumors		
	Total (150)[a]	Male (80)[a]	Female (70)[a]
Sites			
Subcutaneous (site of inoculation)	148 (99%)	80 (100%)	68 (97%)
Internal (total)	65 (43%)	28 (35%)	37 (53%)
Liver	65 (43%)	28 (35%)	37 (53%)
Kidney	1 (0.6%)	1 (1.3%)	0
Diaphragm	1 (0.7%)	1 (1%)	0
Mesentery	1 (0.7%)	0	1 (1%)
Peritoneal wall	1 (0.7%)	1 (1%)	0
Multiplicity			
One site	88 (59%)	53 (66%)	35 (50%)
Two sites	59 (39%)	25 (31%)	34 (49%)
Three sites	2 (1.3%)	1 (1%)	1 (1%)
Four sites	1 (0.7%)	1 (1%)	0

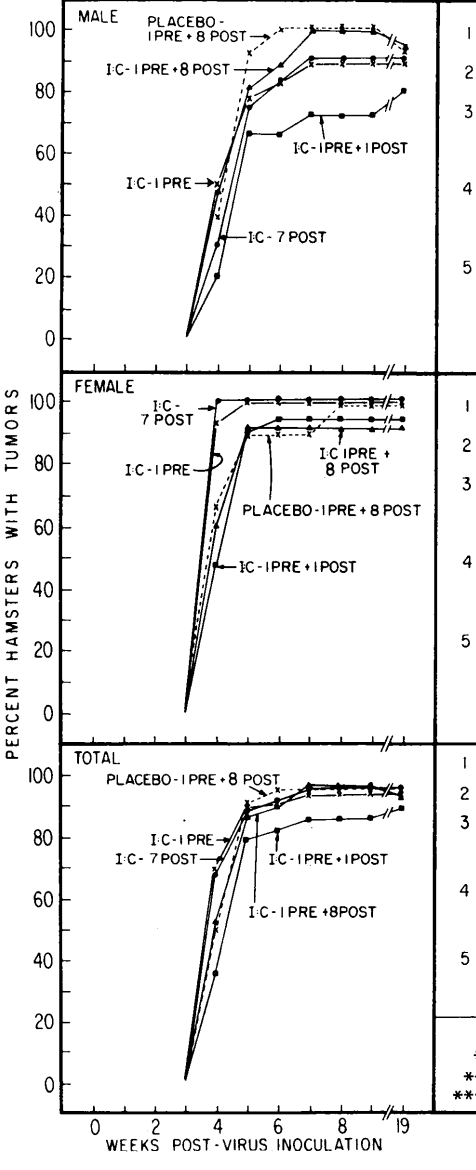
[a] Total numbers of animals from which the percentages were calculated.

Results. Distribution of adenovirus tumors. Table I shows the distribution, according to site and multiplicty, of tumors in the PBS-treated and untreated control hamsters given $10^{9.0}$ $TCID_{50}$ of adenovirus 12 subcutaneously when 20–24 hr old. The overall tumor incidence, regardless of site, was 96% (150/156). Essentially all of the tumor-positive animals developed subcutaneous tumors in the area where the virus was given (99%) and 43% of the animals developed internal tumor also. Internal tumors were usually limited to one site only but sometimes included two or three sites in addition to the subcutaneous tumors. The internal tumors were most often in the liver and were also present infrequently in the kidney, diaphragm, mesentery, or peritoneal wall. Females developed internal tumors (53%) more often than males (35%). Yohn *et al.* (7–9) have also noted viceral tumors in hamsters, especially females, given adenovirus 12 by parenteral routes.

Experiment 1. Newborn hamsters were given Poly I:C prior to and/or after adenovirus 12 in the regimens shown in Fig. 1. This included a single dose of Poly I:C given prior to virus (group 1), one dose given prior to and 1 dose given after virus (group 2), one dose given prior to and multiple doses given after virus (group 3), or multiple doses given only after virus (group 4). Placebo (group 5) was given in the same regimen as for group 3. The findings presented in Fig. 1 show the rate of occurrence of subcutaneous tumors (according to sex and total numbers) and the percentage of protection based on the difference in tumor incidence between the control (group 5) and the treated groups (group 1–4) 19 weeks after virus. Percentage of protection (+) or adverse effect (—) was calculated based on the difference in occurrence of tumor in the test group compared with the controls. There was no substantial difference between the treated and control groups with respect to subcutaneous tumors. The small differences which were found were at random and could not be related to treatment.

The occurrence of internal tumors in these animals is shown in Fig. 2. Though test variation was considerable, it was evident that protection up to 44% was given by the Poly I:C complex against development of internal tumors. This difference was most apparent in female animals and when the complex was given in multiple injections prior to and after virus. With one exception (males, group 4) minimal protection, if any, was apparent (at 19 weeks after virus) when Poly I:C was given in a single dose prior to virus or in multiple doses given only after virus. Although the final outcome was about the same in all groups, there was a temporary delay in detection of internal tumors which was apparent up to 6 weeks after virus in both male and female animals given a single dose of Poly I:C prior to virus.

Experiment 2. In a second experiment, the effect of Poly I and Poly C was compared with that of Poly I:C Figure 3 presents the regimen that was used and shows no effect of any of the substances on subcutaneous tumor, as was found in the previous experiment

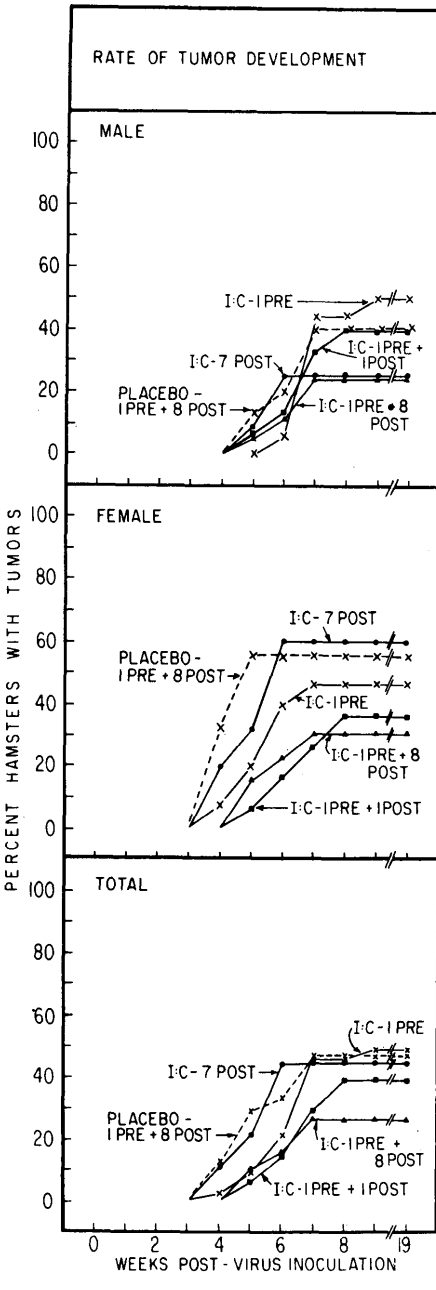


SUBCUTANEOUS TUMOR INCIDENCE AND PERCENT PROTECTION					
TREATMENT		RESULTS			
GROUP	REGIMEN*	TOTAL NO **	NO. (%) WITH TUMOR AT 19 WKS		PERCENT *** PROTECTION
1	1 DOSE I:C, - 6 HR	18	16	(89)	+ 4
2	2 DOSES I:C, - 6 HR., DAY 2	15	12	(80)	+ 14
3	9 DOSES I:C, - 6 HR., DAY 2 AND 4 DAY INTERVALS THROUGH DAY 30	17	16	(95)	- 2****
4	7 DOSES I:C, DAY 6 AND 4 DAY INTERVALS THROUGH DAY 30	12	11	(92)	+ 1
5	9 DOSES PLACEBO, -6 HR, DAY 2 AND 4 DAY INTERVALS THROUGH DAY 30	15	14	(93)	-
1	1 DOSE I:C, - 6 HR.	15	15	(100)	0
2	2 DOSES I:C, - 6 HR., DAY 2	19	18	(95)	+5
3	9 DOSES I:C, - 6 HR., DAY 2 AND 4 DAY INTERVALS THROUGH DAY 30	13	12	(92)	+8
4	7 DOSES I:C, DAY 6 AND 4 DAY INTERVALS THROUGH DAY 30	15	15	(100)	0
5	9 DOSES PLACEBO, -6 HR., DAY 2 AND 4 DAY INTERVALS THROUGH DAY 30	9	9	(100)	-
1	1 DOSE I:C, - 6 HR.	33	31	(94)	+ 2
2	2 DOSES I:C, - 6 HR., DAY 2	34	30	(88)	+ 8
3	9 DOSES I:C, - 6 HR., DAY 2 AND 4 DAY INTERVALS THROUGH DAY 30	30	28	(93)	+ 3
4	7 DOSES I:C, DAY 6 AND 4 DAY INTERVALS THROUGH DAY 30	27	26	(96)	0
5	9 DOSES PLACEBO, -6 HR., DAY 2 AND 4 DAY INTERVALS THROUGH DAY 30	24	23	(96)	-

* I:C dose = 52.5 μg; Virus inoculated at "0" time.
** No. hamsters weaned at 3 weeks.
*** Based on comparison with placebo group #5.
**** Minus sign indicates higher tumor incidence in treated than in control animals.

FIG. 1. Expt. 1. Effect of treatment with I:C on development of subcutaneous adenovirus type 12 tumors in hamsters.

(Fig. 1) with Poly I:C. Figure 4 shows, however, that there was marked reduction by Poly I:C of internal tumor as noted above (Fig. 2) and surprisingly, also, with Poly I and Poly C, even though these substances are not active as interferon inducers when given individually. The effect with Poly I was the same in males as in females. Poly C effect was noted only in females, with a "negative effect" in males.

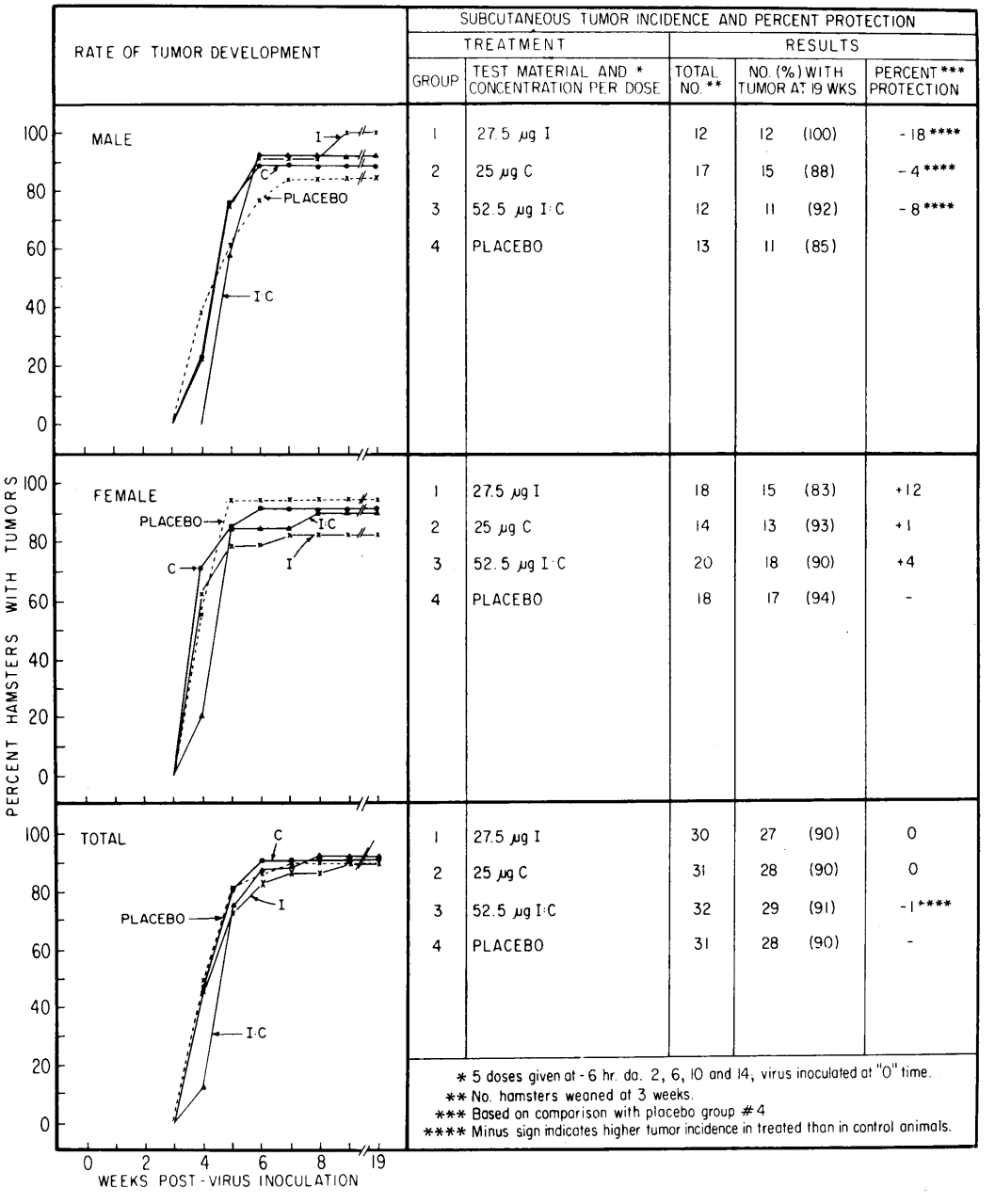


INTERNAL TUMOR INCIDENCE AND PERCENT PROTECTION				
TREATMENT		RESULTS		
GROUP	REGIMEN*	TOTAL NO.**	NO. (%) WITH TUMOR AT 19 WKS.	PERCENT*** PROTECTION
MALE				
1	1 DOSE I:C, -6 HR.	18	9 (50)	-25****
2	2 DOSES I:C, -6 HR., DA.2	15	6 (40)	0
3	9 DOSES I:C, -6 HR., DA.2 AND 4 DAY INTERVALS THROUGH DA. 30	17	4 (24)	+40
4	7 DOSES I:C, DA.6 AND 4 DAY INTERVALS THROUGH DA. 30	12	3 (25)	+38
5	9 DOSES PLACEBO, -6HR, DA. 2 AND 4 DAY INTERVALS THROUGH DA. 30	15	6 (40)	-
FEMALE				
1	1 DOSE I:C, -6 HR.	15	7 (47)	+16
2	2 DOSES I:C, -6 HR., DA. 2	19	7 (37)	+34
3	9 DOSES I:C, -6 HR., DA. 2 AND 4 DAY INTERVALS THROUGH DA. 30	13	4 (31)	+44
4	7 DOSES I:C, DA. 6 AND 4 DAY INTERVALS THROUGH DA. 30	15	9 (60)	-7****
5	9 DOSES PLACEBO, -6 HR., DA. 2 AND 4 DAY INTERVALS THROUGH DA. 30	9	5 (56)	-
TOTAL				
1	1 DOSE I:C, -6 HR.	33	16 (49)	-7****
2	2 DOSES I:C, -6 HR., DA.2	34	13 (38)	+17
3	9 DOSES I:C, -6 HR., DA.2 AND 4 DAY INTERVALS THROUGH DA. 30	30	8 (27)	+41
4	7 DOSES I:C, DA.6 AND 4 DAY INTERVALS THROUGH DA. 30	27	12 (44)	+4
5	9 DOSES PLACEBO, -6 HR., DA. 2 AND 4 DAY INTERVALS THROUGH DA. 30	24	11 (46)	-

* I:C dose = 52.5 μg; Virus inoculated at "0" time.
** No. hamsters weaned at 3 weeks.
*** Based on comparison with placebo group #5.
**** Minus sign indicates higher tumor incidence in treated than in control animals.

FIG. 2. Expt. 1. Effect of treatment with I:C on development of internal adenovirus type 12 tumors in hamsters.

Experiment 3. A third experiment was performed in which hamsters were given one of four interferon inducers in multiple doses prior to and after adenovirus 12. Fig. 5 shows weak, if any, effect of any of the substances on subcutaneous tumors. However, the double-stranded ribonucleic acids, Poly I:C and coliphage MU9-RNA, were highly active in preventing internal tumor, especially in female animals (Fig. 6). Lesser effects were noted



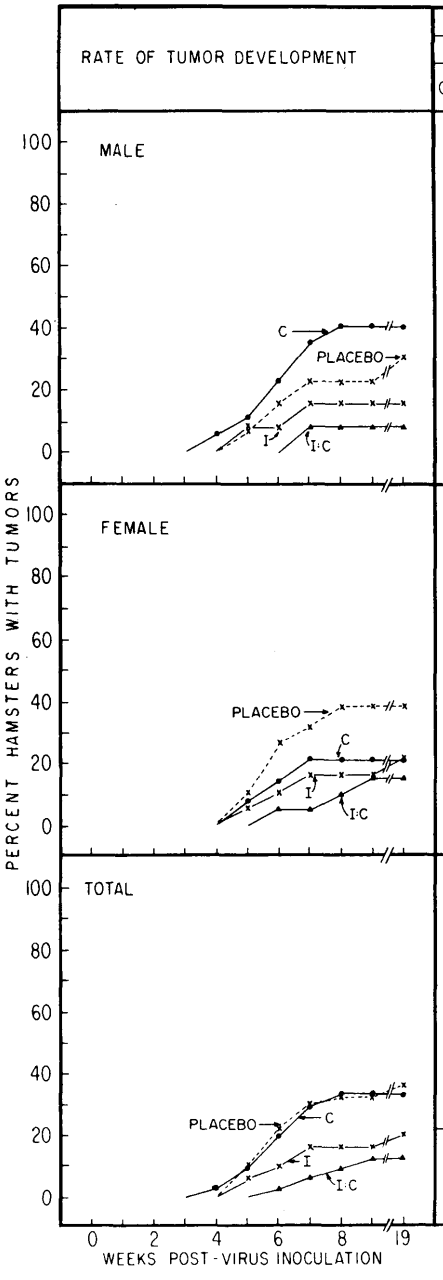
SUBCUTANEOUS TUMOR INCIDENCE AND PERCENT PROTECTION					
	TREATMENT		RESULTS		
	GROUP	TEST MATERIAL AND * CONCENTRATION PER DOSE	TOTAL NO. **	NO. (%) WITH TUMOR AT 19 WKS	PERCENT *** PROTECTION
MALE	1	27.5 μg I	12	12 (100)	-18****
	2	25 μg C	17	15 (88)	-4****
	3	52.5 μg I:C	12	11 (92)	-8****
	4	PLACEBO	13	11 (85)	
FEMALE	1	27.5 μg I	18	15 (83)	+12
	2	25 μg C	14	13 (93)	+1
	3	52.5 μg I:C	20	18 (90)	+4
	4	PLACEBO	18	17 (94)	-
TOTAL	1	27.5 μg I	30	27 (90)	0
	2	25 μg C	31	28 (90)	0
	3	52.5 μg I:C	32	29 (91)	-1****
	4	PLACEBO	31	28 (90)	-

* 5 doses given at -6 hr. da. 2, 6, 10 and 14, virus inoculated at "0" time.
** No. hamsters weaned at 3 weeks.
*** Based on comparison with placebo group #4
**** Minus sign indicates higher tumor incidence in treated than in control animals.

FIG. 3. Expt. 2. Effect of treatment with I, C, and I:C on development of subcutaneous adenovirus type 12 tumors in hamsters.

for pyran and endotoxin.

Discussion. The adenovirus 12 newborn hamster system was used in the present study as a model to measure the effect of double-stranded ribonucleic acids (synthetic Poly I:C and replicative form MU9 coliphage) (2, 4) and of certain other interferon inducers (pyran copolymer and endotoxin) on an oncogenic DNA virus. Subcutaneous tumors appeared in essentially all (99%) hamsters giv-

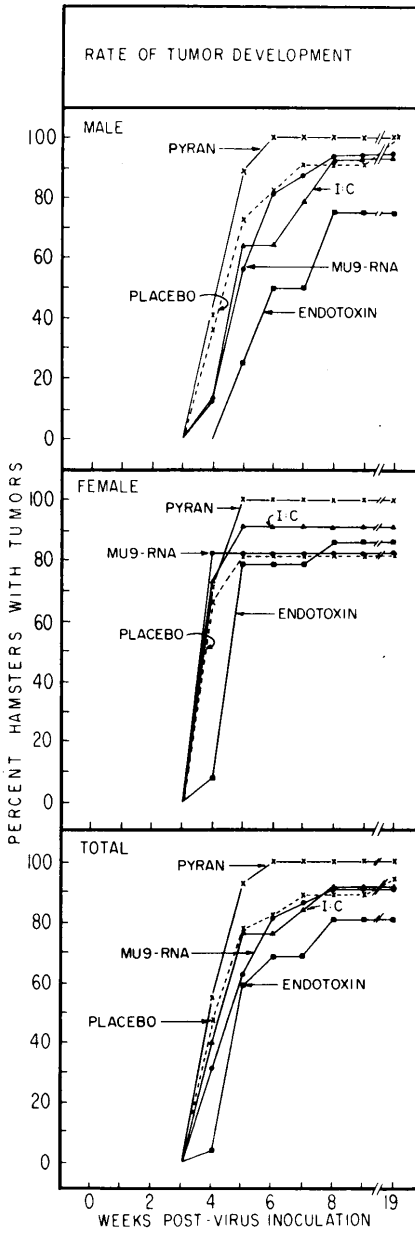


INTERNAL TUMOR INCIDENCE AND PERCENT PROTECTION				
TREATMENT		RESULTS		
GROUP	TEST MATERIAL AND * CONCENTRATION PER DOSE	TOTAL NO. **	NO. (%) WITH TUMOR AT 19 WKS.	PERCENT *** PROTECTION
MALE				
1	27.5 μg I	12	2 (17)	+43
2	25 μg C	17	7 (41)	-37****
3	52.5 μg I:C	12	1 (8)	+73
4	PLACEBO	13	4 (30)	-
FEMALE				
1	27.5 μg I	18	4 (22)	+44
2	25 μg C	14	3 (21)	+46
3	52.5 μg I:C	20	3 (15)	+62
4	PLACEBO	18	7 (39)	-
TOTAL				
1	27.5 μg I	30	6 (20)	+44
2	25 μg C	31	10 (32)	+11
3	52.5 μg I:C	32	4 (13)	+64
4	PLACEBO	31	11 (36)	-

* 5 doses given at -6 hr. da. 2, 6, 10 and 14; virus inoculated at "0" time.
** No. hamsters weaned at 3 weeks.
*** Based on comparison with placebo group #4.
**** Minus sign indicates higher tumor incidence in treated than in control animals.

FIG. 4. Expt. 2. Effect of treatment with I, C, and I:C on development of internal adenovirus type 12 tumors in hamsters.

en the large adenovirus 12 dose employed and internal tumors, especially in the liver, appeared in 43% of the animals. Visceral tumors were present approximately 1.4 times more often in female than in male hamsters. Higher incidence of subcutaneous tumor in female than in male hamsters after adenovirus 12 has been reported by others (8, 10). The reason for the more frequent occurrence of visceral tumor in females is not known but



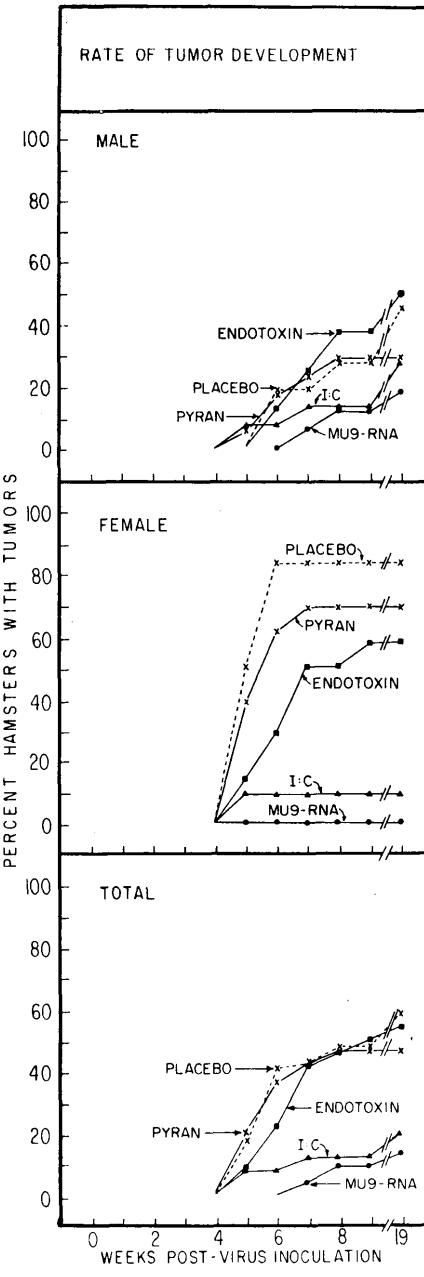
SUBCUTANEOUS TUMOR INCIDENCE AND PERCENT PROTECTION				
TREATMENT		RESULTS		
GROUP	TEST MATERIAL AND * CONCENTRATION PER DOSE	TOTAL NO.**	NO (%) WITH TUMOR AT 19 WKS.	PERCENT*** PROTECTION
1	500 μg PYRAN	17	17 (100)	0
2	1 μg ENDOTOXIN	8	6 (75)	+25
3	52.5 μg I·C	14	13 (93)	+7
4	25 μg MU9-RNA	16	15 (94)	+6
5	PLACEBO	11	11 (100)	-
1	500 μg PYRAN	13	13 (100)	-20****
2	1 μg ENDOTOXIN	14	12 (86)	-4****
3	52.5 μg I·C	11	10 (91)	-10****
4	25 μg MU9-RNA	6	5 (83)	0
5	PLACEBO	6	5 (83)	-
1	500 μg PYRAN	30	30 (100)	-6****
2	1 μg ENDOTOXIN	22	18 (82)	+13
3	52.5 μg I·C	25	23 (92)	+2
4	25 μg MU9-RNA	22	20 (91)	+3
5	PLACEBO	17	16 (94)	-

* 5 doses given at -6 hr. day 2, 6, 10 and 14; virus inoculated at "0" time.
** No. hamsters weaned at 3 weeks.
*** Based on comparison with placebo group #5.
**** Minus sign indicates higher tumor incidence in treated than in control animals.

FIG. 5. Expt. 3. Effect of treatment with pyran, endotoxin, I:C, and MU9-RNA on development of subcutaneous adenovirus type 12 tumors in hamsters.

might be related to hormonal differences. McFarlane *et al.* (11), *e.g.*, have demonstrated marked reduction of adenovirus 12 tumor incidence in gonadectomized female hamsters, suggesting a role of hormone (such as estrogen) in tumor promotion. Whatever the cause, the visceral tumors were often more readily suppressed in females than in males by the various substances which were tested.

The discovery of induction of interferon and of *in vivo* and *in vitro* host resistance to viral infection by double-stranded ribonucle-



INTERNAL TUMOR INCIDENCE AND PERCENT PROTECTION					
TREATMENT		RESULTS			
GROUP	TEST MATERIAL AND * CONCENTRATION PER DOSE	TOTAL NO. **	NO. (%) WITH TUMOR AT 19 WKS.		PERCENT *** PROTECTION
MALE					
1	500 μg PYRAN	17	5	(29)	+36
2	1 μg ENDOTOXIN	8	4	(50)	-11 ****
3	52.5 μg I:C	14	4	(29)	+36
4	25 μg MU9-RNA	16	3	(19)	+58
5	PLACEBO	11	5	(45)	-
FEMALE					
1	500 μg PYRAN	13	9	(69)	+17
2	1 μg ENDOTOXIN	14	8	(57)	+31
3	52.5 μg I:C	11	1	(9)	+89
4	25 μg MU9-RNA	6	0	(0)	+100
5	PLACEBO	6	5	(83)	-
TOTAL					
1	500 μg PYRAN	30	14	(47)	+20
2	1 μg ENDOTOXIN	22	12	(54)	+8
3	52.5 μg I:C	25	5	(20)	+66
4	25 μg MU9-RNA	22	3	(14)	+73
5	PLACEBO	17	10	(59)	-

* 5 doses given at -6 hr., day 2, 4, 6, 10 and 14; virus inoculated at "0" time.
** No. hamsters weaned at 3 weeks.
*** Based on comparison with placebo group # 5.
**** Minus sign indicates higher tumor incidence in treated than in control animals.

FIG. 6. Expt. 3. Effect of treatment with pyran, endotoxin, I:C, and MU9-RNA on development of internal adenovirus type 12 tumors in hamsters.

ic acid was reported by our laboratory in 1967 (1–6). Endotoxin was shown by others (12–14) to release preformed interferon and pyran was observed by Regelson *et al.* (15, 16) to induce interferon *in vivo*. Poly I:C, replicative form coliphage (MU9) RNA, endotoxin and pyran given in multiple doses prior to and after virus were all shown active in the present study in reducing the occurrence of internal but not of subcutaneous tu-

mor. The double-stranded RNA's were the most active and the effect was most readily demonstrated in female animals. The reason for the greater reduction in tumor in female animals is not known. It may be noted, however, that earlier maturation and better immune capacity has been observed in female than in male hamsters (8, 17) and that the nucleotides and endotoxin may enhance immune responses as discussed below.

The mechanism for the action of the double-stranded RNA's in preventing internal tumor is not known and can only be speculated upon at the present time. Poly I:C and MU9-RNA are both excellent inducers of interferon in mice and rabbits (2, 4, 6). Exogenous interferon production has not been demonstrable in hamsters by these substances (18) but this may be only quantitative since induction of resistance to virus by Poly I:C in hamster cells *in vitro* has been shown (5). Additionally, interferon has been demonstrated in hamsters in the viremic stage of viral infection (19). Certain oligodeoxyribonucleotides and ribonucleotide homopolymers, especially complexed double-stranded (*e.g.*, Poly A:U) molecules, may also function as potent stimulators of the immune mechanism; *viz.*, early increase, in the presence of antigens, in numbers of certain antibody-forming cells and stimulation of nucleotide kinase activity in antibody-forming cells (20). The fact that consistent activity against tumor appearance was demonstrated only when Poly I:C was given prior to virus corresponds with the fact that interferon action is principally or entirely prophylactic insofar as the individual infected cell is concerned. On the other hand, the single-stranded homopolymers, Poly I, and to a lesser extent Poly C, were also active. These substances exert very little, if any, interferon-inducing capacity when given alone. They are, however, quite active in stimulating the ordinary immune processes. The exact mechanism of action of double-stranded RNA in adenovirus oncogenesis remains obscure. The fact, however, that the single-stranded homopolymers were active and that the action was most evident in female hamsters suggests a role of ordinary immunologic factors rather than interferon.

The mechanism for the action of pyran on virus-induced tumor has been speculated upon by others (15, 16). It is known to induce interferon in mice and man (15, 16). It is taken up by the nucleated formed elements of the blood and stimulates the reticuloendothelial activity in liver, spleen, and bone marrow. Like many polyanions, it can exert a direct virus-neutralizing effect. Regelson has recorded inhibition of splenomegaly in Friend leukemia possibly due, in part, to direct virus inactivation and, in part, to increase in host resistance (15). It may be noted, based on present data, that pyran exerts weak but detectable activity against adenovirus oncogenesis though the mechanism of action is unknown. Endotoxin may stimulate release of preformed interferon from cells (12–14) and, in addition, exerts very profound effects in altering host resistance and stimulating the reticuloendothelial system (21–23). The mechanism of the weak action of endotoxin against adenovirus oncogenesis is also obscure.

A second report by our group (24) describes the effect of Poly I:C treatment on Friend leukemia virus infection in mice.

Summary. Synthetic double-stranded ribonucleic acid (Poly I:C) and replicative form RNA from MU9 coliphage reduced the occurrence of internal but not subcutaneous tumor after injection of adenovirus 12 subcutaneously into newborn hamsters. The effect was more evident in female than in male animals. Poly I:C treatment was consistently effective only when started prior to virus. The single homopolymers Poly I and Poly C were also protective even though they exert little, if any, interferon-inducing capacity. The action of the ribonucleotides might have been related both to interferon and to ordinary immune mechanisms. The greater activity in females as compared with male animals and the fact that the single-stranded Poly I and Poly C homopolymers were active favors the latter possibility. Pyran copolymer and endotoxin showed a minor reduction in internal but not subcutaneous tumor when given prior to and after adenovirus.

The authors are indebted to G. Beratan, W. Collom, and T. Napier for technical assistance.

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Received March 18, 1969. P.S.E.B.M., 1969, Vol. 131.