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Biological Activity of Polyoma Viral DNA in Mice and Hamsters

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The biological activity of polyoma viral DNA was evaluated in mice and hamsters. Viral DNA administered parenterally is about 4 to 5 logs less efficient than polyoma virions in establishing infection in mice. Supercoiled viral DNA was infectious for mice after parenteral administration, giving mean infective doses of 10^{-3} to 10^{-4} μg. However, animals fed microgram quantities of polyoma DNA I did not become infected. Linearization of viral DNA with R·*Eco*RI or R·*Bam*HI, which are single-cut enzymes cleaving in the early and late regions of the genome, respectively, reduced the infectivity for mice approximately fivefold. Approximately 10% of newborn hamsters inoculated intraperitoneally with polyoma DNA I developed tumors. In contrast, the same amount of viral DNA which had been cleaved in the early region with R·*Eco*RI induced tumors in 50% of inoculated hamsters.

The majority of animal viral DNAs are known to be infectious for tissue culture cells (9, 13), and in the case of many oncogenic viruses, such as simian virus 40 (SV40) and polyoma virus (PY), purified DNA is capable of transforming cells in culture (6, 8). The development of techniques to enhance both the infectivity (24) and transforming ability (2) of viral DNA has further facilitated study of the biological activity of various forms of viral DNA (1, 19) in permissive and nonpermissive tissue culture cell systems.

Much less is known about the biological activity of viral DNA after its inoculation into animals. Ito (20) reported that intradermal inoculation of DNA extracted from papillomatous tissue of wild cottontail rabbits could induce tumors at the inoculation site. Atanasiu and colleagues (3, 27) reported inducing tumors in hamsters by subcutaneous inoculation of DNA extracted from mouse embryo cell cultures infected with PY. DNAs extracted from SV40 (4), bovine papilloma virus (5), simian adenovirus type 7 (7), and oncogenic primate herpesviruses (11) have also been shown to be tumorigenic. Mayne et al. (25) demonstrated that simian adenovirus type 7 DNA fragmented by mechanical shearing retained its ability to induce tumors.

More recently, Sol and van der Noorda (34) found that 11 of 33 newborn hamsters injected with 1 to 2 μg of SV40 DNA I developed tumors during a 6-month observation period. A similar proportion of animals (5/17) injected with R·*Eco*RI-cleaved SV40 DNA also developed tumors. One of these DNA-induced tumors was established in tissue culture and shown to have SV40 T antigen and rescuable SV40.

Mice are known to be highly sensitive to infection by parenterally inoculated PY; a single tissue culture infectious dose is sufficient to initiate an infection that leads to antibody formation (30). Testing inoculated mice for serum hemagglutination-inhibiting (HI) antibodies, the so-called MAP (mouse antibody production) test (30), provides a simple and unambiguous assay for PY infection. In contrast to tumor induction assays in mice, which require inoculation of newborn animals, the infection detected by the MAP test is not age dependent.

To better define the biological activity of viral DNA in animals, we have studied the ability of PY DNA to initiate productive infection in weanling mice, as measured by the MAP test, and to induce tumors in newborn hamsters. Whereas the mouse system constitutes an in vivo test for productive infection, tumor induction in suckling hamsters provides an in vivo equivalent of a transformation assay. Although we found mice to be highly sensitive to parenterally adminstered PY DNA, we were unable to establish infection by feeding viral DNA. Furthermore, we found that the ability of PY DNA to induce tumors is enhanced by cleavage of the viral DNA with R·*Eco*RI, a restriction enzyme which cleaves PY DNA once (15) at a point near

the middle of the early region of the viral genome (21).

MATERIALS AND METHODS

Cell cultures, virus, and viral DNA. A large-plaque variant [PY(LP)] of PY, originally isolated by Vogt and Dulbecco (36), was kindly provided by T. Benjamin. The Toronto strain of PY (23) [PY(T)] was kindly provided by A. Howatson. Plaque-purified virus stocks of these viruses were propagated in secondary mouse embryo cells grown in McCoy 5A medium (GIBCO, Grand Island, N.Y.). After development of extensive cytopathic effects, the mixture of cells and medium was frozen and thawed three times, clarified by low-speed centrifugation, and stored at −60°C.

PY DNA was prepared from infected 3T6 cells by differential salt precipitation (17) followed by two cesium chloride-ethidium bromide equilibrium density centrifugations (12, 28). The supercoiled form of PY DNA was further purified by neutral sucrose gradient centrifugation. Only PY DNA purified in this manner was used in the experiments described. Except where noted, PY(LP) was used in experiments described below.

Restriction enzyme cleavage of PY DNA. PY DNA (50 μg/ml) was cleaved with R·*Eco*RI for 3 h at 37°C in a reaction mixture containing 20 mM Tris-hydrochloride, pH 7.5, 10 mM $MgCl_2$, and 50 mM $(NH_4)_2SO_4$, or with R·*Bam*HI for 3 h at 37°C in a reaction mixture containing 20 mM Tris-hydrochloride, pH 7.5, 7 mM $MgCl_2$, and 2 mM β-mercaptoethanol. In each case, the amount of enzyme added was determined in preliminary experiments to yield complete cleavage of PY DNA incubated under similar conditions. Enzymatic reactions were terminated either by the addition of a 1/20 volume of 0.5 M EDTA, pH 8.0, or by incubating the reaction mixture at 68°C for 6 min. Aliquots of the restriction enzyme digests containing 1 μg of DNA were monitored for complete cleavage of viral DNA by 1.4% agarose slab gel (17 by 12 by 0.3 cm) electrophoresis at 60 V for 16 h (18). Visualization of DNA by shortwave UV illumination in the presence of ethidium bromide (33) invariably revealed a single band which comigrated with full-length linear PY DNA. Such an analysis would allow the certain detection of as little as 2 to 5% contamination with uncleaved DNA. These digests were diluted with physiological saline and stored at −20°C.

Administration of PY virions and DNA to animals. PY virions and DNA suspended in physiological saline were administered to mice by several routes. Weanling mice were inoculated parenterally (intraperitoneally [i.p.] or subcutaneously [s.c.]) with 0.1 ml of the various test preparations. Preparations were administered by the gastric and colonic routes, using a ball-tipped 20-gauge, 1.5-inch (ca. 3.81-cm) stainless-steel catheter attached to a syringe; test animals were lightly anesthesized with ether during the procedure. Intranasal administration was carried out by the installation of 0.01 ml of test materials into the nostrils of lightly anesthesized animals. Mice were individually fed PY DNA either on a cube of bread or in 0.1 ml of milk after an overnight fast.

Newborn hamsters were inoculated i.p. in the right lower quadrant with 0.03 ml of PY or PY DNA (0.5 μg) and observed for 4 or 5 months for the development of tumors.

Evaluation of PY virion and PY DNA infectivity. To determine the susceptibility of mice to PY virions or PY DNA, the MAP test was employed as previously described (30). Five weanling mice from a PY-free colony (Charles River Laboratory, Wilmington, Mass.) were used per dilution. An uninoculated "in-cage" control mouse was included in each cage of inoculated mice, and one or more cages of uninoculated mice were included as room controls. Serum samples were obtained by orbital bleedings on days 21 and 42. The sera were tested for HI antibody in a microtiter test, using 16 hemagglutinating units of virus. Sera were screened at a 1:20 dilution, and positive samples were titrated. Titers greater than 1:20 were considered indicative of PY infection. None of the 134 in-cage control or 19 room control mice developed antibodies during the course of 8 independent experiments.

RESULTS

Susceptibility of mice to infection by PY administered by various routes. The effect of the route of administration of PY virions on the efficiency of infection of mice has been studied in some detail (32). As a preliminary study in the present work, we have repeated some of these tests and examined additional methods of virus administration. Serial dilutions of PY(LP) virions were inoculated by various routes, and the sera were tested for HI antibody. The mean infective dose (ID_{50}; the dilution of virus at which 50% of the mice would be infected) was calculated by the method of Reed and Muench (22, 30). As shown in Table 1, the ID_{50} of PY virions administered by the i.p. route was $10^{-5.5}$, corresponding to about 1 PFU per inoculum. This confirms an earlier observation (30) that the development of cytopathic effect in mouse

TABLE 1. *Comparison of mouse susceptibility to PY infection by various routes of infection*

Virus dilution[a]	Route (no. infected/no. inoculated)			
	Gastric tube (0.1 ml)	Colonic tube (0.1 ml)	i.p. (0.1 ml)	i.n.[b] (0.01 ml)
10^0	9/9	0/3		4/5
10^{-1}				
10^{-2}	5/5	2/5	5/5	
10^{-3}			4/5	
10^{-4}	1/5	1/4	4/5	
10^{-5}			5/5	
10^{-6}			1/5	
10^{-7}			1/5	
ID_{50} (0.1 ml)	$\sim 10^{-3.4}$		$10^{-5.5}$	

[a] Undiluted inoculum: $10^{5.6}$ PFU/0.1 ml.
[b] i.n., Intranasal.

embryo tissue culture cells and the development of HI antibody in mice were of equivalent sensitivity in detecting PY virions.

The administration of PY virions via a colonic tube resulted in scattered positive responses throughout the various dilutions (Table 1). We attribute this pattern to the inapparent penetration of the intestinal mucosa during intubation. Despite all reasonable precautions, intubations of weanling mice with a metal cannula may be traumatic, and we therefore regard the data evaluating the infectivity of PY virions and PY DNA by colonic tube as possibly spurious. The same reservation must be kept in mind for the gastric intubations; however, the uniform dose-response relationship shown in Table 1 suggests that the data may be valid. It should be noted that a previous study evaluating the infectivity of PY virions administered by feeding indicated that this route is about 6 logs less efficient than i.p. inoculation (32), whereas our data show gastric intubation to be only 3 logs less efficient.

Susceptibility of mice to infection by PY DNA. The susceptibility of weanling mice to infection by PY(LP) DNA was initially evaluated with supercoiled viral DNA, using the same type of protocol outlined in the preceding section (Table 2). The data indicate that mice are highly susceptible to infection by PY DNA I administered parenterally (i.p. or s.c.) but resistant to infection by DNA administered by several nonparenteral routes.

These data estimate the s.c. ID_{50} of PY DNA to be about 1.3×10^{-4} μg and the i.p. ID_{50} to be about 1.2×10^{-3} μg (Table 3). Since 1 μg of PY DNA contains about 10^{11} molecules, the ID_{50} by the s.c. route is equivalent to about 1.3×10^{7} molecules. Assuming that 1 PFU of virus corresponds to about 300 virus particles (26) and that each particle contains one viral DNA molecule, the infectivity of PY DNA I in vivo would be 5 and 6 logs below that of intact PY virions. Warren and Thorne (37) have shown that the specific infectivity of PY DNA assayed in cultured mouse embryo cells in the presence of DEAE-dextran is 6×10^{5} PFU/μg. This corresponds to 1.7×10^{-6} μg of DNA/PFU or 1.7×10^{5} molecules/PFU. Thus, the infectivity of viral DNA in mice is approximately 2 logs lower than in an optimally sensitive tissue culture system. We observed no difference in the infectivity of PY DNA for mice when the inoculum was administered in a suspension of calcium phosphate or in physiological saline (data not shown).

Mice showed a high degree of resistance to infection by PY DNA I administered by nonparenteral routes. The infectivity of DNA introduced through colonic and gastric tubes was initially evaluated (Table 2), but since similar experiments with virus (Table 1) suggested that intubation might produce inapparent trauma to mucosal surfaces, direct feeding experiments were carried out as well. Whereas some positive responses occurred in the groups given high doses of DNA by gastric tube, none of the 55 animals consuming 0.5 or 1.0 μg of PY DNA I subsequent to an overnight fast developed HI antibodies (Table 2). Thus, we conclude that orally administered viral DNA is at least 8 to 9 logs less infectious than DNA in whole-virus

TABLE 2. *Susceptibility of mice to infection by PY DNA by various routes of administration*

Inoculum		Route (no. infected/no. inoculated)					
DNA	Dose (ng)	s.c.	i.p.	Oral		i.n.[a]	Colonic (rectal tube)
				Gastric tube	Feeding		
DNA I	1,000		10/10	5/10	0/25		
	500		5/5	1/18	0/30	0/10	
	160						1/4
	50	5/5	5/5				
	16	15/15	12/15				1/4
	5	5/5	8/10				
	1.6	13/15	11/15				
	1		5/5				
	0.5	5/5	1/15				
	0.05	2/5	0/10				
DNA I (DNased)[b]	500		0/10				
	50		0/5				

[a] i.n., Intranasal.

[b] An aliquot of the stock PY DNA preparations was suspended in .01 M Tris-hydrochloride, pH 7.5, 0.01 M $MgCl_2$, and incubated in the presence of 50 μg of DNase (Worthington Biochemicals) per ml for 1 h at 20°C.

particles inoculated parenterally. As is indicated in Table 2, intranasally administered PY DNA I also did not lead to infection.

Pancreatic DNase-treated PY DNA did not evoke HI antibody production in any of the animals inoculated (Table 2).

To further define the biological activity of PY DNA in mice, we evaluated the infectivity of different physical forms of viral DNA (Table 4). The relaxed circular form (DNA II) of PY DNA was nearly as infectious as the supercoiled form, whereas full-length linear forms of viral DNA (DNA I cleaved with the single-cut restriction endonuclease R·*Bam*HI or R·*Eco*RI) retained 15 to 25% of the infectivity determined for DNA I.

Tumorigenicity of PY virions in newborn hamsters. In addition to examining the tumorigenicity of PY(LP), which was used in the mouse experiments described above, we also evaluated the T strain of PY, because of its reported virulence in inducing tumors in hamsters (35). Restriction enzyme digest patterns (R·*Hpa*I, R·*Hin*dII + III, R·*Bam*HI, and R·*Eco*RI) of PY(LP) and PY(T) DNA were not detectably different (our unpublished observations).

We determined the 50% tumor dose of PY(LP) and PY(T) to be 3.8×10^3 and 7.8×10^3 PFU, respectively. The rapidity of tumor development was related to the amount of virus injected and was similar in the two strains. The tumors that appeared had the gross appearance of characteristic PY hemagiomatous and sarcomatous lesions and were located in the kidneys, liver, intestinal wall, omentum, lungs, and thoracic wall.

Tumorigenicity of PY DNA. The tumorigenicity of PY DNA was evaluated by inoculating newborn hamsters with undigested or R·*Eco*RI-digested PY DNA I. As indicated in Table 5 and Fig. 1, 5 to 13% of the animals receiving PY(LP) or PY(T) DNA I and 43 to 50% of hamsters inoculated with R·*Eco*RI-cleaved PY DNA developed tumors. The tumors observed after i.p. administration of PY DNA were invariably located in the abdominal wall near the site

TABLE 3. *Infection of mice by PY DNA*

Route	No. of animals	ID_{50} (μg)
s.c.	50	1.3×10^{-4}
i.p.	90	1.2×10^{-3}
Oral (feeding)	55	No detectable infection
Oral (gastric tube)	28	0.81
Intranasal	10	No detectable infection
Colonic tube	8	0.34

TABLE 4. *Effect of physical configuration on DNA infectivity in mice*

Configuration	Route of infection (ID_{50} [ng])	
	s.c.	i.p.
Supercoiled	0.63	0.4
Relaxed circular	1.05	
R·*Bam*HI linear	2.5	4.2
R·*Eco*RI linear	4.0	1.4

TABLE 5. *Tumorigenesis in newborn hamsters by PY DNA*[a]

DNA configuration	Virus strain	Expt	Incidence of tumors: No.[b]	Total: No.	Total: %
PY supercoiled	PY(LP)	1	0/5	4/30	13
		2	3/12		
		3	1/13		
	PY(T)	1	1/13	1/22	4.5
		2	0/9		
PY R·*Eco*RI linear	PY(LP)	1	1/7	9/21	43
		2	8/14		
	PY(T)	1	6/12	10/20	50
		2	4/8		
PY supercoiled (DNase)[c]				0/4	
pMB 9 DNA I				0/17	
Saline				0/18	
Uninoculated controls				0/6	

[a] In each case, animals were inoculated intraperitoneally with 0.5 μg of PY DNA.

[b] Number of animals developing tumors/number of animals inoculated.

[c] See Table 2 for DNase incubation conditions.

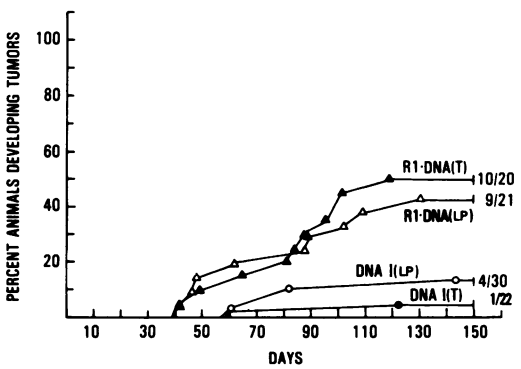

FIG. 1. *Development of tumors in newborn hamsters after inoculation with PY DNA. Same experiments as reported in Table 5. Fractions at the right indicate the number of animals developing tumors compared with the number of animals inoculated with a particular DNA preparation. PY(T) and PY(LP) refer to the strain of virus from which the DNA was isolated. RI·DNA refers to DNA cleaved by R·EcoRI (see Materials and Methods).*

of inoculation. Several tumors were examined histologically and were undifferentiated sarcomas. Many of the tumors induced by either PY DNA I or R·*Eco*RI PY DNA have been established in tissue culture. While all lines examined to date contain PY T antigen as assayed by indirect immunofluorescence, the 105,000-dalton species of T antigen was not detected by immunoprecipitation (M. A. Israel et al., manuscript in preparation).

Development of HI antibodies in hamsters inoculated with PY DNA. One explanation of the tumors appearing in hamsters after parenteral administration of PY DNA is that they resulted as a direct effect of viral DNA inoculation, presumably by a mechanism involving the integration of the input DNA. We also considered the possibility that viral DNA infection resulted in progeny virion production which, in turn, induced the tumors observed. If the latter explanation is correct, the sera from tumor-bearing animals should contain HI antibody.

The sera of 117 hamsters inoculated with PY DNA were examined for the presence of HI antibody. Ten of 76 animals without tumors had measurable serum HI antibody levels (1:20 to 1:160) 3 to 4 months after injection; sera from 5 of 41 tumored hamsters had detectable HI antibody. In another experiment, 12 of 25 nontumored animals had detectable HI antibody 187 days after the injection of 10^3 to 10^5 PFU of PY virions. These results indicate that while the HI test is a relatively insensitive assay for virus production in hamsters (29, 31), the development of antibody cannot be correlated with PY tumor induction.

DISCUSSION

Although a number of investigators have studied the tumorigenicity of viral DNA in animals (3–5, 7, 11, 20, 25, 27, 34), the ability of viral DNA to initiate productive infection in vivo has received little attention. Our experiments indicate that less-than-nanogram amounts of PY DNA can lead to productive viral infection after parenteral inoculation into mice. This level of infectivity is 2 logs below that reported when mouse cells in culture are infected with PY DNA in the presence of DEAE-dextran (37). Whereas addition of calcium phosphate to our inoculum did not enhance its infectivity in mice, the efficiency of infection we observed is adequate to allow the use of mice as a model system in certain experiments requiring expression of the PY genome. In particular, the biological activity of recombinant DNA molecules constructed by linking DNA sequences to a vector derived from the PY genome could be studied in mice.

To the extent that studying the activity of altered viral genomes in animals might represent a potential biohazard, it is interesting to note the wide margin of safety provided simply by purification of DNA from the whole virus. We found PY DNA to be 4 to 5 logs less efficient than PY virions in establishing infection in mice. Furthermore, we were able to establish infection in mice only by the parenteral administration of viral DNA. Experiments in which PY DNA was fed to animals (55 animals) or administered intranasally (10 animals) never resulted in detectable viral infection. Although infection could be established on occasion by administration of viral DNA by either gastric (6/28) or rectal (2/8) tube, we believe this finding may be spurious, reflecting inapparent parenteral inoculation from the trauma of the intubation procedures.

The infectivity of R·*Eco*RI and R·*Bam*HI linear PY DNA molecules for mice is within 10-fold the infectivity of PY DNA I. Although some molecules in the inoculum may have recircularized prior to inoculation, most molecules were no doubt in the linear configuration at the time of injection (see Materials and Methods). Thus, our results indicate that linear DNA molecules do not have a greatly increased susceptibility to degradation and can be efficiently recircularized after their injection into animals.

Stoker (35) reported that as few as 75 to 340 PFU (equivalent to about 2×10^4 to 10×10^4 PY DNA molecules) of the T strain of PY administered i.p. will induce tumors in 50% of newborn hamsters. Our results indicate that 4×10^3 PFU (1.2×10^6 molecules of PY DNA) of PY virions induce tumors in 50% of inoculated hamsters. We also determined that 0.5 μg (5×10^{10} molecules) of PY DNA I, injected by the i.p. route, resulted in tumors in about 10% (5/52) of the inoculated animals (Table 5). On the basis of these experiments, we conclude that PY DNA is at least 4 to 5 logs less tumorigenic than PY virions.

Our results which show that approximately 10% of newborn hamsters develop tumors after i.p. inoculation of PY DNA I contrast sharply with those of Orth et al. (27), who reported the development of tumors in 26 of 34 animals (76%) within 3 months of s.c. inoculation of cellular DNA preparations containing PY DNA. Whether this difference reflects the different routes (i.p. versus s.c.) of inoculation or the possible contamination of the DNA preparations used by Orth et al. (27) with intact PY is presently unclear. It is highly unlikely that our PY DNA preparations contain any virus particles. Purification of the DNA included a Hirt extraction in 0.4% sodium dodecyl sulfate (17), two dye

density equilibrium centrifugations (12, 28), and a final sedimentation through a 5 to 30% neutral sucrose gradient. Furthermore, the same DNA preparation used to inoculate hamsters was not infectious in mice after DNase treatment (Table 2).

A possibly important observation was that the tumorigenicity of PY DNA was significantly enhanced by cleavage of the viral genome at the R·*Eco*RI site. This enhancement was quite unexpected in view of the location of the R·*Eco*RI site in the middle of the early gene region of the PY genome (21). Cleavage at this site would affect the synthesis of PY large T antigen but not PY small t antigen (10). The hr-t function, required for transformation (10) and presumably tumorigenesis, would also remain intact. In a recent experiment, eight of nine newborn hamsters inoculated i.p. with 0.5 μg of S_1 nuclease-treated R·*Eco*RI PY DNA developed tumors (unpublished observation).

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