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Effect of β-Propiolactone Inactivation of Polyoma Virus on Viral Functions

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Polyoma virus was inactivated by treatment with β-propiolactone. T-antigen production, polyoma-RNA synthesis, induction of host DNA synthesis (measured by incorporation of labeled thymidine into the cell culture), and in vitro transforming ability were inactivated to a similar degree by various β-propiolactone concentrations (0.25% β-propiolactone reduced these functions approximately 96%), whereas plaque-forming ability and the ability of the virus to replicate its DNA and to synthesize capsid antigen were inactivated by a given concentration of β-propiolactone to a much greater degree (0.25% β-propiolactone led to a reduction of plaque-forming ability of over 8 logs). The significance of these data and their relationship to previously published experiments are discussed.

Previous studies using a variety of agents to inactivate polyoma virus (i.e., UV, X ray, nitrous acid, P_{32} decay [2], and gamma irradiation [1]) have demonstrated that in vitro transforming ability is inactivated at a slower rate (implying a smaller target size) than is plaque-forming ability. With UV irradiation, Defendi et al. (6) demonstrated that plaque-forming ability is inactivated at a much more rapid rate than induced compliment-fixing antigen (tumor [T] antigen) and that the ability of the virus to induce tumors in animals is essentially unaffected (or may even be enhanced). In this communication, we compare the effect of β-propiolactone (BPL) treatment of polyoma virus with regard to the following properties: (i) the production of virus-specific RNA; (ii) viral and virus-induced cellular DNA synthesis; (iii) the appearance of T and capsid antigens; (iv) in vitro transforming ability; and (v) viral infectivity. Our data show that T-antigen synthesis, early RNA synthesis, in vitro transforming ability, and the ability of the virus to induce host DNA synthesis were inactivated to a similar degree. Plaque-forming ability was inactivated to a much greater extent. The inactivated virus was unable to replicate its DNA or make capsid antigen. These experiments agree in general with the previously performed experiments cited above in which some of these properties were compared.

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MATERIALS AND METHODS

Tissue culture. Throughout the investigation, primary cultures of baby mouse kidney cells prepared as described previously (19, 20) were used. The cells were grown to stationary monolayers in Dulbecco modified (4 times concentration of amino acids and vitamins) Eagle medium (20) supplemented with 10% calf serum. Infected cultures were maintained in the same medium without serum and were incubated at 27 or 37 C.

BPL inactivation of polyoma virus. BPL was obtained from Fluka AG (Buchs; puriss grade). Stock wild-type polyoma virus (19, 20), from which cellular debris was removed, was distributed into portions and treated with various concentrations (see Table 1 and Fig. 1) of BPL diluted with medium at 37 C. Samples were removed at various times, diluted with cold medium, and titered for hemagglutinating (6) and plaque-forming ability (4). For the other studies, the virus was inactivated for 2.5 h at 37 C and was left overnight at 4 C. During BPL treatment, the pH of the 0.125% BPL solution containing the virus falls to 6.4; therefore, following treatment of the virus, the suspension was neutralized with sodium bicarbonate and stored frozen at −20 C until used.

Purified [^{3}H]thymidine-labeled virus was obtained from polyoma-infected cultures that were maintained for 72 h in serum-free medium supplemented with [^{3}H]thymidine (10 μCi of medium per ml; NEN Corp.; specific activity 20 Ci/mmol). Polyoma virus was purified as previously described (3).

The purified virus was inactivated with 0.25% BPL as described above. DNA was extracted from the purified virions by treatment with proteinase K (0.2 mg/ml) and sodium dodecyl sulfate (SDS; 0.2%) at 37

C for 15 min. This DNA was then analyzed by isopycnic centrifugation in ethidium bromide-cesium chloride (13).

T-antigen synthesis, capsid antigen synthesis, and induction of DNA synthesis in the host cell. The ability of BPL-treated polyoma virus to induce T-antigen and capsid antigen synthesis (18, 19) was determined by immunofluorescence, and induction of host DNA synthesis was determined autoradiographically (18, 19). Cover slips containing confluent monolayers of mouse kidney cells were infected and maintained at 37 C for 28 h or at 27 C for 96 h. One-half hour before harvesting the cover slips, the medium was replaced with serum-free medium containing 2 μCi of [^{3}H]thymidine per ml (NEN Corp.; specific activity 20 Ci/mmol). At various times, the dishes were chilled on ice and the labeled medium was removed; the cover-slip cultures were washed three times with isotonic buffer (20) and then the cover slips were cut in half. One-half of the cover slip was fixed in glacial acetic acid-ethanol (1:3) for autoradiography. The other half was fixed at −20 C in acetone-methanol (7:3) for T and capsid immunofluorescent staining. The procedures for both autoradiography and immunofluorescent staining have been previously reported (18, 19). The antisera used for determining T-antigen synthesis was a gift of V. Defendi.

Polyoma-DNA synthesis in infected cultures. Cultures that were infected with untreated or BPL-inactivated polyoma virus were maintained in serum-free medium containing 2 μCi of [^{3}H]thymidine per ml (NEN Corp.; specific activity 20 Ci/mmol) for 96 h at 27 C. Cells were lysed by the addition of SDS and polyoma DNA was selectively extracted with 1 M NaCl (9). The salt-soluble DNA was further separated into component I (supercoiled) and component II (nicked circular and linear) DNA by equilibrium centrifugation in ethidium bromide-cesium chloride (13).

Polyoma-RNA synthesis. Cultures were infected with untreated or BPL-inactivated polyoma virus and were maintained in serum-free medium containing 5-fluorodeoxyuridine (5×10^{-5} M) for 60 h at 27 C (18). The medium was then replaced with serum-free medium containing 250 μCi of [^{3}H]uridine per ml (NEN Corp.; specific activity 25 Ci/mmol), and the cultures were reincubated for an additional 3 h. Conditions for extraction and hybridization of the RNA have been described previously (19).

In vitro transformation. BHK cells were used to assay transforming ability of the untreated and BPL-inactivated polyoma virus. The transformation assay was performed as described by MacPherson and Montagnier (11). BHK-21/13 cells were a gift from I. Maxwell.

RESULTS

The effect of BPL treatment on the plaque-forming ability of polyoma virus is shown in Fig. 1. The loss of hemagglutinating ability (data not shown) was minimal (50% reduction after 16 h of treatment with 0.125% BPL and 75% reduction with 0.25% BPL) except after the most severe treatment. On the other hand, the reduction of plaque-forming ability was drastic (over 4-log drop in titer with 0.125% BPL). There was some variability in the degree of inactivation, by a given concentration of BPL, from preparation to preparation. The results in each of the following experiments, therefore, were obtained by using virus prepared at one time. The control (untreated) polyoma preparations were taken from the concentrated polyoma stock used for BPL treatment before the addition of BPL.

Experiments were performed to determine whether BPL treatment effected virus adsorption. Labeled untreated and BPL-treated virus were allowed to adsorb for 2 h, the monolayers were then washed with isotonic buffer, the cells were solubilized with NaOH, and the radioactivity remaining associated with the cell monolayer was counted. The BPL-treated virus adsorbed to the cells only 60 to 70% as well as the untreated virus.

On SDS-polyacrylamide (15%) slab-gel electrophoresis (12), the mobility of the peptides from the BPL-treated purified virus preparations (0.125 and 0.25%) were unchanged (gel pattern not shown) from that of the untreated

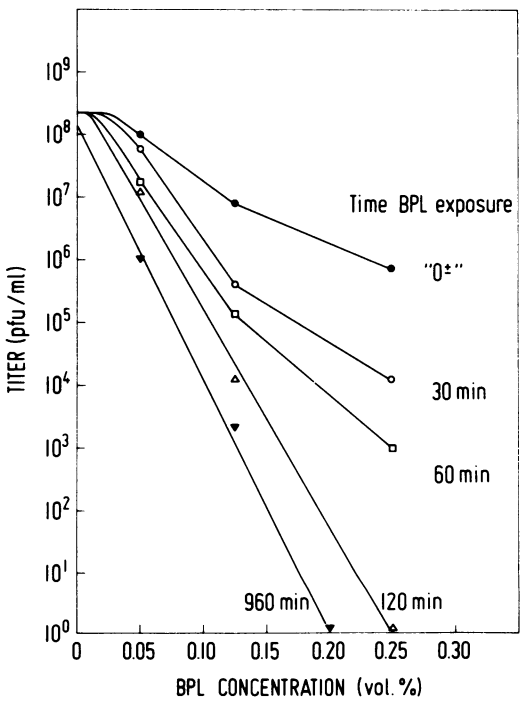

FIG. 1. *The virus was treated with various concentrations of BPL. At the time indicated, samples were removed, diluted promptly with medium, and assayed for plaque-forming activity (4).*

virus. All preparations revealed the seven polypeptides identified in polyoma virions (Consigli, Zabielski, and Weil, manuscript in preparation). Ethidium bromide-cesium gradient profiles of DNA extracted from purified [^{3}H]thymidine-labeled untreated or BPL-treated virus demonstrated that no component I (covalently closed-circular supercoiled DNA) could be seen in the BPL-treated preparation. Preliminary data obtained from alkaline sucrose gradients revealed the DNA from the BPL-treated virus was found fragmented to 8 to 10*S* sized pieces. Further work is in progress characterizing the precise physical effects of BPL upon the virus.

The properties of polyoma virus treated with 0.125 and 0.25% BPL, of untreated polyoma virus, and of untreated virus, diluted to a PFU equivalent to the 0.125% BPL-treated virus, are compared in Table 1. The BPL-treated virus produced a much greater number of T-antigen-positive cells than expected by its infectious titer. No significant amount of capsid antigen was synthesized by any but the untreated, undiluted, virus-infected cells. Similarly, there was no significant stimulation of the number of cells which incorporate [^{3}H]thymidine into their nuclei when infected with diluted, untreated virus or with the two BPL-treated virus preparations when compared with the mock-infected cells. The undiluted, untreated virus under these conditions induced 40% of the cells to incorporate nuclear label.

DNA synthesis in infected cells was also examined by extracting the [^{3}H]thymidine-labeled cellular and viral DNA by the selective SDS-salt method (9) and then by banding the salt-soluble DNA in ethidium bromide-cesium chloride (Table 2 and Fig. 2). The presence of component 1 (supercoiled, closed circular) DNA was considered an index of viral DNA synthesis and could only be detected, in these experiments, in the cells infected with the undiluted, untreated virus preparations. This interpretation was confirmed by hybridization of both the salt-soluble and the insoluble DNA fractions with polyoma DNA (data not shown). Although

TABLE 1. *Properties of BPL-inactivated polyoma virus*[a]

Infection	Titer[b]			T antigen[c] (% positive)		Capsid antigen[c] (% positive)		Autoradiography[d] (% positive)	
	PFU/ml	HA/ml	MOI	27 C/96 h	37 C/28 h	27 C/96 h	37 C/38 h	27 C/96 h	37 C/28 h
Mock	0	0	0	0	0	0	0	2.2	3.5
Untreated virus	2.5×10^8	32,000	10	85.2	62.3	36.8	37.0	37.3	46.9
Untreated diluted	2.5×10^3	<10	10^{-4}	1.3	1.9	0	0.1	4.4	5.0
BPL 0.125%	2.5×10^3	16,000	10^{-4}	25.3	12.9	0	0	2.2	4.9
BPL 0.25%	10	8,000	10^{-6}	3.7	1.0	0	0	2.0	2.2

[a] Data was obtained at 48, 70, and 96 h after infection at 27 C and 12, 18, and 28 h after infection at 37 C. Only the data at the latest times are presented, and at these times the values are maximal.

[b] HA, hemagglutination (reciprocal of the highest dilution of virus suspension which agglutinated guinea pig erythrocytes); MOI, multiplicity of infection (determined by virus titration and cell enumeration).

[c] Determined by indirect immunofluorescence; percentage determined by counting > 1,000 cells.

[d] Percentage determined by counting > 1,000 cells.

TABLE 2. *DNA synthesis in virus-infected cultures*[a]

Infection	Titer	Selective salt extract		
		Insoluble fraction (counts/min)	Soluble fraction (counts/min)	Total counts/min
Mock	0	12,520	16,560	29,080
Untreated virus	2.5×10^8	143,600	157,920	301,520
Untreated diluted	2.5×10^3	13,440	18,560	32,000
BPL 0.125%	2.5×10^3	19,720	24,240	43,960
BPL 0.25%	10	10,720	15,920	26,640

[a] Infected cells were maintained for 96 h at 27 C in serum-free medium containing 2 μCi of [^{3}H]thymidine per ml. Cells were extracted by the selective SDS-salt procedure (9). Salt-insoluble DNA is the high molecular weight (cellular) DNA. The soluble fraction includes viral DNA and fragmented cellular DNA. The salt-soluble fraction was subjected to ethidium bromide-cesium chloride iopycnic centrifugation. Component I (covalently closed circular) DNA was used as an index to the presence of viral DNA. See Fig. 2 for gradient profiles.

these experiments were performed to determine whether viral DNA was synthesized, they may also provide an index of cellular DNA synthesis. These data complement the autoradiography experiment (Table 2), but they also demonstrate the presence of a small anount of induced (over mock levels) cellular DNA synthesis in the preparation infected with virus inactivated with 0.125% BPL (Table 2).

The ability of BPL-inactivated polyoma virus to induce the synthesis of specific polyoma RNA was compared to various multiplicities of infection of untreated polyoma virus. The hybridization data for a series of dilutions of untreated polyoma virus and for virus inactivated with 0.125 and 0.25% BPL are shown in Table 3. As can be seen, more viral RNA was synthesized by the inactivated virus than could be accounted for by infectious titer alone, but, as with the other properties studied, less was synthesized than expected from the total virus particles present.

The transforming ability of the BPL-treated virus preparations was compared with that of various dilutions of untreated virus (Table 4). The results show that the BPL-treated virus preparations also had more in vitro transforming ability than could be related to infectious particles.

DISCUSSION

BPL was used to inactivate polyoma virus because it was previously demonstrated to be an excellent inactivating agent for the paramyxovirus group (10), and because it was also found that BPL quickly broke down into 3-hydroxypropanoic acid, a compound metabolizable by mammalian systems. In addition, it was also demonstrated that BPL treatment of Sendai virus could totally inactivate infectivity while preserving some Sendai genomic functions (16, 17). Thus, it was of interest to investigate the ability of BPL to inactivate a DNA tumor virus like polyoma and to determine the effect of BPL on specific viral functions.

BPL treatment was capable of drastically reducing the infectious titer of polyoma virus. This may be, in part, due to affecting capsid proteins as suggested by the slight reduction of hemagglutinating ability and adsorption of the virus, even though the mobility of virion polypeptides on SDS-polyacrylamide gels were unaltered. Its major effect, however, appears to be alteration of the viral DNA.

Many of the viral particles inactivated with 0.125% BPL were capable of serving as templates for transcription and were capable of eliciting the production of T antigen, even though noninfectious. However, they were not capable of viral DNA replicating or late protein (capsid antigen) synthesis. The data from these and other experiments regarding the degree of inactivation by different BPL concentrations of the various parameters studied are shown in

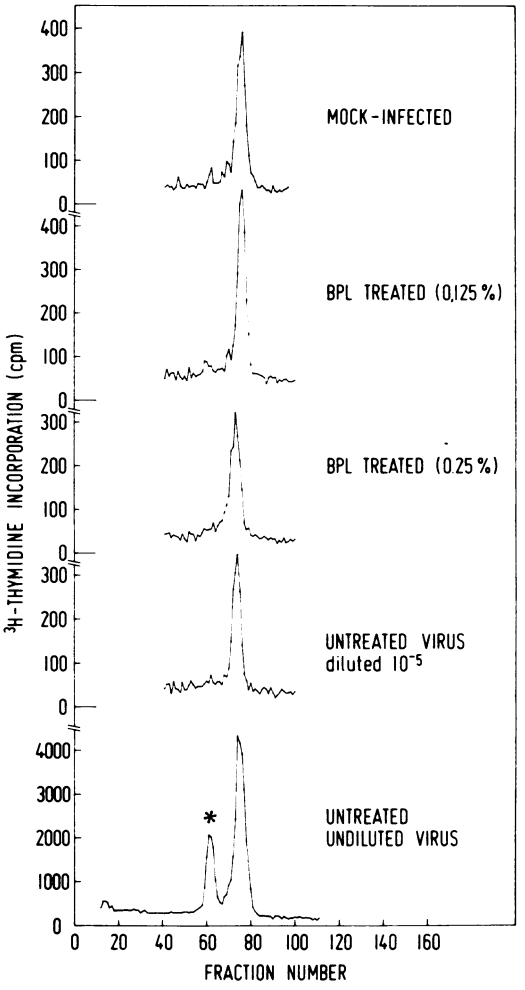

FIG. 2. *Primary mouse kidney cultures were infected (see Table 1 for respective multiplicities of infection) with either untreated stock virus, diluted untreated virus, or virus inactivated with either 0.125 or 0.25% BPL. Control mock-infected cultures were prepared. After viral adsorption, the cultures were incubated for 96 h at 27 C with serum-free medium containing 2 μCi of [^{3}H]thymidine per ml. Cultures were then extracted by the selective SDS-salt procedure (9), and the extractable DNA was subjected to isopycnic ethidium bromide-cesium chloride centrifugation (43,000 rpm for 24 h at 20 C in a Spinco SW50.1 rotor). Fractions were collected from the bottom of the gradient. The starred peak (*) contains component 1 (covalently closed-circular DNA).*

TABLE 3. *Synthesis of polyoma-specific RNA*

Virus	MOI[a] (PFU/ml)	Virus particles[b] per cell	Counts/min added (× 10 ^{6})	Hybridized	
				counts/min	%
Untreated with dilutions	20	2,000	2.5	13,700	0.557
	10	1,000	4.1	9,635	0.237
	2.5	250	3.7	7,982	0.215
	0.02	2	3.2	313	0.010
	0.0001	0.01	1.9	60	0.003
BPL 0.125%	0.02	2,000	2.0	866	0.043
BPL 0.25%	0.00002	2,000	1.7	258	0.015
Mock			1.2	48	0.004

[a] MOI, Multiplicity of infection; arrived at by viral titration and cell enumeration.

[b] Total number of virus particles per cell assuming a 100-fold excess of particles over PFU (4; R. Weil, unpublished data) in the case of the untreated virus preparations. In the BPL-inactivated preparations, the value given for virus particles per cell is based on the viral titer (PFU) before inactivation, while the MOI is based on the titer after inactivation.

TABLE 4. *In vitro transforming ability of BPL-treated polyoma virus*

Infection	MOI[a]	Virus particles per cell	Transformed colonies	Total cells plated (× 10 ^{6})	Transformants per 10^{6} cells
Untreated virus	10	10^{3}	336	6.0	56
Untreated diluted 1/10	1	10^{2}	57	3.25	15
Untreated diluted 1/10^{5}	10^{-4}	10^{-2}	0	6.0	0
BPL 0.125%	10^{-4}	10^{3}	20	9.25	2.2
BPL 0.25%	10^{-6}	10^{3}	3	6.0	0.5
Mock	0	0	2	12.75	0.15

[a] MOI, Multiplicity of infection.

Fig. 3. The degree of inactivation of polyoma-RNA synthesis, induction of host DNA synthesis, and in vitro transforming ability were similar; the synthesis of T antigen was affected to a somewhat lesser degree. However, the reduction in plaque-forming activity was dramatic.

The relative degree of inactivation of various polyoma functions is in general agreement with previous studies (1, 2, 6, 14) in which several of these parameters were compared. The slight difference in the degree of reduction in T-antigen synthesis with increasing BPL concentration and the degree of inactivation of viral induction of DNA synthesis and in vitro transforming ability may be due to (i) the synthesis of immunologically active, but biologically inactive T antigen and (ii) the method of determining T antigen by scoring a cell only as positive or negative.

Preliminary experiments in our laboratory indicate that BPL-treated polyoma virus, which was totally inactivated as to plaque-forming ability and induction of T antigen, was capable of inducing multiple tumors in baby hamsters. These preliminary experiments are in general agreement with the findings of Defendi et al. (6), who used UV inactivation of virus, but is at odds with similar UV experiments of Herberman and Ting (8).

The resistance of the in vivo tumor-stimulating ability of the virus to inactivation by BPL or UV is perplexing, as it is difficult to conceive of the way in which (i) the relative target-size could be so very small or (ii) the effect of recombination or some other repair-reactivation system could be so efficient or selective. The latter possibility is supported by recent reports by Seemayer and Defendi (15) that UV-irradiated Simian virus 40 virions are subject to repair by the host cell system which may actually enhance the oncogenic capabilities of the virions.

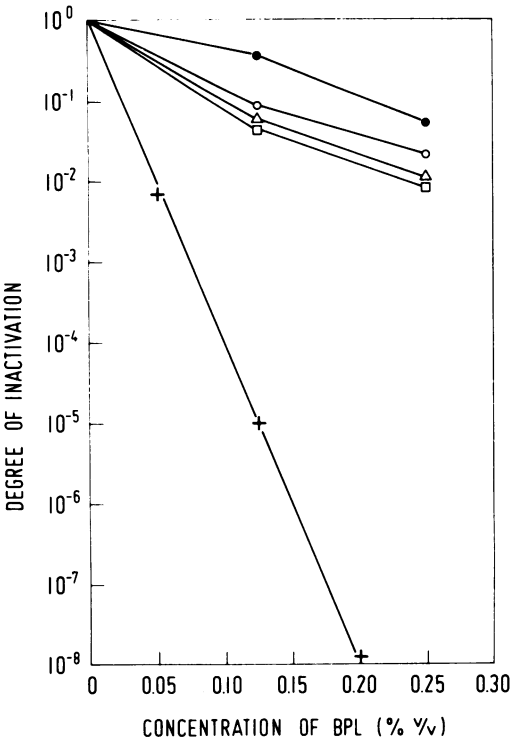

FIG. 3. *Inactivation of polyoma virus functions by BPL treatment of polyoma virions. Symbols: ●, T-antigen synthesis; ○, polyoma-RNA synthesis; △, induction of DNA synthesis; □, in vitro transformation; and, +, plaque-forming titer. The values indicated are the fraction of residual activity as compared with untreated virus. The values represent the averages of several determinations.*

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