JOURNAL OF VIROLOGY, Nov. 1969, p. 626-631
Vol. 4, No. 5
Copyright © 1969 American Society for Microbiology
Printed in U.S.A.

Variation in Properties of Plaque Progeny of PARA (Defective Simian Papovavirus 40)-Adenovirus 7

FRED RAPP[1], SERGIO PAULUZZI[2], AND JANET S. BUTEL

Department of Virology & Epidemiology, Baylor University College of Medicine, Houston, Texas 77025

Received for publication 7 July 1969

One hundred and twelve progeny from double plaque-purified clones were derived from the original PARA (defective simian papovavirus 40)-adenovirus 7 population. These progeny were found to differ in their oncogenic potential in newborn hamsters with progeny from 20 clones not inducing any tumors during 1 year of observation. The varying tumorigenicity of the individual clonal progeny was not related to the titer of PARA (particle aiding replication of adenovirus) in the inoculum. There was a perfect correlation between the tumor antigen content of the tumor cells and the antibody response of the tumor-bearing host. The tumors containing both adenovirus and simian papovavirus 40 (SV40) tumor antigens appeared earlier than those carrying only SV40 tumor antigen. Progeny from clones which induced mixed tumors also produced tumors which contained only SV40 tumor antigen. Three variants of PARA were isolated which induced the synthesis of SV40 tumor antigen in the cytoplasm of infected simian cells; all other clones yielded progeny which induced synthesis of SV40 tumor antigen in the nucleus.

A population of human adenovirus 7 carrying a defective simian papovavirus 40 (SV40) was first reported in 1964 (8, 12, 14) and was subsequently shown to exist as a mixed population. One component is a human adenovirus which can replicate singly in human cells, but can grow in monkey cells only in the presence of SV40. The second particle, PARA (particle aiding replication of adenovirus), is a defective SV40 genome encased in an adenovirus capsid, which is recognized by its ability to induce the synthesis of SV40 tumor (T) antigen. PARA can multiply in monkey kidney cells coinfected with an adenovirus whose presence is required to furnish the coat protein (3, 4, 7, 15). If a heterologous adenovirus is present during the replicative cycle, PARA can be transferred to that population (7, 9, 13) by a process termed "transcapsidation" (9).

The original PARA-adenovirus 7 population was demonstrated to be oncogenic in newborn hamsters (8, 10), and when PARA was transferred to a nononcogenic human adenovirus, such as type 2, that PARA-adenovirus population then became tumorigenic in newborn hamsters (10, 11) and capable of transforming cells in vitro (2). However, there were indications that the degree of oncogenicity conferred by PARA varied from one population to the next (11). It was felt this variation might be due to a random selection of different PARA particles from the original stock of PARA-adenovirus 7.

To establish this point, 112 clonal lines of PARA-adenovirus 7 were derived by two successive plaque purifications in green monkey kidney cells. As will be shown in this report, PARA particles with apparently varying oncogenic potential, as well as variations in other properties, are present in the parental population.

MATERIALS AND METHODS

Viruses. The history of the LL strain of adenovirus 7, referred to hereafter as PARA-adenovirus 7, has been described in detail (12). The original virus, stock SP2, was used in this study after only one additional passage in green monkey kidney (GMK) cells. The adenovirus 7 used as helper virus was derived from the LL strain by three successive plaque purifications in human embryonic kidney cells (3) and then passed six additional times in human embryonic kidney cells. This virus no longer induces the synthesis of SV40 T antigen and cannot replicate in GMK cells.

Virus assay. PARA was titrated in GMK cells grown in plastic petri dishes (60 by 15 mm). Saturating amounts (enough to infect every cell in the culture) of nonreplicating helper adenovirus (6) were

[1] American Cancer Society Professor of Virology. Present address: Department of Microbiology, The Pennsylvania State University College of Medicine, Hershey, Pa. 17033.

[2] Eli Lilly International Fellowship Awardee on leave from the Istituto di Malattie Infettive, Monteluce, University of Perugia, Perugia, Italy.

added to each plate to detect maximum titers of PARA. After adsorption of the viruses at 37 C for 1 hr, 5 ml of overlay containing 1% agar, 10% fetal calf serum, and 0.23% $NaHCO_3$ in Eagles medium was added. A second overlay containing a 1 to 20,000 dilution of neutral red was added 1 week later and PARA plaques were counted 10 to 14 days after inoculation.

Immunofluorescence techniques. Cells were grown on 15-mm round cover glasses in petri dishes and held in an atmosphere containing 5% CO_2. When harvested, cells were washed three times with tris-(hydroxymethyl)aminomethane (Tris) buffer (*p*H 7.4), air dried, and fixed with acetone for 3 min at room temperature. They were stained at room temperature by reacting them with antisera for 30 min, after which they were washed three times with Tris buffer; the cells were then reacted with fluorescein-labeled antiglobulin for 30 min, washed three times again with Tris buffer, air dried, and mounted in Elvanol. The slides were viewed with a Zeiss fluorescence microscope.

The PARA-adenovirus 7 clonal progeny were tested for their ability to induce the synthesis of SV40 T antigen by harvesting GMK cells 24 hr after infection and reacting them with a serum pool from hamsters bearing tumors induced by SV40-transformed cells (H-50 cell line). Tumor cells were tested for the presence of SV40 T antigen using the same serum. Tumor cells were also assayed for adenovirus T antigen by reacting them with a serum pool from hamsters bearing tumors induced by adenovirus type 12. Sera from tumor-bearing animals were reacted with H-50 cells to detect SV40 T antibody and with GMK cells infected 24 hr earlier with adenovirus type 7 to detect adenovirus T antibody. In each test system, the hamster serum pool was followed by fluorescein-labeled anti-hamster globulin baboon globulin.

Animal experiments. The PARA-adenovirus 7 clonal lines were each tested for oncogenicity by subcutaneous inoculation of 0.1-ml volumes into 30 hamsters within 24 hr after birth. The animals were weaned 3 weeks later and checked weekly for tumor development. When tumors measured greater than 10 by 10 mm, the animals were bled out, and the sera were collected and tested for antibody content by the immunofluorescence technique. The tumors were excised, trypsinized, and the cells were grown in 16-oz prescription bottles in Eagles medium containing 10% fetal calf serum. Either primary or secondary cultures of the tumor cells were tested for tumor antigen content by immunofluorescence.

RESULTS

Derivation of clonal lines of PARA-adenovirus 7. Plaque-purification of the original PARA-adenovirus 7 stock was performed following the procedure outlined in Fig. 1. The entire procedure was carried out using GMK cells. Helper adenovirus type 7 was added to the GMK plates to obtain maximum plaque formation by PARA, which, in turn, allowed terminal PARA plaques to be picked. Additional adenovirus was also added to the GMK tubes and bottles to insure that small amounts of PARA would not go undetected due to a failure of a helper adenovirion to infect the same cell as PARA.

The PARA-adenovirus 7 stock was plaqued in GMK cells in 60-mm plates; plaques were picked and passed in GMK cells, and the progeny virus sent through the same procedure again. In this fashion, 112 clonal lines of twice plaque-purified (PP2) progeny from PARA-adenovirus 7 were derived.

The stocks of the PP2 clones were tested by immunofluorescence for their ability to induce the synthesis of SV40 tumor antigen in GMK cells. One hundred and nine of the clones induced the typical particulate, nuclear T antigen. The other three clones failed to induce a nuclear reaction. Rather, there was a diffuse reaction detected in the cytoplasm of infected cells. The properties of these three clones are characterized in detail in the companion paper (5).

The PP2 clones were titrated in GMK cells coinfected with helper adenovirus 7, and, if PARA titered 1×10^5 plaque-forming units (PFU)/ml or greater, 30 newborn hamsters were then inoculated to determine the oncogenic potential of the clone. Adenovirus titers in human embryonic kidney cells were not determined for the clones, since this study was concerned with the PARA component of the mixed populations.

Oncogenicity of the PARA-adenovirus 7 clones. As shown in Table 1, 112 progeny clones were tested for oncogenicity in newborn hamsters.

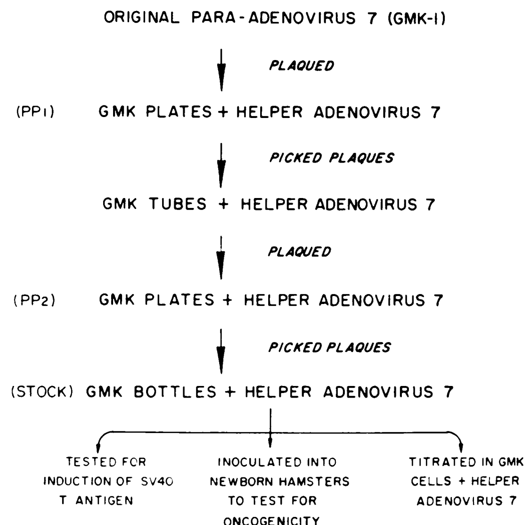

FIG. 1. *Procedure for plaque purification of PARA-adenovirus type 7.*

Ninety-two of the clones produced tumors in one or more animals; thus, 82% of the clones were oncogenic. The titer of PARA did not seem to exert much influence on the oncogenicity. All three clones with a titer of < 5.0 × 10^5 PFU/ml were oncogenic (100%). However, only 19 of 24 clones with a titer of > 1.0 × 10^7 PFU/ml produced tumors (79%).

The effect of the titer of PARA on the tumorigenicity of the clones is examined further in Table 2. The 92 oncogenic clones produced a total of 234 tumors in 1,393 animals which survived to be weaned for an overall tumor induction frequency of 16%. The percentage of tumors in the different groups based on the PARA titer of the clones varied from 11 to 17%. The means of the percentage of tumors for the groups of clones varied from 13 to 20. However, the range of percentage of tumors for individual clones in a specific group varied widely. Even when the data were analyzed in this manner, the titer of PARA did not appear to influence the results. The clone which yielded the lowest percentage of tumors (4%) was one with the highest titer of PARA (> 1.0 × 10^7 PFU/ml).

Effect of titer of PARA on latent period before tumor development. The number of tumors which first became palpable during different observation periods is given in Table 3. A total of 234 tumors developed in the inoculated hamsters which survived to be weaned. Twenty of the tumors appeared between 6 and 10 weeks after inoculation; 57 tumors first became palpable between 11 and 15 weeks; 109 tumors developed during the following 4-week observation period; and the final 48 tumors appeared after a latent period of greater than 20 weeks. All the tumors had appeared by 32 weeks after inoculation of the viruses. As is seen in Fig. 2, in which the percentage of tumors which developed in each group has been plotted against the time in weeks after inoculation, the titer of PARA had little effect on the latent period of the tumors. The tumors in the different groups based on the titers of PARA developed at very nearly the same rate.

TABLE 1. *Oncogenicity of PARA-adenovirus 7 clones*

PARA titer (PFU/ml)	No. of oncogenic clones[a]	No. of clones tested	Percentage of oncogenic clones
<5.0 × 10^5	3	3	100
5.1–10 × 10^5	7	9	78
1.1–5.0 × 10^6	44	54	81
5.1–10 × 10^6	19	22	86
>1.0 × 10^7	19	24	79
Totals	92	112	82

[a] Observation period of 1 year.

TABLE 2. *Lack of correlation between oncogenicity of PARA-adenovirus 7 clones and titer of PARA*

PARA titer (PFU/ml)	No. of oncogenic clones[a]	Totals		Tumors for positive clones	
		No. of tumors/no. weaned	Tumors	Mean	Range
			%	%	%
<5.0 × 10^5	3	5/42	11	13	7–20
5.1–10 × 10^5	7	18/116	15	15	6–29
1.1–5.0 × 10^6	44	120/683	17	18	5–55
5.1–10 × 10^6	19	51/297	17	20	6–67
>1.0 × 10^7	19	40/255	15	18	4–46
Totals	92	234/1393	16	17	

[a] Observation period of 1 year.

TABLE 3. *Lack of correlation between titer of PARA and latent period of tumor development*

PARA titer (PFU/ml)	Latent period				Totals
	6 to 10 wk	11 to 15 wk	16 to 20 wk	21 to 32 wk	
<5.0 × 10^5	1[a]	3	1		5
5.1–10 × 10^5		7	9	2	18
1.1–5.0 × 10^6	8	27	51	34	120
5.1–10 × 10^6	7	10	30	4	51
>1.0 × 10^7	4	10	18	8	40
Total no. of tumors	20	57	109	48	234

[a] Numbers indicate the number of tumors that first became palpable during time period after inoculation indicated at top of the column.

Data pertaining to nononcogenic PARA-adenovirus 7 clones. Twenty of the PP2 PARA-adenovirus 7 clones failed to induce any tumors during 1 year of observation of the inoculated animals. It can be seen in Table 3 and Fig. 2 that all the tumors which appeared had done so by 32 weeks after inoculation. The inocula from the negative clones had titers of PARA comparable to those of the oncogenic clones (Table 4). The number of animals which were weaned after being inoculated with the negative clones varied from 4 to 25 per clone. Although four are relatively few animals and the oncogenicity of that clone might still be in doubt, most of the clones had enough surviving animals to indicate that if the virus

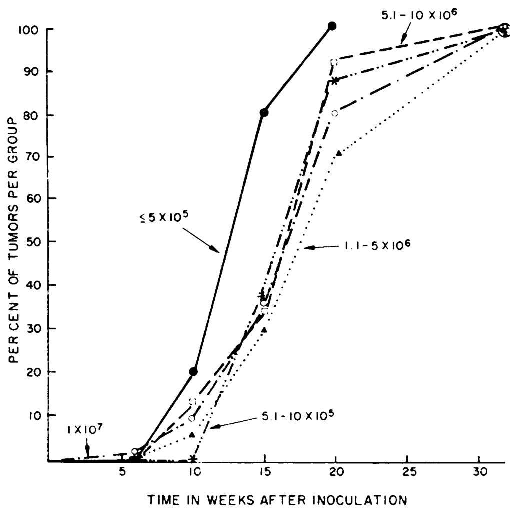

FIG. 2. *Influence of titer of PARA inoculum on latent period for tumor development in hamsters inoculated as newborns.*

TABLE 4. *Nononcogenic PARA-adenovirus 7 clones*

PARA titer (PFU/ml)	No. of negative clones[a]	No. of animals weaned	
		Total	Range
$<5.0 \times 10^5$	0		
$5.1\text{-}10 \times 10^5$	2	24	7-17
$1.1\text{-}5.0 \times 10^6$	10	114	4-25
$5.1\text{-}10 \times 10^6$	3	41	10-17
$>1.0 \times 10^7$	5	78	12-20
Totals	20	257	

[a] Observation period of 1 year.

clones were going to produce tumors, they should have been detected. Indeed, the minimal number of animals weaned in the groups given the highest PARA dose ($> 1 \times 10^7$) was 12.

The three variants which induce the synthesis of SV40 tumor antigen in the cytoplasm failed to induce tumors in this experiment. However, relatively few animals survived to be weaned, so more hamsters have been inoculated with these three clonal progeny to study further their oncogenic potential.

Serological analysis of tumors and sera. Two hundred and nineteen of the tumors which developed were trypsinized and the cells were analyzed for their tumor antigen content. The sera from the tumor-bearing animals were also tested for tumor antibody content (Table 5). The results are correlated in Table 5. One hundred and seventy-one of the tumors contained only SV40 T antigen. The 171 animals bearing these tumors were found to have SV40 T antibody in their sera, but no adenovirus T antibody. Forty-eight tumors were antigenically mixed in that they contained both SV40 and adenovirus T antigens. The sera from these hamsters were found to contain both SV40 and adenovirus T antibodies. No tumors contained only adenovirus T antigen, and no sera contained antibody against only adenovirus T antigen.

The tumor antigen content of the cells was then correlated with the time at which the tumors appeared (Table 6). It was found that the mixed tumors appeared the earliest. All 12 tumors which first became palpable between 6 and 10 weeks after inoculation contained both SV40 and adenovirus T antigens (100%). Of the tumors which appeared during the next 4-week period, 21 of 46 (45%) contained both antigens. However, of the tumors which appeared 16 or more

TABLE 5. *Correlation of antigen content of tumor cells with antibody response of animals*

Antigen(s) in tumor cells	Ratio of positive tumors to tumors tested	Antibody response to T antigens		
		SV40 only	SV40 and adeno	Adeno only
SV40 T only.......	171/219	171[a]	0	0
SV40 T + adeno T..............	48/219	0	48	0
Adeno T only.....	0/219	0	0	0

[a] Numbers indicate the number of sera positive for the antibody (or antibodies) indicated at the top of the column.

TABLE 6. *Correlation of tumor antigen content of tumor cells with latent period before tumor development*

Antigen(s) in tumor cells	No. of tumors	Latent period in weeks				Percentage of total tumors
		6-10	11-15	16-20	21-32	
SV40 T	171	0[a]	25	104	42	78
SV40 T and Adeno T	48	12	21	11	4	22
Totals	219	12	46	115	46	
Percentage of tumors with both antigens in each period		100	45	9	8	

[a] Numbers in the top two rows indicate the number of tumors that first became palpable during the time period after inoculation indicated at the top of the column.

weeks after inoculation, less than 10% were found to contain adenovirus T antigen.

One important point regarding the ability of PARA-adenovirus 7 clones to induce mixed tumors is summarized in Table 7. Virus clones were not derived which induced exclusively mixed tumors. Generally, a virus clone which induced a mixed tumor in one animal also induced tumors in other animals which contained only SV40 T antigen. Progeny derived from 39 virus clones induced at least one mixed tumor (Table 7). Thirty of these clones induced tumors in other hamsters which contained only SV40 T antigen. Of the 39 clones, 8 induced only one tumor (mixed), so it cannot be assumed these 8 clones would not induce some SV40-type tumors if more tumors were surveyed. The remaining clone induced only two mixed tumors, again too few to base a conclusion that the clone would invariably induce tumors containing both SV40 and adenovirus antigens.

DISCUSSION

The purpose of this study was to determine whether PARA particles exist in the original PARA-adenovirus 7 population which vary in their oncogenic potential. One hundred and twelve twice plaque-purified clones were derived from the original stock and it appears that such variation does exist. Ninety-two of the clones were found to be tumorigenic in newborn hamsters; these positive clones produced tumors in 4 to 55% of the inoculated animals, depending on the clone. The reason for this variation between PARA particles is currently not known. The genetic stability of the oncogenic properties of the various clones has not been established, but analysis of the data in several ways indicates that the observed differences are not related to the titer of PARA inoculated into the animals. The possibility exists, of course, that our assay system does not detect all the PARA particles in the population and that PARA particles further defective (and unable to plaque in GMK cells in the presence of a helper adenovirus) are inducing the tumors. Although this raises a question concerning the genetic homogeneity of progeny derived from the plaques, it does not change the conclusion that PARA variants exist which are able to induce SV40 markers in GMK cells but not tumors in newborn hamsters.

TABLE 7. *Ability of PARA-adenovirus 7 clones to induce both mixed and SV40-type tumors*

Property of virus clone	No. of virus clones involved
Induced mixed tumors (SV40 + adeno tumor antigens)	39
Induced SV40 type tumors in other hamsters	30/39
Induced only one tumor (mixed)	8/39
Induced only two tumors (mixed)	1/39

The tumors and sera from the tumor-bearing animals were also analyzed. There was a perfect correlation between the antigen content of the cells and the antibody response of the host. This is in contrast to some previous studies (2, 10) in which adenovirus tumor antibody was more readily detected than the corresponding adenovirus tumor antigen in cells. Of the analyzed tumors, 78% contained only SV40 tumor antigen, whereas the remaining 22% contained both adenovirus and SV40 T antigens. No tumors carried only adenovirus T antigen.

Of interest was the fact that the mixed tumors were the first to appear. All the tumors which appeared during the first 10 weeks after inoculation contained both adenovirus and SV40 T antigen. Tumors which first became palpable after more than a 15-week latent period were primarily (> 90%) SV40-type tumors. It has been suggested from studies with chemically induced tumors that the more antigenic tumors have the shorter latent period (16). Whether increased antigenicity is the explanation for the early appearing tumors containing both adenovirus and SV40 tumor antigens remains to be elucidated.

It should be emphasized that the virus clones which induced the formation of mixed tumors also caused the development of tumors in other hamsters which contained only SV40 tumor antigen. It has been suggested that SV40 and adenovirus deoxyribonucleic acid (DNA) is linked in the hybrid particles (1). It appears, therefore, that there must be some mechanism or mechanisms operating in the hamster cells to suppress the expression of the adenovirus genes in some instances. However, there is no evidence that all PARA particles contain linked DNA. Some could theoretically carry only defective SV40 nucleic acid. Also, it cannot be ruled out that the linked SV40 and adenovirus DNA in the hybrid did not segregate before or during transformation of the hamster cells.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grants CA 04600 and CA 10036 from the National Cancer Institute, National Institutes of Health, Bethesda, Md.

We gratefully acknowledge the excellent technical assistance of Myron Katz.

LITERATURE CITED

1. Baum, S. G., P. R. Reich, C. J. Hybner, W. P. Rowe, and S. M. Weissman. 1966. Biophysical evidence for linkage of

adenovirus and SV40 DNA's in adenovirus 7-SV40 hybrid particles. Proc. Nat. Acad. Sci. U.S.A. 56:1509–1515.

2. Black, P. H., and B. J. White. 1967. In vitro transformation by the adenovirus-SV40 hybrid viruses. II. Characteristics of the transformation of hamster cells by the adeno 2-, adeno 3-, and adeno 12-SV40 viruses. J. Expt. Med 125:629–646.
3. Boeyé, A., J. L. Melnick, and F. Rapp. 1965. Adenovirus-SV40 "hybrids": Plaque purification into lines in which the determinant for the SV40 tumor antigen is lost or retained. Virology 26:511–512.
4. Boeyé, A., J. L. Melnick, and F. Rapp. 1966. SV40-adenovirus "hybrids": Presence of two genotypes and the requirement of their complementation for viral replication. Virology 28:56–70.
5. Butel, J. S., M. J. Guentzel, and F. Rapp. 1969. Variants of defective simian papovavirus 40 (PARA) characterized by cytoplasmic localization of simian papovavirus 40 tumor antigen. J. Virol. 4:632–641.
6. Butel, J. S., J. L. Melnick, and F. Rapp. 1966. Detection of biologically active adenovirions unable to plaque in human cells. J. Bacteriol. 92:433–438.
7. Butel, J. S., and F. Rapp. 1966. Replication in simian cells of defective viruses in an SV40-adenovirus "hybrid" population. J. Bacteriol. 91:278–284.
8. Huebner, R. J., R. M. Chanock, B. A., Rubin, and M. J. Casey. 1964. Induction by adenovirus type 7 of tumors in hamsters having the antigenic characteristics of SV40 virus. Proc. Nat. Acad. Sci. U.S.A. 52:1333–1340.
9. Rapp, F., J. S. Butel, and J. L. Melnick. 1965. SV40-adenovirus "hybrid" populations: Transfer of SV40 determinants from one type of adenovirus to another. Proc. Nat. Acad. Sci. U.S.A. 54:717–724.
10. Rapp, F., J. S. Butel, S. S. Tevethia, M. Katz, and J. L. Melnick. 1966. Antigenic analysis of tumors and sera from animals inoculated with PARA-adenovirus populations. J. Immunol. 97:833–839.
11. Rapp, F., M. A. Jerkofsky, J. L. Melnick, and B. Levy. 1968. Variation in the oncogenic potential of human adenoviruses carrying a defective SV40 genome (PARA). J. Exp. Med. 127:77–90.
12. Rapp, F., J. L. Melnick, J. S. Butel, and T. Kitahara. 1964. The incorporation of SV40 genetic material into adenovirus 7 as measured by intranuclear synthesis of SV40 tumor antigen. Proc. Nat. Acad. Sci. U.S.A. 52:1348–1352.
13. Rowe, W. P. 1965. Studies of adenovirus-SV40 hybrid viruses. III. Transfer of SV40 gene between adenovirus types. Proc. Nat. Acad. Sci. U.S.A. 54:711–717.
14. Rowe, W. P., and S. G. Baum. 1964. Evidence for a possible genetic hybrid between adenovirus type 7 and SV40 viruses. Proc. Nat. Acad. Sci. U.S.A. 52:1340–1347.
15. Rowe, W. P., and S. G. Baum. 1965. Studies of adenovirus SV40 hybrid viruses. II. Defectiveness of the hybrid particles. J. Exp. Med. 122:955–966.
16. Smith, R. T. 1968. Tumor-specific immune mechanisms. N. Eng. J. Med. 278:1207–1214, 1268–1275, 1326–1331.