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EVIDENCE FOR A POSSIBLE GENETIC HYBRID BETWEEN ADENOVIRUS TYPE 7 AND SV40 VIRUSES

BY WALLACE P. ROWE AND STEPHEN G. BAUM

NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES, BETHESDA, MARYLAND

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The accompanying report[1] described an unusual pattern of hamster tumor induction by a monkey kidney adapted strain (L. L.) of adenovirus type 7 (Ad. 7). The tumors contained an antigen (referred to herein as SV40 T antigen) reactive with SV40 tumored hamster sera in complement fixation (CF) and fluorescent antibody (FA) tests, but no infectious SV40 virus could be demonstrated in the inoculum or tumors, and the tumored hamsters did not develop neutralizing antibody to SV40 virus. Since the Ad. 7 strain had been contaminated with SV40 in earlier passages, the contaminant being eliminated by passage with SV40 antiserum,[1] it appeared that there might be a previously unrecognized type of interaction between the Ad. 7 and SV40 viruses.

When it was found that the preparation (E46) of L. L. strain Ad. 7 which induced the hamster tumors also induced FA-stainable SV40 T antigen in acutely infected tissue culture cells, studies were carried out in this system to delineate the specificity of the reactions observed and the nature of the relationship between the Ad. 7 and SV40 genetic material.

Materials and Methods.—Tissue cultures: Fluorescent antibody tests were done with cells grown on 11 × 22 mm coverslips in 60-mm plastic Petri dishes. Primary cultures of human embryonic kidney (HEK) and African green monkey kidney (AGMK) were obtained from Microbiological Associates, Inc., and Flow Laboratories. The cells were dispersed by trypsinization and planted in the Petri dishes using 3.5×10^6 cells in 3.5 ml of growth medium consisting of 10% unheated fetal bovine serum in Eagle's basal medium (BME). Prior to virus inoculation the cultures were washed twice with BME, and 2% heated (56° for 30 min) agammaglobulinic calf serum (Hyland Laboratories) in BME was added as maintenance medium. All media contained penicillin, streptomycin, and glutamine. Plates were held at 37°C in humidified 5% CO_2 in air.

For FA staining of tumor cells, tumors were cultivated in tissue culture as described previously.[2]

Viruses: The origin and characterization of the E46 preparation of the L.L. strain of Ad. 7 have been described in detail.[1] In this report, the term L. L.-E46 will be used to refer to the line

of Ad. 7 virus originating from the preparation, and the term E46 to refer to the particular pool of virus. Subsequent passages from E46 in AGMK are identified as L. L.-E46AG followed by the number of the passage level.

SV40 strain 777[3] was used at the 10th MK passage level.

Fluorescent antibody tests: The procedures for fixation of coverslip preparations and for performance of indirect and direct FA tests have been described.[2] All tests with hamster sera were done by the indirect procedure, using goat antihamster conjugate as described previously.[2] In the present study counterstaining with 1:100 dilution of rhodamine was used. Tests for SV40 T antigen were routinely done with a 1:5 dilution, in normal hamster brain suspension, of peritoneal fluid of a hamster which had been hyperimmunized by repeated intraperitoneal injections of viable SV40 hamster tumor cells; this antibody preparation fixed complement to a dilution of 1:20 against SV40 and E46 hamster tumor suspensions, did not react in CF with SV40 virus antigen, and had no SV40 neutralizing antibody at a 1:5 dilution. Tests for SV40 viral antigen were done by the direct FA procedure, using the conjugated African green monkey hyperimmune anti-SV40 serum used in previous studies.[2]

For determining percentage of cells with specific staining, the coverslips, containing 10,000 cells by count of trypsinized preparations, were first scanned and the percentage was roughly estimated. In the case of high estimated percentage, at least 200 cells were then scored, in random widely dispersed fields; for lower percentages 1000 to 2000 cells were counted, and for very low percentages, the number of positive cells on the entire coverslip was determined. A negative preparation thus had no positive cells among about 10,000 examined.

Cell pack antigens: CF tests for SV40 T antigen in acutely infected HEK cells were done with cell pack antigen preparations.[4] Six HEK culture tubes were inoculated per specimen, and were harvested when definite adenovirus cytopathic effect first appeared; in the case of comparisons of specimens with and without Ad. 7 infectivity, as in neutralization or ultrafiltration studies, the noncytopathic cultures were harvested with the infectivity controls. The tubes were frozen and thawed, the cells shaken or scraped into the fluid, and the contents of the 6 replicate tubes pooled. The cells were then collected by low-speed centrifugation and resuspended in 1 ml of the supernatant fluid. The cell suspensions were then tested in the CF test against SV40 tumored hamster serum.[5]

Results.—General description of the phenomenon: The basic observation on which the present work is based is that cultures infected with L. L.-E46 virus demonstrate an antigen reactive with SV40 tumored hamster sera in FA and CF tests. The appearance of the FA reaction was identical to that seen with SV40-infected cultures[2] and SV40 and E46-tumor cells, i.e., intense, slightly granular staining of the entire nucleus with unstained areas of the size and shape of nucleoli, with no cytoplasmic staining. This nuclear appearance was easily distinguished from a type of nonspecific nuclear fluorescence commonly encountered in adenovirus-infected cultures. When treated with normal or tumored hamster serum in the indirect FA test, the ballooned nuclei of adenovirus-infected cells showed homogenous dull green fluorescence without the sharpness, nucleolar sparing, and restriction to one plane of focus characteristic of the specific staining. The CF reactivity was also like that of SV40-infected cultures in that T antigen was detectable in cell pack preparations but not in culture fluids.[4]

Kinetic data on the formation of FA-stainable antigen in monkey and human tissue culture cells are presented in Figures 1–3, together with similar data for SV40 infection. In these experiments, the multiplicity of infection with E46 was about 0.25, with SV40 about 25, and with L. L.-E46AG5 about 10 $TCID_{50}$ per cell. In monkey kidney cultures (Fig. 1), formation of SV40 T antigen during SV40 and E46 infection followed parallel curves, with that from SV40 10–100-fold higher, reflecting the difference in input multiplicity. However, there was a striking contrast in the antiviral staining; SV40 viral antigen followed closely

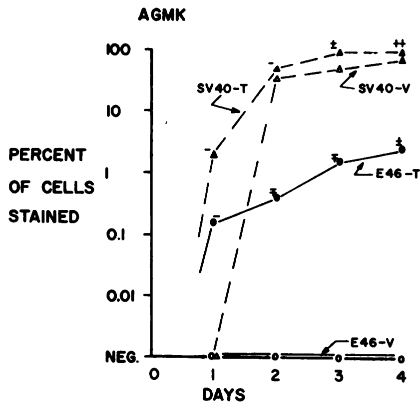

FIG. 1.—Time course of development of immunofluorescent-stainable SV40 tumor reactive (*T*) and viral (*V*) antigens in African green monkey kidney tissue cultures infected with E46 and SV40. Superscripts refer to degree of cytopathic effects at time of harvest.

after T antigen in the SV40-infected cultures, but was absent in the E46-infected cells. In human cells (Figs. 2 and 3) there was a marked contrast in rate of formation of SV40 T antigen, that induced by E46 appearing within 24 hr, while the SV40-infected cultures showed a significant delay. Again, SV40 infection induced SV40 viral antigen, while the E46-infected cultures showed no staining with the monkey antiviral serum. In all tissues, uninoculated controls and cultures infected with other strains of Ad. 7 showed no staining with either reagent.

L. L.-E46 virus which had been carried five additional passages in AGMK (L. L.-E46AG5) was highly efficient in inducing SV40 T antigen (Fig. 3). Antigen appeared after a latent period of 8 or 9 hr, and by 18 hr 50 per cent of cells stained for antigen. The proportion of positive cells decreased between 24 and 48 hr, reaching a plateau at 1–2 per cent; this decline may reflect the possible precursor enzyme nature of the antigen and its instability.[4] No SV40 viral antigen was detectable at any time. Since the peak of antigen formation at 18–24 hr is compatible with the latent period of both adenovirus and SV40 virus infection, it seemed probable that antigen at this time is produced in the first cycle of virus growth, and the frequency of its occurrence would be proportional to virus input. This was borne out by the data in Figure 4, which shows the proportion of stainable cells 20 hr after inoculation of serial dilutions of L. L.-E46AG5. The proportionality indicates that this early harvest procedure will be a useful method for quantitative determination of T antigen-inducing particles.

L. L.-E46AG5 and SV40 also induced SV40 T antigen in chick embryo fibro-

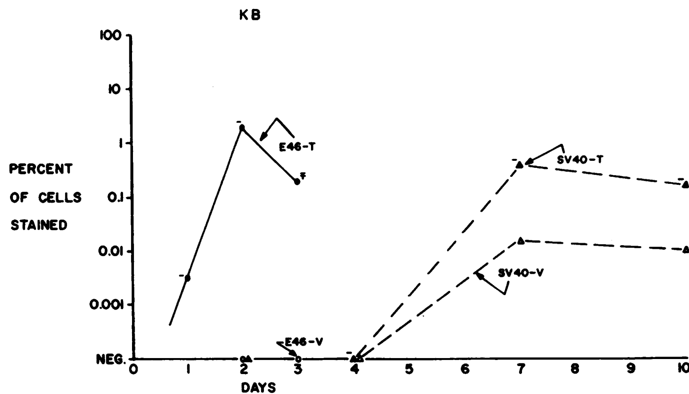

FIG. 2.—Development of immunofluorescent-stainable SV40 antigens in KB cells infected with E46 and SV40 viruses. Legend as in Fig. 1.

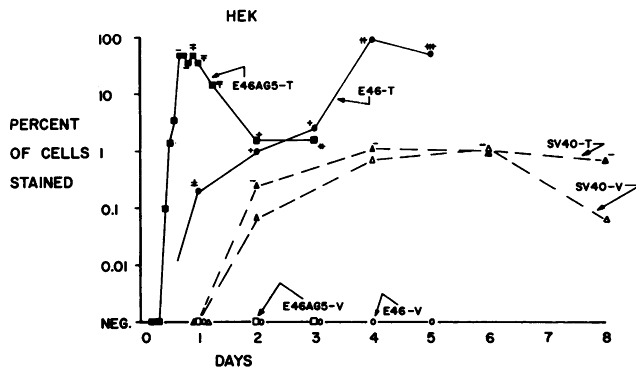

FIG. 3.—Development of immunofluorescent-stainable SV40 antigens in human embryo kidney tissue cultures infected with E46, L. L.-E46AG5, and SV40. Legend as in Fig. 1.

blasts, but only in a small proportion of cells (0.1–0.5%) and after a 2–3 day latent period.

The titer of Ad. 7 infectivity in the E46 preparation was higher than that of its SV40 antigen-inducing component. In a titration of E46 in HEK cultures, in which the Ad. 7 infectivity titer was $10^{4.8}$ $TCID_{50}$/0.1 ml, fluids from the cultures receiving the 10^0 through 10^{-3} dilutions induced SV40 antigen on passage to HEK cells, but the fluid from the set of tubes inoculated with the 10^{-4} dilution did not induce SV40 antigen though containing $10^{7.8}$ $TCID_{50}$ of Ad. 7 per 0.1 ml. This subline of L. L.-E46, freed of SV40 antigen-inducing component by high dilution passage, will be referred to as L. L.-E46$^-$.

The SV40 antigen-inducing component was able to be propagated in porcine cell cultures, since a passage line[6] of L. L.-E46 which had been adapted to porcine kidney cell cultures by 8 serial passages induced SV40 FA antigen, in a small percentage of cells. However, a comparable passage line[6] in bovine kidney cell cultures did not induce antigen in HEK cells at the tenth bovine passage level.

Specificity of the fluorescent staining test: In view of the unusual finding of formation of SV40 T antigen without viral antigen in infected tissue cultures, it is essential to reappraise the evidence that this antigen represents expression of SV40 genetic material. First, Pope and Rowe[2] showed that SV40 tumored hamster sera stained all SV40 tumors and transformed cell lines tested, but did not stain any of a variety of heterologous tumors or

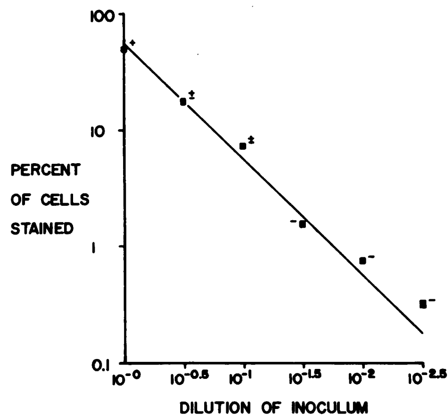

FIG. 4.—Percentage of cells containing immunofluorescent-stainable SV40 T antigen 20 hr after infection of HEK cultures as a function of virus dose. The line is a 45° slope drawn through the $10^{-0.5}$ point. Superscripts refer to degree of cytopathic changes at time of harvest.

TABLE 1

CORRELATION OF CF AND FA ANTIBODY REACTIONS OF HAMSTERS WITH SV40 AND E46 TUMORS

Serum Tested		CF Antibody Titer		FA Staining (1:40 serum)		
Tumor	Animal no.	vs. SV40 tumor	vs. E46 tumor	THK-1*	E46-induced hamster tumor	L. L.-E46 infected HEK
SV40 (THK-1)						
transplant	2090-1 37 days†	<10	<10	±(0.1%)‡	0	±(1%)
"	" 65 "	80	40	±(100%)	1–2+(98%)	2+(20%)
"	" 76 "	>160		2–3+(100%)	2+(98%)	3–4+(20%)
E46 primary	1518-2	10	<10	±(100%)	±(30%)	±(2%)
	1518-7	40	20	2+(100%)	2–3+(95%)	3–4+(20%)
	1518-4	>40	>40	1–2+(100%)	±(30%)	3–4+(25%)

* THK-1 is a cell line of SV40 transformed hamster kidney cells.[11]

† Days after inoculation of cells.

‡ The 0 to 4 + scale refers to the average intensity of nuclear fluorescence, and the percentage gives the estimated proportion of cells showing specific nuclear fluorescent staining.

control cells. Second, the fluorescent-stainable antigen has been shown to be formed by all three SV40 strains tested. Third, the hamster antibody preparation used in the present tests has given consistently negative results when used to stain control HEK, KB, HEp-2, BSC-1, hamster kidney, and numerous batches of AGMK tissue culture cells; a hamster tumor induced with Ad. 7 strain 14500,[7] and tissue cultures infected with prototype strains of adenovirus types 2, 3, 4, 7, 7a, 8, and 12, Pinckney strain[8] of Ad. 7, and four field strains[6] of Ad. 7 with no laboratory passage in monkey cell cultures. Fourth, cultures infected with L. L.-E46 virus gave no staining with sera from normal hamsters or hamsters bearing tumors induced by polyoma, bovine papilloma, or Schmidt-Ruppin strain of Rous virus. Sera from Ad. 12 tumor-bearing hamsters gave nuclear staining of E46 infected cells, of the type observed with Ad. 7-infected cells,[9] but did not stain cells from tumors induced by E46 virus. Fifth, staining of L. L.-E46 infected cultures by the SV40 hamster antibody was blocked by pretreatment of the peritoneal fluid with SV40-infected AGMK cell pack or SV40 hamster tumor extract but not by similar treatment with control AGMK cell pack, Ad. 12 tumor extract, or a standard SV40 viral preparation not containing demonstrable T antigen.

Further evidence of the identity of the FA and CF antigens induced by L. L.-E46 and SV40 is shown in Table 1. Sera from hamsters carrying SV40 or E46-induced tumors showed comparable CF and FA antibody reactivity to both SV40 and E46 tumor cells and L. L.-E46-infected HEK cells, and with one exception, the degree of reactivity in the FA test paralleled the CF antibody titer.

These data leave no alternative but that the SV40 T antigen is a specific response to SV40 infection. The evidence that it is formed from information in the viral genome rather than the host genetic apparatus has rested primarily on the ability of the SV40 to induce the antigen in diverse mammalian species.[2, 5] The finding that the antigen also appeared in SV40 and L. L-E46-infected chicken cells gives further support to this view.

State of the SV40 genetic material in L. L.-E46 virus preparations: The failure to recover infectious SV40 virus from the E46 virus pool and from hamster tumors induced by E46,[1] as well as the failure of various L. L.-E46 preparations to induce SV40 viral antigen, strongly indicated that the E46 virus pool was not a simple mixture of Ad. 7 and SV40 viruses. Further, artificial mixtures of SV40 and Ad. 7 did not produce the anomalous staining results observed with L. L.-E46.

TABLE 2

NEUTRALIZATION TEST WITH L. L.-E46AG 1. HEK CELLS, HARVESTED AT 48 HR

Serum	Dilution of serum	Adeno. CPE	Reaction with SV40 Hamster Serum FA, %	Reaction with SV40 Hamster Serum CF (cell pack antigen 1:4)
None		1–2+	3*	4+†
Ad. 7 rabbit antiserum	1:20	—	0	0
SV40 " "	1:20	1–2+	3	4+
SV40 tumored hamster serum	1:5	1+	2	4+
Ad. 12 " " "	1:5	1+	1	4+
E46 " " "	1:5	1+	2	AC§
Normal hamster serum	1:5	1–2+	2	4+
Ad. 7 infected human volunteer R. S.‡—pre	1:10	1+	4	
" " post	1:10	—	0	
" W. B.—pre	1:10	1+	2	
" " post	1:10	—	0.01	

* Approximate per cent of cells showing specific nuclear staining.
† Degree of complement fixation; 4+ = complete fixation.
‡ These volunteers were infected with an Ad. 7 strain which had been isolated and propagated only in HEK cells. These sera were kindly provided by Dr. R. M. Chanock.
§ AC = anticomplementary.

A number of lines of evidence showed that integrity of the Ad. 7 infectious particle was essential for SV40 antigen induction by L. L-E46. First, neutralization tests, of which the test summarized in Table 2 is representative, showed that antigen induction was prevented by rabbit or human antibody to Ad. 7, but not by SV40 rabbit antiserum (homologous neutralizing antibody titer >1280) or by SV40 or E46 tumored hamster sera with high CF antibody to SV40 T antigen. These findings not only indicated that Ad. 7 infectivity was required, but also provided evidence that the SV40 genetic material is not contained within the SV40 capsid protein. Second, heating E46 virus at 56°C for 10 min inactivated the adenovirus infectivity and the ability to induce SV40 T antigen; infectivity of intact SV40 virus would not be significantly affected by this treatment.[10]

In view of these findings, several hypotheses on the location of the SV40 genetic material were considered: (1) as free SV40 DNA, (2) as nonencapsidated SV40 DNA associated with an internal protein (nucleoids), (3) as DNA or nucleoids adsorbed onto the Ad. 7 virion, or (4) SV40 DNA enclosed in the Ad. 7 capsid. Free or adsorbed DNA was rendered an unlikely possibility by failure of DNAase treatment (50 γ pancreatic DNAase per ml, with 0.001 M magnesium acetate, 37°C, 45 min) to affect the SV40 antigen-inducing capacity of E46. Two types of experiment were done to evaluate the second hypothesis. It was postulated that

TABLE 3

GRADACOL FILTRATION OF L. L.-E46 PREPARATIONS, TESTED IN HEK CULTURES

Inoculum	Reaction with SV40 Hamster Serum — Expt. 1 L. L.-E46AG1 FA	Expt. 1 L. L.-E46AG1 CF (cell pack antigen, 1:4)	Expt. 2 L. L.-E46AG7 FA
None	0*	0†	0*
Starting material (670 mmu filtrate)	3%	2+	10%
110 mmu filtrate	0	0	0‡
" " membrane	1%	4+	
48 " filtrate	0	0	
" " membrane	1%	4+	
110 mmu filtrate + L. L.-E46⁻	0		0
L. L.-E46⁻	0		

* Approximate percentage of nuclei showing specific fluorescence.
† Degree of complement fixation; 4+ = complete fixation.
‡ After the L. L.-E46 filtration, this filter was tested and found to pass SV40 virus.

removal of the Ad. 7, if it were acting as a helper virus for subinfectious SV40 particles, would provide material which could be activated by another helper virus. First, Ad. 7 infectivity was inhibited by addition of Ad. 7 rabbit antiserum, and Ad. 3 virus was added to the neutral mixture to serve as a potential helper; this mixture did not induce SV40 T antigen in HEK cells. Second, E46 and L. L.-E46AG5 preparations were passed through Gradacol filters (Table 3), the 110 mmu filter being chosen as appropriate for eliminating Ad. 7 but not SV40 or smaller particulates. The 110 mmu filter retained both the Ad. 7 infectivity and SV40 T antigen-inducing activity, and admixture of the 110 mmu filtrate with nonantigen-producing L. L.-E46$^-$ virus did not yield an antigen-inducing mixture. Thus, both experiments confirmed the integral association of the SV40 genetic material with the Ad. 7 virion.

In efforts to remove superficially adsorbed material on the virion, L. L.-E46 preparations were treated with trypsin (0.1% for 1 hr at 37°C) or chloroform (10%, 10 min at 4°); antigen induction was not affected by either treatment.

Another possibility that must be considered is the presence in L. L.-E46 preparations of a serologically distinct serotype of SV40, sharing T antigen but not capsid antigen and producing no cytopathic changes in AGMK cells; however, this hypothesis does not explain the necessary role of adenovirus infectivity and the negative results of the "helper virus" experiments.

Discussion.—The above data indicate that the most probable explanation of the phenomena observed with L. L-E46 virus is that a proportion of the virus population consists of Ad. 7 capsids containing both Ad. 7 and SV40 genetic material, i.e., a genetic hybrid. While it cannot be excluded that the SV40 genetic material is on the surface of the Ad. 7 virion, it does not seem likely that a high proportion of the Ad. 7 particles would contain adsorbed nucleoids without detectable nucleoids being present unattached to Ad. 7 particles. Biophysical and homology studies of DNA from the AGMK passage line of L. L.-E46 may resolve this point and also provide evidence whether the SV40 DNA is integrated into the Ad. 7 DNA strand.

The available evidence suggests that only a portion of the SV40 genome is being carried by the hybrids. At no time has capsid antigen been detected by FA tests, hamsters with E46-induced tumors do not develop neutralizing antibody to SV40, and infectious SV40 cannot be recovered from L. L.-E46 virus preparations or E46-induced hamster tumors.[1] However, the SV40-transformed hamster cell line THK-1 shows no viral antigen staining with monkey anti-SV40 serum, but does contain the complete SV40 genome as evidenced by the retrievability of whole virus by the overlay procedure.[2, 11]

The observation by Rabson *et al.*[12] that adenoviruses 12 and 5 replicate poorly, if at all, in monkey kidney cell cultures unless SV40 virus is present (in preliminary experiments we have found that the same phenomenon holds true with Ad. 7 strains) provides a working hypothesis on some aspects of the origin of the L. L.-E46 hybrid. It can be postulated that efficient adenovirus replication in monkey cells requires induction of one or more enzymes by SV40; a mixture of the two viruses could be serially propagated by this "helper virus" effect of SV40. Unless highly monkey-adapted adenovirus mutants arise, this requirement for SV40 would mean that dual infection of cells is necessary for adenovirus growth, and consequently

there may have been an enormous number of obligatory dual infections during the long passage series of E46, with opportunity for a hybrid to occur. When the SV40 is neutralized, as was done during the passage history of E46, those Ad. 7 particles which have hybridized with the essential portion of the SV40 genome would have high selective advantage in further passages. This hypothesis is difficult to reconcile with the observation that nonhybrid particles were in the majority in the E46 preparation; however nonhybrid Ad. 7 could develop by reversion from the hybrid, by superinfection of hybrid-infected cells by nonhybrid virus, or if there were monkey-adapted mutants in the population. It is noteworthy that a preliminary experiment indicates that L. L.-E46$^-$, which does not induce the SV40 antigen, is poorly adapted to AGMK.

It is not known whether the SV40 T antigen is itself the required enzyme or a protein produced by a gene near that producing the enzyme(s).

Summary.—A passage line of Ad. 7 which had been freed of SV40 contamination induces an SV40 antigen in acutely infected tissue culture cells. The SV40 antigen-inducing capacity is integrally associated with the Ad. 7 infectious particle, suggesting incorporation of a portion of the SV40 genome into the Ad. 7 virion.

The studies reported here were made possible by the generosity of many people. Drs. B. A. Rubin and R. M. Chanock provided the E46 virus and some of the passage lines, Dr. J. H. Pope prepared the hamster SV40 antibody preparation, Dr. R. Wilsnack prepared the antihamster conjugates, Dr. M. D. Hoggan conjugated the monkey antiviral serum, and Dr. R. J. Huebner supplied E46 tumors and sera from E46 tumored hamsters. Mr. Wendell E. Pugh provided invaluable technical assistance.

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