Type 12 Adenovirus Proteins: Influence on Tumor Development in Offspring of Immunized Pregnant Hamsters[1] (35784)

G. R. DREESMAN,[2] H. PINKERTON, E. S. HOLTWICK, AND J. R. SURIANO[3]

The Institute for Molecular Virology and Department of Pathology, St. Louis University School of Medicine, St. Louis, Missouri 63104

There are two immunologic approaches which can be used for the prevention of tumor induction by an oncogenic virus. The first involves the immunization of the host, in which the oncogenic process has already started, with either the virus or nonviable virus-transformed cells (1–5). This is effective only when the tumors induced are antigenic and the developing tumor mass is small enough to be eliminated by immune lymphocytes. The second approach involves the neutralization of oncogenic virus before it transforms cells *in vivo*. Neutralization can be achieved by preimmunizing the host with the virus. Since only newborn animals are susceptible to DNA viral oncogenesis, it is necessary to immunize the mothers with the virus (6). This method has been successfully applied in Ad 12, SV40, and murine leukemia-sarcoma model systems (7–9). However, immunization with the whole virion is not desirable since it has been demonstrated that inactivated oncogenic viruses may not only retain their complete oncogenic potential, but also show enhanced oncogenicity (10) probably because only a fraction of the viral genome is required for transformation.

We report below the effectiveness of maternal immunization with purified protein components of Ad 12 in repressing the development of Ad 12 oncogenesis in newborn hamsters. As a model, this virus has the advantages that it produces tumors rapidly in newborn hamsters (11, 12) and that its components have been well characterized (13, 14).

Materials and Methods. Propagation of the virus. The Huie strain of Ad 12 (kindly supplied by Dr. Raymond Gilden, Flow Laboratories, Bethesda, Md.), was propagated in a continuous human cell line (KB) grown in suspension culture in Eagle's minimal essential medium with 5% horse serum. Viral infectivity was determined by plaque assay on monolayer cultures of KB cells (15). Virus used in all experiments was free of adenovirus-associated satellite virus as evidenced by electron microscopy.

Purification of virions and capsomer antigens. Virus was purified from infected KB cells by sonic disruption of the cells, treatment with genetron, concentration on a layer of CsCl, and density gradient centrifugation in CsCl (16, 17). Hexon was purified from the supernatant fluid obtained from the CsCl concentration step during virion purification. Ammonium sulfate was added to the supernatant fluid at 4° to 66% of saturation at pH 7.2. The precipitated proteins, including hexon antigen, were suspended in a minimal volume of 0.05 *M* Tris–HCl buffer, pH 8.4, and further fractionated by gel filtration in Sepharose 2B (Pharmacia Fine Chem., Inc.) followed by zonal centrifugation in preformed sucrose gradients (5–20% sucrose). Details of these purification steps are described under "Results."

Isolation of core proteins. Purified preparations of Ad 12, labeled with ^{14}C-thymidine, were disrupted by treatment with acetone (18). The disrupted, precipitated material was resuspended in a minimal volume of water, layered over 4.5 ml of CsCl (density

[1] Supported by research contract PH 43-67-692 from the National Cancer Institute and by USPHS Grants CA 11629, CA 07972, and CA 10630.

[2] Present address: Department of Virology and Epidemiology, Baylor College of Medicine, Houston, Texas 77025.

[3] Present address: Department of Microbiology, Medical College of Ohio at Toledo, Toledo, Ohio 43614.

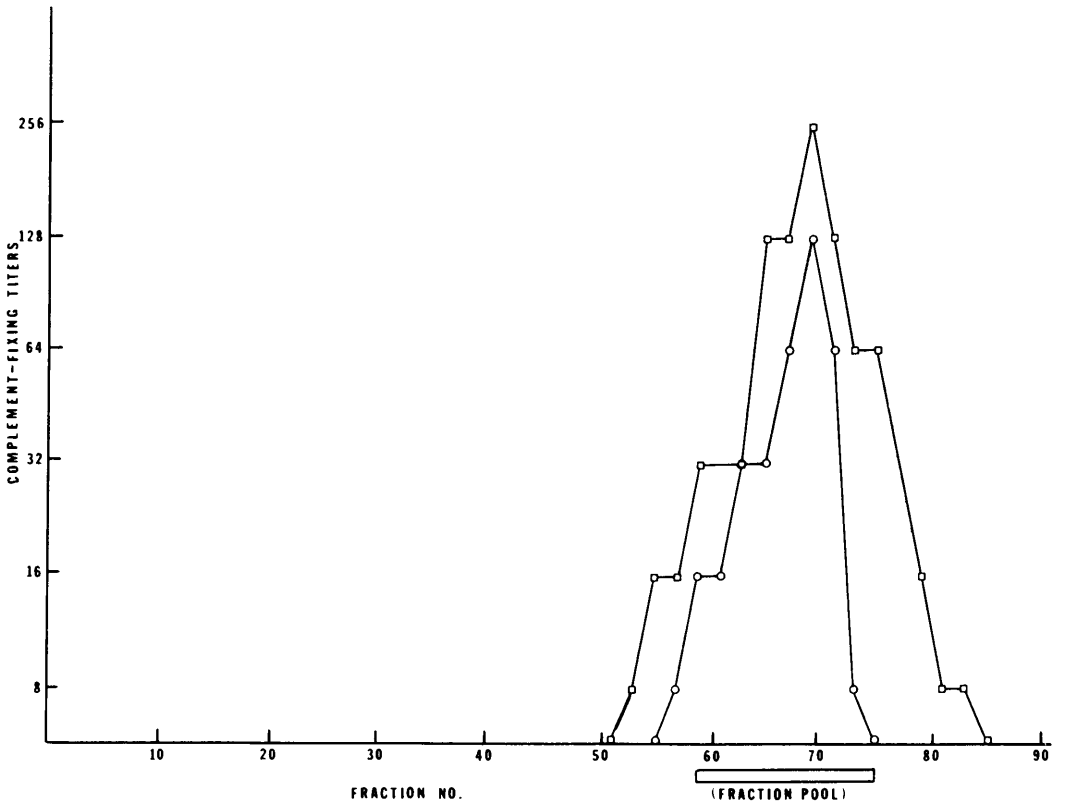

FIG. 1. Gel filtration of Ad 12 soluble antigens in Sepharose 2B with CF activities determined with anti-Ad 12 serum (□); and with anti-Ad 7 serum (○).

of 1.36 g/cm^3) and centrifuged at 44,000 rpm for 48 hr at 4° in a Spinco Ti-50 rotor. Four-drop fractions were collected from the bottom of the tube; and the density of every other fraction was computed from measurements of the refractive index obtained with an Abbe 3L refractometer.

Polyacrylamide gel electrophoresis. Analytical polyacrylamide gel electrophoresis was performed, utilizing gels containing 7.5% acrylamide and 1% *N,N'*methylenebisacrylamide. Electrophoresis was conducted in 75 × 8-mm tubes at 3 mA/tube for 3 hr. Gels were stained with amido schwarz for 1 hr and excess stain removed with 7.5% acetic acid.

Protein assay. Protein was assayed by a micromodification of the Lowry *et al.* (19) method using crystalline bovine serum albumin as standard.

Animal experiments. Pregnant hamsters obtained from the Lakeview Hamster Colony were divided into 5 groups. On the third day of pregnancy three groups were immunized with either hexon, core protein, or purified virus containing 160, 26, and 260 μg of protein/ml, respectively. These preparations were emulsified in an equal volume (0.5 ml) of Freund's complete adjuvant and injected subcutaneously. The fourth group was injected with phosphate-buffered saline emulsified in the adjuvant, and the fifth group was untreated. Similar injections were repeated on day 10 of pregnancy. The majority of the hamsters delivered on day 16. All offspring were injected with 4 × 10^5 plaque-forming units of Ad 12 within 12 hr after birth.

The appearance and development of tumors were recorded; and tumor weights were estimated by palpation and/or caliper measurement. All tumor-bearing animals were sacrificed on day 65, the tumor weights were recorded, and selected tumors were fixed for histologic study. Animals without tumors remained negative until day 72, when the ex-

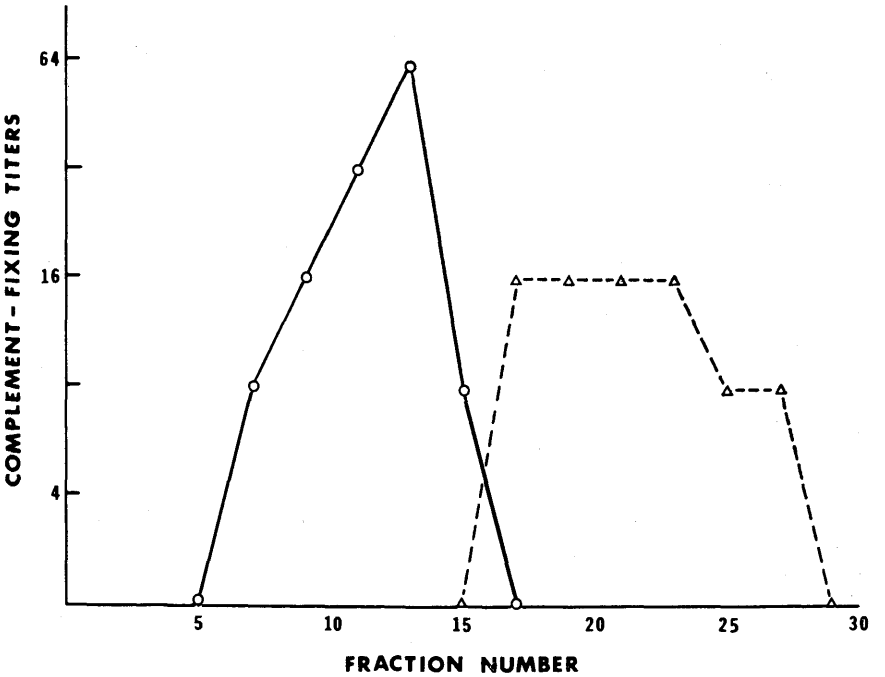

FIG. 2. Zonal centrifugation in preformed sucrose gradients (5–20%) of Ad 12 hexon pool obtained from Sepharose 2B column. Centrifugation was carried out in a Spinco SW-25 rotor at 25,000 rpm for 30 hr. CF activity was determined with anti-Ad 7 serum (O), and with anti-KB cell serum (△).

periment was terminated. Statistical comparisons were made by a standard t test using unequal variance.

Complement-fixation (CF) assay. Ad 12 antigens were assayed by a micro-CF technique (20) using 4 units of rabbit anti-Ad 12 or anti-Ad 7 sera. The CF test utilizing anti-Ad 12 serum detects both penton and hexon antigens, while the anti-Ad 7 serum reacts only with hexon. Host-cell proteins were assayed with 4 units of antibody made in rabbits. Serum antibody titers in the immunized hamsters were measured using either 4 units of Ad 12 purified soluble virion antigens, or 4 units of Ad 12 T-antigen (Flow Laboratories, Bethesda, Md.).

Results. Purification of viral subunits. Gel filtration of the proteins precipitated from the soluble antigen pool yielded the group-specific hexon (Fig. 1). The contaminating cellular proteins in the hexon pool derived from the CF positive gel filtration fractions were effectively removed by zonal centrifugation (Fig. 2). Analytical polyacrylamide gel electrophoresis confirmed the purity of the preparation, showing only a single protein component (Fig. 3), which was eluted and shown to have group-specific CF activity. There was no evidence for any contamination of the hexon preparation by penton or fiber antigens.

Purified Ad 12 virions were disrupted with acetone and fractionated by ultracentrifugation on a CsCl gradient. A DNA-protein fraction (core protein) was obtained with a buoyant density of 1.37 g/cm^3. Electron microscopy revealed that the bulk of this material had the appearance of poorly stained core material. Small quantities (less than 10%) of aggregated capsomers were also observed.

Inhibition of tumor growth by maternal immunization. All maternally immunized animals responded with a low but significant serum antibody titer against the group-specific virion antigens (Table I). Significant anti-T antigen titers were not observed. The effect of maternal immunization on the inci-

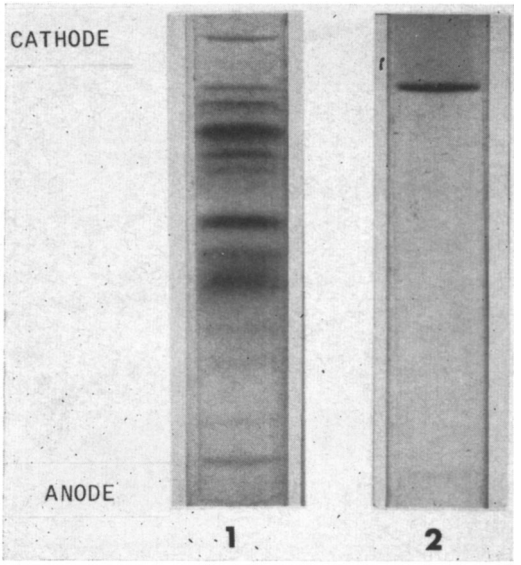

FIG. 3. Polyacrylamide gel electrophoresis of (1) concentrated Ad 12 soluble antigen preparation; and (2) Ad 12 hexon purified by gel filtration and sucrose gradient centrifugation. Approximately 100 μg of protein were applied to each with migration going from cathode to anode.

dence of Ad 12-induced tumors in the offspring is shown in Table II. At 5 weeks after inoculation, 55% of the control offspring had developed subcutaneous tumors. Immunization of mothers with purified virion and hexon provided clear cut protection to the offspring since only 6 and 12% of the animals had developed palpable tumors by 5 weeks. The core protein and Freund's adjuvant failed to provide significant protection; in these two groups 20 and 33% of the animals developed tumors, respectively. The incidence of tumors at 10 weeks was similar in all groups except the untreated control group, in which 66% of the animals developed tumors, thus showing that maternal immunization with hexon and purified virions retarded rather than prevented the development of tumors by Ad 12.

When the tumor weight in each group was plotted against time after challenge (Fig. 4), a much clearer picture emerged. At autopsy, the average tumor weight in the adjuvant controls was 8.9 g compared with 2.5 and 3.1 g in groups immunized with purified virions and hexons, respectively. This data supports the conclusion reached from the figures on tumor incidence (Table II) that maternal immunization with hexon and purified virions resulted in the inhibition of growth of primary tumors induced by Ad 12.

TABLE I. Serum Antibody Activity in Maternal Hamsters.

Immunizing antigen	Complement-fixing titers[a]			
	Anti-Ad 12		Anti-T antigen	
Days:	17	30	17	30
Purified virus	256	512	8	4
Hexon	256	512	<2	<2
Core proteins	128	128	<2	<2
Freund's adjuvant	<2	<2	<2	<2
No treatment	<2	<2	<2	<2

[a] Titers given are average values for all animals which bore young. The titers are given for 17 and 30 days after the second injection.

TABLE II. Incidence of Adenovirus Type 12 Tumors in Offspring of Immunized Hamsters.

Immunizing antigen	No. of animals inoculated	Animals with tumors (%)	
		35 days[a]	72 days
Purified virus	34	6	44
Hexon	32	12	37
Core proteins	25	20	44
Freund's adjuvant	30	33	37
No treatment	29	55	66

[a] Days after challenge of offspring with 4×10^5 plaque-forming units of Ad 12.

Autopsy findings showed that the spleens of tumor-bearing offspring were 2–6 times normal size; other organs showed no changes and no metastases were noted. The larger tumors showed the typical histologic picture of Ad 12-induced tumors, with marked hemorrhage and necrosis. Several tumors, which had been stationary for 15–20 days, were composed of elongated cells rather than the typical "epitheloid" cells. Subcutaneous cysts as large as 0.8 cm in diameter were found in most of the animals injected with complete Freund's adjuvant, but granulomatous reactions were not observed.

Discussion. As noted above, maternal im-

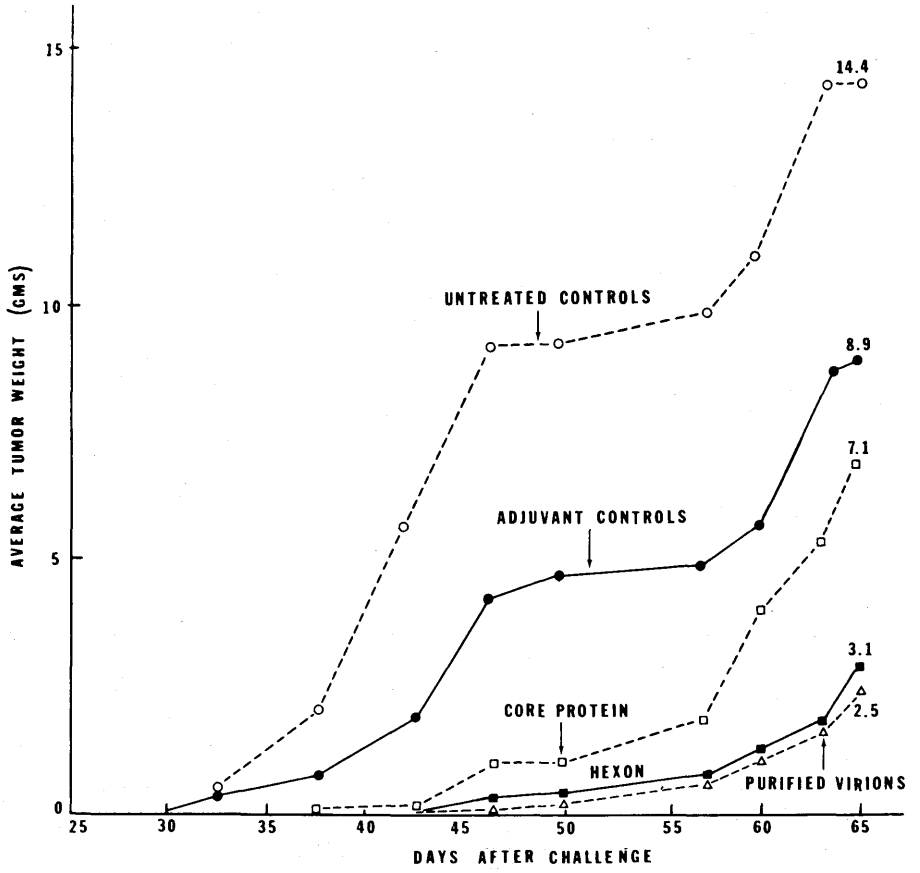

FIG. 4. Average tumor weight in offspring of animals immunized with complete Freund's adjuvant, Ad 12 core proteins, Ad 12 hexon, and purified virion, and in offspring of untreated control mothers.

munization prior to pregnancy has been found to prevent SV40 and Ad 12 tumorigenesis in the offspring (7, 8). However, these animals were immunized with complete virus. A recent report (21) showing that SV40 DNA in sperms could be recovered from fertilized ova, suggests that the use of DNA-containing viral material for maternal immunization would be potentially dangerous.

Our results indicate that the purified capsid proteins (hexon), which are devoid of viral nucleic acid, can repress Ad 12 tumorigenesis in newborn hamsters when maternally injected during pregnancy. The finding of repression, rather than complete prevention, is not unexpected. The animals were immunized on days 3 and 10 of pregnancy. With a hamster gestation period of only 16 days, it is probable that only a small amount of IgG 7S viral antibody is synthesized and thus, very little would pass through the placenta to neutralize the virus administered to newborns. The results, however, probably indicate that some antibody crossed the placenta and neutralized the virus to a degree that resulted in the differential growth rate of tumors in the various groups. The latent period and the growth of tumors have been shown to depend upon the virus dose (22).

The lower incidence of the tumors in the group immunized with Freund's adjuvant alone needs explanation. It is possible that in these animals there is enhanced synthesis of antibody directed against fetal antigens, which have been shown to cross-react with virus-specific surface antigen(s) present in SV40-transformed cells (23, 24). A similar phenomenon may have been responsible for the lower incidence of tumors in this group.

The retardation of tumor growth that we

observed in the offspring of hamsters immunized with hexon suggests that an antibody elicited by this capsomer is protective. The hexon is known to be antigenically complex and to elicit the formation of a number of different antibodies, including one which cross-reacts with all members of the adenovirus group and one which is type specific and neutralizes viral infectivity (14).

The presence of internal proteins within the core of the adenovirion is now well established (25, 26). These core proteins share some of the characteristics of adenovirus-induced T-antigens (25), and it has been suggested that they are closely related to the T-antigens, since hamsters immunized with highly purified Ad 7 virus, which was disrupted by aging at 4° for 3 weeks, produced antibody to T-antigen (27). However, the hamsters in our study, which were immunized with the core proteins of Ad 12, did not produce any detectable antibodies against the T-antigen and the retardation of tumor growth noted in their offspring was slight compared with that caused by purified virus and by hexon proteins.

Summary. Using Freund's complete adjuvant, groups of pregnant hamsters were immunized with purified type 12 adenovirus (Ad 12), core proteins isolated from this virus, and hexon proteins isolated from unassembled soluble viral proteins. Control groups were untreated, or given the adjuvant alone. The progeny of all groups were challenged soon after birth with a highly oncogenic strain of Ad 12. Retardation of tumor growth in the maternally immunized, as compared with the control groups, was evidenced by delayed appearance, slower growth, and significantly lower average tumor weights at the termination of the study. On the basis of these criteria, purified virus and purified hexon protein both appeared to be highly and equally effective in repressing tumor growth in the progeny. The protection afforded by core proteins was of a much lower order. These results suggest the possibility of immunizing against Ad 12 by using highly purified, DNA-free, and presumably harmless hexon proteins.

We thank Miss Sharon Gant and Miss Kay Weiss for their technical assistance.

1. Goldner, H., Girardi, A. J., Larson, V. M., and Hilleman, M. R., Proc. Soc. Exp. Biol. Med. **117**, 851 (1964).

2. Coggin, J. H., Larson, V. M., and Hilleman, M. R., Proc. Soc. Exp. Biol. Med. **124**, 774 (1967).

3. Girardi, A. J., Proc. Nat. Acad. Sci. U.S.A. **54**, 445 (1965).

4. Jensen, F., and Defendi, V., J. Virol. **2**, 173 (1968).

5. Tevethia, S. S., Diamandopoulos, G. T., Rapp, F., and Enders, J. F., J. Immunol. **101**, 1192 (1968).

6. Melnick, J. L., Clarke, N. A., and Kraft, L. M., J. Exp. Med. **92**, 496 (1950).

7. Trentin, J. J., *in* "Viruses Inducing Cancer" (W. J. Burdette, ed.), p. 203. Univ. of Utah Press, Salt Lake City (1966).

8. Larson, V. M., Raupp, W. G., and Hilleman, M. R., Proc. Soc. Exp. Biol. Med. **126**, 674 (1967).

9. Otten, J. A., Tyndall, R. L., and Upton, A. C., J. Nat. Cancer Inst. **45**, 227 (1970).

10. Defendi, V., and Jensen, F., Science **157**, 703 (1967).

11. Trentin, J. J., Yabe, Y., and Taylor, G., Science **137**, 835 (1962).

12. Huebner, R. J., Rowe, W. P., and Lane, W. T., Proc. Nat. Acad. Sci. U.S.A. **48**, 2051 (1962).

13. Norrby, E., and Ankerst, J., J. Gen. Virol. **5**, 183 (1969).

14. Norrby, E., J. Gen. Virol. **5**, 221 (1969).

15. Green, M., Piña, M., and Kimes, R., Virology **31**, 562 (1967).

16. Green, M., and Piña, M., Virology **20**, 199 (1963).

17. Green, M., and Piña, M., Proc. Nat. Acad. Sci. U.S.A. **51**, 1251 (1964).

18. Laver, W. G., Suriano, J. R., and Green, M., J. Virol. **1**, 723 (1967).

19. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193**, 265 (1951).

20. Dreesman, G. R., and Benyesh-Melnick, M., J. Immunol. **99**, 1106 (1967).

21. Brackett, B. G., Baranska, W., Sawicki, W., and Koprowski, H., Proc. Nat. Acad. Sci. U.S.A. **68**, 353 (1971).

22. Eddy, B. E., Progr. Exp. Tumor Res. **4**, 1 (1969).

23. Duff, R., and Rapp, F., J. Immunol. **105**, 521 (1970).

24. Coggin, J. H., Ambrose, R. R., and Anderson, N. G., J. Immunol. **105**, 529 (1970).

25. Russell, W. C., and Knight, B. E., J. Gen. Virol. **1**, 523 (1967).

26. Prage, L., Pettersson, U., and Philipson, L., Virology **36**, 508 (1968).

27. Jordan, G. W., Riggs, J. L., and Lennette, E. H., Proc. Soc. Exp. Biol. Med. **132**, 10 (1969).

Received April 21, 1971. P.S.E.B.M., 1971, Vol. 137.