

19. Gerber, G., personal communication.
20. Potter, V. R., in *The Kinetics of Cellular Proliferation*, F. Stohlman, Jr., ed., Grune & Stratton, New York, London, 1959, p104.
21. Gerber, G. B., Remy-Defraigne, J., *Z. Naturforsch.*, 1963, v18b, 216.
22. Goutier, R., *Euratom Report*, 2201 f., 1965, p24.
23. Feinendegen, L. E., Bond, V. P., *Exp. Cell Res.*, 1962, v27, 474.
24. Stone, G. E., Miller, O. L., Prescott, D. M., *J. Cell. Biol.*, 1965, v25, 171.
25. Cleaver, J. E., Holford, R. M., *Biochim. Biophys. Acta*, 1965,
26. Bryant, B. J., personal communication.
27. Feinendegen, L. E., Bond, V. P., Hughes, W. L., in preparation.
28. Oehlert, W., Magnusson, B., *Z. f. Krebsforsch.*, 1965, v66, 455.
29. Rothstein, H., Weinsieder, *Exp. Eye Res.*, 1965, v4, 1-3.
30. Rubini, J. R., Cronkite, E. P., Bond, V. P., Fliedner, T. M., *J. Clin. Invest.*, 1960, v39, 909.
31. Zimmerman, M., *Biochem. Biophys. Res. Comm.*, 1962, v8, 169.
32. Odartchenko, N., Bond, V. P., Feinendegen, L. E., Cottier, H., in *Cell Proliferation*, A Guinness Symposium, Blackwell Scientific Publications, Oxford, England, 1963, 172.
33. Quastler, H., Sherman, F. G., *Exp. Cell Res.*, 1959, v17, 420.
34. Ottolenghi, E., Hotchkiss, R. D., *J. Exp. Med.*, 1962, v116, 491.
35. Fox, B. W., Prusoff, W. H., *Cancer Res.*, 1965, v25, 234.

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Human Adenoviruses: Tumor Production in Hamsters by Types 12 And 18 Grown from Single Plaques.* (31160)

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Human adenoviruses reported to date to cause tumors in hamsters(1-5) were from uncloned virus seedstocks. Thus, it is possible that the tumors resulted from agents in the stocks other than the adenovirus or that the tumors resulted from the combined action of the adenovirus and a second agent. Two sets of observations support these possibilities. First, small virus-like particles have been detected by electron microscopy in stocks of human adenovirus types 2, 5, 7 and 12(6) and of simian adenovirus type 15(7). Second, Huebner *et al*(8) reported the induction by adenovirus type 7 of tumors in hamsters having the antigenic characteristics of SV40 virus. With this exception, however, only adenovirus-specific antigens have been de-

tected in the adenovirus-induced virus-free tumors(2,3,5), a finding that suggests the tumors were induced by adenoviruses acting alone. In addition, Trentin *et al*(1) found that only human sera which neutralized the cytopathic effects in tissue culture of adenovirus type 12 also neutralized the tumor-inducing capacity of the virus.

In this study we sought further evidence on the point of whether or not adenoviruses acting alone caused tumors by inoculating hamsters with adenoviruses prepared from single plaques of types 12 and 18. The hamsters which developed tumors also developed complement-fixing serum antibodies to both adenovirus tumor antigens(3) and the cell associated T antigens or neoantigens(9-11). The induced tumors contained antigens that reacted in the complement fixation test with specific reference antisera from hamsters bearing either type 12- or type 18-induced tumors. In carrying out these studies, we found that plaque methods described for cer-

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tain other adenoviruses, such as types 2, 4 and 5(12-15), required modification for the oncogenic adenovirus types 12 and 18. This report therefore also describes a simple and workable method for the production of plaques by these serotypes on cell cultures of human embryo kidney and of HeLa.

Materials and methods. Cells. Primary cultures of human embryo kidney cells (HEK) used in these studies were cultivated in 16 oz prescription bottles or milk dilution bottles. These cultures were maintained in Eagle's medium containing 10% inactivated calf serum. For monolayer preparation, the cells were washed with Hanks' balanced salt solution (HBSS), trypsin-dispersed and suspended in new medium in amounts to give approximately 2×10^5 cells per ml.

Virus. Adenovirus type 12 (Huie strain) and type 18 (DC strain) were supplied by one of the authors (R.H.).

Growth media and solutions used in the plaque assays. Basal Eagle's medium was modified to contain 4 times the normal concentration of vitamins and 2 times the normal concentration of amino acids.

Antibiotics were included in all growth media: penicillin, 100 units/ml, and streptomycin, 50 μ g/ml. The agar overlays used had the following composition per 100 ml:

A. White agar overlay: Agar (special agar—Noble, Difco, 1 g; basal Eagle's medium (modified), 80 ml; agamma calf serum (Hyland), 20 ml.

B. Red agar overlay: Same as A with addition of 0.015% (W/V) neutral red.

Method of carrying out the plaque assay. The monolayers were prepared by adding about 10^6 cells in 5 ml of modified Eagle's medium containing 10% inactivated calf serum (for HEK) or 15% inactivated calf serum (for HeLa) per plate (plastic petri dish, 60 mm diameter). The plates were incubated at 37°C in an atmosphere constantly gassed with approximately 5% CO₂ in humidified air. After 4 days, the plates were refed with 5 ml of the same medium and incubated another 1 to 2 days. Before inoculation the monolayers were rinsed with 3 ml HBSS. The standard virus inoculum was 0.1 ml per plate, and the time allowed for adsorption was 1

hour. The plates were again washed before they were overlaid with 5 ml white agar nutrient medium (A above). After 2 to 3 days, a second overlay of 2.5 ml of agar nutrient was added and additional overlays of 2.5 ml were added every 2 to 3 days for 17 days. The red agar overlay (B above) was substituted for white agar on the seventh day after infection. The first visible plaques appeared 6 to 7 days after infection and the number of plaques increased with time up to 20 days. For the virus strains tested, the plaques produced on HEK cultures were larger and more distinct than those produced on HeLa cultures.

A linear relationship existed between virus input and number of plaques produced.

No further increase in the number of plaques occurred after 60 minutes' virus adsorption time. The first new virus appeared in infected cells at 24 hours and reached a maximum at 48 hours with a yield of about 10^2 PFU per cell. Extracellular virus first appeared at 48 hours.

Preparation of virus clones. To obtain virus clones, plaques were selected from dishes containing 1 or a few well-separated plaques. A plug of agar was removed from over the plaque and was dropped into a culture tube containing a monolayer of HEK cells. Agar plugs were taken a few millimeters from plaques to test for possible spread of virus into the agar; none of these yielded infective virus.

The HEK cultures inoculated with the agar plug from over the plaques were allowed to develop advanced viral CPE. They were then frozen and thawed and the fluids centrifuged to remove cellular debris. The supernatant fluids were stored at -60° . These stocks had titers in HEK cells of 1.7×10^6 to 4.2×10^7 plaque-forming units (PFU) per ml. The identity of the virus in each stock was tested in a plaque reduction neutralization experiment(16), using standard prototype viral rabbit antiserum prepared by the Research Reference Reagents Branch, National Institute of Allergy and Infectious Diseases.

Hamster inoculation. Newborn hamsters (California Caviary Co., Los Angeles), less than 48 hours after birth, were injected sub-

cutaneously with 0.05 ml of undiluted virus. Four plaque purified virus inocula were used; type 12 virus cloned on HEK and on HeLa cells and type 18 virus cloned on the same cell types. The hamsters were weaned at 3 weeks of age and were kept in separate cages in an isolation room. They were examined at frequent intervals for tumors. When large tumors had developed in the inoculated hamsters, the following were collected: (1) Whole blood was obtained from the heart at post-mortem examination; the serum was separated and frozen (undiluted). (2) Tumor fragments were minced and cultivated in tissue culture by methods described in another report(17). After 1 to 2 passages, the cells were scraped from the culture vessel and a 10% cell pack suspension prepared in Eagle's medium. (3) Tumor fragments were trypsin-dispersed and a 20% cell pack prepared in Eagle's medium. (4) Tumor cells were homogenized and a 10% suspension prepared in Eagle's medium and clarified at 1500 RPM for 15 minutes. (5) Tumor fragments were fixed in formalin for pathological study.

Serological methods and reagents. Tests for complement-fixing antigens in the tumor cells, as well as hamster serum antibodies to both tumor and T antigens, were carried out as described in other reports(3,5,8).

Results. Tumor formation in hamsters. Eleven of 27 hamsters inoculated with adenovirus type 12 developed subcutaneous tumors from 38 to 93 days after injection of virus. Tumors developed in hamsters inoculated with adenovirus type 12 cloned on HeLa cells (7/14) and on HEK cells (4/13).

Similarly, 13 of 28 hamsters inoculated with adenovirus type 18 developed subcutaneous tumors from 30 to 70 days after inoculation. Again, tumors developed in hamsters inoculated with adenovirus type 18 cloned on HeLa (8/13) and on HEK cells (5/15).

The numbers of infectious doses of type 12 virus and the time of appearance of tumors were similar to those reported previously(1-4). Larger doses of type 18 virus were used in our studies than in previous studies, and the latent period before the appearance of

tumors was shorter than that previously reported (60-280 days).

Histological findings. The dominant cell type of the tumors induced by type 12 and by type 18 was a moderately large polygonal cell with a large vesicular nucleus and scanty cytoplasm. Growth was usually patternless, but occasionally perivascular palisading was seen. Areas of smaller, rounder cells with small, opaque, round nuclei were commonly present; although these areas often resembled lymphoma, their association with areas of necrosis suggested that the small round-cell appearance was an early stage of necrosis. Bands and areas of necrosis were common and occasionally were massive, with only small residual areas of viable tumor. Multinucleate tumor giant cells were infrequent, but atypical mitoses (tri- and tetra-polar, etc.) were moderately common.

Reticulum and van Gieson stains of the tumors showed scanty connective tissue stroma arranged in a sarcoma-pattern. Phosphotungstic acid hematoxylin stain gave no evidence that the tumors were rhabdomyo- or leiomyosarcomas, and periodic acid-Schiff stains showed very little glycogen in the tumor cells. A definite conclusion as to the cell type of these virus-induced tumors could not be drawn, but the general microscopic appearance did not suggest mesothelioma or angiosarcoma. They must be considered for the moment as undifferentiated "fibrosarcomas." The only significant difference between tumors induced by the 2 viruses was that type 18 tumors were less likely to show areas of small, round cells than were type 12 tumors; namely, they were less likely to have areas suggesting lymphoma.

Serological findings. The hamster tumors induced by both viruses (10% homogenates and 20% trypsin-dispersed cell packs), as well as tissue culture-grown tumor cells (10% cell pack), contained antigens that were reactive in the complement fixation test with serum from tumored hamsters (whose tumors were induced by either type 12 or 18).

The tumored hamsters developed relatively high titers (>1:80) of serum antibodies to both tumor and T antigens of adenovirus types 12 and 18. Similar reactions, including

cross reactions between types 12 and 18 antigens and antisera, have been reported(3,5).

Discussion. Virus stocks prepared from plaques on cell cultures containing only 1 to 3 well-separated plaques most likely contain a population of adenovirus particles of common genetic origin. The possibility cannot be excluded that a contaminating virus, noncytopathic for HeLa or HEK cells, and in higher titer in the seed than the adenovirus, was in the same agar plug as the adenovirus. To obtain enough material for hamster inoculation as well as for plaque assay and plaque neutralization tests, the adenovirus stocks were passaged 1 to 3 times in HEK cell cultures, the fewest passages possible, in order to lessen the possibility of "picking up" viruses known to be indigenous in HEK cells (18).

With the exception of these unlikely possibilities for viral contamination, the present data suggest that adenovirus types 12 and 18 are capable of inducing tumors in hamsters without aid of other viruses. The tumors induced by both viruses contained tumor antigens, and the tumored hamsters developed antibodies to both tumor antigen and T antigen.

Although only 4 clones (2 of type 12 and 2 of type 18) were tested and found to be oncogenic, these results suggest that all type 12 and type 18 particles may carry the oncogenic determinant.

Summary. Clonally derived adenovirus types 12 and 18 induced tumors in hamsters. Specific complement fixing antigens were demonstrated in the tumors, and the tumored hamsters developed specific complement fixing antibodies to both tumor and T antigens. The histologic characteristics of the tumors

resembled those previously described for tumors induced by these viruses. These data support the conclusion that adenovirus types 12 and 18 can induce tumors in hamsters without the aid of other agents.

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1. Trentin, J. J., Yabe, Y., Taylor, G., *Science*, 1962, v137, 835.
2. Huebner, R. J., Rowe, W. P., Lane, W. T., *Proc. Nat. Acad. Sci.*, 1962, v48, 2051.
3. Huebner, R. J., Rowe, W. P., Turner, H. C., Lane, W. T., *ibid.*, 1963, v50, 379.
4. Girardi, A. J., Hilleman, M. R., Zwickey, R. E., *Proc. Soc. Exp. Biol. & Med.*, 1964, v115, 1141.
5. Huebner, R. J., Casey, M. J., Chanock, R. M., Schell, K., *Proc. Nat. Acad. Sci.*, 1965, v54, 381.
6. Hoggan, M. D., *Fed. Proc.*, 1965, v24, 248.
7. Atchison, R. W., Casto, B. C., Hammon, W. McD., *Science*, 1965, v149, 754.
8. Huebner, R. J., Chanock, R. M., Rubin, B. A., Casey, M. J., *Proc. Nat. Acad. Sci.*, 1964, v52, 1333.
9. Hoggan, M. D., Rowe, W. P., Black, P. H., Huebner, R. J., *ibid.*, 1965, v53, 12.
10. Huebner, R. J., *Perspectives in Virology IV*, Harper & Row, New York, 1965, 142.
11. ———, Presentation in May 1965 at International Conference on Tumor Antigens in Sukhumi, U.S.S.R., sponsored by Union Internationale Contre le Cancer, Geneva, Switzerland, in press.
12. Bonifas, V., Schlesinger, R. W., *Fed. Proc.*, 1959, v18, 560.
13. Kjellén, L., *Virology*, 1961, v14, 234.
14. Philipson, L., *ibid.*, 1961, v15, 263.
15. Rouse, H. C., Bonifas, V. H., Schlesinger, R. W., *ibid.*, 1963, v20, 357.
16. Kjellén, L., *ibid.*, 1962, v18, 448.
17. McAllister, R. M., Landing, B. H., Goodheart, C. R., *Lab. Invest.*, 1964, v13, 894.
18. Benyesh-Melnick, M., Rosenberg, H. S., Watson, B., *Proc. Soc. Exp. Biol. & Med.*, 1964, v117, 452.

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