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An Oncogenic Virus Carried by Hamster Kidney Cells

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SUMMARY

A filterable oncogenic factor was isolated from ascitic tumors induced in X-irradiated hamsters by inoculation of BHK 21 clone 13 cells. This agent reproduces a tumor in hamsters 22 days after inoculation. Its oncogenic characteristics are preserved even after the ascitic tumor cells have been subcultured *in vitro*.

The occurrence of R or H virus-like particles, both in tumor cells and in cultures, suggests a probable participation of such structures in the oncogenetic mechanisms of the aforementioned neoplasm.

INTRODUCTION

The BHK 21[1] line, originated in 1961 (9) from the kidney fibroblasts of a newborn Syrian hamster, is widely used in the study of cell and virus biology.

As previously described in the literature (5, 6), BHK cells are capable both of reproducing themselves in hamster tissues and of provoking tumors. Although no filterable oncogenic factor was obtained from BHK cells (1), electron microscopy has shown virus-like particles in their cytoplasm (2, 16, 18).

The presence of virus-like particles in this cell line, approximately 100 nm in diameter with a nucleoid 50 nm in diameter and with a typical series of radiating structures, is described therein as well. Filamentous and budding elements were also noted by these authors.

These particles were also observed in several hamster tumors and in a bovine cell line (13, 17).

In this paper we describe: (*a*) the production of an ascitic tumor by a series of i.p. transfers starting with an initial inoculation of BHK 21 clone 13 cells in whole-body-irradiated animals; (*b*) the obtention of acellular material from the tumor; and (*c*) the production, induced by this acellular suspension, of new tumors, similar in their characteristics to the original ascitic tumor.

MATERIALS AND METHODS

Tissue Cultures. Cells from the BHK 21 clone 13 line and new cell line 793, originated from the ascitic tumors, were used in these experiments.

Both lines were cultivated in Eagle's basal medium containing lactalbumin and 10% bovine serum.

[1] The abbreviations used are: BHK, baby hamster kidney; BHKAT, baby hamster kidney ascites tumor.

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Animals. The studies were carried out on 12- to 20-week old adult hamsters (*Mesocricetus aureatus*) of both sexes.

Tumors. Those animals were used to maintain 2 types of tumors, that is, s.c. and ascitic tumors, both caused by the inoculation of the BHK 21 clone 13 cells.

The s.c. Tumor. BHK 21 cells (10^7) were inoculated on the back of each animal for production of the s.c. tumor (BHK fibrosarcoma). The ensuing transplantations took place every 10th or 12th day after the tumor had grown to 2 cm in diameter.

After excision of the tumor, the tissue was cut in small fragments, suspended in Hanks' solution, and inoculated into the back of the animal.

The Ascitic Tumor. The BHKAT (11) was obtained by i.p. inoculation of 5×10^6 BHK 21 clone 13 cells in animals that had previously been whole-body irradiated with 700 rads. The 1st transplantation took place 3 weeks afterward. In all transplantations, cells suspended in the ascitic medium were used. Those cells were obtained from the animal by peritoneal puncture with the use of heparinized syringes.

Ascitic fluid was extracted from the 9th to 14th day following transplantation. The cells were usually inoculated i.p. in the receiver animals at the rate of 4×10^7 cells/animal. After 3 transfers through whole-body-irradiated animals, subsequent transplantations were carried out in nonirradiated animals. When irradiated animals were used, inoculation took place 24 hr after irradiation. Irradiated animals were treated with antibiotics for a period of 10 days (10). The average survival time of these ascitic tumor-bearing animals was 9 days. Transfers containing 5×10^5 cells also produced BHKAT's with the same characteristics, but the animals showed a longer survival time (13 days). Inoculation of 10^5 cells produced only a very few nodular solid i.p. tumors, with no ascitic components and with a much longer survival time (30 days).

Total Irradiation. Animals were irradiated with an X-ray-therapy apparatus (250/25; N.V. Philips Gloeilampenfabrieken, Eindhoven, Holland), under the following conditions: 200 kV; 8 ma; half-value layer, equivalent to 1.3-mm Cu focus-skin distance, 34 cm; whole-body irradiation dose, 700 rads.

Acellular Material. Direct extraction of viral particles from tumor tissues was performed with the use of BHKAT. For this study, animals included in transfer Generations 22 to 37 were used. Eight g of neoplastic tissues were obtained from the animals 8 days after inoculation. These tissues were homogenized with 20 ml ascitic BHKAT fluid, with mechanical (sand) or ultrasonic homogenization. Hanks' solution, pH 7.2, was added to complete 50 ml. This was followed by centrifugation of the supernatant at 3,000,

7,000, and 37,000 × *g* (Centrifuge RC2-B, Rotor 5534-SS-1; Ivan Sorvall, Inc., Norwalk, Conn.).

The supernatant was then filtered through Millipore filters HA, 0.45 μm; and GS, 0.22 μm (Millipore Corp., Bedford, Mass.) (15). Centrifugation and filtration procedures were controlled by light and electron microscopy of the sediments of the homogenates and of the filters. No recognizable fragmented or whole cells could be seen in the sediments, while the filters contained cell debris on the upper filtration surface.

Escherichia coli cultures were also used for filtration checkings, and the filtrates produced proved to be sterile.

After filtration, the acellular material was concentrated with polyethylene glycol (Carbowax 6000; Fluka AG, Buchs S.G., Switzerland) to one-tenth of the original volume (3). The animals received a single injection of the concentrated suspension.

The amount used for each animal was 0.3 ml in the pouch or 1 ml either i.p. or s.c.

Electron Microscopy. Electron microscope observations of cell pellets and tumor tissues were carried out as follows: (*a*) fixation with 4% glutaraldehyde in cacodilate buffer and postfixation in 1% osmium tetroxide in phosphate buffer; (*b*) embedding in Epon 812; (*c*) staining of fine sections with uranyl acetate and lead citrate (E. F. Fullam, Inc., Schenectady, N. Y.).

Direct observation of the acellular material obtained through 0.45 μm pore Millipore filters was carried out by placing the material on carbon nitrocellulose-coated copper grids and staining it with 2% uranyl acetate or 3% phosphotungstic acid (Fullam).

A Philips EM 300 electron microscope was used (N. V. Philips Gloeilampenfabrieken).

RESULTS

BHK Cells and BHK Fibrosarcoma. Inoculation (s.c.) of 10^7 BHK 21 clone 13 cells produced a fibrosarcoma which killed the animals in a 40-day span (BHK fibrosarcoma).

Successive transplantations performed on both normal and whole-body-irradiated animals resulted in the shortening of the animal's survival.

The ultrastructural investigation of BHK 21 and s.c. BHK fibrosarcoma cells revealed the presence of several virus-like particles resembling those described by Thomas *et al.* (18) and Shipman *et al.* (16) as H or R particles, respectively (Fig. 1, *A* and *B*).

The acellular suspensions of s.c. tumors obtained in both normal and irradiated animals failed to produce new tumors after their inoculation in normal animals.

When inoculation of BHK 21 cells in normal animals was i.p., solid tumors were produced.

BHK Ascitic Tumor. The tumor, consisting of multiple pinkish nodules 2 to 6 mm in size, was located on the parietal peritoneum, the omentum, and the mesenterium, Interstitial hemorrhagic lesions and atelectasia were frequent lung findings, from Day 5 after inoculation, and thereafter.

Liver and lung metastases were also observed.

Histological studies revealed that the BHKAT was an undifferentiated sarcoma. Ascites or hemoperitoneum and hemothorax containing 4 × 10^7 cells/ml were observed. Virus-like particles were seen in all transfer generations studied (Fig. 2*A*).

Acellular Material. The acellular material, obtained from BHKAT through filters with 0.45-μm pores, was injected into the pouch and s.c., producing solid tumors that killed the animals in 33 and 39 days, respectively.

When the same material was injected into the peritoneal cavity, ascitic tumors developed that killed the animals in 21 days (Table 1). As with the BHKAT, these tumors proved to be transplantable by i.p. inoculation of acellular material. The same virus-like particles were also seen in this induced tumor (Fig. 2*B*).

It was further noted that particle concentration was a necessary condition for the production of tumors. The acellular material injected showed spheric particles 110 to 120 nm in diameter. The inoculation of normal animals with acellular material driven through filters with 0.22-μm pores proved negative.

When animals were inoculated with acellular suspensions obtained from BHK s.c. and i.p. fibrosarcoma or irradiated hamster tissues, no tumors developed during the 150-day period of study. The issue was the same when animals received injections of the acellular suspension's solvent and of the acellular suspension derived from BHK 21 clone 13 cells. No particles were observed in acellular material obtained through 0.22-μm pore filters.

Production of a New Cell Line. The BHKAT cells were cultured *in vitro*. By this procedure, new cell line 793 was obtained. The cells were morphologically similar to BHK 21 clone 13 cells and grew in monolayers. Up to the present time, this line has been kept for 70 consecutive subcultures. When inoculated in the peritoneum of hamsters a neoplasm quite similar to the original BHK fibrosarcoma was noted, which killed the animal after 9 days.

The cytoplasm of both 793 line cells and the tumor cells obtained by their inoculation contained virus-like particles (Fig. 2*B*).

Virus-like particles were also isolated by the previously described filtration and concentration techniques (through filters with 0.45-μm pores). These structures were found also in the culture medium of the 793 cells. Tumors developed after this material was injected i.p. into adult hamsters. On the other hand, no tumors appeared after the same procedure was applied, with filters with 0.22-μm pores. The inoculation of acellular material from line 793 culture medium resulted in the appearance of i.p. tumors.

Table 1 shows the comparative mean survival time of animals given injections of the different suspensions.

DISCUSSION

Our experiments indicate that an oncogenic virus, which may be identified with R particles, preexists in BHK 21 clone 13 cells.

Successive transfers in hamster tissues would activate these viruses. This phenomenon was observed when cells

Table 1

Comparative mean survival time of hamsters inoculated with tumor cells and acellular material

Cell type used	Inoculation	No. of whole cells[a]	Animals inoculated with cells		Animals inoculated with acellular material[c]	
			No.	Survival time (days)[b]	No.	Survival time (days)[b]
BHKAT	i.p.	4×10^7	86	9.8 ± 1.6	14	21.2 ± 4.1
	Pouch				5	33.4 ± 5.2
	s.c.				3	38.6 ± 0.5
Line 793	i.p.	4×10^6	7	9.8 ± 2.8	8	24.1 ± 7.9

[a] Data on number of whole cells are corrected, taking into account that 25% of the inoculated ascitic cells proved to be nonviable by the Nigrosin test (14).

[b] Mean ± S.D.

[c] Approximately 2×10^9 tumor cells were used for producing the acellular material. With line 793, tumors were produced by the use of culture media without cells.

containing virus-like particles were injected in the peritoneal cavity of irradiated animals. Various forms of immunosuppression, including X-irradiation, have been proved capable of enhancing viral infections and virus-induced oncogenesis (4, 7).

In addition, after the initial transfers, activation appears to be self-perpetuating. Our studies revealed that viruses remained active even after 70 subcultures *in vitro*. This would suggest that after the initial transfers in irradiated animals, viral production, retaining its oncogenic activity, is maintained independently of the animal.

It was also shown that oncogenic material can be extracted, not only from cells, but also from ascitic fluid and from the culture medium.

The viral nature of the oncogenic material is supported by the fact that the active suspensions have kept their characteristics through the successive techniques of centrifugation, filtration, and concentration. Such techniques are normally known to eliminate cell components, except viruses. This point of view is further supported by the electron microscopic observation of virus-like particles in the acellular material.

Electron microscopy of the isolated particle purified in sucrose density gradient has shown a slightly irregular spheric structure with an electron-dense core and a peripheral zone with round projections. Using column chromatography with different agarose gels and sucrose density gradient centrifugation, it was determined that the molecular weight of the particle is higher than 4×10^7 daltons. Additional work with biochemical and radioactive tracer techniques (uridine-^{3}H) showed the RNA nature of the viral nucleic acid (C. E. Nollmann, J. H. Lombardo, and J. Mayo, unpublished data).

Taking into account the ultrastructural localization of the virus-like particles in ultrathin sections, the morphology and size of the related particle, and the nature of the nucleic acid (and although determination of virus behavior is under way), we consider that the virus of the BHKAT tumor could possibly be included in the group of coronaviruses (12).

An interesting fact was the absence of tumor production after inoculation of acellular suspensions obtained from BHK 21 clone 13 cells and solid s.c. tumors cells whereas, using the same experimental procedures, we did obtain tumors when the acellular material was obtained from BHKAT cells. This phenomenon could be due to variations in the viral agent's characteristics acquired during successive i.p. transfers. As has been mentioned, radio-induced immunosuppression of the animals in the course of BHKAT production could account for the suggested virus-activity modification.

These findings provide an interesting insight into the field of foot and mouth disease of cattle. Vaccines prepared from BHK cells assure effective control of the disease (8). However, our findings seem to indicate that, despite the beneficial effects, such vaccines may carry material which is oncogenic in hamsters.

REFERENCES

1. Aramburu, M. S., and Rivenson, S. Acción Tumorigena de las Células BHK 21 Clona 13 Utilizadas en la Producción de Virus Aftoso. Rev. Inv. Agrop. INTA, Sec. 4, Patologia, Anim. Vol. 5, No. 4, pp. 27-31, 1968.
2. Bernhard, W., and Tournier, P. Infection Virale Inapparente de Cellules de Hamsters Deceleé par la Microscopie Eléctronique. Ann. Inst. Pasteur, *107:* 447-452, 1964.
3. Fayet, M. T. Concentration du Virus de la Fiévre Aphteuse par le Polyéthylene Glycol. Ann. Inst. Pasteur, *118:* 356-366, 1970.
4. Glasgow, L. A. Immunosuppression, Interferon and Viral Infections. Federation Proc., *30:* 1846-1851, 1971.
5. Gotlieb-Stemasky, T., and Shilo, R. Studies on the Tumorigenic Properties of Baby Hamster Kidney Cell Lines and a Method of Selection of High and Low Tumorigenic Clones. Virology, *22:* 314-320, 1964.
6. Hale, J. H., Goffe, A. P., Tomkinson, B. E., Inghan, H. R., Waller, M. P., and Selkon, J. B. Studies on Tumours Developing in Hamsters following Inoculation of SV 40 Virus and BHK 21 Cells. Brit. J. Exptl. Pathol., *46:* 598-606, 1965.
7. Hisch, M. S., Black, P. H., and Proffitt, M. R. Immunosuppression and Oncogenic Virus Infections. Federation Proc., *30:* 1852-1857, 1971.
8. Kane, G. J., Pay, T. W. F., and Bracewell, C. D. Some Investigations and Control Procedures of Foot and Mouth Disease Vaccines Produced from Virus Cultivated on BHK Clone 13 Cells. Bull. Offic. Int. Epiz., *64:* 225-230, 1965.
9. Macpherson, I., and Stocker, M. Polyoma Transformation of Ham-

ster Cell Clones. An Investigation of Genetic Factors Affecting Cell Competence. Virology, *16:* 147-151, 1962.

10. Mayo, J., Carranza F. A., and Cabrini, R. L. Comparative Study of the Effect on Antibiotics, Bone Marrow and Cysteamine in Oral Lesions Produced in Hamsters by Total Body Irradiation. Experientia, *20:* 403, 1964.
11. Mayo, J., Moreira, J. L., Lombardo, J. H., Conti, C. J., and Rivenson, S. Aszitischer Tumor durch BHK-21 Zellen (Klon 13) bei Hamstern nach Röntgenganzkörperbestrahlung. Arch. Exptl. Veterinaermed., *25:* 853-862, 1971.
12. Melnick, J. L. Summary of Classification of Animal Viruses, 1969. Progr. Med. Virol., *11:* 451-453, 1969.
13. Mussgay, M., Reczko, E. and Ahl, R. J. Demonstration of Virus-like Particles in a Bovine Cell Line. J. Gen. Virol., *4:* 445-447, 1969.
14. Paul, J. *In:* Cell and Tissue Culture. London: E. & S. Livingstone, Ltd., p. 357, 1970.
15. Perry, P. V., and Vicent, M. M. *In:* K. Maramorsch and H. Koprowsky (eds.), Methods in Virology Vol. 2, p. 371. New York: Academic Press, Inc., 1967.
16. Shipman, C., Vander Weide, G. C., and Il Ma, B. Prevalence of Type R. Virus-like Particles in Clones of BHK 21 Cells. Virology, *38:* 707-710, 1971.
17. Stenback, W. A., Van Hoosier, G. L., and Trentin, J. J. Virus Particles in Hamster Tumors as Revealed by Electron Microscopy. Proc. Soc. Exptl. Biol. Med., *122:* 1219-1223, 1966.
18. Thomas, J. A., Delain, E., and Hollander, E. Morphogenese d'un Virus du Hamster Associé a la Souche BHK ou a des Tumeurs. Compt. Rend. Acad. Sci. Paris, *264* (D): 785-788, 1967.

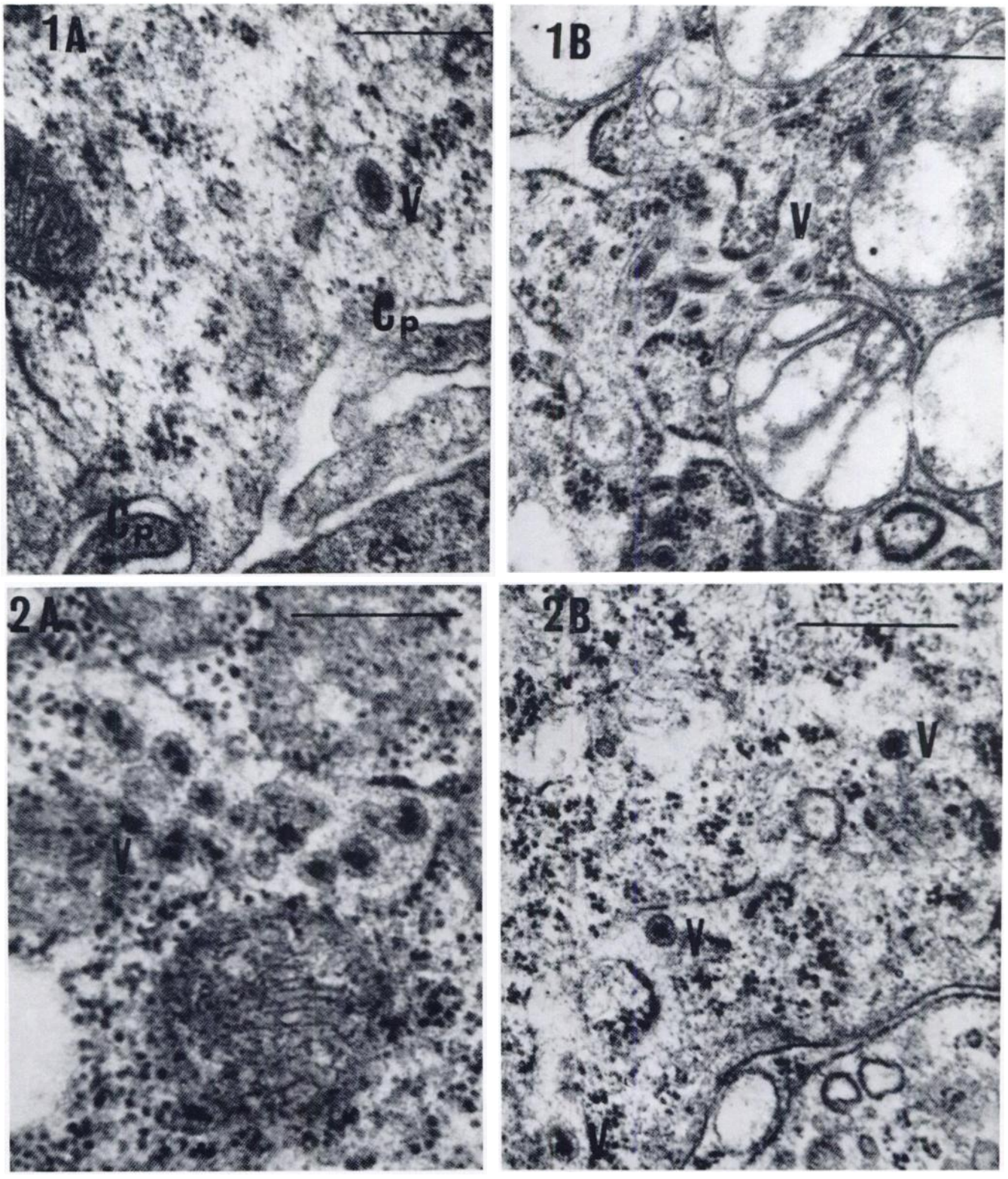

Fig. 1. *A*, BHK cells with their respective cytoplasmic processes (*Cp*) can be noted. In one of them, an R or H particle (*V*) with its characteristic nucleoid and peripheral radiations can be seen (scale represents 500 nm). × 50,000. *B*, cytoplasm of BHK fibrosarcoma. Note numerous virus-like particles (*V*) in dilated endoplasmic reticulum. Both round and filamentous structures can be seen (scale represents 1 μm). × 30,000.

Fig. 2. *A*, cytoplasm of BHK ascitic tumor cell showing virus-like particle (*V*) in a rough endoplasmic reticulum cisternae (scale represents 500 nm). × 60,000. *B*, type R or H particle (*V*) in cytoplasm of a tumor induced by inoculation of acellular material obtained from BHK ascitic tumor (scale represents 500 nm). × 56,000.