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Activation and Isolation of Hamster-Specific C-Type RNA Viruses from Tumors Induced by Cell Cultures Transformed by Chemical Carcinogens

(hamster-embryo fibroblasts/3-methylcholanthrene/cigarette-smoke condensate)

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ABSTRACT **Cell cultures of Syrian hamster embryo were treated for 7 days with selected chemicals. Certain cultures were morphologically transformed by three different chemical preparations and yielded cell lines that subsequently produced malignant tumors in hamsters. Although the cell lines were negative for infectious virus before inoculation into animals, hamster-specific C-type RNA virus was isolated from tumors or from cell lines derived from the tumors. Since infectious C-type viruses are usually not demonstrable in hamster tissues of normal or tumor origin, we conclude that the chemical treatment and activation of the viruses are related events.**

Infectious leukemia viruses (C-type, RNA) have been isolated many times from different strains of chickens, mice, and cats (1). In contrast, infectious viruses capable of serial propagation have been isolated from hamsters only on rare occasions, mainly from tumors induced by murine sarcoma viruses (2–5). These viruses, termed "hamster specific", have an altered host range and do not share envelope or group-specific (gs) antigens with the murine C-type viruses (6, 7). Nonsarcomagenic C-type viruses, isolated from stocks of the hamster sarcoma virus by endpoint dilution (5) or cloning techniques (8), share a gs antigen (7) with a noninfectious hamster C-type virus that was detected in a spontaneous lymphoma (9). The above data were interpreted as indicating *in vivo* rescue of the murine sarcoma virus genome by a hamster-specific leukemia virus (HaLV). Leukemia virus is used here in the generic sense to indicate a nonsarcomagenic C-type virus independent of other possible pathogenic effects. A survey was made to determine prevalence of HaLV by the use of antiserum against hamster virus gs prepared in guinea pigs (10). No convincing antigenic evidence of the virus was found in hamster-embryo fibroblasts, the organs of adult hamsters, or hamster tumors induced by SV40 or adenoviruses[¶]; however, as in the mouse (11), specific gs reactions were detected with embryonic extracts. It seemed clear that active HaLV infections rarely occur in hamsters, but certain questions remained open. Does HaLV exist in a latent or integrated state in hamsters, and if so, how prevalent is the viral genome? In this paper we present evidence that the latent or repressed HaLV genome may be widely disseminated in hamster colonies, but can be activated and isolated only under certain conditions.

Abbreviations: CF, complement fixation; HaLV, hamster leukemia virus; MCA, 3-methylcholanthrene; DMBA, 7,12-dimethyl-benz[a]anthracene; gs, group-specific (antigen).

[¶] Kelloff, G. J., R. J. Huebner, W. T. Lane, and R. V. Gilden, in preparation.

PROCEDURES AND RESULTS

Cell transformation and tumor induction

Cell cultures prepared from an early passage of hamster (LSH and NIH strains)-embryo cells were treated for 7 days with 0.1 μg/ml of the chemical carcinogen 3-methylcholanthrene, or with 0.1 or 1.0 μg/ml of certain fractions of cigarette-smoke condensate (12) dissolved in acetone and diluted in Eagle's Minimal Essential Medium with 10% fetal-calf serum. After the initial 7-day treatment, the chemicals were removed permanently and the cultures were subdivided as needed. Complete details of culture derivation and chemical treatment have been published (13). As summarized in Table 1, six of the chemically treated cell lines became transformed, based on changes in growth rates and morphological alterations after 5–10 subcultures (Figs. 1 and 2). The control cell lines remained normal; however, there was a marked variation in the growth characteristics of the cell pools used, with the F695 control culture becoming terminal after a few passages, and the F559 and F839 control cultures continuing to grow for at least 30 passages. Due to their lack of growth in culture, large numbers of F695 control cells were not available for inoculation into newborn hamsters. On the other hand, F839 and F559 control cells were available in large numbers and did not produce tumors when 2×10^6 cells were inoculated into newborn hamsters. Cell cultures transformed by methylcholanthrene or by cigarette-smoke fractions 6 and 9 produced malignant tumors at the site of inoculation after 43–140 days. New cell lines were established from representative tumors.

Demonstration of specific HaLV antigen

When 20% cell extracts of control and transformed cultures were tested in a complement fixation (CF) test against specific guinea-pig antiserum to HaLV antigen, none were clearly positive. One of the transformed cultures, however, did produce an occasional positive reaction when low dilutions were tested, but the CF titer of these cultures was not

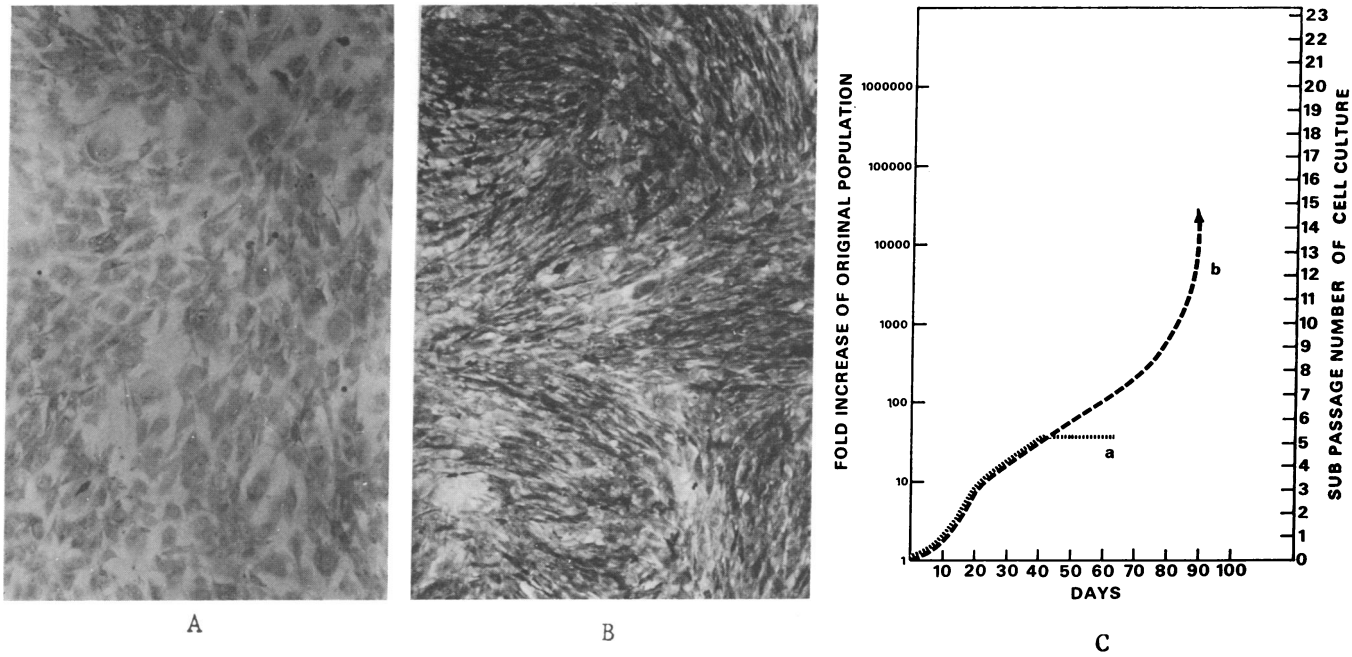

FIG. 1. (*A*) Normal terminal phase of NIH hamster-embryo cells, (F695) passage 5. ×129. (*B*) NIH hamster-embryo cells morphologically altered (spindle-shaped cells, loss of cell orientation, piling up of cultures by 0.1 μg of MCA/ml, passage 10. ×129. (*C*) Growth curves of *a* and *b*.

enhanced by further subdivision of the cell lines and tests for infectious virus were consistently negative.

Tumors induced by each of five transformed cell cultures were tested for gs antigen; of these, two tumors contained HaLV antigen at a titer of 1:4–1:8; the other 20 tumors were CF-negative. Extracts of the spleen, thymus, kidney, liver, small intestine, and colon of control and tumor-bearing hamsters were negative for HaLV antigen.

After 5–10 tissue-culture subpassages, cell cultures established from representative tumors induced by each agent were tested for the presence of HaLV antigen. As seen in Table 2, 6 of the 9 tumor lines contained significant quantitites of HaLV gs antigen, with titers up to 1:16.

To verify these reactions, cultures were labeled with tritiated uridine and the supernatant fluids were removed 24 hr later and analyzed for the presence of radioactivity at the

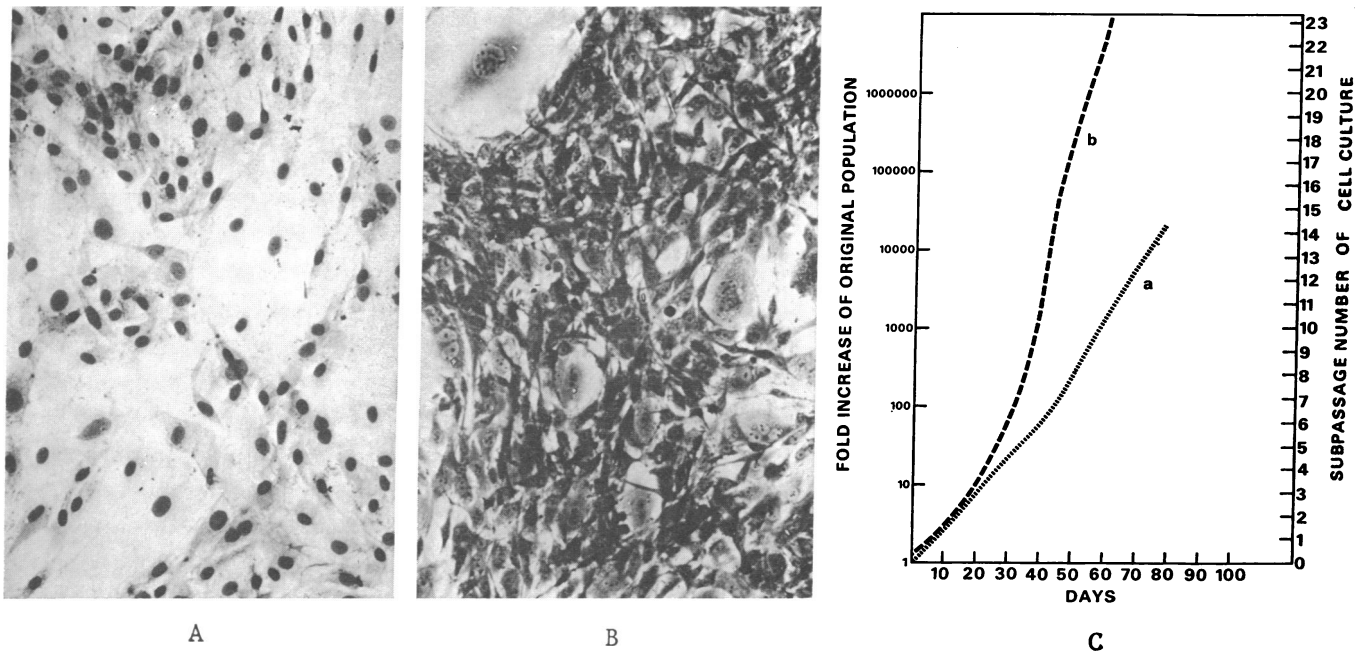

FIG. 2. (*A*) Normal LSH hamster-embryo cultures, (F839) passage 10. ×129. (*B*) Morphologically altered culture from LSH hamster embryo (spindle shaped and giant cells, loss of cell orientation, piling up of cultures) by 1.0 of μg/ml cigarette-smoke condensate fraction 9, passage 10. ×129. (*C*) Growth curves of *a* and *b*.

TABLE 1. *Transformation of hamster-embryo cell cultures by 3-methylcholanthrene and condensates selected from cigarette smoke*

Hamster strain	Treatment	Cells in culture: No. of cell-line subculture at time of treatment	Cells in culture: No. of cell lines transformed*/no. of attempts	Tumor induction: No. of cell-line subculture at time of inoculation into hamsters	Tumor induction: No. of tumors induced in 150 days: No. of tumors/no. of inoculated	Tumor induction: No. of tumors induced in 150 days: No. of days until appearance of tumors†
LSH (F839)	None	3	0/3	13	0/3	NA‡
				16	0/4	NA
	1.0 μg/ml of no. 6§	3	1/1	20	4/13	57–140
				23	3/8	56–84
	1.0 μg/ml of no. 9§	3	1/1	21	10/10	78–99
				23	8/8	50–90
NIH (F695)	0.1% Acetone	5	0/1	NT¶	NA	NA
	0.1 μg/ml of MCA	5	1/1	10	4/6	77–125
NIH (F559)	0.1% Acetone	3	0/6	11	0/6	NA
				13	0/7	NA
			3/6			
	0.1 μg/ml of MCA	3	⌊-Line 1:	12	6/8	43–68
			⌊-Line 2:	12	3/5	77–125

* Based on morphological changes and ability to produce transplantable tumors.
† Based on readings once a week.
‡ Not applicable.
§ Selected cigarette-smoke condensate fractions (12).
¶ Terminal cultures, cells unavailable for inoculation into animals.

buoyant density of the virus in sucrose gradients (about 1.16 g/cm^3). The CF-positive lines showed the presence of virus by this procedure, while CF-negative lines did not show evidence of RNA-containing particles (Table 2). Further studies were performed on viral isolates obtained from a single tumor induced by fraction 9 transformed cells from fraction 9 (Table 2). In gel-diffusion assays, reactions of identity were obtained with Tween 80–ether disrupted pellets of the current isolates and HaLV gs antigen. C-type viruses were also seen in these pellets by electron microscopy. Filtrates of the positive cell line were used in a CF–gs-antigen induction test; it was shown that these new isolates were infectious for hamster-

TABLE 2. *Detection of HaLV in cell lines derived from tumors*

Hamster strain	Source of tumor	CF: No. positive/no. tested	CF: Subculture no. when tested	CF: Titer* of positives	Uptake of tritiated uridine	Demonstration of particles by electron microscopy	CF antigen induction test: titer†
LSH cell line (F839)	Cells transformed by 1.0 μg/ml no. 6‡	1/2	3	8	yes	NT§	NT
	Cells transformed by 1.0 μg/ml no. 9‡	2/2					
		⌊-Line 1:	9	8	yes	yes	$10^{0.5}$
		⌊-Line 2:	9	16	yes	NT	NT
		1/3	3	4	yes	NT	NT
NIH cell line (F695)	Cells transformed by 0.1 μg/ml MCA	2/2					
		⌊-Line 1:	18	8	yes	yes	NT
		⌊-Line 2:	18	4	yes	NT	NT
NIH cell line (F2241)	Transplants of SV40 hamster tumor	1/26	8–22	2	no	no	10^0
LSH	Tumor induced *in vivo* by DMBA	1/3	3	2	NT	NT	NT
			11	2	NT	NT	NT
			26	4	yes	NT	NT

*Reciprocal of CF titer. 0 indicates a titer <1:2.
† Logs of infectivity.
‡ Selected cigarette-smoke condensate fractions (12).
§ Not tested.

embryo cultures, however, the infectivity titers were low, never exceeding $10^{1.0}$. All tumor-cell lines were negative in gel diffusion (14) and CF tests with antisera specific for murine leukemia viruses (15).

In order to test whether infectious HaLV could be detected by a similar protocol with hamster cells transformed by other means, we inoculated several litters of NIH hamsters with a single cell-line derived from an SV40-induced hamster tumor. 20% tumor extracts and extracts of cell lines derived from 25 of 26 different tumors were found to be negative for HaLV as determined by the CF test. One tumor cell line frequently gave a low CF antigen titer (1:2 or less); however, further subculture did not enhance that titer and the ancillary procedures of viral identification previously described failed to produce evidence of infectious virus (Table 2).

We also induced tumors in the LSH, NIH, and Graffi strains of hamsters with 3-methylcholanthrene (MCA) or dimethylbenzanthracene (DMBA). Complement fixation tests of 20% extracts from eight different tumors yielded no evidence of HaLV. However, one of the cell lines derived from a DMBA-induced tumor from hamster (strain LSH) was found to be positive in the CF test, with a titer of 1:2. In this case, the CF titer did increase with subculturing of the cell line and additional evidence that this culture contained infectious HaLV was obtained by the tritiated uridine method (Table 2).

HaLV antigen has also been detected in 4 of 17 cell lines derived from additional tumors. These include cell lines derived from one MCA and two DMBA tumors induced in Graffi hamsters, and one cell line derived from a DMBA tumor induced in an NIH hamster.

DISCUSSION

The serological and supportive data show that a significant percentage of tumors produced by implantation of cells transformed by chemical carcinogens contain infectious C-type particles. It is clear that this virus is of hamster origin and not merely a host-adapted murine leukemia virus because (*a*) the viruses contained hamster specific gs antigen, and (*b*) no mouse sarcoma viruses were used as activation agents. Since virus or viral antigens were undetectable in the cell lines before implantation, it appears relatively certain that overt synthesis of virus was provoked *in vivo* and that the positive results were not due to contamination with HaLV from previous experiments in this laboratory (13). In addition, these particular experiments were performed in an isolated area that had never been used for hamster studies.

One important question remaining was whether the synthesis of HaLV proceeds from information contained within the transformed cells or results from infection of the tumor cells *in vivo* with indigenous HaLV. Since the incidence of HaLV was not found in 26 SV40 tumors, but was found in 6 of 9 tumors induced by chemically-transformed cells and in 1 of 8 tumors induced by chemicals *in vivo*, it follows that our isolation of virus from the tumor cells was not dependent upon a "vacuum cleaner effect," but was related to the nature of the treatment itself. However, several alternative explanations still remain: The chemically-transformed cultures may contain only a few HaLV-positive cells that are selected for during the development of the tumor; the chemically transformed cells may carry HaLV in an incomplete form that is completed by additional viral information available *in vivo;* the chemically transformed cells may be specifically susceptible to growth of infectious HaLV that is usually latent in the host animal. In any case, expression of virus was linked to the chemical treatment of the cells. We favor the interpretation that HaLV exists in a repressed state in most, if not all, normal hamster cells. Derepression of viral antigens of infectious virus occurs under certain normal conditions such as aging (18), and can be accelerated by certain environmental factors such as irradiation or treatment with chemical carcinogens; but, even under these enhancing conditions, activation of infectious virus particles appears to be a rare event in many strains and species of animals.

The presence of infectious virus in the tumor lines was demonstrated by inoculation of fresh embryo fibroblasts; however, infectivity titers were exceedingly low. If we consider the relatively large number of physical particles present in these lines (based on estimates from radioactive labeling), the intrinsic infectivity of these isolates for hamster cells is evidently poor. This behavior and the previous lack of a specific serologic test for hamster C-type viruses could readily account for the lack of isolations by more routine methods. C-type particles have been seen in a number of tumor-cell lines from hamster derived previously by treatment with DNA tumor viruses (16); however, in our experiments the tumor lines induced by DNA viruses and passaged *in vitro* for long periods of time were negative for HaLV gs antigen¶. These data suggest that most hamster cells possess strong inhibitors for expression of infectious C-type virus or viral antigens. The ability to recover HaLV from hamsters derived from the London School of Hygiene (LSH) colony and the National Institutes of Health (NIH) colony shows that the HaLV genome is probably widespread in laboratory strains of Syrian hamster, while the unusual procedures required to isolate the virus suggset that it usually exists in a repressed state.

Since HaLV is representative of the RNA tumor virus family, the relevance of the presence of the RNA virus genome to the transformed state must be considered. We believe that derepression of an "oncogene" (1, 17) is not dependent upon the synthesis of infectious viruses. Transformed cell lines that were HaLV-negative before implantation and the HaLV-negative tumor cell lines are cases in point that support this view, although it is true that negative findings may only indicate a lack of sensitivity of the test procedures or that appropriate test procedures were not used. Activation of viruses could occur as the end result of an extended state of derepression with the aid of factors provided *in vivo*.

There are several considerations that preclude direct application of these findings to the mechanisms of cancer induction in man. First of all, we are using hamster cells that may react to chemicals differently or with a different order of magnitude than human cells. Secondly, our work is based in large part upon transformation *in vitro*, which has not yet been shown to correlate with tumor induction *in vivo*. What we have shown, however, is that chemical treatment, cell transformation, tumor induction, and viral activation are closely related events in our system and that studies with carcinogens must take into consideration the widespread occurrence of oncogenic virus genomes.

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1. Huebner, R. J., G. J. Todaro, P. Sarma, J. W. Hartley, A. E. Freeman, R. L. Peters, C. E. Whitmire, H. Meier, and R. V. Gilden, *Proc. 2nd Int. Symp. on Tumor Viruses, Paris* (1969).
2. Klement, V., J. W. Hartley, W. P Rowe, and R. J. Huebner, *J. Nat. Cancer Inst.*, **43**, 925 (1969).
3. Kelloff, G., R. J. Huebner, Y. K. Lee, R. Toni, and R. Gilden, *Proc. Nat. Acad. Sci., USA*, **65**, 310 (1970).
4. Sarma, P., T. Log, and R. V. Gilden, *Proc. Soc. Exp. Biol. Med.*, **133**, 718 (1970).
5. Bassin, R. H., P. J. Simons, F. C. Chesterman, and J. J. Harvey, *Int. J. Cancer*, **3**, 265 (1968).
6. Kelloff, G., R. J. Huebner, N. H. Chang, Y. K. Lee, and R. V. Gilden, *J. Gen. Virol.*, **9**, 19 (1970).
7. Kelloff, G., R. J. Huebner, S. Oroszlan, R. Toni, and R. V. Gilden, *J. Gen. Virol.*, **9**, 27 (1970).
8. Kelloff, G. J., R. J. Huebner, and R. V. Gilden, *J. Gen Virol.*, in press.
9. Stenback, W. A., G. L. Van Hoosier, and J. J. Trentin, *J. Virol.*, **2**, 1115 (1968).
10. Oroszlan, S., C. Foreman, G. Kelloff, and R. V. Gilden, *Virology*, in press.
11. Huebner, R. J., G. J. Kelloff, P. T. Sarma, W. T. Lane, H. C. Turner, H. Meier, D. D. Myers, R. V. Gilden, and S. Oroszlan, *Proc. Nat. Acad. Sci. USA*, **66**, 366 (1970).
12. Swain, A. P., J. E. Cooper, and R. L. Stedman, *Cancer Res.*, **29**, 579 (1969).
13. Freeman, A. E., P. J. Price, R. J. Bryan, R. J. Gordon, R. V. Gilden, G. J. Kelloff, and R. J. Huebner, *Proc. Nat. Acad. Sci. USA*, **68**, 445 (1971).
14. Geering, G., L. J. Old, and E. A. Boyse, *J. Exp. Med.*, **124**, 753 (1966).
15. Hartley, J. W., W. P. Rowe, W. I. Capps, and R. J. Huebner, *J. Virol.*, **3**, 126 (1969).
16. Stenback, W. A., G. L. Van Hoosier, and J. J. Trentin, *Proc. Soc. Exp. Biol. Med.*, **122**, 1219 (1966).
17. Huebner, R. J., and G. J. Todaro, *Proc. Nat. Acad. Sci. USA*, **64**, 1087 (1969).
18. Aaronson, S. A., J. W. Hartley, and G. J. Todaro, *Proc. Nat. Acad. Sci. USA*, **64**, 87 (1969).