PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 148, 891–896 (1975)

Malignant Transformation of Hamster Cells following Infection with a Bovine Herpesvirus (Infectious Bovine Rhinotracheitis Virus) (38655)

F. MICHALSKI AND G. D. HSIUNG

Department of Laboratory Medicine, Yale University School of Medicine, New Haven, Connecticut 06510, and Virology Laboratory, Veterans Administration Hospital, West Haven, Connecticut 06516

Several herpesviruses have been reported to be capable of transforming primary hamster embryo cells *in vitro*. These include human herpes simplex virus types 1 and 2, human cytomegalovirus, and a guinea pig herpes-like virus (1–4). The presence of infectious bovine rhinotracheitis (IBR) virus, a common bovine herpesvirus, as a contaminant in bovine sera has been reported (5, 6). Since bovine serum is commonly used in tissue culture media, it was considered relevant to determine whether or not bovine herpesvirus is capable of transforming cells *in vitro*. This report demonstrates that malignant transformation of hamster embryo cells did occur following infection with IBR virus.

Materials and Methods. Cell Culture. Bovine embryonic kidney cell suspensions (BEK) were obtained commercially and resuspended in a modified growth medium, HK, with 10% fetal bovine serum (7). After confluent cell sheets were obtained, the medium was changed to Eagle's medium containing 2% fetal bovine serum. These cultures were used for the preparation of virus stocks and for virus assay.

Hamster embryo fibroblast (HEF) cells were prepared from 11- to 14-day old embryos as previously described (7). Briefly, the embryos were minced and treated with 0.25% trypsin to disperse the cells. After centrifugation, cell pellets were resuspended in Eagle's basal medium with Hanks' salts containing 10% fetal bovine serum, two times concentrated amino acids, vitamins and glutamine, 10% tryptose phosphate broth, one time concentrated nonessential amino acids and 0.11% $NaHCO_3$. For maintenance, additional $NaHCO_3$ was added to a concentration of 0.33%. The HEF cell suspensions, 1:1000, were seeded in 8 oz prescription bottles for monolayer cultures.

Virus strain. Four strains of IBR virus, two oral and two genital isolates, were used (Table I). The reference IBR virus, Cooper strain, seventh passage (8), was obtained from Dr. T. L. Chow of the University of Colorado and passaged twice in BEK in our laboratory. URI-NH6, a Colorado strain (9), was received from Dr. P. W. Chang of the University of Rhode Island, and passaged six times in BEK. Two genital IBR virus isolates, K22 and CE (10), were obtained in the 21st and 27th passage respectively from Dr. J. A. Baker of Cornell University and passaged twice in BEK.

All virus stocks were prepared in BEK cell cultures. Ultraviolet-inactivated virus suspension was prepared by exposure to ultraviolet light (uv) at a distance of 20 cm. An uv lamp with a Sylvania G30T8 bulb was used to inactivate the virus in 2.0 ml aliquots which were placed in 60 mm plastic petri dishes. Varying exposure times, ranging from 1 to 16 min, were used for different experiments.

Virus assay were made by the tube dilution method using BEK cell cultures. Final reading of virus-induced cytopathic effect (CPE) was made after 14 days at 34°, and the virus infectivity titer was calculated by the method of Reed and Muench (11). Virus titers in uv-inactivated samples were determined before irradiation.

Transformation experiment. Both infectious and uv-inactivated preparations of both the oral and genital virus strains were used. Aliquots of IBR virus suspension were inoculated into HEF cells either in monolayer or in suspension at input multiplicities of one to ten. After virus adsorption, the infected cells were resuspended in fresh medium at 1:4 dilution and subcultured either in 8 oz prescription bottles or in 75 cm^2 Falcon plastic flasks.

Detection of IBR virus antigen or antibody. Both direct and indirect immunofluorescent techniques were used. In the direct immuno-

TABLE I. TRANSFORMATION OF HAMSTER EMBRYO CELLS INFECTED *in Vitro* WITH IBR VIRUS.

Virus strain	Original source of isolation	uv inactivation (minutes)	Total no. expt. performed	No. expt. show cell transformation	Designation of cell line
K22-NH_2	Genital	0	3	1	YH-1
		1–8	8	0	
		10–12	2	0	
CE-NH_2	Genital	0	2	0	
		1–8	8	0	
		10–16	7	2	YH-2, 3
		Total	30	3	
Cooper-NH_2	Oral	4–8	4	0	
		10–12	2	0	
URI-NH_6	Oral	0	3	1	YH-4
		8	2	0	
		12–16	2	0	
		Total	13	1	

fluorescent method, IBR-specific antiserum produced in goats was labeled with fluorescein isothiocyanate. The latter reagent was obtained from Colorado Serum Co., Denver, CO. In the indirect immunofluorescent method, IBR-specific antiserum produced in rabbits and serum of tumor bearing hamsters were used. Fluorescein-conjugated anti-hamster and anti-rabbit globulins were obtained from Antibodies Inc., Davis, CA. Both fixed cells and living cells were used. The latter was done following the procedure by Albrecht and Rapp for the detection of membrane fluorescence (3).

Sera obtained from hamsters bearing tumors were also tested for neutralizing antibody to IBR virus. This was done by mixing equal volumes of serial twofold dilutions of hamster serum and virus suspension, containing approximately 100 $TCID_{50}$/0.1 ml. The mixtures were incubated at room temperature for one hour. Then 0.2 ml of the virus-serum mixture was inoculated into BEK tube cultures. After virus adsorption for 1 hr at room temperature, one ml of Eagle's medium with 2% fetal bovine serum was added. The culture tubes were read at frequent intervals for virus induced CPE.

Hamster inoculation. The random-bred golden Syrian hamsters used in most of the experiments were purchased from Dennen, Gloucester, MA, and the LSH inbred golden Syrian hamsters were from Lakeview Hamster Colony, Newfield, NJ and the white NIH inbred were purchased from Trenton Experimental Laboratories Animal Co., Bar Harbor, Maine. Both random-bred and inbred hamsters of all ages were inoculated subcutaneously with 10^5–10^7transformed cells or 10^3–10^7 tumor cells and observed for tumor induction.

Results. In vitro transformation of hamster cells following inoculation. Infection of primary hamster embryo cells (HEF) at multiplicities of one to ten with bovine herpesvirus was found to destroy most of the cells, although in some cases the few surviving cells would eventually regrow to confluency. To prevent cell destruction, uv-inactivated virus suspensions were used. IBR virus irradiated for less than eight minutes usually destroyed cells with typical herpesvirus cytopathic effects. The same virus suspension showed no effect on cells when irradiated for 10–16 min. In all experiments noninfected cultures of the same lot of embryo cells were observed in parallel. There was no evidence of transformation noted in the noninfected cell cultures.

Among the 13 experiments using the oral strains of IBR virus and 30 experiments using genital strains, foci of transformed cells were obtained in four separate experiments, three in cells infected with genital isolates of IBR and once in cells infected with the oral strain (Table I). One cell line, YH-1, was derived from a culture flask which had been infected with the nonirradiated K22 strain of IBR at an input multiplicity of approximately 10.

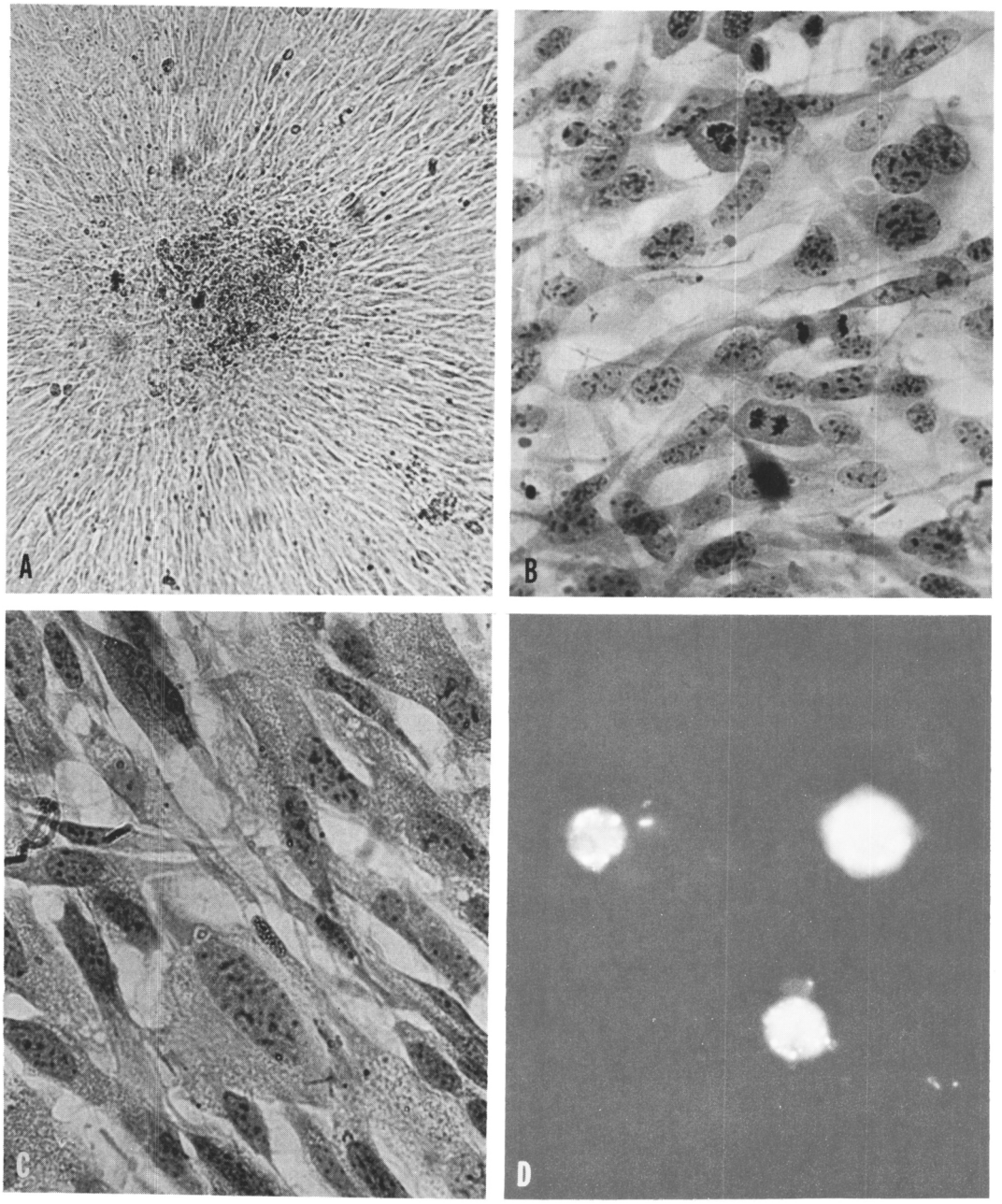

FIG. 1. A. A transformed focus of hamster embryo cells, first passage, 3 days after subcultivation, unstained preparation, × 100. B. Transformed hamster cells, YH-1, eight passages, showing mitosis, multiple nucleoli, and clumped basophilic chromatin; hematoxylin and eosin preparation, × 430. C. Primary tumor cells 7 days *in vitro*; hematoxylin and eosin preparation, × 430. D. Unfixed primary tumor cells 3 days *in vitro*; stained with fluorescein-conjugated goat anti-IBR serum showing granular membrane fluorescence, × 430.

Initially this culture was almost completely destroyed by the virus but had regrown. Heavily confluent cell monolayers were obtained 97 days after initial infection without passage. Multilayer foci (Fig. 1A) were observed after subsequent subculture. Further subcultivation led to the establishment of this cell line which is now in the 68th passage.

TABLE II. TUMOR INDUCTION IN HAMSTERS BY TRANSFORMED HAMSTER CELLS.

Cell line designation	Cell line passage no.	Strain of hamsters inoculated	No. hamsters with tumors / No. inoculated[a]			First evidence of tumor (weeks postinoculation)		
			No. Newborn	Weanling	Adult	No. Newborn	Weanling	Adult
YH-1	4–12	Random-bred Syrian	38/38	26/29	1/1	7–10	6–12	10
		Inbred NIH white	ND	6/6	0/1		8–10	
	12–68	Random-bred Syrian	35/35	30/30	2/2	5–8	5–9	2–4
		Inbred NIH white	ND	10/10	ND		3–7	
		LSH inbred Syrian	ND	ND	15/17			5–6
YH-2	3–19	Random-bred Syrian	0/15	0/4	ND			
	20–25	Random-bred Syrian	ND	5/5	11/11		3	2–5
		LSH inbred Syrian	36/36	ND	10/10	3–8		2–3

[a] The amount of cells each hamster received varied from 10^5 to 10^7 cells; the number of newborn that survived 3 wk until weanling was considered the total number inoculated. ND = not done.

Morphologically, the transformed cells consisted primarily of fibroblast-like cells (Fig. 1B). There were abundant mitotic figures, many cells with multiple nucleoli and clumped basophilic chromatin. Attempts to rescue IBR virus from the transformed cells by cocultivation with BEK cells have failed.

Similar foci of morphologically transformed cells were noted in two experiments using uv-irradiated CE genital strain IBR virus (Table I). One of the cell lines, YH-2, derived following infection with the CE strain inactivated by UV irradiation for 12 minutes, showed morphological changes with a high saturation density at about the 19th passage. The other cell line, YH-3, derived following infection with CE strain inactivated by irradiation for 10 min, has been passaged 31 times.

Only once following infection at an input multiplicity of approximately two with the oral strain, URI-NH6, did morphological change in the cells occur. This line, YH-4, is now in the 12th passage.

Tumor induction in hamsters by the transformed cells. Results obtained from hamsters inoculated with the two transformed cell lines, YH-1 and YH-2, are shown in Table II. Tests of YH-3 and YH-4 cell lines are still in progress. Between the fourth and 12th passage of the YH-1 cells, small solid tumors near the site of inoculation were noted in most of the animals. At higher passages of the cell line, 12–68, almost 100% of the surviving inoculated animals developed tumors following latent periods of 2–9 wk. Tumors were also obtained in two inbred strains, white NIH and LSH golden Syrian hamsters. A total of 163 of the 169 inoculated hamsters that survived showed tumors. Thus, the transformed cells were shown to be highly oncogenic. No tumors were found in hamsters inoculated with the uninfected control HEF cells which were originated from the same lot of cultures used for the YH-1 transformation experiments.

During the first 19 passages YH-2 cells showed no capacity to induce tumors when inoculated into newborn or weanling hamsters (Table II). After the cells showed morphological changes all of the inoculated animals developed tumors with a shorter incubation period than that for YH-1.

Preliminary examination of the histopathology of the YH-1 cell induced tumors suggested that they were undifferentiated

sarcomas. Metastases to the lungs were found in 15% of 73 tumors examined. Cells derived from the tumors propagated rapidly *in vitro* and showed a morphological resemblance to the transformed cells (Fig. 1C). Electron microscopic examination of the transformed cells, and tumor cells, both with and without 5-bromo-2′-deoxyuridine induction have not revealed any C-type oncornavirus or other virus particles. Tests for reverse transcriptase in the YH-1 cell line have also been negative. Attempts to recover infectious virus from the tumor cells by cocultivation with BEK cells have been unsuccessful. When 10^3–10^7 tumor cells were inoculated subcutaneously into weanling golden Syrian or white hamsters, all surviving animals developed tumors within 2 wk.

Antigenic properties of the transformed hamster cells and tumor cells. Using labeled IBR antiserum, both transformed and tumor cells fixed in acetone showed perinuclear and cytoplasmic fluorescence in about 5% of the cells. Primary tumor cells without acetone fixation showed brilliant granular membrane fluorescence in 10% of the cells using the same labeled IBR antiserum (Fig. 1D). Only about 1% of the fixed transformed cells showed immunofluorescence when the rabbit anti-IBR sera was used. HSV-1 transformed hamster cells did not react with the IBR antisera. Although sera obtained from hamsters with tumors induced by YH-1 transformed cells did not neutralize IBR virus at a dilution of 1:2, a granular membrane fluorescence was observed in 5% of the unfixed, IBR infected BEK cells, and 40% of the unfixed tumor cells. Sera obtained from hamsters bearing SV40 and SV20 tumors showed no fluorescence when added to YH-1 or YH-2 transformed cells.

Discussion. The Syrian hamster embryo cells which were transformed following IBR IBR virus infection were highly oncogenic when injected into hamsters of all ages, both inbred white or golden Syrian and random-bred golden Syrian hamsters. No infectious IBR virus has yet been found in either the transformed cells or in the tumor cells following cocultivation with the sensitive BEK cells. Similarly, infectious virus has not been recovered from herpes simplex virus types 1 and 2 and cytomegalovirus transformed hamster cells (1–3).

Membrane fluorescence was observed in 10% of the YH-1 induced tumor cells when IBR antiserum was used and sera obtained from YH-1 tumor-bearing hamsters showed membrane fluorescence on BEK cells infected with IBR virus, the antigenic relationship of the IBR virus and the transformed and tumor cells was evident. Using acetone fixed cells only 1–5% of the transformed or tumor cells showed evidence of IBR antigen either by direct or indirect fluorescent antibdy. The membrane fluorescence of live cells appeared to be a more sensitive test than the acetone fixed cells method.

No evidence for the presence of an oncornavirus in the IBR transformed cells was found by either electron microscopy or reverse transcriptase assay. Since spontaneous transformation of hamster embryo cells has been reported (12–14), experiments designed in the present studies have been carried out with careful controls including serially passaged noninfected cells and inoculation into hamsters in parallel with the transformed cells. Apparently the capacity of herpesvirus to transform cells *in vitro* is not a rare event and bovine herpesvirus is now added to the group.

Summary. Hamster embryo cells, following infection with IBR virus, showed malignant transformation. Hamsters of all ages, inbred or random bred, inoculated with two of the transformed cell lines developed solid tumors. Preliminary characterization of the tumors induced by one of the cell lines has indicated undifferentiated sarcomas. Viral specific antigen was detected in about 5% of the transformed cells and 10% of primary tumor cells in culture. Viral specific antibody was detected in the serum of tumor-bearing hamsters by the indirect immunofluorescent method, but no neutralizing antibodies were found. Infectious virus has not been recovered from either the transformed or tumor cells by cocultivation with bovine embryonic kidney cells.

This investigation was supported by NIH Contract No. DBS-72-2105 from the Food and Drug Administration and USPHS Grant No. AI 08648-05

from the Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md.

The authors are grateful to Dr. T. W. Reid, Yale University School of Medicine for the reverse transcriptase assay. The excellent technical assistance of Edileà Ellinger and Andrea Tomanik is appreciated.

The HSV-1 transformed hamster cells were obtained from Dr. William Summers of Yale University who had originally obtained them as line 14-012-8-1 from Dr. Fred Rapp of Pennsylvania State University.

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Received November 4, 1974. P.S.E.B.M. 1975, vol. 148.