Spontaneous Transformation of Rat Cells After Long-term *in Vitro* Cultivation and the "switch-on" of a New Complement-fixing Antigen[1] (36470)

JOHNG S. RHIM, M. L. VERNON, ROBERT J. HUEBNER, H. C. TURNER, W. T. LANE, AND RAY V. GILDEN

Microbiological Associates, Inc., and National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014, and Flow Laboratories, Inc., Rockville, Maryland 20852

During recent studies on long-term *in vitro* cultivations of rat embryo (RE) cells (1, 2) we found that RE cell lines exhibited morphological alteration spontaneously, and also were demonstrated to be tumorigenic when inoculated *in vivo*. The transformed cells began to produce a new complement-fixing (CF) antigen when tested against rat antiserum which has broad reactivity with various intraspecies and interspecies antigens of the RNA tumor viruses (3–8). The antigen was found to be not only immunologically distinct from the murine intraspecies and interspecies group-specific (gs) antigens but also to have properties which distinguish it from these antigens. It was similar to those found in RE cells transformed in early subcultures by polyoma virus (9).

Materials and Methods. Rat embryo cell lines. The RE cell lines (S-1193h and F-111) derived from inbred Fischer RE used in this study have been described in detail elsewhere (1, 10). Cell lines were tested for infectious virus by the CF test for murine leukemia virus, the COMUL test (3, 4).

Complement fixation. Complement-fixation tests were carried out using the microtiter technique described for tumor and viral antigen studies (11). Titers were recorded as reciprocals of the highest dilution giving 3+–4+ fixation of 1.8 units of complement. Cell pack preparations of CF antigens from RE cells were made as previously described (3, 4).

Antisera. Rat antisera used in the CF test were produced in Fischer rats carrying transplanted sarcoma induced by Moloney sarcoma virus (M-MSV). An antiserum prepared in guinea pigs with purified gs antigen of the viruses of murine leukemia sarcoma group (5–8) was also used.

Results and Discussion. In the S-1193h line, morphological alteration of the cells and an abnormal pattern of growth were noted in the 45th subculture. The morphological changes observed in these cultures were similar to those observed in previous studies where RE cells were transformed by virus alone (12), or by virus plus chemical carcinogens (2). Foci of transformed cells consisted largely of criss-crossing, randomly oriented, spindle-shaped cells with nuclear and cytoplasmic overlapping, heavily stained with Giemsa (Fig. 1B). Increased growth rate was observed in the S-1193h transformed cells and they had to be subcultured much more frequently. The CF antigen reactive with MSV rat antiserum was first detected by the 51st subculture, and was positive in CF at a titer of 1:8 (Fig. 2). Similar morphological alteration and the appearance of MSV rat antiserum reactive CF antigen were also observed in the F-111 line after a longer period (Fig. 1D and Fig. 2): (a) Morphological transformation was noted in the 65th subculture; (b) CF antigen reactive with MSV rat serum was first demonstrated in a cell pack preparation at the 69th subculture. Attempts to isolate virus from CF positive materials in the COMUL test have failed so far, nor

[1] This investigation was supported by Contract PH 43-70-2068A from the National Cancer Institute, National Institutes of Health, Bethesda, MD. We thank Dr. Louise Rabstein for assistance in the interpretation of histological sections of rat tumors.

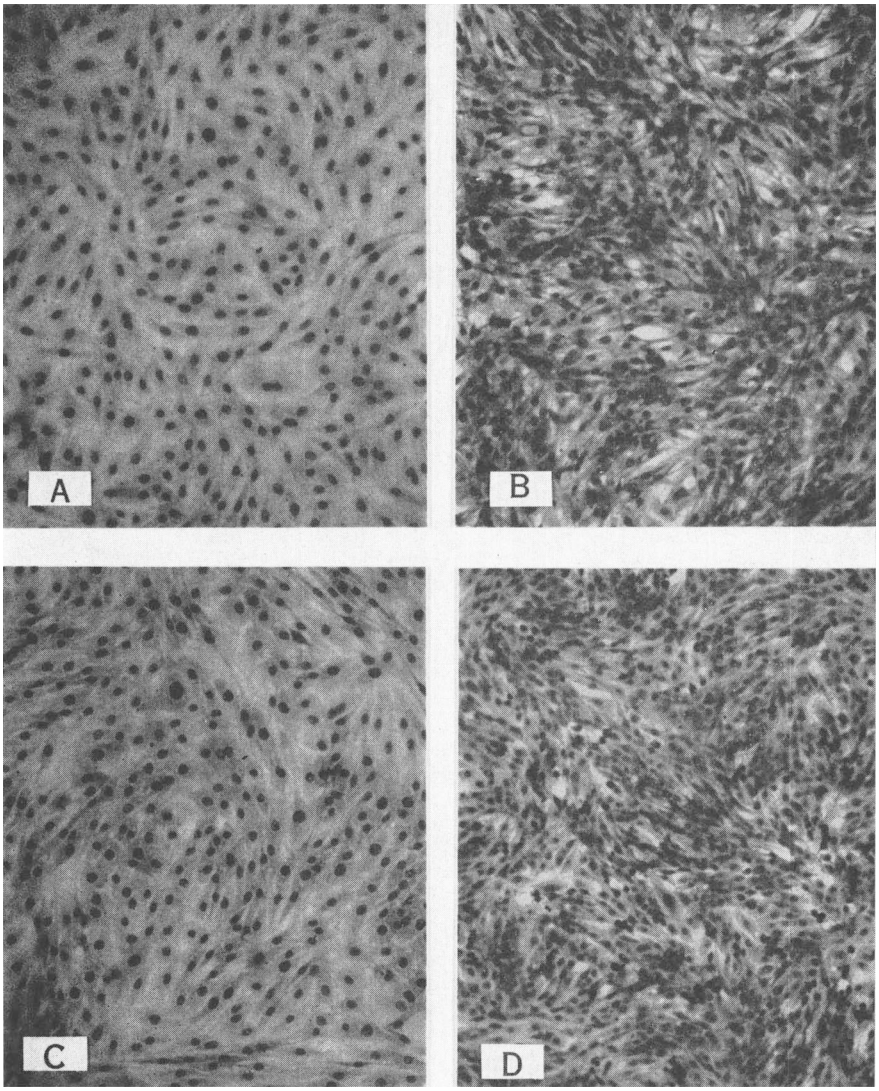

FIG. 1. Normal rat embryo cultures; Giemsa stain (×72). (A) Normal rat embryo culture (S-1193h) at the 27th passage. (B) Transformed rat embryo culture (S-1193h) at the 51st passage. Note criss-crossing spindle-shaped cells and nuclear and cytoplasmic overlapping. (C) Normal rat embryo culture (F-111) at the 44th passage. (D) Transformed rat embryo culture (F-111) at the 70th passage.

have viruses or virus-like particles been seen in these cell lines by electron microscopic examination.

Progressive, transplantable tumors were readily produced in newborn rats by inoculation of the transformed cells (Table I). Both the cultured tumor cells and rat tumors contained antigens reactive with MSV rat antisera but not with gs-1 specific guinea pig antisera. In addition, rat tumors reacted with the antiserum derived from rats bearing tumors induced by polyoma transformed RE (F-111) cells. The polyoma virus transformed RE (F-111) cells had also been found to contain a new CF antigen which was detectable with MSV rat antiserum (9). The sera of tumor-bearing rats developed antibodies in high titer when tested against trans-

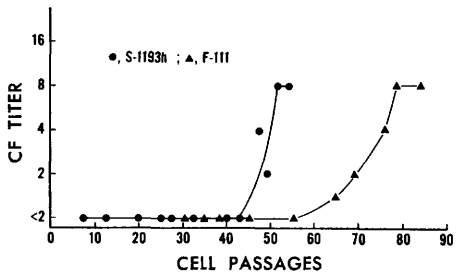

FIG. 2. Mouse leukemia–sarcoma virus CF antigen in rat embryo cell lines as a function of the number of passages in culture. MSV rat serum (#20).

formed rat embryo (TRE) (F-111) induced antigen: although the antibodies were not present in early tumor subtransplants at titers high enough to detect they did appear in later tumor transplants (Table I).

The present study describes the first demonstration of *in vitro* spontaneous transformation of rat cells. The following criteria established the *in vitro* transformation had occurred in the RE cell lines described: (a) The cells were morphologically altered and grew as randomly oriented multilayers. (b) The cells showed increased growth rate and lost contact inhibition. (c) Inoculation of the altered cells into unconditioned rats produced malignant, progressive, transplantable tumors.

In addition, it is quite interesting in the present study to find the spontaneous "switched on" of a new CF antigen in rat cells after long-term *in vitro* cultivation. We have shown that spontaneous transformation of rat cells by long-term *in vitro* cultivation was accompanied by the development of new antigens with properties different from gs antigens detectable in CF test with MSV rat antisera but not with the gs-1 specific guinea pig antiserum. At the same time, the transformed rat cells when transplanted into rats, produced tumors which also contained the new antigen. This antigen was also "switched on" much earlier when the same cell line was transformed by polyoma virus (9). This new rat cell antigen and the spontaneously and induced transformed cell lines are currently under study. Such spontaneous derepression in the cultured rat cells was not unexpected since various strains of mouse cell lines have been shown to transform spontaneously after prolonged subcultures, yielding in many cases gs antigen and infectious RNA tumor viruses (13, 14).

Some characteristics of the new rat cell antigen so far obtained as follows: (a) The antigen was nonsedimentable at 58,000*g* for 1 hr and ether resistant. (b) The antigen reacted with MSV rat serum and also with the sera from rats bearing tumors induced in early passaged RE (F-111) cells transformed by polyoma virus (Table I). (c) There was always the reciprocal exclusion of TRE (F-111) and Ki-MSV antibody: every sera positive for TRE (F-111) antigen was negative for Ki-MSV antigen and vice versa. (d) In studies using MSV rat antisera to characterize the TRE (F-111) antigen and which is induced rapidly in early passage cells infected with polyoma virus (9) it was found that the antigen was stable to Tween-80 ether and deoxycholate–genetron extraction. This is consistent with properties of authentic virion-derived gs antigens; however, in contrast to the latter category of antigen the activity in rat cells was not recoverable after precipitation with 5 vol of 95% ethanol (Table II). (e) In addition, sera from rats bearing tumors induced by polyoma-F-111 cells did not react in the CF or gel-diffusion assays concentrated C-type virus of mouse or rat origin (the virus originally termed M–MSV (6) was used in these tests based on recent evidence that this is a rat pseudotype sarcoma virus (15)). These sera do, however, react with antigens (*i.e.*, ether stable, alcohol labile). Until the antigen is charactertized in more detail, its relationship to virus or viral information remains to be determined. However, expressions of the antigen seems to be related to the transformed state.

In this regard, the new rat antigen may belong to that class of antigens which are cryptical in normal cells and only become exposed during the course of transformation (16). At least one member of this class, the Forsmann antigen is known to be detectable in certain hamster cells (BHK) transformed by polyoma virus (17, 18) while nontransformed BHK cells generally do not show

TABLE I. Presence of CF Antigen in Tumors and Organs of Rats Inoculated Subcutaneously with Transformed Rat Embryo Cells.[a]

Donor (passage no.) (No. of cells/rat)	No. of tumor / No. inoculated	No. of tumor transplanted	T-number	Specimen (extract)	CF titers *vs.* rat serum			
					MSV (#21)	MSV (#26)	Polyoma F-111 (#1)	Homologous (F-111)
F-111 (P-79) 2.9×10^6	5/5(22)	Primary	T-49171	Tumor	> 4	—	1:4	0
		1	T-49440	Tumor	—	> 8	> 8	> 16
		2	T-49561	Tumor	—	> 8	> 8	—
		3	T-49803	Spleen	—	> 8	1:8	—
			T-49804	Thymus	—	1:4	1:4	—
			T-49805	Kidney	—	1:8	> 8	—
			T-49806	Muscle	—	0	0	—
			T-51926	Tumor	—	> 16	> 16	> 16
S-1193h(p-49) 1.0×10^6	5/5(21)	Primary	RA-750	Tumor	—	1:4	—	—

[a] CF titers = Reciprocal of dilution giving 3+ to 4+ fixation of 1:8 units of complement. 0 = 0/2; — = not done. Polyoma-F-111 (#1) = Serum derived from polyoma transformed rat embryo (F-111) cell induced rat tumor.

TABLE II. Characterization of Antigenic Reactivity in F-111 Rat Tumors[a] and Polyoma-F-111 Rat Tumors[b] with MSV and Polyoma Rat Serum.

Treatment	Reciprocal of CF titer			
	MSV 26[c]		Polyoma 2[d]	
	T-51926	T-57927	T-51926	T-51927
20% homogenate	> 16	> 16	> 16	> 16
Sonicate	> 16	> 16	> 16	> 16
Tween–ether	> 16	> 16	> 16	> 16
Alcohol precipitate (cold)	lac[f]	1	lac	2
Deoxycholate–genetron	> 16	> 16	> 16	> 16
Tween–ether 60° 30 min	< 1	< 1	< 1	< 1
Alcohol ppt.[e] 37° 30 min	< 1	< 1	< 1	< 1
Deoxycholate–genetron 60° 30 min	lac	< 1	< 1	2
Tween–ether, alcohol ppt.[e]	< 1	< 1	< 1	< 1

[a] F-111 rat tumors (T-51926) = rat tumors induced by spontaneously transformed rat embryo (F-111) cells after prolonged passage.

[b] Polyoma F-111 rat tumors (T-51927) = rat tumors induced by polyoma transformed rat embryo (F-111) cells.

[c] MSV 26 = rat antisera containing antibodies to gs and envelope antigen.

[d] Polyoma 2 = serum derived from tumor-bearing rat induced by polyoma transformed rat embryo (F-111) cells.

[e] Reconstituted in equal volumes of buffer.

[f] ac = Anti-complementary.

this antigen unless treated with proteases (18). Our attempts to detect Forsmann antigen in the polyoma transformed rat cells by inhibition of rabbit anti-sheep RBC hemolytic activity have been negative and the CF reactive rat sera have not reacted with sheep RBC even at low dilutions, indicating that the Forsmann system is not responsible for the CF activity in the rat system as previously reported (9). Whether or not other membrane alterations may account for this reactivity remains to be critically tested.

Summary. Rat embryo (RE) cells maintained in tissue culture for long periods developed into tumorigenic lines that exhibited morphological alteration. When tested with broadly reactive anti-MSV rat serum, the spontaneously transformed RE (TRE) cells and tumor cells induced by the TRE cells were positive, however, these cells did not react with guinea pig antiserum which is reactive only with the gs-1 antigen of C-type RNA viruses. While the antigen derived from the TRE line appeared similar to that derived from polyoma transformed RE cell line, it was detected much earlier in the latter cell line. Some characteristics of the new rat cell antigens were described.

1. Rhim, J. S., Joglekar, M. H., Richardson, E. S., Nelson, N. A., and Huebner, R. J., Proc. Soc. Exp. Biol. Med. **135**, 160 (1970).

2. Rhim, J. S., Vass, W., Cho, H. Y., and Huebner, R. J., Int. J. Cancer **7**, 65 (1971).

3. Hartley, J. W., Rowe, W. P., Capps, W. I., and Huebner, R. J., Proc. Nat. Acad. Sci. U.S.A. **53**, 931 (1965).

4. Hartley, J. W., Rowe, W. P., Capps, W. I., and Huebner, R. J., J. Virol. **3**, 126 (1969).

5. Oroszlan, S., Fisher, C. L., Stanley, P. B., and Gilden, R. V., J. Gen. Virol. **8**, 1 (1970).

6. Gilden, R. V., and Oroszlan, S., J. Amer. Vet. Med. Ass. **158**, 1099 (1971).

7. Oroszlan, S., Foreman, C., Kelloff, G., and Gilden, R. V., Virology **43**, 665 (1971).

8. Gilden, R. V., Oroszlan, S., and Huebner, R. J., Nature (London) New Biol. **231**, 107 (1971).

9. Rhim, J. S., Lengel, C. R., Cho, H. Y., Takemoto, K. K., Turner, H. C., Huebner, R. J., and Gilden, R. V., Nature (London) New Biol. **235**, 188 (1972).

10. Freeman, A. E., Price, P. J., Igel, H. J., Young, J. C., Maryak, J. M., and Huebner, R.

J., J. Nat. Cancer Inst. **44**, 65 (1970).

11. Huebner, R. J., Rowe, W. P., Turner, H. C., and Lane, W. T., Proc. Nat. Acad. Sci. U.S.A. **50**, 379 (1963).

12. Rhim, J. S., Huebner, R. J., Lane, W. T., and Vernon, M. L., Proc. Soc. Exp. Biol. Med. **133**, 914 (1970).

13. Hall, W. T., Anderson, W. F., Sanford, K. K., Evans, V., and Hartley, J. W., Science **156**, 85 (1967).

14. Aaronson, S. A., Hartley, J. W., and Todaro, G. J., Proc. Nat. Acad. Sci. U.S.A. **64**, 87 (1969).

15. Gilden, R. V., Oroszlan, S., and Huebner, R. J., Virology **43**, 722 (1971).

16. Burger, M. M., Proc. Nat. Acad. Sci. U.S.A. **62**, 994 (1969).

17. Fogel, M., and Sachs, L., Exp. Cell Res. **34**, 448 (1964).

18. Burger, M. M., Nature (London) New Biol. **231**, 125 (1971).

Received Jan. 26, 1972. P.S.E.B.M., 1972, Vol. 140.