In Vitro Transformation Assay of Major Fractions of Cigarette Smoke Condensate (CSC) in Mammalian Cell Lines[1] (37162)

JOHNG S. RHIM AND ROBERT J. HUEBNER[2]

Department of Virus Research, Microbiological Associates, Inc., and Viral Carcinogenesis Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014

Sensitive quantitative *in vitro* assay systems suitable for studies of potential carcinogenic agents found in the human environment are much needed. A number of *in vitro* assay systems utilizing hamster and mouse cells have been described (1–5).

Recently a number of our associates and we described *in vitro* systems utilizing cell cultures infected with nontransforming Type C RNA tumor virus (6–12). Rat and mouse cells were readily transformed by urban smog when they were preinfected with nontransforming Type C RNA viruses (13, 14). Hamster embryo cultures infected with hamster leukemia viruses were found to be sensitive to the transforming effects of smog and although uninfected hamster cultures were also transformed, 10-fold higher doses of the smog extract were required (13). Thus, the combined effects of RNA tumor viruses and carcinogenic chemicals in producing transformation of various animal cells (mouse, rat and hamster) would seem to provide highly sensitive and reproducible test systems for quantitative *in vitro* assays of suspected environmental carcinogens.

Transformation of hamster cells by selected cigarette smoke condensate (CSC) fractions has recently been reported (15). This communication reports an *in vitro* study of the transforming activity of 12 major fractions of CSC on rat and mouse cells infected with C-type RNA tumor viruses and on uninfected hamster cells; the results are compared to those obtained with the same fractions in an *in vivo* study by Bock, Swain and Stedman (16).

Materials and Methods. Media. Eagle's minimal essential medium supplemented with 10% fetal bovine serum (FBS) 2 m*M* glutamine, 100 unit/ml penicillin, and 100 μg/ml streptomycin (EMEM + 10% FBS) was used for growing and maintaining cells.

Cell lines. Cell lines used in this study were as follows:

1. Mouse embryo cell lines; AKR virus-infected and uninfected NIH Swiss mouse embryo tissue culture (NIH-METC) lines have been described in detail (10–12).

2. Rat embryo cell lines; Rauscher leukemia virus-infected (F-119) and uninfected (F-111) Fischer rat embryo (RE) cell lines (39th subculture level) used in this study were established by Freeman *et al.* (6).

3. Hamster embryo cell lines; A normal hamster cell line (HE ER-6) established from NIH syrian embryo cells was used at fifth subculture (12).

The rat and mouse normal cultures, when infected with murine leukemia viruses, became continuously infected carrier cultures which regularly produced complement-fixing (CF) antigens characteristic of the murine leukemia sarcoma virus complex and yielded infectious virus (3.0–4.0 logs of virus/ml) when tested in the CF test for murine leukemia virus, the COMUL test (17). Microtiter complement-fixation (CF) tests used for group-specific (gs) antigen were performed as previously described (18). Titers were recorded as reciprocals of the highest dilution giving 3+ to 4+ fixation of 1.8 units of com-

[1] This work was supported by the Council for Tobacco Research Contract 2221 and in part by Contract NIH 70-2068A, National Cancer Institute, National Institutes of Health, Bethesda, MD 20014.

[2] We thank Dr. Ansel P. Swain, Eastern Marketing and Nutritional Research Division, Agricultural Research Service, USDA Philadelphia, PA for his kindness in providing us the cigarette smoke condensate fractions. Excellent technical assistance was provided by Mr. William Vass and Han Y. Cho.

plement.

Fractions of cigarette smoke condensate (CSC). Fractions of CSC as coded samples were obtained from Dr. A. P. Swain, Tobacco laboratory, Eastern Marketing and Nutritional Research Division, Agricultural Research Service, USDA, Philadelphia, PA. The detail of fractionation of CSC for chemical and biologic investigations has been described by Swain, Cooper and Stedman (19). The results of bioassays of fractions of CSC using mice previously treated with 7,12-dimethylbenz(a)anthracene (DMBA) have been reported by Bock, Swain and Stedman (16) (Table I). The weak acid (phenol) fraction (No. 8) and two neutral fractions Nos. 13 and 14 had statistically significant carcinogenic activity. The neutral fraction (No. 12) showed suggestive activity, and the ether-soluble base fraction (No. 5) was found to have possible activity. Cigarette smoke condensate fractions were dissolved in dimethyl sulfoxide (DMSO) to give stock solution of 100 μg/ml, which were stored in the dark at 4°. Benzo[*a*]pyrene (BP) was obtained from Eastman Organic Chemicals and was dissolved in 1 ml of DMSO to yield a stock solution of 100 μg/ml and also was stored at 4° in the dark.

Transformation assay. One day after 5×10^4 cells/ml from the cultures were planted in Falcon plastic petri dishes, the medium was removed and fresh medium containing CSC (1 μg/ml) was added. The control medium contained 0.5% DMSO. The media with CSC or BP were changed twice in 7 days. After incubation of the cells with experimental media the cultures were washed and refed with nutrient medium and subdivided by trypsin treatment. Additional subcultures were prepared every 7 or 10 days thereafter. When changes in morphology and growth patterns appeared, representative cultures were fixed in alcohol and stained with Giemsa for further microscopic examination.

Results. The AKR leukemia virus-infected mouse cells were transformed by 4 CSC fractions (Nos. 3, 8, 13, and 14) and also by BP (Table I). The altered foci (Fig. 1A) consisted of randomly oriented, spindle-shaped cells showing a loss of density inhibition of growth and were similar to those observed previously in AKR-infected NIH-ME cells transformed by various chemical car-

TABLE I. Correlation of *in Vitro* Cell Transformation Assay with *in Vivo* Bioassay of Fractions of Cigarette Smoke Condensate (CSC) Using Mice Previously Treated with BP.

Fraction no.	Group or fraction	*In vivo*[a] total no. of skin tumors in mice	*In vitro*[b] cell transformation		
			Mouse + virus	Rat + virus	Hamster, no virus
	BP	3	+	+	+
3	Bases before, insoluble	3	+	+	−
4	Bases after, insoluble	3	−	−	−
5	Bases, ether-soluble	6	−	−	−
6	Bases, water-soluble	1	−	−	−
7	Weak acids, insoluble	2	−	−	−
8	Weak acids, ether-soluble	48	+	+	+
9	Strong acids, ether-soluble	1	−	−	−
10	Strong acids, ether-soluble	2	−	−	−
11	Strong acids, water-soluble	2	−	−	−
12	Neutrals, 80% methanol-soluble	2	−	−	−
13	Neutrals, cyclohexane-soluble	8	+	−	+
14	Neutrals, nitromethane-soluble	38	+	+	+

[a] Data from Bock, Swain and Stedman (16).

[b] Cell lines used: mouse + virus = AKR-infected NIH Swiss mouse embryo (NIH-ME) cell line; rat + virus = RLV-infected rat embryo cell line; hamster, no virus = hamster embryo cell line. All CSC fractions and BP were also tested in uninfected NIH-ME and uninfected RE cell lines and no transformation was observed.

cinogens (10, 12) (Fig. 1B). Approximately 4 wk after treatment, morphological changes were observed in the second subculture of cells treated with fractions 8 and 14. Transformation appeared about 7 days later in cells treated with fractions 3 and 13 (Table II).

The RLV-infected rat cells were transformed by 3 CSC fractions (Nos. 3, 8, and 14) and by BP; no transformation was noted in the uninfected rat cells by either CSC or BP (Table I). Transformation was first observed in the fourth subculture of infected cells exposed to fraction 8 approximately 42 days after treatment. Morphological alteration appeared 7 days later in cells treated with fractions 3 or 14 (Table II). Foci were made up of randomly oriented spindle-shaped cells, piled up like jack straws, showing nuclear and cytoplasmic overlapping, and were similar to those observed previously in chemical induced transformed RLV-infected rat cells (8, 12).

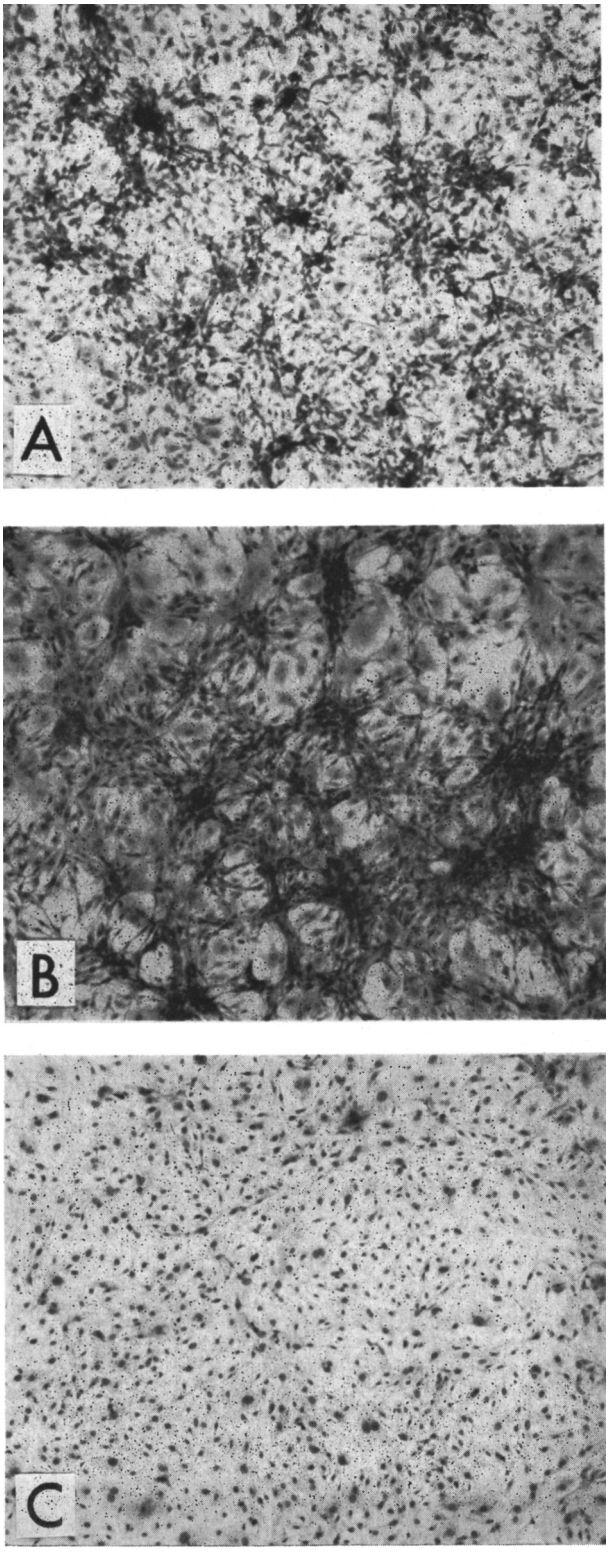
Hamster cells were transformed by 3 fractions (Nos. 8, 14 and 13) of CSC and BP (Table I). After 3 subcultures the hamster cells exposed to fractions 8 or 14 exhibited morphological changes: similar changes were observed in the cells exposed to fraction 13 2 wk later (Table II). Dense, multilayered foci noted were similar to those observed in cells treated with BP (12).

Discussion and Summary. It is interesting that the data from our *in vitro* studies corroborate the *in vivo* results obtained by Bock, Swain and Stedman (16). Bock, Swain and Stedman (16) reported that the weak acid (phenol) fraction (No. 8) and two neutral fractions (Nos. 13 and 14) had statistically significant tumor inducing activity. In addition, the third neutral fraction, 12, gave results suggesting activity, and the ether-soluble base fraction (No. 5) exhibited possible activity. We found cell transforming activity with four fractions (Nos. 3, 8, 13 and 14) of CSC. Fractions 8 and 14 exhibited early transformation in infected mouse and rat cells and also subsequently in uninfected hamster cells suggesting that they were most active. Fraction 13 showed transformation in infected mouse cells and uninfected hamster cells. In addition, fraction 3 demonstrat-

TABLE II. Time of Appearance of *in Vitro* Transformation in Mouse, Rat and Hamster Embryo Cells[a] Treated with Cigarette Smoke Condensate Fractions[b] and BP.[c]

Subculture level	Cumulative no. of days after treatment	Morphological changes in cells treated with CSC fraction or chemical															
		AKR-infected NIH-METC					RLV-infected RE					Hamster embryo					
		No. 3	No. 8	No. 13	No. 14	BP	No. 3	No. 8	No. 13	No. 14	BP	No. 3	No. 8	No. 13	No. 14	BP
1	21	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−
2	28	−	+	−	+	+	−	−	−	−	−	−	+	−	+	+
3	34	+	+	+	+	+	−	−	−	−	−	−	+	−	+	+
4	42	+	+	+	+	+	−	+	−	−	+	−	+	−	+	+
5	49	+	+	+	+	+	+	+	−	+	+	−	+	+	+	+

[a] Cells were treated with CSC or chemicals for 7 days.
[b] Cigarette smoke condensate fraction, 1 μg/ml.
[c] BP, 1 μg/ml.


A
B
C

ed changes in infected mouse and rat cells. However, none of the fractions exhibited cell alteration in uninfected mouse and rat cells. These findings are consistent with previous reports that rat and mouse cells were readily transformed by chemical carcinogens (6–12) and smog extract (13, 14) when these cells were preinfected with C-type RNA viruses, whereas cells treated with chemical or virus alone were not transformed.

A number of bioassays of major fractions of CSC have been reported (16, 20, 21). However, the *in vivo* assay systems are time consuming. Bock, Swain and Stedman (16) detected the positive fractions by their *in vivo* accelerated technique after 40 wk. These and previous findings suggest that *in vitro* transformation systems such as described herein have certain distinct advantages, particularly for rapid quantitative assays of putative carcinogens in tobacco smoke as well as in other suspect environmental materials.

In a preliminary study Freeman *et al.* (15) reported transforming activity in hamster cells by three fractions, Nos. 9, 6, and 13. We have no explanation for the differences since we observed no transformation effects by fractions 6 and 9 which were the weakest fractions in the *in vivo* test. Studies are underway to determine the optimum doses of the CSC fractions for inducing transformation particularly in the infected mouse cell system. It is obvious that subfractionations be done particularly on fractions 8 and 14 for further study.

1. Berwald, Y., and Sachs, L., J. Nat. Cancer Inst. **35**, 641 (1965).

2. Borenfreund, E., Krim, M., Sanders, F. K., Sternberg, S., and Bendich, A., Proc. Nat. Acad. Sci. USA **56**, 672 (1966).

3. DiPaolo, J. A., and Donovan, P. J., Exp. Cell Res. **48**, 361 (1967).

4. Huberman, E., and Sachs, L., Proc. Nat. Acad. Sci. USA **56**, 1123 (1966).

5. Chen, T. T., and Heidelberger, C., J. Nat. Cancer Inst. **42**, 915 (1969).

6. 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).

7. Price, P. J., Freeman, A. E., Lane, W. T., and Huebner, R. J., Nature (London) New Biol. **230**, 144 (1971).

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

9. Rhim, J. S., Vass, W., Cho, H. Y., and Huebner, R. J., Proc. Amer. Ass. Cancer Res. **12**, 23 (1971).

10. Rhim, J. S., Creasy, B., and Huebner, R. J., Proc. Nat. Acad. Sci. USA **68**, 2212 (1971).

11. Rhim, J. S., Creasy, B., Joglekar, M. H., Vass, W., and Huebner, R. J., Bacteriol Proc., p. 217 (1971).

12. Rhim, J. S., Cho, H. Y., Joglekar, M. H., and Huebner, R. J., J. Nat. Cancer Inst. **48**, 949 (1972).

13. Freeman, A. E., Price, P. J., Bryan, R. J., Gordon, R. J. Gilden, R. V., Kelloff, G. J., and Huebner, R. J., Proc. Nat. Acad. Sci. USA **68**, 445 (1971).

14. Rhim, J. S., Cho, H. Y., Rabstein, L., Gordon, R. J., Bryan, R. J., Gardner, M. B., and Huebner, R. J., Nature (London) **239**, 103 (1972).

15. Freeman, A. E., Kelloff, G. J., Gilden, R. V., Lane, W. T., Swain, A. P., and Huebner, R. J., Proc. Nat. Acad. Sci. USA **68**, 2386 (1971).

16. Bock, F. G., Swain, A. P., and Stedman, R. L., Cancer Res. **29**, 584 (1969).

17. Hartley, J. W., Rowe, W. P., Capps, W. I., and Huebner, R. J., Proc. Nat. Acad. Sci. USA **53**, 931 (1965).

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

19. Swain, A. P., Cooper, J. E., and Stedman, R. L., Cancer Res. **29**, 579 (1969).

20. Wynder, E. L., and Hoffman, D. A., Cancer **14**, 1306 (1961).

21. Wynder, E. L., and Wright, G., Cancer **10**, 255 (1957).

Received Oct. 12, 1972. P.S.E.B.M., 1973, Vol. 142.

FIG. 1. AKR leukemia virus-infected NIH Swiss mouse embryo cells. The cultures were treated for 7 days with cigarette smoke condensate (CSC) fraction 8 or BP, followed by 3 subcultures (34 days) in nutrient medium. Giemsa stain (×32). (A) Cells treated with CSC fraction 8 (1 μg/ml); (B) cells treated with BP (1 μg/ml); (C) cells, no treatment (0.5% DMSO). Note the morphological changes in AKR-NIH-ME cells treated with CSC fraction 8 or BP; criss-crossing, spindle-shaped cells with nuclear and cytoplasmic overlapping (A, B).