PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 167, 572–575 (1981)

Rat Hepatocyte-Mediated Mutagenesis of Human Cells by Carcinogenic Polycyclic Aromatic Hydrocarbons but Not Organochlorine Pesticides[1] (41217)

CHARLES TONG,[2] MARYELLEN FAZIO, AND GARY M. WILLIAMS

Naylor Dana Institute for Disease Prevention, American Health Foundation, Valhalla, New York, 10595

Abstract. A hepatocyte primary culture-mediated human cell hypoxanthine–guanine phosphoribosyl transferase mutagenesis assay was established. This assay provides an *in vitro* system that combines a realistic activation capability and an endpoint of clear biological significance in a target cell line relevant to human risk. The genotoxicity of 7,12-dimethylbenz[*a*]anthracene and benzo[*a*]pyrene were demonstrated in this assay. In the same assay, DDT, chlordane, and mirex were not genotoxic. An epigenetic mechanism for the carcinogenicity of these organochlorine pesticides is suggested.

At least 10 commonly used organochlorine pesticides have been shown to be carcinogenic for rats and mice (1–6). Many of these have, however, been negative in a variety of *in vitro* short-term assays (7, 8) including those tests involving bacteria (9, 10). The reason for this discrepancy has frequently been assumed to be a lack of adequate metabolism in the *in vitro* test systems (11). Since certain of the organochlorine pesticides are carcinogenic primarily or exclusively for the liver of rodents, we have been systematically evaluating their activity in liver-derived intact cell systems developed in our laboratory (12–13). Thus far, the pesticides tested have been negative in the adult rat liver epithelial cell/hypoxanthine–guanine phosphoribosyl transferase (ARL/HGPRT) mutation assay (14, 15) and in the hepatocyte primary culture/DNA repair test using hepatocytes from rats (14, 15), mouse, and hamster (16, 17). As a further measure, we have adapted the approach of hepatocyte-mediated mutagenesis (18) to examine whether these compounds are mutagenic to human cells in the presence of an active metabolizing system from the target organ of the carcinogens. In these studies, we have used members of the structurally similar polycyclic aromatic hydrocarbons (PAH) as positive controls because these compounds are highly lipophilic like the organochlorine pesticides, but not strongly carcinogenic for liver. We report here that human skin fibroblasts, in the presence of the rat hepatocyte primary cultures as the metabolizing system, can be mutated by benzo[*a*]pyrene and 7, 12-dimethyl-benz-[*a*]anthracene at the HGPRT locus, but not by DDT, chlordane, or mirex.

Materials and Methods. The preparation of rat hepatocyte primary cultures (HPC) from male Fischer rats has been described extensively (19, 20). Routinely, these primary cultures have a greater than 90% viability as shown by trypan blue exclusion. The target cell line, Detroit 550 (D-550), is a human foreskin fibroblast line obtained from the American Type Culture Collection and is maintained frozen in this laboratory at around its 22nd passage. The cells were usually revived at the initiation of any experiments and discarded afterward.

Benzo[*a*]pyrene and 7, 12-dimethylbenz[*a*]anthracene were purchased from Sigma (St. Louis, Mo.); DDT was purchased from Aldrich Chemical Corporation (Metuchen, N.J.), and chlordane and mirex were kindly provided by Velsicol Chemical Corporation (Chicago, Ill.).

In the HPC-mediated D-550 HGPRT mutation assay, 1×10^6 D-550 were generally coplated with 20×10^6 freshly prepared HPC. The cocultures were allowed to at-

[1] This work was supported by Grant ES-01-724-03 from the National Institute of Environmental Health Sciences and Contract NO1-CP-55705 from the National Cancer Institute.

[2] Recipient of the Young Environmental Scientist Health Research Grant Program, NIEHS.

0037-9727/81/080572-04$01.00/0

tach for 2 hr. They were then washed and fresh media containing the carcinogens or appropriate controls were added for a 24 hr exposure. The cultures were then washed and allowed a mutant expression period of 10 days. During this period, the cultures were trypsinized and replated whenever they reached 50% confluency. Trypsinization and replating also removed any remaining HPCs as they do not survive replating. In general, by Day 4 of the 10-day expression period, no viable HPCs were observed.

6-Thioguanine (TG) was used for the selection of HGPRT mutants in the human foreskin fibroblast cell line D-550. TG, at a concentration of 20 μg/ml of culture medium, was used and a minimum of 1×10^6 D-550 were selected at a seeding density of 1×10^3 cells/cm^2. A 3-week selection period with five changes of the TG-containing medium was allowed. 6-Thioguanine-resistant (TG^r) colonies that developed were fixed and stained for counting. A parallel plating of the D-550 at a lower seeding density and in the regular growth medium, Williams' Medium E, provided the colony-forming efficiency of the cell culture for computation of mutant incidence. These procedures were described in detail in a previous publication (21) for the adult rat liver epithelial cell/HGPRT mutation assay.

Results. The human foreskin fibroblast, D-550, is highly sensitive to the toxic effects of 6-thioguanine with an incidence of spontaneous TG-resistant mutants of less than 1 per 10^6 colony-forming cells. Exposure of D-550 cells to the direct acting mutagen/carcinogen, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, results in a dose-dependent increase in the TG^r mutant incidence (unpublished observation), as described for the ARL/HGPRT mutagenesis assay (21). D-550 cells, however, were not mutated by activation-dependent mutagens/carcinogens such as 2-acetylaminofluorene or benzo[*a*]pyrene (unpublished observation) in contrast to the metabolically active ARL cells (21). Thus, HPCs can be used as a metabolizing system to provide activation of xenobiotics for study of the effects on D-550 cells.

In the presence or absence of HPCs, D-550 cells were exposed to representative PAHs. Exposure of D-550 alone to benzo[*a*]pyrene or 7, 12-dimethylbenz[*a*]anthracene did not result in any increase in TG^r mutant incidence over the parallel-run control (Table I). However, a significant increase in the TG^r mutant incidence over that in control cultures was observed in D-550 when the carcinogen exposure was in the presence of HPCs. These data thus support previous findings in which HPCs were successful in providing metabolism for mutagenesis in ARL cells (18), bacteria (22), and hamster V79 cells (23) and transformation in hamster embryo cells (24).

The effect of DDT, chlordane, and mirex was then examined in this system. At the log dose below the toxic dose, no significant increase in TG^r mutant incidence was observed in the exposed human cells either in the absence or presence of HPC. The studies were repeated and the averages of three separate experiments were determined (Table II). Student *t* test analyses of mutant incidences in this series of experi-

TABLE I. RAT HEPATOCYTE PRIMARY CELL CULTURE (HPC)-MEDIATED MUTAGENESIS OF HUMAN FORESKIN FIBROBLAST (DETROIT-550) BY THE POLYCYCLIC AROMATIC HYDROCARBONS[a]

Cell culture	Carcinogen	No. of TG^r colonies/ 10^6 colony-forming cells
Human fibroblast	DMBA(10^{-4} *M*)	0
Human fibroblast + HPC	DMBA(10^{-5} *M*)	Toxic
Human fibroblast + HPC	DMBA(10^{-6} *M*)	76
Human fibroblast	B[*a*]P(10^{-4} *M*)	0
Human fibroblast + HPC	B[*a*]P(10^{-4} *M*)	Toxic
Human fibroblast + HPC	B[*a*]P(10^{-5} *M*)	142
Human fibroblast + HPC	DMSO(0.1%)	0

[a] These experiments were repeated three times, yielding similar results each time.

TABLE II. SUMMARY OF ASSAY RESULTS WITH THE ORGANOCHLORINE PESTICIDES

Chemicals	Dosage (*M*)	TG^r mutants/10^6 CFC[a]	
		Without HPC	With HPC
DDT	10^{-4}	13.0 ± 4.0	22.3 ± 22.0
	10^{-5}	ND[b]	0
Chlordane	10^{-4}	4.3 ± 7.5	0
	10^{-5}	ND	0
Mirex	10^{-4}	0	0
	10^{-5}	ND	0
DMBA	10^{-4}	11.7 ± 20.2	Toxic
	10^{-5}	ND	Toxic
	10^{-6}	ND	119.8 ± 51.0[c]
Control		10.5 ± 10.0	5.0 ± 11.2

[a] Average mutant incidence with standard deviation of at least three assays.
[b] Not done.
[c] $P < 0.05$.

ments with the organochlorine pesticides (Table II) indicated that only the parallel-run positive control, DMBA in the presence of HPCs, induced mutant incidences that are significantly above those of the parallel-run controls. The relatively high mutant incidence occasionally observed in the other populations probably is due to the selection in one particular experiment of a subpopulation with high mutant incidences.

Discussion. The present report of an HPC-mediated human cell mutagenesis assay provides an *in vitro* system that combines a realistic activation capability and an endpoint of clear biological significance in a target cell line relevant to human risk (25, 26). The absence of any mutagenicity of the organochlorine pesticides in this human cell system, together with negative findings in the bacterial tests (9, 10), in the hepatocyte primary culture/DNA repair test (7, 8, 14, 15), and in the ARL/HGPRT mutation assay (14, 15) must be considered relevant to the mechanism of the carcinogenicity of these agents. Based on these and other findings, we have suggested that certain organochlorine pesticides are carcinogens that are not genotoxic, but rather operate by epigenetic mechanisms including tumor-promoting effects (14, 15, 27). Recently, we have demonstrated (6, 28) that DDT behaves in a manner similar to that of other tumor promoters in inhibiting intercellular communication (15, 29, 30). In the case of DDT and other lipophilic organochlorine pesticides, this may be a result of their deposition within the lipid layer of the cell membrane. Inhibition of intercellular communication is visualized to interrupt transmission of signals that are required for maintaining normal growth regulation. The loss of normal growth control signals would permit the progressive growth of initiated cells into neoplasms. This concept would account for the promoting effect of DDT when administered after a genotoxic carcinogen (31) and for the carcinogenicity of DDT when given to strains of rodents with a spontaneous incidence of liver tumors (32, 33) and, therefore, presumably a spontaneous background of initiated cells. According to this hypothesis (34), the carcinogenicity of nongenotoxic organochlorine pesticides would depend upon a substantial level of exposure (in order to saturate membranes and inhibit intercellular communication) and exposure for a prolonged duration (in order for transformed cells to proliferate beyond the cell number that would be subject to reimposition of growth control). If the concept described is correct, exposure to certain carcinogenic organochlorine pesticides at levels below those sufficient to cause sustained interruption of intercellular communication would not be oncogenic. Clearly more research in this area is warranted.

In summary, a hepatocyte-mediated human cell mutagenesis system has been

established in which the genotoxicity of representative members of the class of polycyclic aromatic hydrocarbon carcinogens was demonstrated. In the same system, DDT, chlordane, and mirex were not genotoxic. An epigenetic mechanism for the carcinogenicity of these organochlorine pesticides is, therefore, suggested.

1. Sternberg, S. S., Pharm. Ther. **6**, 147 (1979).
2. Kimbrough, R. D., Ann. N.Y. Acad. Sci. **320**, 415 (1979).
3. Saffiotti, U. *in* "IARC Scientific Publication No. 25" (W. Davis and C. Rosenfeld, eds.), p. 151. IARC, Lyon (1979).
4. Sugar, J., Toth, K., Csuka, O., Gati, E., and Somfai-Relle, S. J., Toxicol. Environ. Health **5**, 183 (1979).
5. Cebral, J. R. P., *in* "Toxicology in the Tropics" (R. L. Smith and E. A. Bababunmi, eds.), p. 162. Taylor & Francis, London (1980).
6. Williams, G. M., *in* "The Pesticide Chemist and Modern Toxicology. American Chemical Society Symposium Series," (S. K. Bandal, G. J. Marco, M. L. Leng, and L. Goldberg, eds). ACS, Washington, D.C. (1981).
7. Swenberg, J. A., Petzold, G. L., and Harbach, P. R., Biochem. Biophys. Res. Commun. **72**, 732 (1976).
8. Grant, W. F., *in* "A Rational Evaluation of Pesticidal vs. Mutagenic/Carcinogenic Action" (R. W. Hart, H. F. Kraybill, and F. J. DeSerres, eds.), p. 72. DHEW Publication (NIH) 78-1306, Washington, D.C. (1978).
9. McCann, J., Choi, E., Yamasaki, E., and Ames, B. N., Proc. Nat. Acad. Sci. USA **72**, 5135 (1975).
10. Koda, T., Moriya, M., and Shirasu, Y., Mutat. Res. **26**, 243 (1974).
11. IARC Monograph No. 20, p. 35. IARC, Lyon (1979).
12. Williams, G. M., *in* "Short-Term Tests for Chemical Carcinogens" (R. H. C. San and H. F. Stich, eds.), p. 581. Springer-Verlag, New York (1980).
13. Williams, G. M., *in* "Chemical Mutagens" (F. J. DeSerres and A. Hollaender, eds.), Vol. 6, p. 61. Plenum, New York (1980).
14. Williams, G. M., *in* "Advances in Medical Oncology, Research and Education" (G. P. Margison, ed.), Vol. I, p. 273. Pergamon Press, Oxford/New York (1979).
15. Williams, G. M., Ann. N. Y. Acad. Sci. **349**, 273 (1980).
16. Maslansky, C. A., and Williams, G. M., J. Toxicol. Environ. Health, in press (1981).
17. Williams, G. M., and Maslansky, C. J., *in* "Abstracts of 19th Annual Meeting, A76." Soc. Toxicol., Washington, D.C. (1980).
18. San, R. H. C., and Williams, G. M., Proc. Soc. Exp. Biol. Med. **156**, 534 (1977).
19. Williams, G. M., Bermudez, E., and Scaramuzzino, D., In Vitro **13**, 809 (1977).
20. Williams, G. M., Bermudez, E., San, R. H. C., Goldblatt, P. J., and Laspia, M. F., In Vitro **14**, 824 (1978).
21. Tong, C., and Williams, G. M., Mutat. Res. **74**, 1 (1980).
22. Green, M. H. L., Bridges, B. A., Rogers, A. M., Horspool, G., Murial, W. J., Bridges, J. W., and Fry, J. R., Mutat. Res. **48**, 287 (1977).
23. Langenbach, R., Freed, H. J., Raven, D., and Huberman, E., Nature (London) **276**, 277 (1978).
24. Poiley, J. A., Raineri, R., and Pienta, R. J., J. Nat. Cancer Inst. **63**, 519 (1979).
25. DeMars, R., *in* "Banbury Report on Mammalian Cell Mutagenesis—The Maturation of Test Systems" (A. W. Hsie, J. P. O'Neill, and V. K. McElheny, eds.) Vol. 2, p. 329. Cold Spring Harbor Laboratory, New York (1979).
26. Thilly, W. G., and Liber, H. L., *in* "Critical Reviews in Toxicology—Strategies for Short-Term Testing for Mutagens/Carcinogens" (B. Butterworth, eds.) p. 13. CRC Press, West Palm Beach, Fla. (1979).
27. Williams, G. M., Biochim. Biophys. Acta (Rev. Cancer) **605**, 167 (1980).
28. Williams, G. M., Telang, S., and Tong, C., Cancer Lett. **11**, 339 (1981).
29. Yotti, L. P., Chang, C. C., and Trosko, J. E., *Science* **206**, 1089 (1979).
30. Murray, A. W., and Fitzgerald, D. J., Biochem. Biophys. Res. Commun. **28**, 395 (1979).
31. Peraino, C., Fry, R. J. M., Staffeldt, E., and Christopher, J. P., Cancer Res. **35**, 2884 (1975).
32. Innes, J. R. M., Ulland, B. M., Valerio, M. C., Petracelli, L., Fishbein, L., Hart, E. R., Pallotta, A. J., Bates, R. R., Falk, H. L., Gaut, J. J., Klein, M., Mitchel, I., and Peters, S., J. Nat. Cancer Inst. **42**, 1101 (1969).
33. Tomatis, L., Turusov, V., Day, N., and Charles, R. T., Int. J. Cancer **10**, 489 (1972).
34. Williams, G. M. Food Cosmet. Toxicol., in press (1981).

Received January 12, 1981. P.S.E.B.M. 1981, Vol. 167.