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Mutagenic Activity of Tumor-associated Macrophages in *Salmonella typhimurium* Strains TA98 and TA100[1]

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ABSTRACT

Suspensions of cells from a series of strain BALB/cfC3H mouse mammary tumors, and adherent and nonadherent cells from the tumors, were tested for their ability to increase the mutation rate of *Salmonella typhimurium* tester strains TA98 and TA100. Significant increases were seen with cells from three of four tumor lines tested on the TA98 strain and with one of four tested on the TA100 strain. The mutagenic activity was due primarily to cells in the adherent fractions which were greatly enriched for macrophages.

INTRODUCTION

Weitzman and Stossel (19) and Weitberg *et al.* (18) have shown that human peripheral phagocytes are capable of increasing the mutation rate in standard bacterial tester strains and of producing cytogenetic lesions in mammalian cells. Barak *et al.* (3) have reported human polymorphonuclear neutrophil-mediated mutagenicity detected by a new bioluminescence assay. The mechanism of mutagenicity appears to be oxygen-reactive metabolites, such as superoxide anions, H_2O_2, and hydroxyl radicals, which are released by inflammatory cells and which are known mutagens (3, 20). Although the endogenous production of mutagens by phagocytic cells may be involved in the association of chronic inflammation and the initiation of cancer (1, 2, 12, 17), genetic change is not limited to the early stages of carcinogenesis but is a feature of neoplastic progression as well. Progression is thought to be due to the production and growth of variant neoplastic populations over time (14). The mechanisms of variant production are probably multiple, but mutation is likely to be involved. Solid tumors are comprised not only of tumor cells but also of inflammatory cells, primarily macrophages and lymphocytes. This suggests that tumor-associated phagocytes (macrophages) might be a source of mutagen that could fuel variant production. As a beginning to exploring this hypothesis, we have tested the ability of tumor suspensions, and of adherent and nonadherent populations isolated from them, to enhance the mutation rate of bacteria in an Ames assay. Our tumor system is a series of subpopulation variant lines originally derived from a single, heterogeneous BALB/cfC3H mouse mammary tumor (4, 5).

MATERIALS AND METHODS

Mice. BALB/c mice were obtained from Charles River Breeding Laboratories (Wilmington, MA) or bred in our Animal Care Facility from breeding pairs obtained from the Cancer Research Laboratory (University of California, Berkeley, CA). They weighed approximately 20 g when inoculated with tumor. The mice were housed 6/cage and maintained on standard laboratory chow and tap water *ad libitum*. Mice were acclimated at least 2 weeks prior to experimental use.

Tumors. Lines 66, 67, and 168 were isolated from a single mammary tumor that arose spontaneously in a strain BALB/cfC3H breeding female (4). Line 410 was derived from a metastatic nodule isolated from the lung of a BALB/cfC3H mouse bearing the tenth s.c. passage of the original parent tumor (5). Line 410.4 is derived from the fourth transplant generation of the 410 tumor (11).

Media. Cell culture reagents were purchased from Grand Island Biological Co. (Grand Island, NY). Tumor cells were maintained in Waymouth's medium supplemented with 15% serum (7% horse, 7% newborn calf, 1% heat-inactivated fetal bovine), L-glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml) and buffered with $NaHCO_3$.

Cell Cultures. Tumor cell lines were maintained *in vitro* in 25-sq cm flasks (Corning Glass Works, Corning, NY), incubated at 37° in a humidified atmosphere of air and 5% CO_2, and passaged biweekly following exposure to 0.25% trypsin:EDTA. Tumor cells were found to be free of *Mycoplasma* and of the following murine viruses: pneumonia virus; reovirus type 3; Sendai virus; encephalomyelitis virus; K virus; polyoma virus; minute virus; mouse adenovirus; mouse hepatitis virus; lymphocytic choriomeningitis virus; and ectromelia virus (Microbiological Associates, Bethesda, MD).

Dissociation of Tumors. Mice were given injections s.c. in the inguinal region with 0.2 ml of a tumor cell suspension. When a tumor reached about 15 mm in diameter, which, depending upon the cell line and inoculum size, took 3 to 6 weeks, it was removed aseptically and kept in ice-cold minimal essential medium with Earle's salts. The tumor was quartered, necrotic material was removed, and the remainder was cut into small fragments with scissors. Disposable 11 surgical blades (Bard Parker, Rutherford, NJ) were used to reduce the fragments to 1-cu mm pieces, which were rinsed twice before being placed into a Prosil (PCR Research Chemicals, Gainesville, FL)-treated 100-ml round bottle. The digestion medium was HBSS[3] containing 0.15% dispase (type IX protease; Sigma Chemical Co., St. Louis, MO), 0.075% collagenase (type III; Millipore Corp., Freehold, NY), and 0.001% DNase (type I; Sigma). The fragments were digested at 37° with continuous shaking in an orbital water bath shaker (250 rpm). Four 20-min digestion periods were used, resulting in almost complete digestion of fragments and the production of a single cell suspension. Following each digestion period, the fragments were allowed to settle, and the supernatant material was poured through 3 layers of sterile gauze. The cells were washed in minimal essential medium with Earle's salts (10% horse serum), suspended in Ca^{2+}-Mg^{2+}-free HBSS, and kept on ice in polypropylene tubes. Viability (trypan blue exclusion) was usually greater than 85% in each fraction, so the fractions were pooled and counted.

Macrophage Isolation and Characterization. The tumor cell suspension (10^7 cells) was plated in 60-sq mm tissue culture dishes. Every 15 min, the plates were swirled to detach any loosely attached cells. At the end of 1 hr, nonadherent cells were removed by vigorous pipetting in HBSS. Trypsin:EDTA was added to the remaining cells for 5 min to remove adhering tumor cells and to select for the trypsin-resistant macrophages. The adherent cells were gently removed with a Teflon

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[3] The abbreviation used is: HBSS, Hanks' balanced salt solution.

policeman, washed, and counted. Most of the adherent cells were classified as macrophages by morphology, and >90% were positive for latex bead phagocytosis, Fc receptor, and nonspecific esterase, indicating a successful purification for cells with macrophage characteristics (9).

Ames Assay. This well-documented procedure (8) is a sensitive bacterial assay for detecting mutagens in which histidine-requiring auxotrophs of *Salmonella typhimurium* are plated in histidine-deficient agar. Revertant bacteria are detected by their ability to form colonies, reflecting a mutation at the histidine locus. Two tester strains were used; TA98 detects frame-shift mutations, whereas TA100 detects base-pair substitutions (8). Bacteria were maintained as frozen permanent cultures at −80° and on master plates at 4°. Tester strain cultures were inoculated into Oxoid Nutrient Broth 2 and incubated overnight at 37° in a shaker water bath. Cultures were allowed to grow to a density of 1 to 2×10^9 cells/ml.

Tumor cell suspensions in 0.1 ml were added to 0.1 ml of bacteria. Two ml of top agar (containing trace L-histidine and biotin) were added to each tube, mixed, and plated in a 100-mm-diameter Petri plate containing a bottom layer of minimal glucose agar. The trace of histidine in the top agar allows revertant and nonrevertant bacteria to undergo several divisions and form a lawn, whereas only revertant bacteria grow to form large, distinct colonies. The number of replicates for each test group was at least 3. Controls included plates with bacteria alone (spontaneous mutation) and plates with bacteria exposed to an appropriate mutagen for the different tester strains (positive controls). Four nmol of 2-naphthohydroxanic acid gave 518 ± 73 TA98 revertant colonies, whereas 15 nmol of sodium azide gave 266 ± 18 TA100 revertant colonies. All plates were incubated at 37° for 48 hr, at which time, revertant colonies were enumerated. A revertant index was calculated by dividing the number of revertant colonies observed in the presence of mammalian cells by the number of spontaneous revertant colonies.

Statistical Analysis. Comparison of the mean number of revertant colonies for different treatment groups was carried out using Student's *t* test. A test was considered positive for mutagenicity when the probability of significance for the difference between test and control values was <0.01.

RESULTS

Whole tumor cell suspensions, as well as adherent and nonadherent cells isolated from them, were tested for mutagenic activity on 2 *S. typhimurium* tester strains, TA98 and TA100. The number of cells tested per plate ranged from 2×10^5 to, in most cases, 5×10^6. Tumors of 4 different mammary tumor lines were tested. These lines, 410.4, 66, 67, and 168, were originally derived as subpopulations of one BALB/cfC3H mammary cancer.

TA98 data from 5 experiments with the most active line, 410.4, are presented in Table 1, and a summary of all TA98 experiments is shown in Table 2. As can be seen, whole suspensions of 410.4 tumors were consistently mutagenic, tumors of line 67 were mutagenic at the higher cell numbers, and tumors of lines 66 and 168 were mutagenic at only the highest numbers of cells tested. Overall, the frequency of positive tests declined with decreasing numbers of cells from whole tumors, from 68% (13 of 19) at the highest numbers tested to 27% (3 of 11) at the lowest. Table 2 also shows that the major mutagenic activity in the tumor suspensions was associated with the adherent cell population, of which >90% were macrophages. Although some activity was seen with the nonadherent cells, this was not consistent and was significant only with higher cell numbers. Macrophages from line 67 and 410.4 tumors were mutagenic at all cell numbers tested, whereas line 66 macrophages were mutagenic only at the lowest cell number, and line 168 macrophages were essentially nonmutagenic.

In order to show that the TA98 colonies that grew out in the above assays were indeed true revertants, individual bacterial colonies from plates of histidine-deficient agar were inoculated into histidine-deficient or whole nutrient broth and observed for 48 hr. Scrapings of agar containing the background lawn of nonrevertant bacteria were also tested. Bacterial growth was observed only in histidine-deficient media inoculated with the colonies. Both colonies and lawn grew in histidine-supplemented media. Thus, the growth of TA98 in the presence of cells from tumors was not the result of histidine supplementation by the mammalian cells.

Results of tests with the TA100 tester strain of *S. typhimurium* were different from those with TA98. Mutagenic activity was not detected with any cells from Tumors 66, 168, or 410.4. Significant activity was seen, however, with higher numbers of 67 adherent cells (Table 3).

DISCUSSION

The association between inflammation and the development of cancer is once again the subject of active research. Emphasis is being placed on the role of phagocytic cells in carcinogenesis, principally through the production of oxygen metabolites such as superoxide anions, H_2O_2, and hydroxy radicals (1). These metabolites are released by phagocytic cells (13) and are also suspected to be involved in carcinogenesis, both as initiators

Table 1
Mutagenicity of cells from line 410.4 tumors assessed by S. typhimurium TA98

		No. of revertants/plate in the presence of					
		Unfractionated cells		Adherent cells		Nonadherent cells	
Experiment	No tumor cells	6×10^5	2×10^5	6×10^5	2×10^5	6×10^5	2×10^5
1	106, 116	181, 183, 193[a]	ND[b]	116, 120, 127	143, 151, 154[c]	151, 154, 158[c]	131, 134, 146
2	112, 122	171, 177, 181[a]	ND	177, 183, 191[a]	121, 131, 133	114, 119, 128	134, 138, 150
3	94, 101	159, 162, 171[a]	144, 161, 163[c]	ND	121, 129, 132[c]	131, 140, 141[c]	111, 123, 129
4	88, 96	148, 149, 165[c]	142, 159, 160[c]	ND	119, 126, 131[c]	ND	114, 118, 132
5	90, 103	159, 162, 171[a]	158, 161, 167[a]	ND	122, 131, 136[c]	ND	116, 117, 126

[a] $p < 0.001$.
[b] ND, not determined.
[c] $p < 0.01$.

Table 2

Summary of mutagenicity assays on S. typhimurium TA98 with cells from tumors of 4 mammary tumor lines

Tumor line	No. of cells	Revertant index: Unfractionated cells	Adherent cells	Nonadherent cells
410.4	1–5 × 10^6	2.5 ± 0.5[a] (4/5)[b]	ND[c]	1.1 (0/1)
	6 × 10^5	1.7 ± 0.03 (5/5)	1.4 ± 0.2 (1/2)	1.3 ± 0.1 (2/3)
	2 × 10^5	1.7 ± 0.03 (3/3)	1.3 ± 0.05 (4/5)	1.2 ± 0.02 (0/5)
67	2 × 10^6	1.6 ± 0.03 (2/2)	2.2 (1/1)	ND
	6 × 10^5	1.5 ± 0.06 (3/3)	2.0 ± 0.06 (3/3)	1.3 (0/1)
	2 × 10^5	1.1 ± 0.04 (0/3)	1.4 ± 0.1 (3/3)	1.0 ± 0.05 (0/3)
168	5 × 10^6	1.6 ± 0.4 (1/2)	1.0 (0/1)	1.4 (0/1)
	5–6 × 10^5	1.1 ± 0.05 (0/5)	1.4 ± 0.3 (1/3)	1.2 ± 0.08 (0/4)
	2 × 10^5	1.0 ± 0.1 (0/4)	1.0 ± 0.001 (0/2)	0.8 ± 0.04 (0/3)
66	1–5 × 10^6	1.9 ± 0.3 (6/10)	1.1 ± 0.1 (0/4)	1.1 ± 0.2 (1/3)
	5–6 × 10^5	1.2 ± 0.1 (0/3)	1.2 ± 0.04 (0/2)	1.0 ± 0.1 (0/2)
	2–2.5 × 10^5	1.2 (0/1)	1.6 ± 0.1 (2/2)	ND

[a] Mean ± S.E. of the ratio of the number of revertants in test to the number of revertants in "no cells" control.
[b] Numbers in parentheses, number of experiments in which the number of revertants in the test was significantly greater ($p < 0.01$) than in the control/total number of experiments performed.
[c] ND, not determined.

Table 3

Mutagenicity of cells from line 67 tumors assessed by S. typhimurium TA100

Experiment	No tumor cells	No. of revertants/plate in the presence of: Unfractionated cells 2 × 10^6	Unfractionated cells 6 × 10^5	Unfractionated cells 2 × 10^5	Adherent cells 6 × 10^5	Adherent cells 2 × 10^5	Nonadherent cells 6 × 10^5	Nonadherent cells 2 × 10^5
1	169, 178	ND[a]	171, 173, 183 (1.0)[b]	161, 162, 177 (1.0)	243, 246, 261 (1.4)[c]	183, 191, 196 (1.1)	ND	181, 186, 194 (1.1)
2	170, 182	196, 199, 208, (1.1)	191, 197, 199 (1.1)	176, 190, 191 (1.1)	251, 252, 273 (1.5)[c]	189, 191, 206 (1.1)	214, 218, 226 (1.2)[c]	191, 197, 211 (1.1)
3	169, 186	198, 203, 209 (1.1)	184, 191, 194 (1.1)	181, 190 (1.0)	254, 259, 271 (1.5)[c]	192, 198 (1.1)	ND	189, 200, 201 (1.1)

[a] ND, not determined.
[b] Numbers in parentheses, mean revertant index, *i.e.*, ratio of the number of revertants in test to number of revertants in "no cells" control.
[c] $p < 0.001$.

and promoters (1). Weitzman and Stossel (19) showed that phagocytes from normal human peripheral blood were mutagenic to bacteria in the Ames assay and presented evidence in support of the role of hydroxyl radicals in the process (20). Similar data have been published recently by Barak *et al.* (3). Weitberg *et al.* (18) extended these findings by showing that human blood phagocytes are able to induce DNA strand breaks in cultured mammalian cells, again apparently through a mechanism involving oxygen metabolites.

We here show that tumor cell suspensions can increase mutation frequency in a standard bacterial test system and that the source of the mutagenic activity is tumor-associated macrophages. The magnitude of activity is very similar to that reported by Weitzman and Stossel (19, 20), although in our results, there is a divergence between the frame-shift tester strain TA98 *versus* the base-pair substitution strain TA100. We observed mutagenic activity more frequently with the TA98 strain, and one of the tumor lines most active on TA98, namely line 410.4, was inactive on TA100. Furthermore, significant mutagenic activity was only seen with isolated adherent cells from the TA100 active line 67 and not with the whole tumor cell suspensions, suggesting an enrichment for the active cell. These results suggest that more than one mutagen may be involved, with different tumors being the source of different mutagens. We have reported previously considerable heterogeneity in macrophages isolated from the same tumors used in the present experiments (10). These macrophages were found to differ in physical and biochemical properties (10), as well as in their state of activation (9). Experiments are in progress to see whether different macrophage subpopulations produce different mutagens and whether these mutagens are oxygen metabolites. Preliminary results indicate that activated peritonal macrophages are mutagenic to mammalian cells by reason of release of OH^-.[4]

The fact that macrophages from different sources (*e.g.*, Tumor 67 *versus* 168; Table 2) are not equally active against different bacterial tester strains (TA98 *versus* TA100) argues against a nonspecific explanation, such as a histidine feeder effect as the mechanism responsible for our observations. Parenthetically, it should be pointed out that the TA98 and TA100 experiments were run simultaneously using the same cell preparations. In addition, we have shown that the bacterial mutants can be subcultured in histidine-free medium and hence are true revertants.

Our experiments were initially carried out with tumor lines

[4] K. Yamashina and G. H. Heppner, unpublished observations.

410.4, 66, and 168, and the results suggested that mutagenic activity was more often associated with macrophages from metastatic than from nonmetastatic mammary tumors (6). Both tumor lines 410.4 and 66 metastasize spontaneously to the lung at high frequency, whereas line 168 tumors are essentially nonmetastatic from a s.c. or mammary fat pad site (11). We now know the correlation is an imperfect one, in that line 67 tumors are also nonmetastatic, although their macrophages are mutagenic. Furthermore, comparison of the data reported here with those on the tumoricidal activity of tumor-associated macrophages (9) shows that cytotoxic activity is also not necessarily correlated with mutagenic activity, although the oxygen metabolite, H_2O_2, is released by activated macrophages and is also cytotoxic (13, 15). Again, Tumor 67 is the "oddball" in that its macrophages are not cytotoxic but are mutagenic, whereas both activities are coordinate in the other tumors.

Clearly, a causal association between tumor progression and production of mutagens by macrophages is only speculative at this point. Although our data indicate that tumors are differentially associated with mutagenic macrophages, we do not know the role this activity plays in the biology of the different tumors. Although we have preliminary evidence that mammalian tumor cells are susceptible to the activity of macrophage-produced mutagens, see above, we do not know whether these mutagens will be active in the tumor environment. The inverse relationship noted in the line 66 experiments (Table 2) between the number of adherent cells and detection of mutagenicity is similar to that reported by Barak *et al.* (3) and suggests the presence of a minor subpopulation of inhibitory cells in the adherent population. Weitzman and Stossel (20) also noted that mutagenicity can be altered by the cellular environment. Nevertheless, the increasing evidence linking inflammatory events with initiation, the data showing that the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate is chemotactic for macrophages and able to alter their function (7, 16), and now our demonstration of macrophage mutagenic activity in growing neoplasms all focus attention on the continuing role of inflammatory cells in cancer development.

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REFERENCES

1. Ames, B. N. Dietary carcinogens and anticarcinogens. Science (Wash. DC), *221:* 1256–1264, 1983.
2. Argyris, T. S., and Slaga, T. J. Promotion of carcinomas by repeated abrasion in initiated skin of mice. Cancer Res., *41:* 5193–5195, 1981.
3. Barak, M., Ulitzur, S., and Merzbach, D. Phagocytosis-induced mutagenesis in bacteria. Mutat. Res., *121:* 7–16, 1983.
4. Dexter, D. L., Kowalski, H. M., Blazar, B. A., Fligiel, Z., Vogel, R., and Heppner, G. H. Heterogeneity of tumor cells from a single mouse mammary tumor. Cancer Res., *38:* 3174–3181, 1978.
5. Heppner, G. H., Dexter, D. L., DeNucci, T., Miller, F. R., and Calabresi, P. Heterogeneity in drug sensitivity among tumor cell subpopulations of a single mouse mammary tumor. Cancer Res., *38:* 3758–3763, 1978.
6. Heppner, G. H., Loveless, S. E., Miller, F. R., Mahoney, K. H., and Fulton, A. M. Mammary tumor heterogeneity. *In*: G. L. Nicolson and L. Milas (eds.), Cancer Invasion and Metastasis, pp. 209–221. New York: Raven Press, 1984.
7. Laskin, D. L., Laskin, J. D., Kessler, F. K., Weinstein, I. B., and Carchman, R. A. Enhancement of macrophage-induced cytotoxicity by phorbol ester tumor promoters. Cancer Res., *41:* 4523–4528, 1981.
8. Levin, D. E., Hollstein, M., Christman, M. F., Schwiers, E. A., and Ames, B. N. A new *Salmonella* tester strain (TA 102) with A·T base pairs at the site of mutation detects oxidative mutagens. Proc. Natl. Acad. Sci. USA *79:* 7445–7449, 1982.
9. Loveless, S. E., and Heppner, G. H. Tumor-associated macrophages of mouse mammary tumors. I. Differential cytotoxicity of macrophages from metastatic and nonmetastatic tumors. J. Immunol., *131:* 2074–2078, 1983.
10. Mahoney, K. H., Fulton, A. M., and Heppner, G. H. Tumor-associated macrophages of mouse mammary tumors. II. Differential distribution of macrophages from metastatic and nonmetastatic tumors. J. Immunol., *131:* 2079–2085, 1983.
11. Miller, F. R., Miller, B. E., and Heppner, G. H. Characterization of metastatic heterogeneity among subpopulations of a single mouse mammary tumor: heterogeneity in phenotypic stability. Invasion Metastasis, *3:* 22–31, 1983.
12. Murasaki, G., and Cohen, S. M. Effect of sodium saccharin on urinary bladder epithelial regenerative hyperplasia following freeze ulceration. Cancer Res., *43:* 182–187, 1983.
13. Nathan, C. F., Silverstein, S. C., Brukner, L. H., and Cohn, Z. A. Extracellular cytolysis by activated macrophages and granulocytes. II. Hydrogen peroxide as a mediator of cytotoxicity. J. Exp. Med., *149:* 100–113, 1979.
14. Nowell, P. C. The clonal evolution of tumor cell populations. Science (Wash. DC), *194:* 23–28, 1976.
15. Simon, R. H., Scoggin, C. H., and Patterson, D. Hydrogen peroxide causes the fatal injury to human fibroblasts exposed to oxygen radicals. J. Biol. Chem., *256:* 7181–7186, 1981.
16. Sturm, R. J., Smith, B. M., Lane, R. W., Laskin, D. L., Harris, L. S., and Carchman, R. A. Antagonist of phorbol ester receptor-mediated chemotaxis in mouse peritoneal macrophages. Cancer Res., *43:* 4552–4556, 1983.
17. Vasiliev, J. M., and Moizhess, T. G. Tumorigenicity of sarcoma cells is enhanced by the local environment of implanted foreign body. Int. J. Cancer, *30:* 525–529, 1982.
18. Weitberg, A. B., Weitzman, S. A., Destrempes, M., Latt, S. A., and Stossel, T. P. Stimulated human phagocytes produce cytogenetic changes in cultured mammalian cells. N. Engl. J. Med., *308:* 26–30, 1983.
19. Weitzman, S. A., and Stossel, T. P. Mutation caused by human phagocytes. Science (Wash. DC), *212:* 546–547, 1981.
20. Weitzman, S. A., and Stossel, T. P. Effects of oxygen radical scavengers and antioxidants on phagocyte-induced mutagenesis. J. Immunol., *128:* 2770–2772, 1982.