BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **251,** 182–189 (1998)
ARTICLE NO. RC989428

# Morphological Transformation of C3H/M2 Mouse Fibroblasts by Extracts of Human Mammary Lipid

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Received September 4, 1998

**Mammary lipid may act as a reservoir for genotoxins. Mammary lipid extracts (MLEs), obtained from eight UK women (21–41 years) undergoing reduction mammoplasty, were examined for their abilities to morphologically transform C3H/M2 mouse fibroblasts. Resultant transformation rates were 0.27, 0.33, 0.07, 0.29, 0.21, 0.00, 0.07, and 0.13 transformed foci/treated dish, respectively. Although the lipid-extraction procedure used was originally designed to extract heterocyclic aromatic amines (HAAs), liquid chromatography/mass spectroscopy (LC/MS) with selective ion monitoring has failed to detect HAAs in any of the lipid extracts so far examined. Genotoxicities were also assessed in *S. typhimurium* TA98 and in metabolically competent human (MCL-5) cells by the micronucleus and by the alkaline single-cell gel ("comet") assays. The MLEs induced bacterial mutagenicity rates ranging from 0 to 498 revertants/plate/g-lipid equivalent and micronucleus-formation rates from 0 to 20 micronuclei/500 binucleate cells/g-lipid. Median comet tail lengths (induced with MLEs of 8.0 g-lipid equivalent) ranged from 6.0 to 74.0 $\mu$m. The results demonstrate the presence of as-yet-unidentified transforming agents in mammary lipid.** 

Breast cancer is a major cause of cancer-related deaths in women resident in the Western world (1). The majority of these cases are considered sporadic, as distinct from the small proportion (~5–10%) of familial breast cancers that result from inherited high-penetrance breast cancer susceptibility genes (2). The only environmental agent proven to induce breast cancer in women is ionizing radiation (3). However, global differences in breast cancer incidence and studies of migrating populations suggest that environmental and/or dietary factors play a significant role (4).

The morphological structure of the breast is unique in that functional elements are embedded in a variable fatty stroma (70–90% breast mass). At the ends of these elements are small saccular evaginations lined with a single layer of epithelial cells. It is from these cuboidal or low columnar cells that most breast cancers arise. Throughout adult life, the glandular tissue of the breast in non-lactating women secretes and reabsorbs fluid (5). Such events may result in exposure of mammary epithelial cells to xenobiotics (6). Compounds of endogenous or exogenous origin have been detected in breast fluids of adult women and some of these compounds may be genotoxic (7).

Malignant transformation *in vivo* is a multistage process involving initiation and promotional events driven by genetic and epigenetic alterations (8). Chronic exposure to xenobiotics is most likely pivotal in these processes. During promotion, a clonal population of irreversibly-initiated cells is expanded and ultimately progresses to malignancy (8). Numerous chemicals present in the human environment are rodent mammary carcinogens, including heterocyclic aromatic amines (HAAs; the 'food mutagens') formed during the cooking of protein-rich foods, polycyclic aromatic hydrocarbons (PAHs), nitro-PAHs and several halogenated pesticides (9–11).

Sporadic breast cancers are associated with mutations clustered within exons 5-8 of the *p*53 gene, and

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Abbreviations used: PAH, polycyclic aromatic hydrocarbon; DMSO, dimethyl sulfoxide; MLE, mammary lipid extract; SSB, single-strand break; HAA, heterocyclic aromatic amine; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; MCA, 3-methylcholanthrene; 7,8-DiMeIQx, 2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; 4-MeIQ, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline; LC/MS, liquid chromatography/mass spectrometry.

0006-291X/98 $25.00

include point mutations uncharacteristic of endogenous mutagenic processes alone; these mutations are believed to be xenobiotic-induced mutagenic events (2, 12). Although a number of risk factors have been associated with breast cancer the aetiology of this disease still remains obscure (13). Total cumulative exposure to oestrogen may play a significant role but oestrogens are most probably incomplete carcinogens that act as tumour promoters (1).

We have recently detected genotoxic components in mammary lipid extracts (MLEs) obtained from healthy pre-menopausal women undergoing elective reduction mammoplasty (14, 15). In the present study MLEs prepared from eight donors have been examined for their ability to morphologically transform C3H/M2 mouse fibroblasts and extracts have been tested for the presence of HAAs. The MLEs were also examined for their abilities to induce (a) reverse mutation in *S. typhimurium* TA98, (b) micronucleus formation in metabolically-competent human (MCL-5) cells, and (c) DNA single-strand breaks (SSBs) in MCL-5 cells, detected by the alkaline single-cell gel ('comet') assay. The results demonstrate the activity of MLEs in inducing the morphological transformation of mammalian cells *in vitro* and the apparent absence of HAAs from any of the MLEs examined to date.

## MATERIALS AND METHODS

*Donor tissues.* Tissues were obtained from UK-resident women (21–41 years) undergoing elective reduction mammoplasty. Excised tissues were examined for macroscopic pathological abnormalities. Only tissues found to be normal were used.

*Mammary lipid extracts.* Mammary lipid was obtained following enzymatic digestion of tissues (16) and MLEs prepared by subjecting lipid (4-g equivalent) to a solid-phase tandem extraction procedure (17). Lipid was first homogenised with an Ultra Turrax homogeniser in NaOH (1 M, 10 ml) at high speed for 1-2 min, after which the saponified lipid was extracted (17). The column eluates in methanolic ammonia (9:1 (v/v) 3 ml) were evaporated to dryness in a Savant Speed Vac concentrator and the residues resuspended in dimethyl sulfoxide (DMSO) prior to analyses. MLEs or DMSO alone, as a control, were incorporated into all assays at a DMSO concentration that did not exceed 5% (v/v).

*Morphological transformation of C3H/M2 mouse fibroblasts in vitro.* Cells harvested from logarithmically-growing stock cultures (between passages 5 and 20) were plated on day 0 into 60-mm dishes containing basal Eagle's medium supplemented with 10% fetal calf serum to determine either their plating efficiency (100 cells/dish) or their transformation rate (1000 cells/dish) (8). After 24 h, the cultures were treated for a further 24 h with individual MLEs. Initiating agents variously used as positive controls were *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (0.5 $\mu$g/ml) or 3-methylcholanthrene (MCA) (10.0 $\mu$g/ml). DMSO (5%) was used as a solvent control. Media were changed twice a week. The cells were fixed and stained after 2 weeks to determine plating efficiency or after 8 weeks to determine the transformation rate. Morphologically-transformed foci of C3H/M2 mouse fibroblasts have been previously shown to form tumours and to metastasise in nude mice (18). Increases in transformation frequencies were tested for significance using a $\chi^2$ test for trend. Individual transformation frequencies were tested for statistical significance using a Fisher exact test (one-tailed *P*-values).

*Liquid chromatography/mass spectrometry (LC/MS) analysis of MLEs.* Extracts in methanolic ammonia [9:1 (v/v), 3 ml] were evaporated to dryness under a stream of nitrogen at 40°C and the residues resuspended in 200 $\mu$l of buffer B (see below). MLEs were examined using LC/MS with selective ion monitoring in conjunction with the retention times of known HAAs as previously described (19). Briefly, HPLC was performed with a Waters 600-MS pump, a Waters 486-MS UV detector and a Waters 717 autosampler injecting 20 $\mu$l of the sample. The column was a ToyoSoda TSK ODS-Super HPLC column (100 × 4.6 mm i.d., particle size 2 $\mu$m), protected with a pre-column containing the same stationary phase. The ternary mobile phases were composed as follows: solvent A, 10 mM ammonium acetate adjusted with HCl to pH 3.2; solvent B, 10 mM ammonium acetate adjusted with HCl to pH 4.0; solvent C, acetonitrile. The mobile phase gradient program (time: %A-%B-%C) was as follows: 0 min 95-0-5; 2 min 92-0-8; 2.1 min 0-92-8; 13 min 0-70-30; 20 min 0-40-60. The flow rate was 1 ml/min and was split 1/10 before entering a Finnigan TSQ-700 mass spectrometer (Bremen, Germany) equipped with a Finnigan electrospray interface working at the high voltage of 4.5 kV. The manifold temperature was 70°C and the heated capillary was set at 250°C. Nitrogen was used as sheath gas at a pressure of 4.8 bar. Compounds were detected by monitoring the protonated molecular ions $[M + H]^+$.

*Bacterial mutagenicity.* MLEs were assessed for mutagenicity towards *S. typhimurium* TA98 by the plate-incorporation assay. Assays were performed in the presence of Aroclor 1254-induced rat-liver S9 prepared from male Wistar rats with the final S9 mix (30% v/v) containing 33 mM KCl, 8 mM $MgCl_2$, 5 mM glucose-6-phosphate, 3.3 mM $NADP^+$, 0.1 M phosphate buffer, pH 7.4 (20). Revertants/plate were recorded following 72 h incubation at 37°C, using a calibrated Biotran II Automatic Colony Counter. MLEs were considered positive if revertants/plate on treated plates were at least double spontaneous rates, with a dose–response effect.

*Clastogenicity.* MCL-5 cells, a line of human lymphoblastoid cells genetically engineered to express enzymes that metabolise a wide range of xenobiotic compounds, were obtained under license from the Gentest Corporation (Woburn, MA). Micronucleus induction in cells blocked at cytokinesis by cytochalasin-B was used as an indicator of chromosomal damage (21). Cell cultures were treated with a minimum of two concentrations of MLEs in DMSO (maximum 100 $\mu$l in 10 ml medium) or with DMSO alone (solvent control) for 24 h at 37°C. They were then treated with 6 $\mu$g/ml cytochalasin-B for a further 24 h, fixed and stained (21). Five hundred binucleate cells were scored for micronuclei. The percentage of binucleate cells was used as an index of cytotoxicity. Increases were tested for significance using a $\chi^2$ test for trend (21).

*Single-cell gel electrophoresis ('comet') assay.* MCL-5 cell suspensions in PBS ($\sim 1 \times 10^5$ cells/75 $\mu$l) were incubated in the presence of the DNA-repair inhibitors, hydroxyurea (HU, 10 mM) and cytosine arabinoside (ara-C, 1.8 mM), at 37°C for 30 min (15) with or without individual MLEs. Cell lysis and alkaline nuclear electrophoresis were performed exactly as described (15). Frosted microscope slides (Curtin Matheson Scientific, Inc., Houston, TX), on which cells were embedded in an agarose sandwich, were submerged in cold alkaline lysis solution (2.5 M NaCl, 100 mM EDTA disodium salt, 10 mM Tris, 1% N-lauroyl sarcosine, adjusted to pH 10 with NaOH; then made up to 1% Triton X-100 and 10% DMSO before use), protected from light and stored at 4°C for at least 1 h. Under red light, slides were transferred to a horizontal electrophoresis tank, covered in alkaline electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH 12.3), and stored in a chilled incubator at 10°C for 40 min to allow unwinding of the DNA before electrophoresis at 0.8 V/cm and 300 mA for 36 min. Slides were neutralised (Tris, 0.5 M, pH 7.5), stained with ethidium bromide (20 ng/ml) and nuclear material was visualised by

**TABLE 1**

Induction of Morphological Transformation in C3H/M2 Mouse Fibroblasts by Human Mammary Lipid Extracts

| Donor No. | 1[e] | | 2[e] | | 3 | | 4 | | 5 | | 6 | | 7 | | 8[i] | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| mg-lipid equivalent[a] | PE[b] | Tx[c] | PE | Tx | PE | Tx | PE | Tx | PE | Tx | PE | Tx | PE | Tx | PE | Tx |
| 0 | 49 | 0/14 (0.00)[d] | 38 | 0/14 (0.00) | 38 | 0/15 (0.00) | 38 | 0/15 (0.00) | 41 | 0/15 (0.00) | 46 | 0/13 (0.00) | 43 | 0/15 (0.00) | 43 | 0/14 (0.00) |
| 30 | 50 | 2/15 (0.13) | 41 | 2/15 (0.13) | 32 | 1/14 (0.07) | 36 | 2/15 (0.13) | 41 | 3/14 (0.21) | 51 | 0/10 (0.00) | 39 | 0/15 (0.00) | 39 | 0/7 (0.00) |
| 70 | 40 | 4/15[f] (0.27) | 34 | 5/15[f] (0.33) | 34 | 0/14 (0.00) | 30 | 3/15 (0.20) | 33 | 3/14[f] (0.21) | 52 | 0/15 (0.00) | 34 | 1/15 (0.07) | 34 | 1/10 (0.10) |
| 80 | n.d. | n.d. | n.d. | n.d. | 36 | 0/14 (0.00) | 34 | 2/7[f] (0.29) | 37 | 2/15 (0.13) | 48 | 0/13 (0.00) | 36 | 1/15 (0.07) | 36 | 2/15 (0.13) |
| 110 | 0 | — | 33 | 2/14 (0.14) | 30 | 0/14 (0.00) | 29 | 2/15 (0.13) | 34 | 1/14 (0.07) | 41 | 0/14 (0.00) | 31 | 0/14 (0.00) | n.d. | n.d. |
| MNNG (0.5 μg/ml) | 40 | 11/14[h] (0.78) | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 33 | 8/15[g] (0.53) | 46 | 5/14[f] (0.36) | 33 | 5/15[f] (0.33) | 33 | 4/10[g] (0.4) |
| MCA (10 μg/ml) | n.d. | n.d. | 38 | 6/13[g] (0.46) | 25 | 4/15[f] (0.27) | 25 | 4/15[f] (0.27) | 34 | 4/12[f] (0.33) | 48 | 3/9[f] (0.33) | n.d. | n.d. | n.d. | n.d. |

[a] Human mammary lipid samples were obtained following collagenase digestion of mammary tissue (16) and mammary lipid extracts (MLEs), prepared from different individuals using the tandem extraction procedure (17), were added as solutions in DMSO.
[b] PE, plating efficiency (%).
[c] Tx, number of transformed foci per treated dishes.
[d] Numbers in parentheses, numerical values of the ratio (transformed foci:treated dish). n.d., not determined; —, cell survival too low due to cytotoxicity.
[e] Significance in the $\chi^2$ test for trend, $P < 0.05$.
[f] Significance in the Fisher exact test, $P < 0.05$.
[g] Significance in the Fisher exact test, $P < 0.01$.
[h] Significance in the Fisher exact test, $P < 0.0001$.
[i] Indicates a dose response over the range 0, 5, 25, and 50 mg-lipid equivalent.

epifluorescence using a Leitz Laborlux S microscope. Images were digitized, and DNA damage was expressed as comet tail length (μm) (15). A total of 50 nuclei/data point from two slides was scored. Increases were assessed for significance using the Mann–Whitney test.

## RESULTS

*Morphological transformation.* The transforming potential of MLEs *in vitro* were studied using C3H/M2 mouse fibroblasts and compared in different experiments with positive controls, MNNG and MCA (Table 1). Interindividual variations were observed in the ability of MLEs to induce morphological transformation. Transformation rates in C3H/M2 mouse fibroblasts of 0.27 (70 mg), 0.33 (70 mg), 0.07 (30 mg), 0.29 (80 mg), 0.21 (30 mg), 0.00 (110 mg), 0.07 (70 mg), and 0.13 (50 mg) transformed foci/treated dish (mg-lipid equivalent), respectively were obtained from donors 1 to 8. Of these, MLEs from donors 1 and 2 gave significant evidence of a dose response ($\chi^2$ test for trend, $P < 0.05$). Transformation rates at individual MLE mg-lipid equivalents were tested for significance (Fisher exact test, $P < 0.05$) and significant increases were obtained with MLEs from donors 1, 2, 4 and 5 (Table 1).

*LC/MS analysis.* Three MLEs (71, 51, and 43 g-lipid equivalent) that had been found to be mutagenic (including donors 2 and 6) and 2 inactive MLEs (46 and 53 g-lipid equivalent) (including donor 8) were investigated by LC/MS analysis for the presence of known HAAs. Parallel samples were spiked with 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ; 5.0 μg) or 2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline (7,8 DiMeIQx; 2.3 μg), or a combination of both, either before or after extraction. As already described (19), HPLC-MS is able to separate and detect 13 mutagenic HAAs, viz. IQ, IQx, 4-MeIQ, MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx, PhIP, Glu-P-1, Glu-P-2, Trp-P-1, Trp-P-2, amino-α-carboline and methyl-amino-α-carboline as well as the non-mutagens, norharman and harman. For each HAA, the detection limit by selective ion monitoring is ~100 pg (pure compound injected). LC/MS analysis indicated the presence of both IQ and 7,8 DiMeIQx in all the samples that had been spiked either before or after extraction. However, LC/MS analysis of the unspiked MLEs for the presence of recognised HAAs, carried out under the same conditions, failed to detect any of the above-mentioned HAAs (data not shown).

*Bacterial mutagenicity.* MLEs from eight women were investigated for mutagenic activity towards *S. typhimurium* TA98. MLEs obtained from 5 of the 8

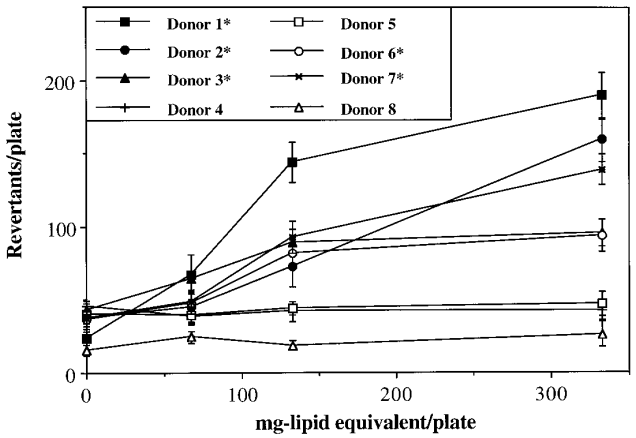


**FIG. 1.** Dose–response curves for bacterial mutagenicity of mammary lipid extracts (MLEs) obtained from donated breast tissues. The respective ages of each donor at the time of tissue donation were as follows: donor 1 (age 41 years); donor 2 (38 years); donor 3 (29 years); donor 4 (41 years); donor 5 (21 years); donor 6 (41 years); donor 7 (31 years); donor 8 (24 years). Bacterial mutagenicity assays were performed in the presence of S9 as described in Materials and Methods using *S. typhimurium* TA98. Each point represents mean revertants per plate ± SD from three plates. *Mutagenic MLEs.

donors examined elicited a significant mutagenic response whereas MLEs from the other 3 did not (Fig. 1). An exogenous metabolic activation system (Aroclor 1254-induced rat-liver S9) was found to be a requirement for the positive bacterial mutagenicity, since no mutagenic activity was observed in its absence (data not shown).

Normalized values (revertants/plate/g-lipid equivalent) were calculated by taking revertant rates (189, 159, 95, 42, 46, 93, 139, and 26 from donors 1–8, respectively) obtained with donor MLEs, subtracting the corresponding spontaneous revertant rates (24, 37, 43, 45, 40, 36, 38, and 15 from experiment 1–8, respectively) and correcting for the dilution factor and g-lipid equivalent. Inspection of these normalized values revealed appreciable inter-individual variations in bacterial mutagenicity, ranging from 157 to 498 revertants/plate/g-lipid equivalent obtained with mutagenically-positive MLEs. A range of 0 to 32 revertants/plate/g-lipid equivalent was obtained with the three MLEs classed as inactive in mutation assays.

*Micronucleus formation in MCL-5 cells.* Dose–response curves for micronucleus formation and cytotoxicity following treatment of MCL-5 cells with MLEs obtained from the eight donors are given in Figs. 2A and 2B. Six of the eight MLEs tested showed significant micronucleus-forming activity as judged by a $\chi^2$ test for trend in a dose response. Induction of micronucleus formation was normalized by taking micronucleus rates (22, 19, 25, 7, 5, 14, 26, and 31 from donors 1–8, respectively) obtained with the donor MLEs examined, subtracting corresponding spontaneous micronucleus rates in 500 binucleate cells (6, 6, 5, 6, 5, 5, 5, and 5 from experiment 1–8, respectively) and correcting for dilution factor and g-lipid equivalent. Normalized micronucleus-forming activities showed considerable variation between individuals, with significant values ranging from 7 to 20 micronuclei/500 binucleate cells/g-lipid equivalent, and values ranging from 0 to 1 micronuclei/500 binucleate cells/g-lipid equivalent in MLEs that were classed as inactive. Values for cytotoxicity, expressed as the percentage of binucleate cells (21), were 47, 71, 61, 51, 80, and 42% of control values for positive MLEs and, 92 and 95% for MLEs that were classed as inactive.

*Comet formation in MCL-5 cells.* Incorporation of the DNA-repair inhibitors HU and ara-C enhances

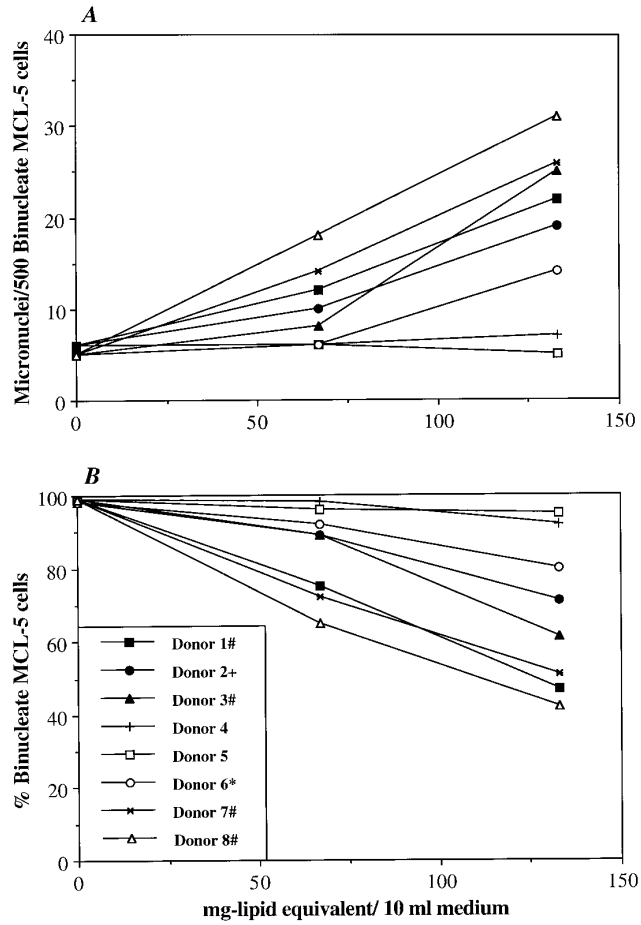


**FIG. 2.** Dose–response curves for clastogenicity of mammary lipid extracts (MLEs) obtained from donated breast tissues. A, Micronucleus formation (micronuclei/500 binucleate cells) and B, cytotoxicity (% binucleate cells) were determined in MCL-5 cells following treatment as described under Materials and Methods. *Significance in the $\chi^2$ test for trend, $P < 0.05$. +Significance in the $\chi^2$ test for trend, $P < 0.01$. #Significance in the $\chi^2$ test for trend, $P < 0.005$.

the sensitivity of the alkaline elution assay for SSBs in DNA (15). Untreated MCL-5 cells in the presence of HU/ara-C exhibit a very low level of DNA migration (Fig. 3B) that was not significantly different from that observed in the absence of repair inhibitors (Fig. 3A) (median comet tail lengths 5.0 and 3.0 $\mu$m respectively). A large inter-individual variation in comet-forming activity of MLEs was observed following treatment of MCL-5 cells, in the presence of HU/ara-C (Figs. 3C–3J). MLEs from donors 1-5, 7 and 8 (Figs. 3C–3G, 3I, and 3J) induced significant increases in median comet tail lengths (53.5, 62.0, 16.0, 14.0, 8.5, 32.5 and 74.0 $\mu$m, respectively). The remaining MLE (donor 6, Fig. 3H) did not induce significant increases in comet formation (median comet tail length 6.0 $\mu$m).

*Comparison between morphological transformation and genotoxicity.* An apparent lack of correlation between any one of the three genotoxic end-points and morphological transformation was observed (Fig. 4). In some instances, high levels of genotoxicity, as measured by any or all of the end-points, was associated with morphological transformation (donors 1 and 2). In other instances, MLEs that exhibited low levels of genotoxicity induced morphological transformation (donors 4 and 5) whereas an MLE from donor 6 was inactive in the transformation assay despite being mutagenic and clastogenic. In addition, an MLE from donor 8 induced both SSBs and micronucleus formation while an MLE from donor 3 was only clastogenic: however MLEs from both donors induced morphological transformation. MLEs from donors 1-6 were previously tested for bacterial mutagenicity and clastogenicity in MCL-5 cells (14), and MLEs from donors 1, 7 and 8 were previously tested for comet-forming activity (15). In all instances, the present results concur with previous observations.

## DISCUSSION

Our working hypothesis is that human mammary lipid contains fat-soluble carcinogens, thus exposing adjacent epithelial cell populations to their effects (14, 15). Although many studies have focused on tissues removed from breast cancer patients (22, 23), genotoxic events occurring years before the detection of overt disease may be involved in the initiation of sporadic breast cancers. There is already evidence (bacterial mutagenicity and micronucleus formation in human cells) that genotoxic components existed in some 40% of mammary lipid samples obtained from young, healthy women (14). We now show that MLEs from 7 out of 8 such donors are capable of inducing morphological transformation in C3H/M2 mouse fibroblasts but that none of the MLEs so far examined, using LC/MS with selective ion monitoring, appeared to contain detectable concentrations of any of 15 HAAs.

Mammalian cell transformation assays have been developed as *in vitro* test systems which may predict the carcinogenic potential of chemical, biological and physical agents (24). Enhanced expression of the protooncogenes *c-myc* and *c-fos* is associated with genotoxic mechanisms (25) and persistent *c-myc* levels correlate with anchorage-independent growth and transformed focus formation in chemically-transformed C3H 10T1/2 mouse embryo fibroblasts (26).

The transformed foci of C3H/M2 cells obtained in the present studies following treatment with MLEs have not, so far, been tested for their abilities to form tumours in nude mice, but this was established for similar transformed foci in earlier work (18).

Although the induction of morphological transformation in mouse cells does not prove that the genotoxic components detected in breast lipid are involved in mammary tumour initiation, it does heighten the suspicion that they might be.

The MLEs found to induce transformation of C3H/M2 cells did so in the absence of an exogenous metabolic activation system. This suggests that C3H/M2 mouse fibroblasts possess the enzymes required to activate the genotoxic components of these MLEs. In the absence of cytochrome P450s, enzymes such as peroxidases have been shown to catalyze sequential one-electron oxidations of aromatic amines and diamines to generate reactive electrophiles which can bind to DNA (27). MCA, a compound that requires metabolic activation, induces morphological transformation in C3H/M2 mouse fibroblasts. The MLEs examined here for their abilities to transform cells were also variously active as bacterial mutagens and as inducers of micronucleus and comet formation in MCL-5 cells. The lack of any obvious correlation between the results obtained in the four assays probably indicates the presence, in active MLEs, of a variety of genotoxic components.

The extraction procedure used in all our lipid studies to date was originally developed for the extraction of HAAs (17). HAAs are xenobiotics formed during the cooking of food, particularly proteinaceous materials, at high temperatures. Many are mutagenic in the Ames/*Salmonella* assay, especially towards frameshift-detecting strains such as TA98. Certain HAAs such as 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), IQ, and 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (4-MeIQ) are rodent mammary carcinogens (9). Work carried out in our laboratories has shown that they are also comet-forming and clastogenic in MCL-5 cells and that, in terms of morphological transformation of C3H/M2 mouse fibroblasts, the most mutagenic HAAs are not always the most active in transformation assays (unpublished observations). Their formation during cooking processes commonly used in the Western world, i.e. frying, barbecuing etc. (9), may

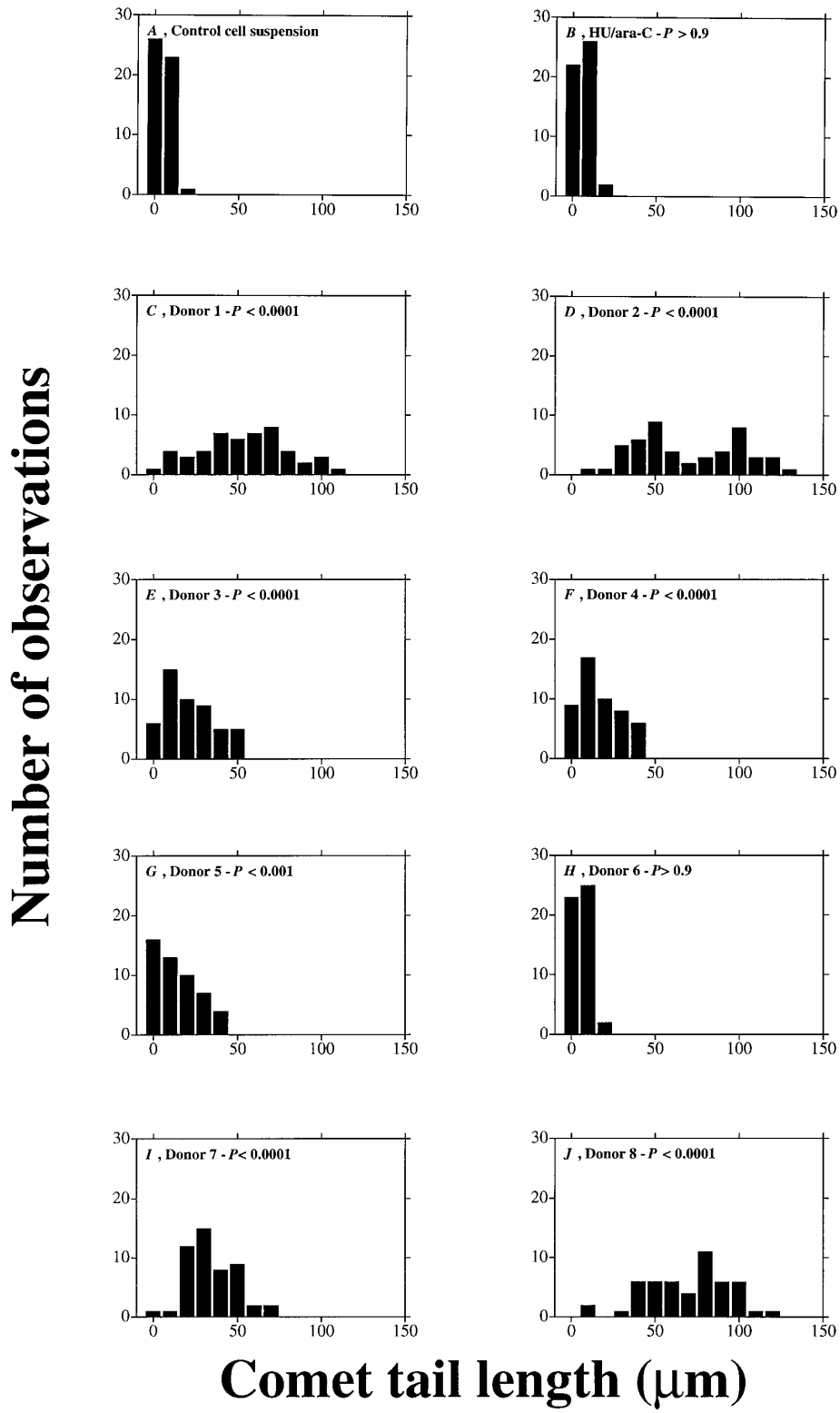


**FIG. 3.** Comet-forming activities of human mammary lipid extracts (MLEs) measured in MCL-5 cells. Cell suspensions in PBS ($\sim 1 \times 10^5$ cells/75 $\mu$l) were incubated in the presence of the DNA-repair inhibitors, hydroxyurea and cytosine arabinoside (HU/ara-C) (10 mM/1.8 mM final concentration), at 37°C for 30 min. Comet tail length ($\mu$m) was used as a measure of DNA damage. MCL-5 cells were treated with vehicle control (DMSO): A, in the absence of HU/ara-C; B, in the presence of HU/ara-C. Cells were also treated in the presence of HU/ara-C with MLEs (8 g-lipid equivalent) added as solutions in DMSO as follows: C, donor 1; D, donor 2; E, donor 3; F, donor 4; G, donor 5; H, donor 6; I, donor 7; J, donor 8. Incubations and the comet assay were carried out as described under Materials and Methods.

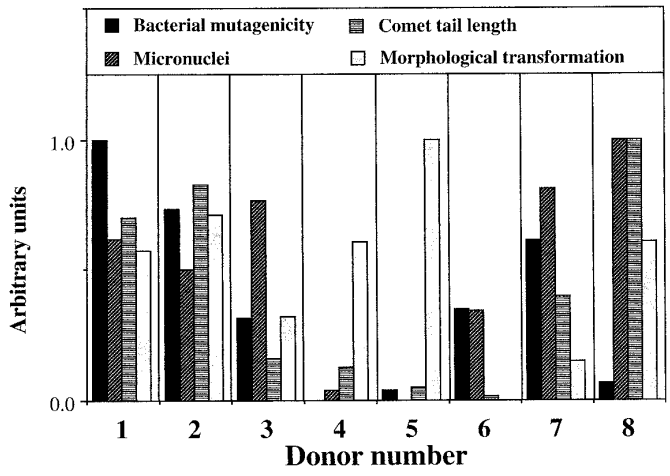


**FIG. 4.** Interindividual variations in biological activity obtained with mammary lipid extracts (MLEs) in each of three genotoxicity assays and in the morphological transformation assay. Values are normalized to the most active MLE in each assay, assigned an arbitrary value of 1.00.

implicate them in the aetiology of malignancies such as breast cancer that are more prevalent in the West. Studies measuring the distribution of tritiated HAAs (28) and HAA-DNA adducts (29) in animals have shown a wide tissue distribution. The fact that we have not detected, using LC/MS with selective ion monitoring (detection limit ~ 100 pg), the presence of any of 15 HAAs in genotoxic MLEs suggests that HAAs are not responsible for the biological activities observed. Alternatively, metabolites and/or chemically-altered HAAs may be present. These would not be detected by selective ion monitoring. It is also possible that other classes of yet-to-be-identified genotoxins are present in active MLEs. It may be worth noting that unidentified hydrophobic adducts have been detected in human mammary DNA (22).

Many rodent carcinogens are lipid soluble (6) and there is now increasing evidence to suggest that such compounds are present in human fat (11, 14, 15). Examination of the spectrum of *p*53 gene mutations in breast cancer cases reveals mutational patterns different from those attributable to endogenous/background mutagenic processes, suggesting that genotoxic events and malignant transformation of human mammary epithelial cells could result from exposure to a wide range of xenobiotics (12, 30).

The present work has extended previous studies which demonstrated that human mammary lipid could act as a reservoir for mutagenic/genotoxic components (14, 15) since we have now found that most MLEs from UK women also possess the ability to induce the morphological transformation of mammalian cells in culture. It may be important to characterise and identify the genotoxic components concerned, to determine if they play a role in the aetiology of breast cancer and then, perhaps, to devise strategies to reduce human exposures to such compounds.

## ACKNOWLEDGMENTS

We thank Professor B. A. Gusterson for supplying mammary tissue samples. This study was supported by research grants from the Association for International Cancer Research and the DFG (Grant pf283/1-3).

## REFERENCES

1. Higginson, J., Muir, C. S., and Muñoz, N. (1992) *in* Cambridge Monographs on Cancer Research, pp. 377–387, Cambridge Univ. Press, Cambridge, UK.
2. MacDonald, F., and Ford, C. H. (1997) *in* Biology of Cancer, pp. 117–130, BIOS Scientific, UK.
3. Tokunaga, M., Land, C. E., Yamamoto, T., Asano, M., Tokuoka, S., Ezaki, H., and Nishimori, I. (1987) *Radiat. Res.* **112,** 243–272.
4. Ziegler, R. G., Hoover, R. N., Pike, M. C., Hildesheim, A., Nomura, A. M., West, D. W., Wu-Williams, A. H., Kolonel, L. N., Horn-Ross, P. L., Rosenthal, J. F., and Hyer, M. B. (1993) *J. Natl. Cancer Inst.* **85,** 1819–1827.
5. Petrakis, N. L., Maack, C. A., Lee, R. E., and Lyon, M. (1980) *Cancer Res.* **40,** 188–189.
6. El-Bayoumy, K. (1992) *Chem. Res. Toxicol.* **5,** 585–590.
7. Wrensch, M. R., Petrakis, N. L., Gruenke, L. D., Miike, R., Ernster, V. L., King, E. B., Hauck, W. W., Craig, J. C., and Goodson, W. H., III (1989) *Cancer Res.* **49,** 2168–2174.
8. Wolfle, D., and Marquardt, H. (1996) *Carcinogenesis* **17,** 1273–1278.
9. Snyderwine, E. G. (1994) *Cancer Suppl.* **74,** 1070–1077.
10. Dunnick, J. K., Elwell, M. R., Huff, J., and Barrett, J. C. (1995) *Carcinogenesis* **16,** 173–179.
11. Obana, H., Hori, S., Kashimoto, T., and Kunita, N. (1981) *Bull. Environm. Contam. Toxicol.* **27,** 23–27.
12. Biggs, P. J., Warren, W., Venitt, S., and Stratton, M. R. (1993) *Mutagenesis* **8,** 275–283.
13. Bowlin, S. J., Leske, M. C., Varma, A., Nasca, P., Weinstein, A., and Caplan, L. (1997) *Int. J. Epidem.* **26,** 915–923.
14. Martin, F. L., Carmichael, P. L., Crofton-Sleigh, C, Venitt, S., Phillips, D. H., and Grover, P. L. (1996) *Cancer Res.* **56,** 5342–5346.
15. Martin, F. L., Venitt, S., Carmichael, P. L., Crofton-Sleigh, C., Stone, E. M., Cole, K. J., Gusterson, B. A., Grover, P. L., and Phillips, D. H. (1997) *Carcinogenesis* **18,** 2299–2305.
16. O'Hare, M. J., Ormerod, M. G., Monaghan, P., Lane, E. B., and Gusterson, B. A. (1981) *Differentiation* **46,** 209–221.
17. Gross, G. A. (1990) *Carcinogenesis* **11,** 1597–1603.
18. Marquardt, H., Grover, P. L., and Sims, P. (1976) *Cancer Res.* **36,** 2059–2064.
19. Johansson, M. A. E., Fay, L. B., Gross, G. A., Olsson, K., and Jägerstad, M. (1995) *Carcinogenesis* **16,** 2553–2560.
20. Venitt, S., Crofton-Sleigh, C., and Forster, R. (1984) *in* Mutagenicity Testing: A Practical Approach (S. Venitt and J. M. Parry, Eds.), pp. 45–98, IRL Press, Oxford, Washington, DC.
21. Crofton-Sleigh, C., Doherty, A., Ellard, S., Parry, E. M., and Venitt, S. (1993) *Mutagenesis* **8,** 363–372.
22. Perera, F. P., Estabrook, A., Hewer, A., Channing, K., Rundle, A., Mooney, L. A., Whyatt, R., and Phillips, D. H. (1995) *Cancer Epidemiol. Biomarkers Prevent.* **4,** 233–238.

23. Li, D., Wang, M., Dhingra, K., and Hittelmann, W. N. (1996) *Cancer Res.* **56,** 287–293.
24. Ridder, G. M., Stuard, S. B., Kerckaert, G. A., Cody, D. B., LeBoeuf, R. A., and Isfort, R. J. (1997) *Carcinogenesis* **18,** 1965–1972.
25. Amsted, P., and Cerutti, P. (1990) *Environ. Health Perspect.* **88,** 77–82.
26. Shuin, T., Billings, P., Lillehaug, J., Patierno, S., Roy-Burman, P., and Landolph, J. (1986) *Cancer Res.* **46,** 5302–5311.
27. Josephy, P. D. (1996) *Mutagenesis* **11,** 3–7.
28. Dragsted, L. O., Frandsen, H., Reistad, R., Alexander, J., and Larsen, J. C. (1995) *Carcinogenesis* **16,** 2785–2793.
29. Thorgeirsson, S. S., Davis, C. D., Schut, H. A. J., Adamson, R. H., and Snyderwine, E. G. (1995) *in* Heterocyclic Amines in Cooked Foods: Possible Human Carcinogens, pp. 85–92, Princess Takamatsu Symposium, Princeton Scientific, Princeton, NJ.
30. Hartmann, A., Blaszyk, H., Kovach, J. S., and Sommer, S. S. (1997) *Trends Genet.* **13,** 27–33.