Heightened susceptibility of fish oil polyunsaturate-enriched neoplastic cells to ethane generation during lipid peroxidation

C. Patrick Burns[1] **and Brett A. Wagner**

Department of Medicine, University of Iowa College of Medicine, Iowa City, IA 52242

Abstract We have studied the generation of volatile hydrocarbons by fatty acid-modified L1210 leukemia cells in tissue culture as a measure of lipid peroxidation. There was considerable generation of ethane, and this was dependent on cell number and Fe^{2+} concentration; it was eliminated by antioxidants and augmented by ascorbic acid. The assay was sensitive and reproducible; ethane was detected when as little as 0.03% of the cellular n-3 (omega-3) fatty acids were peroxidized. To gain further understanding we used a lipid modification model that allows study of cells enriched with fatty acids of different degrees of unsaturation. The quantity of ethane generated was greatest by cells modified with fatty acids of the n-3 family, and there was a high direct correlation of percentage of n-3 fatty acids contained in cellular lipids with peroxidation as measured by ethane generation. Ethane generation was more sensitive in detecting peroxidation than loss of polyunsaturated fatty acids. We conclude that lipid-supplemented leukemic cells produce ethane, and that the rate of generation is a sensitive, quantitative, and highly useful measure of lipid peroxidation when small amounts of iron are present.—**Burns, C. P., and B. A. Wagner.** Heightened susceptibility of fish oil polyunsaturate-enriched neoplastic cells to ethane generation during lipid peroxidation. *J. Lipid Res.* 1991. **32:** 79–87.

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There is increasing interest in lipid peroxidation as a mechanism for cytotoxicity in cancer therapy. The generation of volatile hydrocarbons has been proposed as a sensitive measure of lipid peroxidation (1). Ethane and pentane have been studied since they are the products of peroxidation of fatty acids of the n-3 and n-6 families, respectively (2, 3). There has been a particular interest in the generation of hydrocarbons as a measure of peroxidation in vivo in the intact animal or of the isolated perfused organs since exhaled breath or gasses can be collected in closed chambers and analyzed. There also have been reports of its use to study peroxidation by intact cells in tissue culture (4). For example, there is information on homogenates of liver and brain (1), hepatocytes (5–9), granulocytes (3), endothelial cells (10), platelets (11), and peritoneal macrophages (12).

However, these studies were carried out on normal cells, and pro-oxidant compounds were often added. We were interested in studying hydrocarbon generation as an assay for lipid peroxidation of neoplastic cells. It is established that cancer cells have a low susceptibility to peroxidative attack (13). This low peroxidizability is especially characteristic of rapidly growing tumors like the L1210 leukemia, and it might be anticipated that limited amounts of hydrocarbons would be generated. In addition, many details of the process of hydrocarbon generation as a measure of lipid peroxidation are not completely understood, including its ultimate place in the study of cellular membrane lipid damage.

We undertook a study to quantitate hydrocarbon generation by cells enriched with fatty acids having variable degrees of susceptibility to peroxidative attack. No pro-oxidant drugs or compounds that produce oxygen radicals or stimulate redox cycling were added except near-physiologic concentrations of iron. We report that, using this model, detection of ethane generation is a precise, specific, and generally useful method for measuring peroxidation in the intact cell. The results also suggest that fatty acids of the fish oil (n-3) family, when incorporated into neoplastic mammalian cells, result in a considerable overall susceptibility to peroxidation. However, a lower percentage of total n-3 fatty acids is peroxidized as the cellular content increases.

MATERIALS AND METHODS

Cell handling, fatty acid modification of cells, and lipid analysis

Stock murine leukemia L1210 cells, mycoplasma-free, were maintained in RPMI 1640 with 5% heat-inactivated

Abbreviations: FBS, fetal bovine serum; HBSS, Hank's buffered salt solution; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; MDA, malondialdehyde.

[1]To whom reprint requests should be addressed.

fetal bovine serum (FBS) (Sigma Chemical Co., St. Louis, MO) at 37°C in a humidified 95% air/5% CO_2 atmosphere. Fatty acid modification of cells (14) was achieved by growing cells for 3 days in media supplemented with various fatty acids at 32 μM. Media was enriched with the following fatty acids (Nu Chek Prep, Inc., Elysian, MN): oleic (18:1n-9), linolenic (18:3n-3), γ-linolenic (18:3n-6), arachidonic (20:4n-6), eicosapentaenoic (20:5n-3), docosatetraenoic (22:4n-6), docosahexaenoic (22:6n-3). After 3 days of incubation in fatty acid-enriched media, cells were pelleted at 300 *g* for 17 min, washed, then resuspended in Ca^{2+}- and Mg^{2+}-free Hank's buffered salt solution (HBSS) containing 15 mM HEPES buffer and adjusted to 1×10^7 cells/ml. Cell numbers were determined with a Coulter Model Z_f Counter (Coulter Electronics, Inc., Hialeah, FL). Cells were used within 1 h of preparation and maintained at 5°C before initiation of peroxidation. Gas-liquid chromatography was used to determine the fatty acid composition of cellular lipids (15). Total cellular content of fatty acids also was determined (16).

Measurement of lipid peroxidation by ethane generation

Lipid peroxidation was measured by ethane generation into head spaces during cell incubation in a closed plastic syringe. Before the start of each experimental reaction, a new 10-ml plastic disposable syringe (Becton Dickinson and Co., Rutherford, NJ) was flushed with a stream of low hydrocarbon compressed air (Air Products Inc., Allentown, PA), adjusted to contain 5 ml of compressed air, and then closed using a 1-ml syringe plunger septum (Becton Dickinson and Co., Rutherford, NJ) placed over the needle barrel. Then the prepared cells in 5 ml Ca^{2+}-, Mg^{2+}- free HBSS containing 15 mMN-2-hydroxyethylpiperazine-N'-2-ethanesulfonic (HEPES) buffer were added to reaction syringe through the septum of the reaction syringes using a 20-gauge needle. Some early experiments were carried out in 0.9% NaCl and the results were similar. Peroxidation was initiated by injecting 50 μl of $FeSO_4 \cdot 7H_2O$ in 0.9% NaCl through the reaction syringe septum. The syringes were then shaken and incubated for 2h at 37°C before injection of the headspace containing peroxidation-generated hydrocarbons into the gas chromatograph.

After incubation, headspace gas was collected and analyzed for ethane using gas-liquid chromatography as a major modification of the methods of Muller and Sies (4) and Roberts, Rendak, and Bucher (17). Briefly, the 5-ml head space from the reaction syringe was injected into a heated (50°C) external sample loop containing 60/80 mesh Alumina F-1 (Supelco, Inc., Bellefonte, PA) using a 15-cm 18-gauge cannula after removal of the reaction syringe septum. The external sample loop was attached to a Hewlett-Packard model 5710A gas chromatograph (Avondale, PA) modified for hydrocarbon head space analysis and use of the heated external sample loop. Quantification of ethane production was accomplished by separation of C1-C4 hydrocarbons in sample head spaces on a 5 m × 32 mm stainless steel column packed with 80/100 mesh Poracil C (Supelco, Inc.) run at 50°C with N_2 (20 ml/min) as the carrier gas. Detection of hydrocarbons was by flame ionization at 150°C. Standard curves were established using 0.5 ppm ethane, 0.49 ppm n-pentane in N_2 primary standards (Matheson Gas Products, Inc., Joliet, IL) or C1-C6 n-paraffins (10-20 ppm each) in N_2 (Supelco, Inc.) for quantification of ethane peaks. Sensitivity for ethane was about 20 pmol. Compressed air in the headspace contained some methane which served as an internal standard for the positive identification of peaks from peroxidation experiments as being ethane. This allowed confirmation that all of the headspace was injected into the sample loop by monitor ing amplitude of the methane peak area, thus reducing errors in ethane quantification due to loss or improper injection of the sample headspaces.

Malondialdehyde (MDA) determination

For comparison, MDA was also measured using a 2-thiobarbituric acid assay (18, 19). For these studies the MDA contained in cells was determined at the completion of the 2-h time period. MDA equivalent standards were prepared using serially diluted 1,1,3,3-tetramethoxypropane (Fluka Chemical Corp., Ronkonkoma, NY).

RESULTS

Fatty acid modification

Table 1 shows the fatty acid composition of L1210 cells after growth for 72 h in media containing supplemented fatty acids. Each supplemented cell type was enriched at least to some extent in the fatty acid added to the culture. This enrichment ranged from 62% for 18:1n-9 to 3% in the case of 18:3n-6. In several instances there was metabolic conversion, for example, elongation of 20:4n-6 and 20:5n-3, or elongation and desaturation of 18:3n-6. The resultant cells varied in total polyunsaturated fatty acids (2-46%) and in mean number of double bonds per fatty acid molecule (0.7-1.8). There was also a wide range of proportions of n-3 (0.3-35%) and n-6 (2-41%) classes contained in the various enriched cells. This diversity of fatty acid modification, which is similar to our previous report (14), provided the model for the quantitative study of relative lipid peroxidation.

Effect of fatty acid modification on ethane generation and dependency on iron

Fig. 1 shows the amount of ethane generation by L1210 cells enriched with the polyunsaturated fatty acid, 22:6

TABLE 1. Fatty acid composition of L1210 cells

Fatty Acid	Fatty Acid Composition (%)						
	18:1n-9	18:3n-3	18:3n-6	20:4n-6	20:5n-3	22:4n-6	22:6n-3
Individual acids							
14:0	1.6 ± 0.6	0.9 ± 0.3	2.2 ± 0.6	2.3 ± 0.5	1.4 ± 0.2	2.8 ± 0.1	1.5 ± 0.2
16:0	13.9 ± 0.3	12.8 ± 1.8	18.6 ± 1.3	22.4 ± 1.6	22.8 ± 1.3	25.7 ± 0.4	17.9 ± 0.7
16:1	4.2 ± 0.5	1.1 ± 0.6	2.7 ± 0.2	1.7 ± 0.9	0.8 ± 0.8	3.6 ± 0.4	1.6 ± 0.1
18:0	11.7 ± 2.0	18.5 ± 1.4	13.4 ± 1.7	16.0 ± 0.8	26.7 ± 2.3	17.8 ± 0.2	19.4 ± 1.1
18:1n-9	61.6 ± 3.3	19.5 ± 0.8	15.5 ± 0.6	15.0 ± 0.8	23.9 ± 1.0	16.6 ± 1.4	16.0 ± 0.2
18:2n-6	0.6 ± 0.3	1.2 ± 0.4	1.2 ± <0.1	0.8 ± 0.4	0.7 ± 0.4	1.2 ± <0.1	0.3 ± <0.1
18.3n-6	0	0	3.0 ± 0.3	0.1 ± <0.1	0.3 ± 0.3	<0.1 ± <0.1	<0.1 ± <0.1
18:3n-3	0	33.0 ± 2.1	0.6 ± 0.3	0.2 ± 0.2	0	0	0.9 ± 0.8
20:3n-6	0.5 ± <0.1	0.2 ± 0.1	21.5 ± 3.7	0.4 ± 0.2	0.2 ± 0.2	0.5 ± <0.1	0.8 ± 0.1
20:4n-6	0.9 ± 0.9	9.2 ± 0.8	10.9 ± 2.2	25.8 ± 3.0	3.6 ± 0.6	9.8 ± 0.2	3.0 ± 0.1
20:5n-3	<0.1 ± <0.1	1.8 ± 0.1	0	0	7.8 ± 1.3	0	1.7 ± 0.4
22:4n-6	0	<0.1 ± <0.1	4.3 ± 2.0	11.6 ± 1.8	0.2 ± <0.1	15.1 ± 1.3	0.2 ± <0.1
22:5n-3	<0.1 ± <0.1	0.3 ± 0.2	0.2 ± <0.1	0.4 ± <0.1	9.6 ± 2.3	0.7 ± 0.1	1.3 ± 0
22:6n-3	<0.1 ± <0.1	<0.1 ± <0.1	0.2 ± <0.1	0.7 ± 0.4	0	0.9 ± 0.4	30.7 ± 1.5
Other	4.5	1.2	5.7	2.5	2.0	3.8	4.6
Classes							
% PolyU	2.2 ± 1.3	45.9 ± 3.4	41.7 ± 1.0	40.0 ± 3.5	22.4 ± 4.4	28:3 ± 0.6	39.1 ± 1.3
% MonoU	65.8 ± 3.3	20.6 ± 0.5	18.3 ± 0.4	16.7 ± 0.5	24.7 ± 1.0	20.2 ± 1.3	19.2 ± 0.3
% Sat	27.7 ± 4.5	32.4 ± 3.4	34.6 ± 3.1	41.6 ± 2.9	51.6 ± 4.0	47.0 ± <0.1	38.7 ± 1.4
Do.Bds.	0.735	1.726	1.670	1.790	1.313	1.370	2.37
% n-3	0.30	35.10	1.00	1.30	18.70	1.60	34.7

L1210 cells were grown for 72 h in medium supplemented with various fatty acids at 32 μM. Cells were washed and extracted with $CHCl_3$–CH_3OH 2:1 (v/v). After alkaline hydrolysis, fatty acids in the saponifiable fraction were methylated and the methyl esters were separated by gas-liquid chromatography. Values are the mean and SE of three independent experiments. Abbreviations: PolyU, polyunsaturated fatty acids; MonoU, monounsaturated fatty acids; Sat, saturated fatty acids; Do.Bds., average number of double bonds per fatty acid molecule.

n-3. There was considerable hydrocarbon generated at all iron concentrations. In contrast, only trace amounts of ethane could be detected even at the highest iron concentrations in the case of 18:1-modified or unmodified cells. Fig. 1 demonstrates an essential role of iron in lipid peroxidation as measured by ethane generation. There was an increase in hydrocarbon product of lipid peroxidation by 22:6-modified cells as the iron concentration increased. The direct relationship of amount of ethane generation and iron concentration was particularly steep between 0 and 20 μM; as the concentration was raised above 20 μM, there was some slowing of the rate of increase of ethane with increasing iron, but no plateau was evident at the highest iron concentration studied. We used 20 μM iron for most further studies since concentrations of iron above that level resulted in cell death (>15%) as measured by trypan blue dye exclusion (data not shown). In addition, the chosen concentration approximates that of iron bound to transferrin in human blood. These studies were done using ferrous iron; incubation in medium containing ferric iron as 20 μM $FeCl_3$ yielded no hydrocarbon gas.

Relationship of ethane generation with time and number of 22:6-modified cells

We examined the effect of increasing the number of cells in the incubation syringe on hydrocarbon generation. **Fig. 2** shows that there was an increase in ethane generation as cell number increased. The relationship was linear to the greatest concentration studied which was 75 × 10^6/cells.

Fig. 3 shows the time course of ethane generation at four concentrations of iron. A relationship of incubation time and ethane generation was evident at all iron concentrations. A plateau was present at the lower concentrations; after 120 min of incubation time, there was little additional lipid peroxidation at 2 or 20 μM iron. There-

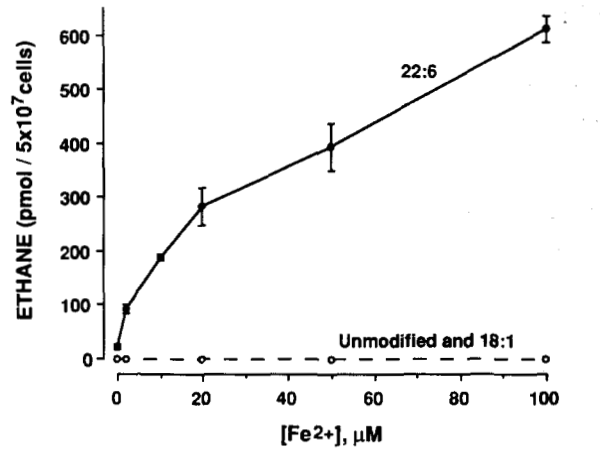

Fig. 1. Effect of fatty acid modification on iron concentration-dependent ethane generation. 22:6n-3 or 18:1n-9-modified or unmodified L1210 cells (5 × 10^7) were incubated for 2 h at 37°C in Ca^{2+}-, Mg^{2+} free HBSS containing 15 mM HEPES in the presence of 0–100 μM $FeSO_4 \cdot 7H_2O$ and ethane production was measured. Values of ethane generation are per 2 h.

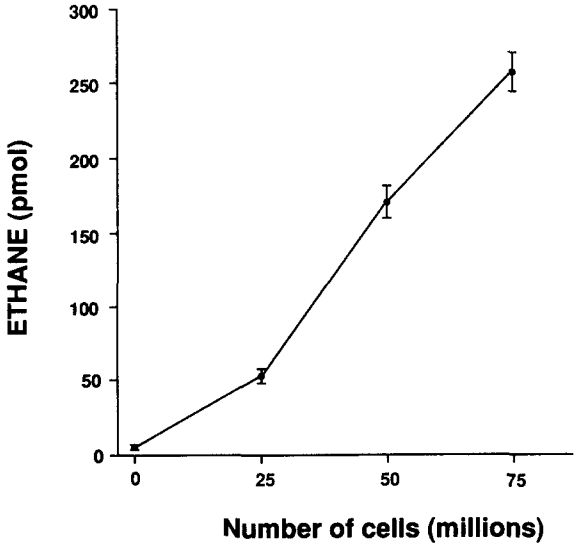

Fig. 2. Relationship of ethane generation and cell number. L1210 cells enriched with 22:6n-3 were incubated at 37°C in 0.9% NaCl containing 20 μM $FeSO_4 \cdot 7H_2O$. The ethane released into the headspace gas of a closed syringe during a 2-h period was measured. Values of ethane are pmol per 2 h and are expressed as means and SE of four replicates.

fore, subsequent studies were carried out using these conditions. To extend these experiments, cells were incubated for 2 h in 20 μM iron prior to the addition of further iron to the media. Figure 3 inset shows that there was additional ethane produced to exceed the plateau level, and the magnitude of this increment was a function of the added iron concentration. This suggests that the plateau is a result of oxidation or metabolic depletion of the iron since augmentation of hydrocarbon production resulted from its replenishment. Therefore, the plateaus demonstrated in Fig. 3 are not due to a limited source of lipids that are susceptible to oxygen radical attack.

Requirement for membrane incorporation of polyunsaturated fatty acid.

We next examined whether it was essential that the polyunsaturated fatty acid responsible for the generation of the lipid peroxidation reactant, ethane, be incorporated into biologic membranes. **Table 2** shows that when 22:6 was added to the incubation medium in the absence of cells, there was no appreciable ethane generation even in the presence of iron. Unmodified cells failed to generate ethane even in the presence of iron. Most importantly, the addition of 22:6 to the incubation medium containing unmodified cells immediately before the 2-h ethane collection period resulted in little hydrocarbon generation due to oxidation of the fatty acid in the medium prior to membrane incorporation. This indicates a fundamental biological difference in the effect of fatty acid in the media as compared to fatty acid incorporated into cells.

We also examined the effect of the addition of 22:6 to incubation media containing 22:6-modified cells. In the presence of iron the generation of ethane was decreased by about half when 22:6 was added to the media. This may be due to an antioxidant in the serum containing the added 22:6, or to an effect of the fatty acid on cellular peroxidative processes.

If cell or membrane-associated fatty acid is responsible for the hydrocarbon production, there should be a relationship of ethane generation and time in lipid-supplemented media. Therefore, we incubated L1210 cells in media containing 22:6 for various times prior to measurement of ethane. **Fig. 4** shows that there is a direct correlation of ethane generation and the time of incubation in supplemented media until about 24 h at which time the rate of hydrocarbon production plateaus.

MDA production

MDA content is a frequently used estimate of lipid peroxidation. We studied the generation of MDA by L1210 cells modified with the polyunsaturated 22:6 (**Fig. 5**). In the absence of iron only small amounts of MDA were detected. As the amount of iron in the medium was increased, MDA generation increased in a linear manner. For comparison, the relationship of ethane generation is also shown in Fig. 5. Under these conditions, the two measures of lipid peroxidation gave similar results.

For the seven fatty acid-modified cell types studied, the MDA generation during the study period comparable to

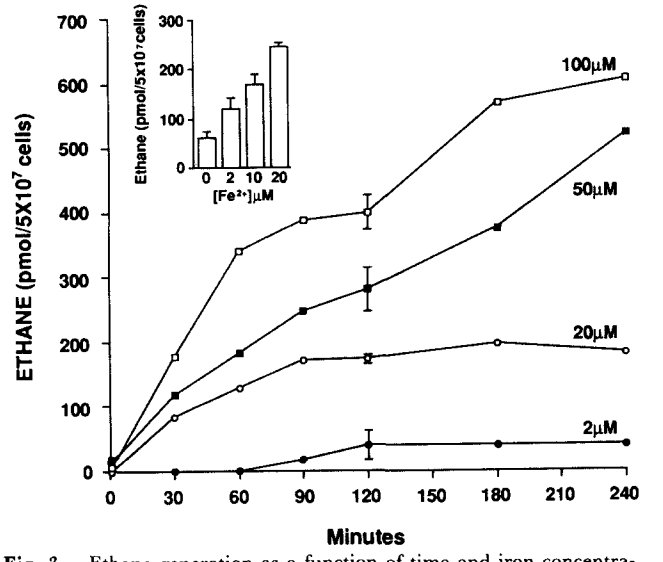

Fig. 3. Ethane generation as a function of time and iron concentration. L1210 cells (5×10^7) enriched with 22:6n-3 were incubated with various concentrations of $FeSO_4 \cdot 7H_2O$. Aliquots were taken at the time points shown. The bars at 120 min are the SE of three to six replicates; each mean is significantly different from that value for contiguous iron concentrations. The values at the other time points are the means of two closely agreeing duplicates. Inset: In some experiments, additional iron was added after 120 min to those cells originally exposed to 20 μM iron. This further iron was added after the ethane generation had plateaued. The bar graph shows that ethane generation during a subsequent 120 min could be restored and that the amount recovered was related to the concentration of supplemental iron.

TABLE 2. Requirement for membrane incorporation of polyunsaturated fatty acids to generate ethane

Material	Additions	Ethane[a]
22:6-No cells	None	tr
22:6-No cells	+ Iron[b]	tr
Unmodified cells	+ Iron	tr
Unmodified cells	+ 22:6[c]	tr
Unmodified cells	+ Iron, + 22:6	tr
22:6-Modified cells	None	tr
22:6-Modified cells	+ 22:6	tr
22:6-Modified cells	+ Iron	270 ± 36
22:6-Modified cells	+ Iron, + 22:6	122 ± 34

Determination of ethane generation was begun immediately after the material and additions shown were placed in media.
[a]pmol/2 h per 5 × 10^7 cells; tr, trace.
[b]20 μM $FeSO_4 \cdot 7H_2O$.
[c]320 μM.

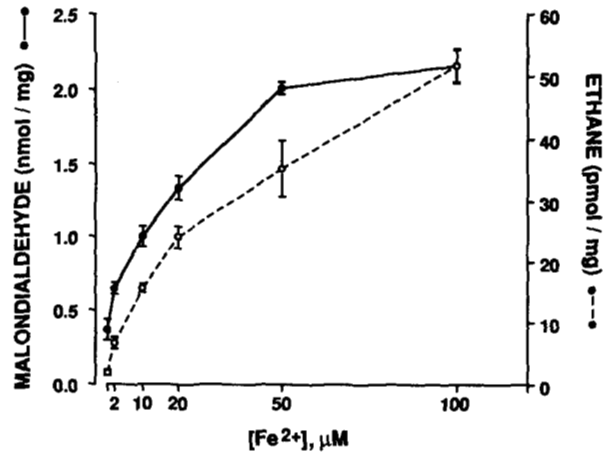

Fig. 5. Relationship of malondialdehyde and ethane generation. L1210 cells were enriched with 22:6. Values are expressed as product per mg cellular protein per 2 h.

the time utilized for ethane generation, ranged from 0(18:1n-9, 18:3n-3, 18:3n-6) to 17.98 nmol/5 × 10^7 cells per 2-h time period (22:6n-3). There was little correlation of MDA generation during this 2 h with ethane generation. In fact, the MDA proved to be an apparently poor assay of lipid peroxidation since it failed to correlate with average number of double bonds or with the percentage of fatty acids that were polyunsaturated. The MDA contained within the cells at time zero was also not correlated with percentage of fatty acids that were polyunsaturated, or with average number of double bonds. These observations suggest that measurement of MDA has limited value as an estimate of lipid peroxidation by the neoplastic L1210 cells when quantitative or qualitative comparisons of different peroxidizable fatty acids are studied.

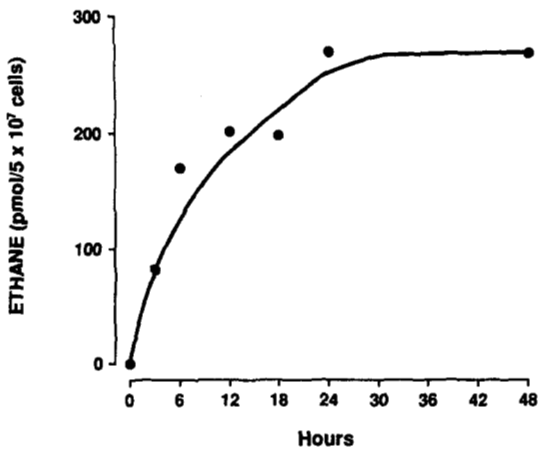

Fig. 4. Relationship of ethane generation by L1210 cells and time of incubation in serum containing 22:6. Cells were placed in RPMI 1640 media containing 5% FBS and 32 μM fatty acid. Aliquots were removed at times shown, washed, and the ethane generated during a subsequent 2-h period was measured. Values of ethane are pmol per 2 h.

Loss of polyunsaturated fatty acids

The extent of peroxidation was also estimated by the loss of unsaturated fatty acids analyzed by gas-liquid chromatography. L1210 cells (5 × 10^7) were subjected to the identical conditions utilized for measurement of ethane generation. The proportion of n-3 and of total unsaturated fatty acids remained unchanged after the 2 h of incubation in both 20 μM Fe^{2+} and the more intense conditions of 100 μM Fe^{2+} and ascorbic acid (data not shown); thus no peroxidation was detected by this alternative method which proved to be less sensitive than ethane generation.

Ethane generation by L1210 cells modified with various fatty acids

L1210 cells enriched with various fatty acids by growth in media supplemented with seven different acids were studied. Ethane generation for each group is shown in **Fig. 6**. The values varied from 27 to 262 pmol and there were clearly quantitative differences in ethane generation by n-3 and n-6 family fatty acids. Furthermore, there was a correlation of ethane production with percentage of n-3 fatty acids incorporated into cellular lipids from Table 1 (**Fig. 7**). It is noteworthy that there was no correlation of ethane production with percentage of n-6 fatty acids, average number of double bonds per fatty acid molecule, or percentage of polyunsaturated fatty acids. This indicates that lipid peroxidation primarily of the n-3 fatty acids is being detected.

The potential capacity of ethane generation can be calculated for each specific fatty acid-modified population. The total fatty acid content of each modified cell type was determined and this was multiplied by the percentage of n-3 fatty acid contained in each cell from

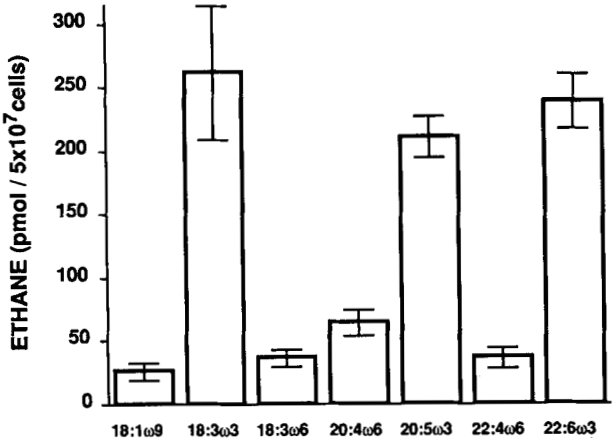

Fig. 6. Ethane generation by L1210 cells modified by growth in media enriched with various fatty acids. L1210 cells were grown for 72 h in RPMI 1640 and 5% FBS enriched with 32 μM fatty acids of diverse degrees of unsaturation prior to determination of ethane generation. Values are pmol ethane per 5 × 10^7 cells per 2 h and are the mean and SE of 8–12 independent determinations.

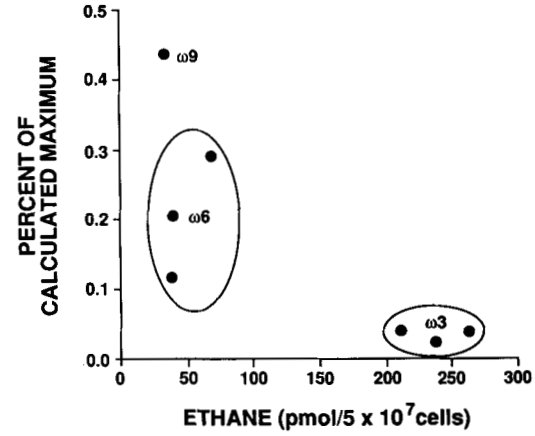

Fig. 8. Relationship of actual ethane generation by L1210 cells enriched with fatty acids of various families (abscissa) and a measure of efficiency of hydrocarbon production (ordinate). The latter is the ratio of actual to calculated theoretical maximum possible ethane generation. The results clustered by fatty acid families as shown by the ellipses.

Table 1. **Fig. 8** shows the relationship of this potential yield to actual ethane generation. Although the relationship was not linear, the cells with the greatest capacity had the least efficient conversion of fatty acids to ethane. There was a clustering of n–3 and n–6 fatty acids. These data also indicate the sensitivity of the assay since ethane generation from 0.03–0.4% of the cellular n–3 fatty acids could be detected.

Effect of antioxidants, inhibitors and pro-oxidants

To further demonstrate that hydrocarbon generation is a result of a peroxidative reaction, the studies of 22:6-modified cells were repeated in the presence of several antioxidants or metabolic inhibitors of different potency (**Table 3**). The strong antioxidant butylated hydroxytoluene virtually eliminated ethane generation; the chemically related butylated hydroxyanisole had a similar effect. Retinoic acid (all-*trans*), which may quench reactions of singlet oxygen, decreased the production of ethane by about 26%. The cyclooxygenase and lipoxygenase inhibitors, ibuprofen and nordihydroguaiaretic acid, inhibited ethane generation by 24% and 69%, respectively.

Augmentation of ethane production was attempted using ascorbic acid. The combination of ascorbic acid and iron that has been used as a maximum peroxidative challenge (20) resulted in a 3.4-fold increase in hydrocarbon production. The reducing agent, dithiothreitol, had a similar augmenting effect of lesser magnitude and this remains unexplained. To further analyze the system, H_2O_2 was added to the incubations. In these studies no iron was added in order to compare with the controls. There was no generation of ethane. Since ethanol was used to constitute some of the chemicals added to incubations, its effect was studied. At a concentration of 1.0%, there was no appreciable effect as compared to controls.

Serum was not added to most of our incubations since a component might influence lipid peroxidation. To test its effect, ethane generation was measured in the presence of 20 μM iron and various proportions of FBS. Ethane production was inhibited 5% ± 4% (mean and SE of triplicates) in 1% FBS, 16% ± 5% in 5% FBS, 29% ± 11% in 10% FBS, and 66% ± 11% in 15% FBS. This indicates that there is an antiperoxidant component of serum that inhibits ethane generation in a concentration-dependent fashion. This demonstrates the value of working in a serum-free medium as we have done in our usual incubations. However, if necessary, small amounts of serum can be added with only a minor effect on hydrocarbon generation.

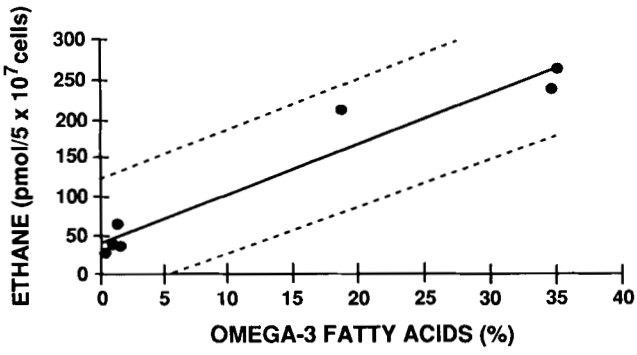

Fig. 7. Correlation of ethane generation and proportion of n–3 family fatty acids. Shown are the regression line and 95% confidence limits: r = 0.97; P = 0.0003. Ethane values are pmol per 5 × 10^7 cells per 2 h.

TABLE 3. Effect of antioxidants and oxidants on ethane generation

Addition	Ethane
	pmol
None	192.2 ± 9.1
Butylated hydroxytoluene (20 μM)	tr[a]
Butylated hydroxyanisole (20 μM)	tr
Retinoic acid (10 μM)	149.2 ± 16.4
Ibuprofen (50 μM)	145.2 ± 27.7
Nordihydroguaiaretic acid (10 μM)	60.3 ± 30.2
Ascorbic acid (500 μM)	692.1 ± 145.6
Dithiothreitol (1 mM)	372.3 ± 93.1
H_2O_2 (500 μM)	tr
Ethanol (1%)	190.1 ± 11.6

Ethane generation by L1210 cells enriched with 22:6 was measured. All studies were carried out in the presence of 20 μM $FeSO_4$ except in the H_2O_2 experiment. The decrease in cell viability by trypan blue exclusion after 2 h incubation with various compounds ranged from 5 to 16% (mean 10%). Values are in pmol/2 h per 5×10^7 cells and are means and SE of three to seven experiments.

[a]tr, Trace; value is < 25 pmol.

DISCUSSION

We have demonstrated that neoplastic murine leukemia cells generate ethane during peroxidation of appropriate fatty acids incorporated into cellular structures. Previous studies of ethane generation have been performed on cells of nonmalignant origin (1, 3–12). We found that ethane production by L1210 leukemia cells was dependent on time, cell and iron concentration. Many studies have demonstrated that the susceptibility of tumor cells to lipid peroxidation is lower than that of normal cells (13). These studies, which have been performed mostly with hepatomas, show an inverse relationship of growth rate and lipid peroxidizability. We were able to detect appreciable hydrocarbon generation by this rapidly growing undifferentiated leukemia cell. We conclude that this technique is quantitative, precise, reproducible, and specific for the measurement of peroxidation by neoplastic cells in culture.

The efficiency of peroxidation can be estimated for each modified cell type from the proportion of susceptible fatty acids that generate a hydrocarbon molecule. Paradoxically, the efficiency of ethane generation was greatest by the modified cells with the lowest proportion of n–3 molecules even though the total ethane generation for those n–6 and n–9 acids was the lowest. Conversely, the efficiency of n–3 fatty acid peroxidation was lowest for those fatty acids with the highest total ethane generation, and these were all of the n–3 family. This lower efficiency for cells with highest total peroxidation may be due to a rate-limiting step in the peroxidative chemical process such as iron concentration or differences in constitutive antioxidant activity.

Lipid peroxidation is a result of free radical reactions in cellular membranes containing polyunsaturated fatty acids. We used a model that allows fatty acid modification of the structure of cellular membranes (21, 22). Previous studies of lipid peroxidation in tissue culture have used a drug or agent to stimulate peroxidation (1, 4–8, 10, 12). We added only iron, and our studies were done at iron concentrations similar to those found in vivo (23). Iron promotes the generation of reactive species such as hydroxyl and superoxide radicals, but perhaps most importantly for our study, it facilitates the degradation of lipid peroxides to hydrocarbon gases and aldehydes (23). The fatty acids are not acting as a detergent or oxidant since the presence of 22:6 in media failed to generate peroxidative products prior to incorporation into membrane. The range of values obtainable demonstrates the power of this lipid model for studies of peroxidation. Augmentation of the total proportion of n–3 fatty acids supplementing the culture medium offers a means of studying cells with unusual susceptibilty to peroxidative attack. This might be a useful method for studying the role of oxygen radical damage to membranes by antineoplastic drugs or in the process of carcinogenesis.

The addition of polyunsaturated fatty acids to the media with or without unmodified cells resulted in no appreciable generation of ethane. This indicates that cell or membrane incorporation is necessary for both ethane generation and peroxidation. This suggests that the incorporation of the fatty acid in the membrane or proximity to cellular components allows the enzymes of the peroxidative process to function properly.

Lipid peroxidation is frequently measured by the formation of thiobarbituric acid-reactive compounds. MDA is the major carbonyl product formed by peroxide breakdown, and this forms an adduct with thiobarbituric acid which can be measured colorimetrically. It is useful as a measure of extent of oxidation of unsaturated fatty acids with three or more double bonds (19). In the fatty acid-modified L1210 cells, MDA production at baseline or during a 2-h time period identical to that used for hydrocarbon generation was at low levels and could not be correlated with anticipated peroxidative susceptibility. This made it a much less useful and specific measure of peroxidation as compared to hydrocarbon generation. This is likely explained by the fact that MDA is reactive with cell components (24), unstable in the presence of H_2O_2 (25), and metabolized by certain cells including liver and macrophages (12, 26). Thus, MDA formation may measure only a small proportion of total polyunsaturated fatty acids lost. Consonant with our study, Smith et al. (5) concluded from studies of hepatocytes exposed to ethylmorphine and aminopyrine that ethane is more sensitive and useful than MDA and most other measures of peroxidation.

Analysis of fatty acids by gas-liquid chromatography provides a direct method used to detect biological lipid peroxidation. It has been considered very useful for

peroxidation stimulated by metal complexes (27). We have identified conditions under which hydrocarbon gas generated from lipid peroxide decomposition is more sensitive and useful than the loss of total polyunsaturated fatty acids or n-3 fatty acids, which failed to detect peroxidation under the conditions studied. This indicated that ethane generation can be more sensitive as a measure of lipid peroxidation in tissue culture as compared to the specific loss of polyunsaturated fatty acids.

There is considerable general interest in fatty acids of the n-3 family because of their cardiovascular effects (28). There is also evidence that animals exposed to carcinogens may have a lower incidence of tumors if they are fed n-3 fatty acids (29). In our study L1210 murine neoplastic cells enriched with the n-3 fatty acids, 18:3, 20:5, and 22:6 generated large amounts of the volatile hydrocarbon ethane, whereas cells enriched with fatty acids of the n-9 or n-6 family such as 18:1, 18:3, 20:4, and 22:4, or unmodified cells generated only small amounts. This lipid peroxidation was mediated by iron but not by H_2O_2. The extent of ethane generation indicates the considerable susceptibility to peroxidation that results from n-3 fatty acids contained in cells. This correlation of brisk lipid peroxidation with the proportion of n-3 fatty acids raises the possibility that ingestion of this type of fatty acid by animals and humans increases the peroxidation rate of cells and may have implications for cancer susceptibility and cancer treatment.

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