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Research Article

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Resistance of Human Tumor Cells In Vitro to Oxidative Cytolysis

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Abstract

Nine human cell types, six of them malignant, displayed a marked resistance to lysis by hydrogen peroxide (LD_{50}, 2–20 mM). Of the reactive oxygen intermediates generated extracellularly, only H_2O_2 lysed all the cell types. $OH^{\cdot}$ was lytic to one of four, OI^- to one of one, and O_2^- to none of four cell types tested. Resistance to oxidative lysis did not correlate with specific activity of catalase, glutathione (GSH) peroxidase, other peroxidases, or glutathione disulfide reductase, or with specific content of GSH. Resistance to H_2O_2 seemed to occur via mechanisms distinct from those responsible for cellular consumption of H_2O_2. Consumption was inhibitable by azide and was probably due to catalase in each cell type. In contrast, resistance to oxidative lysis occurred via distinct routes in different cells. One cell type used the GSH redox cycle as the primary defense against H_2O_2, like murine tumors previously studied. Other cells seemed to utilize catalase as the major defense against H_2O_2. Nonetheless, with both catalase and the GSH redox cycle inhibited, all the human cells tested exhibited an inherent resistance to oxidative lysis, that is, resistance independent of detectable degradation of H_2O_2.

Introduction

Hydrogen peroxide, a secretory product of activated macrophages and granulocytes, lyses murine tumor cells under some experimental conditions (1). A major defense of murine tumors against lysis by H_2O_2 is the glutathione (GSH)[1] redox cycle (2–4). Thus, threefold to 10-fold smaller fluxes of H_2O_2 were required to lyse tumor cells after inhibition of their GSH peroxidase by deprivation of selenium (2), inactivation of GSSG reductase with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) (2), blockade of γ-glutamylcysteine synthetase with buthionine sulfoximine (BSO) (3), or acute GSH depletion with chlorodinitrobenzene (3) or sulfhydryl-reactive natural products including sesquiterpene lactones (4). In contrast, murine tumor cell catalase seemed to play a secondary role in defense against oxidative lysis (2, 3).

In this report, we have attempted to define which reactive oxygen intermediates are rapidly lytic for various human cells (mostly tumors) and at what concentrations, to correlate relative resistance with the activity of the GSH redox cycle and catalase, and to analyze the role of these antioxidant enzymes in the consumption of H_2O_2 by intact cells and in the resistance of the cells to lysis by H_2O_2. The results indicate that the specific activities of catalase and the GSH redox cycle components in cell lysates predict neither the H_2O_2 consumption nor the resistance of the intact cell. Moreover, H_2O_2 consumption and resistance can occur by apparently different mechanisms. These findings may be relevant for pathophysiologic concepts and therapeutic designs based on oxidant injury of human cells.

Methods

Cells. Three breast adenocarcinoma cell lines were used. SK-BR-1-III, derived from a pleural effusion (5), was grown as a suspension in RPMI 1640 medium (KC Biologicals, Lenexa, KS) supplemented with 1 mM pyruvate, 300 μg/ml glutamine, 0.1 mM nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin (P/S_2) (Gibco Laboratories, Grand Island, NY), and 20% heat-inactivated fetal bovine serum (FBS_{20}) (HyClone Laboratories, Logan, UT). This medium is designated R-S. SK-BR-2-III, explanted from ascites, was grown in suspension in R-S. CAMA-I, from a pleural effusion, was grown as a monolayer in α-modified Eagle's minimum essential medium (KC Biologicals) with 0.1 mM nonessential amino acids, 15% heat-inactivated fetal bovine serum (FBS), and P/S_2. This medium is designated M-S. SK-OV-3, an ovarian adenocarcinoma cell line derived from ascites (5–7), was grown as a monolayer in R-S. The above lines were obtained from Dr. J. Fogh, Sloan-Kettering Institute, Rye, NY. B0467, an Epstein-Barr virus-induced B cell line grown in suspension in R-S, was obtained from Dr. N. Chiorazzi, The Rockefeller University, New York, NY. HSB, a T cell line from a patient with acute lymphocytic leukemia, was obtained from the Human Genetic Mutant Cell Repository, Institute for Medical Research, Camden, NJ, and was grown in suspension in R-S. CCD-21SK, a human skin fibroblast line, was obtained from American Type Culture Collection, Bethesda, MD, and was grown as an adherent monolayer in M-S. All cell lines were periodically checked for mycoplasma and found to be negative by fluorescent bisbenzimide staining (Hoechst 33342; Aldrich Chem. Co., Milwaukee, WI). Normal human lymphocytes were isolated from buffy coats purchased from the New York Blood Center. Mononuclear leukocytes were obtained as described (8) with collection of the cells that were nonadherent to glass after 2–4 h incubation in RPMI 1640 medium containing 25% human serum. The nonadherent mononuclear cells, which were 82% positive by direct immunofluorescence with monoclonal anti-Leu-4 antibody (Becton-Dickinson & Co., Oxnard, CA), were incubated in R-S for 17–30 h before use. Normal human erythrocytes were isolated from heparinized (30 U/ml) venous blood and washed by centrifugation in RPMI and used immediately. Each experiment with normal human erythrocytes and/or lymphocytes used

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1. *Abbreviations used in this paper:* AT, 3-amino-1,2,4-triazole; BCNU, 1,3-bis(2-chlorethyl)-1-nitrosourea; BSO, buthionine sulfoximine; FBS, heat-inactivated fetal bovine serum; GR, glutathione reductase; GSH, glutathione; KRPG, Krebs-Ringer phosphate buffer containing 7.2 mM phosphate and 5.5 mM glucose; LD_{50}, 50% specific lysis; M-5% HS, α-modified Eagle's minimal essential medium with 5% heat-inactivated horse serum; M-S, α-modified Eagle's minimal essential medium with 0.1 mM nonessential amino acids, 15% FBS and 100 U/ml penicillin and 100 μg/ml streptomycin; MEM, minimal essential medium; P/S_2, 100 U/ml penicillin, 100 μg/ml streptomycin; R-S, RPMI 1640 medium supplemented with 1 mM pyruvate, 300 μg/ml glutamine, 0.1 mM nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and heat-inactivated FBS.

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a different donor as the cell source. Mouse mastocytoma (P815) and lymphoma cells (L1210, P388, TLX9) were as described (2, 9).

Cytolysis assay. To determine susceptibility to lysis by H_2O_2, nonadherent cells, usually numbering 2–5 × 10^6, were suspended in 2 ml α-modified minimal essential medium (MEM)-5% heat-inactivated horse serum (HyClone Laboratories) (M-5% HS) with 200 μCi of $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, MA), incubated at 37°C in 5% CO_2/95% air for 60 min, and washed four times by centrifugation in M-5% HS. Cell concentration and viability were determined by counting trypan blue-excluding cells in a hemocytometer. 4 × 10^4 ^{51}Cr-labeled cells in 0.2 ml M-5% HS were added to round-bottomed microtest plate wells (Linbro Division, Flow Laboratories, McLean, VA) containing graded amounts of H_2O_2 (Superoxol; Fisher Chemical Co., Fairlawn, NJ) or glucose oxidase (Sigma Chemical Co., St. Louis, MO), diluted in 0.9% NaCl. When glucose oxidase was used, the glucose concentration was raised to 40 mM. H_2O_2 generated by glucose oxidase was measured using the O_2 monitor as described below for H_2O_2 consumption. Plates were incubated at 37°C in 5% CO_2/95% air for 3 h. For murine tumor cells, non-α-modified MEM was used as before (9). After centrifugation at 550 *g* for 5 min, 0.1 ml supernate was removed for gamma counting. Results were similar when the assay time was extended to 4.5, 6, or 16 h, or when 5% horse serum was replaced with 5% human serum or 0.005% gelatin (Fisher Chemical Co.). For adherent cells, 2 × 10^5 cells were plated on 13-mm diam glass coverslips in 24-well Costar plates (Data Packaging, Inc., Cambridge, MA), labeled by the addition of 1 ml M-5% HS containing 2 μCi $Na_2{}^{51}CrO_4$ for 60 min at 37°C in 5% CO_2/95% air, and washed four times in M-5% HS. The cells were then incubated in 1 ml M-5% HS with or without H_2O_2 for 3 h, centrifuged as above, and 0.5 ml supernate was removed as sample. The residual supernate was pooled with the 0.5 N NaOH lysate of the remaining cells and both the sample and residual were used for gamma counting. The concentration of H_2O_2 causing 50% specific lysis (LD_{50}) was calculated by interpolation as previously described (9). Determination of cell susceptibility to lysis by superoxide and hydroxyl radicals was performed as above with modifications to reduce the scavenging effect of serum on these oxygen intermediates. ^{51}Cr-labeled cells were placed in serum-coated borosilicate glass tubes (12 × 75 mm) in Krebs-Ringer phosphate buffer containing 7.2 mM phosphate and 5.5 mM glucose (KRPG) (8) in the presence of graded amounts of KO_2 (Sigma Chemical Co.), or H_2O_2 together with an equimolar amount of $FeSO_4$ (chelated by a threefold molar excess of EDTA) (Sigma Chemical Co.) for 30 min at 37°C in air. M-5% HS was then added and the tubes were incubated in 5% CO_2/95% air for an additional 2 h to allow for completion of the ^{51}Cr release. Adherent cells were treated similarly except that cell-bearing coverslips were placed in serum-coated 24-well plates. Effects of OI^- were determined by a 3-h incubation in microtest plates of 4 × 10^4 cells in 200 μl KRPG containing 0.005% gelatin together with lactoperoxidase (Sigma Chemical Co.), iodide, and H_2O_2 as indicated. Lysis as estimated by the specific release of ^{51}Cr was closely comparable to lysis estimated from hemocytometer counts of cells excluding 0.2% trypan blue.

Biochemical assays. Total cell glutathione (GSH plus GSSG) was measured according to Tietze (10) in the 2.5% sulfosalicylic acid-soluble fraction of 0.1% Triton X-100 (Sigma Chemical Co.) lysates. GSSG reductase (GR) was assayed according to Roos et al. (11), and GSH peroxidase according to Paglia and Valentine (12). Catalase was measured by two methods. The colorimetric assay of Baudhuin et al. (13) detects the interference by cell lysates with the oxidation of titanium sulfate by exogenous H_2O_2. For more sensitive determinations, polarimetry was employed with an oxygen electrode (Model 53; Yellow Springs Instrument Co., Yellow Springs, OH) coupled to a magnetically stirred, water-jacketed vessel. The system was calibrated at 37°C using air-saturated phosphate-buffered saline (9.53 mM phosphate, 0.15 M sodium, 0.14 M chloride, 4.15 mM potassium, pH 7.4) (PBS). Then 2.95 ml PBS containing 5 mM H_2O_2 was purged with N_2 until <5% of the base-line O_2 concentration was detected. 50 μl of 0.2% Triton X-100 cell lysate (4 × 10^7 cells/ml) was added and catalase activity was determined from the nanomoles of O_2 generated per minute per cell number or cell protein. Cellular peroxidases were measured with three different cosubstrates (Sigma Chemical Co.): guaiacol by the method of Maehly and Chance (14), NADH by a modified method of Avigad (15), and ascorbate as described (16). Protein was determined by the method of Lowry et al. (17) with bovine serum albumin as the standard. H_2O_2 was measured by the horseradish peroxidase-catalyzed oxidation of fluorescent scopoletin (8), O_2^- by the superoxide dismutase-inhibitable reduction of ferricytochrome c (8), and $OH^\cdot$ by the production of formaldehyde from dimethylsulfoxide (18).

H_2O_2 consumption. Cells were incubated at 37°C in 5% CO_2/95% air at 1 × 10^6 cells/ml M-5% HS for nonadherent cells and ~5 × 10^5 cells per coverslip in 1 ml M-5% HS for adherent cells. At time 0, 5 mM H_2O_2 was added. At 0, 30, 60, and 180 min, the H_2O_2 concentration was determined on aliquots by a modification of the method of Schroy and Biaglow (19). The oxygen electrode system described above was calibrated with air-saturated PBS. 2.8 ml PBS containing 200 U/ml bovine liver catalase (Sigma Chemical Co.) were purged with N_2. A 200-μl aliquot of the reaction medium was added with a Hamilton syringe and the generation of O_2 was followed to completion. H_2O_2 was calculated as twice the generated O_2.

Enzyme inhibitors. Sodium azide (Fisher Chemical Co.) was dissolved in 0.9% NaCl and added to cells at 0.5 mM immediately before assay. 3-amino-1,2,4-triazole (AT; Sigma Chemical Co.) was dissolved in M-5% HS and added to cells at 50 mM for 60 min during ^{51}Cr-labeling as well as during assays. BSO (Chemical Dynamics Corp., S. Plainfield, NJ) was dissolved in H_2O at 20 mM and cells were exposed to 0.2 mM BSO for 17 h before ^{51}Cr-labeling and throughout the assays. BCNU (Bristol Laboratories, Syracuse, NY) was dissolved in absolute ethanol at 100 mg/ml, diluted to 1 mg/ml in MEM, and added to cells at 100 μg/ml for the last 10 min of ^{51}Cr-labeling.

Results

Identification of cytolytic oxygen reduction products. To determine which extracellularly generated oxygen reduction products rapidly lyse human cells, and therefore which enzymes might be involved in defense against oxidative cytolysis, we compared the susceptibility of fibroblasts, SK-BR-1-III, SK-BR-2-III, erythrocytes, and CAMA-I to H_2O_2, O_2^-, and $OH^\cdot$. Lysis first was attempted with the H_2O_2 generating system of glucose oxidase and glucose, which lysed most murine tumors tested (1). However, fluxes of H_2O_2 (nanomoles per minute per 0.2 ml) up to 3 for fibroblasts, 14 for SK-BR-1-III, 15 for SK-BR-2-III, 21 for erythrocytes, and 105 for CAMA-I failed to produce substantial lysis (not shown). Consistent with this, there was no significant lysis of SK-BR-2-III or fibroblasts by phorbol myristate acetate-stimulated human granulocytes or monocytes at a 10-fold or 20-fold excess over target cells during 6 h of co-incubation (not shown). We therefore tested these cells' susceptibility to preformed H_2O_2. As shown in Fig. 1 for SK-BR-2-III, H_2O_2 was lytic in a dose-dependent, catalase-inhibitable manner. 50% lysis by 3 h required 1.2 mM H_2O_2. Results were similar with the other four cell types.

We next attempted to lyse these cells with the O_2-generating system of xanthine oxidase and acetaldehyde. Using each agent at the maximal nontoxic concentration, only μM concentrations of O_2^- were detected. As these concentrations were not cytolytic, we employed preformed O_2^- (as KO_2). Fig. 1 shows that lysis of SK-BR-2-III by <10 mM O_2^- was inhibitable by catalase and thus presumably due to dismutation of O_2^- to H_2O_2. At 10 mM O_2^-, catalase was no longer inhibitory, but the same extent of cytolysis was seen by bringing the pH of the medium to 10.5, the pH attained with 10 mM O_2^-, as anticipated from the reaction: $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$ (20). Results were similar for CAMA-I, fibroblasts, and erythrocytes (not shown).

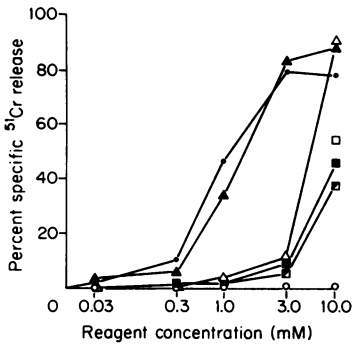

Figure 1. Effect of oxygen intermediates on SK-BR-2-III viability. 2×10^5 ^{51}Cr-labeled SK-BR-2-III cells in KRPG were incubated in a 37°C H_2O bath in serum-coated borosilicate glass tubes in the presence of the indicated concentrations of H_2O_2, KO_2, or H_2O_2 with EDTA-chelated $FeSO_4$, all in the presence or absence of 2,000 U/ml catalase for 30 min. M-5% HS was then added to all tubes and they were transferred to a 37°C incubator with 5% CO_2/95% air for an additional 2 h. Percent specific ^{51}Cr release was then measured. (●), H_2O_2; (▲), KO_2; (■), H_2O_2 + EDTA-chelated $FeSO_4$; (◩), EDTA-chelated $FeSO_4$; (○), H_2O_2 + catalase; (△), KO_2 + catalase; (□), H_2O_2 + EDTA-chelated $FeSO_4$ + catalase. Values on the abscissa represent the concentration of added oxygen intermediate and/or $FeSO_4$. EDTA was present in threefold molar excess over $FeSO_4$. Points are means of triplicates in two or more experiments.

To generate OH·, we added EDTA-chelated $FeSO_4$ to H_2O_2. This resulted in rapid loss of H_2O_2 and generation of formaldehyde from added dimethylsulfoxide (18), indicating the production of at least micromolar concentrations of OH·. However, as shown in Fig. 1 for SK-BR-2-III, only marginal cell lysis was attained with these reagents, and this could be reproduced with EDTA-chelated $FeSO_4$ alone. Results were similar with CAMA-I and erythrocytes. The sensitivity of fibroblasts to H_2O_2, however, was enhanced by EDTA-$FeSO_4$ and this enhancement was abolished by catalase, suggesting that this cell type was susceptible to OH· derived from H_2O_2.

As first shown for bacteria by Klebanoff (21) and for mammalian cells by Edelson and Cohn (22), the hypohalite generated by H_2O_2, peroxidase, and halide is extremely cytotoxic. Table I illustrates the finding that H_2O_2-dependent lysis of SK-BR-2-III was augmented ~100-fold by lactoperoxidase and iodide. Catalase prevented lysis. Azide, which was expected to inhibit lactoperoxidase, nonetheless augmented lysis two- to threefold. The tumor cells seemed to be using an azide-sensitive pathway for protection against H_2O_2.

The foregoing results suggested that the more reduced forms of O_2, primarily H_2O_2 and IO^-, and in some cases OH·,

Table I. Potentiation of H_2O_2 Lysis of SK-BR-2-III by Peroxidase and Iodide

Treatment	LD_{50}
	mM
H_2O_2	5.35
H_2O_2 + LPO/KI	0.05
H_2O_2 + LPO/KI + catalase	>30
H_2O_2 + LPO/KI + azide	2.05
H_2O_2 + azide	2.25

4×10^4 ^{51}Cr-labeled SK-BR-2-III cells in 200 μl KRPG containing 0.005% gelatin were incubated for 3 h at 37°C in air in the presence of H_2O_2 (ranging from 0.03 to 30 mM) with and without 1,000 U/ml lactoperoxidase, 0.1 mM KI, 2,000 U/ml catalase, and 1.5 mM azide. Specific ^{51}Cr release was measured and concentrations of H_2O_2 resulting in 50% lysis of cells (LD_{50}) were calculated by interpolation.

were lytic to human cells, while the less reduced O_2^- was not. As there is no known enzymatic defense against IO^- or OH·, we next focused on the cells' enzymatic defenses against H_2O_2.

Quantification of susceptibility of human cells to H_2O_2 and relation to levels of catalase, GSH redox cycle components, and other peroxidases. Study of human cell susceptibility to and defense against H_2O_2 was undertaken with the following cell types: three breast adenocarcinomas, of which two (SK-BR-2-III and SK-BR-1-III) were nonadherent and one (CAMA-I) was adherent, an adherent ovarian adenocarcinoma (SK-OV-3), a nonadherent B cell line (B0467), a nonadherent leukemic T cell line (HSB), an adherent, contact-inhibited diploid skin fibroblast line (CCD-21-SK), erythrocytes, and peripheral blood lymphocytes. All nine of these human cell types exhibited far greater resistance to lysis by H_2O_2 than previously studied murine cells, with LD_{50}s ranging from 2 to 20 mM (Table II). In contrast to a number of earlier reports on oxidative lysis of human cells, these experiments were conducted in media of physiologic tonicity, pH, and glucose concentration.

Several potential scavengers of H_2O_2 were measured in each cell type (Table II). Although human tumors have been said to lack catalase (23, 24), we found catalase activities varying over a 38-fold range for tumors and over a 3,000-fold range for nonmalignant cells. GR activities varied 250-fold and GPO activities varied 65-fold. No correlation between susceptibility to H_2O_2 and the activity of any of these enzymes was observed. GSH content was similar among all the cell types. No peroxidases were detected that utilized guaiacol, NADH, or ascorbate as substrates.

Effect of inhibition of antioxidant defenses on lysis by H_2O_2. To understand better the role that these H_2O_2 scavengers might play in protecting cells from oxidative stress, we measured sensitivity of the cells to lysis by H_2O_2 in the presence of inhibitors or depletors of the antioxidants. Thus, the GSH redox cycle was interrupted by 75–100% inhibition of GR with BCNU, and by inhibition of GSH synthesis with BSO, resulting in 70–98% depletion of GSH. To inhibit cellular catalase, we first used AT. By the colorimetric method of Baudhuin et al. (13), AT apparently afforded 100% inhibition of catalase. However, a fivefold more sensitive assay of catalase based on the polarigraphic determination of the generation of O_2 from H_2O_2 indicated that inhibition by AT was variable (85–100%), even in the presence of exogenous H_2O_2 (25). In contrast, 0.5 mM sodium azide resulted in 100±5% (mean±SEM, 12 experiments) inhibition of human cell catalase, as determined polarigraphically.

The effect of the above agents on the LD_{50} of H_2O_2 for each of nine human cell types is shown in Table III. BCNU and BSO reduced the LD_{50} of H_2O_2 for fibroblasts by 10- to 20-fold. In contrast, the GSH redox cycle in two other nonmalignant cell types (lymphocytes and erythrocytes) and in all the tumors studied except SK-BR-1-III, appeared unable to substantially protect cells from lysis by H_2O_2. In contrast to the other cell types studied, in SK-BR-1-III BSO and BCNU treatments had different effects on H_2O_2 sensitivity. It is possible that other effects of BCNU besides inhibition of GR may be more prominent in this cell type. Inhibition of catalase with azide reduced LD_{50}s by 66–70% for lymphocytes, erythrocytes, and all the tumors except B0467 and HSB. Similar results were often seen with AT implicating an effect on catalase rather than on mitochondrial respiration. B0467 and

Table II. Antioxidant Defenses in Human Cells

Cell type	LD_{50}	Catalase	GSH	GPO	GR
	mM				
Erythrocytes	1.7±0.1*	2,300±942	9.5±2.3	16.1±5.2	30.5±3.2
SK-BR-2-III	3.3±0.7	22.6±2.5	30.0±0.8	0.0±0.0	51.1±2.7
Fibroblasts	4.7±0.8	0.7±0.1	9.3±3.3	13.3±4.8	1.5±0.5
SK-BR-1-III	5.2±1.3	13.2±0.7	43.8±3.6	0.0±0.0	45.6±2.8
HSB	6.0±0.8	0.6±0.4	4.3±1.7	11.8±7.7	13.8±4.8
Lymphocytes	9.3±0.9	7.7±2.8	16.8±0.4	23.7±4.6	248±78
B0467	9.4±1.3	1.3±0.3	18.3±4.5	65.0±7.6	18.9±2.6
SK-OV-3	19.4±0.9	1.6±0.3	34.4±12.9	4.5±1.1	1.7±0.9
CAMA-I	19.7±1.6	1.1±0.1	21.8±4.6	15.9±2.3	5.5±1.8

Levels of various antioxidants were measured in 0.2% Triton X-100 lysates of cells at a concentration of 10–20 × 10^6 cells/ml. Values represent the means of triplicates of 2–3 experiments±SEM. Catalase is expressed in Baudhuin units per milligram protein × 10^3, GSH as nanomoles per milligram protein, GPO and GR as nanomoles of NADPH oxidized per minute per milligram protein. LD_{50}s are calculated as described in the legend of Table I. * This value represents LD_{30} (mM H_2O_2).

HSB were not substantially sensitized by any of the inhibitors tested. The combination of azide and BSO was no more effective than the more effective agent alone. In apparently utilizing the GSH redox cycle for defense against H_2O_2, fibroblasts resembled murine cells (2, 3), although the LD_{50} of H_2O_2 for fibroblasts (4.7 mM) was approximately two orders of magnitude higher than that of the murine cells. Thus, the level of sensitivity or resistance to H_2O_2 exhibited by a cell does not appear to predict whether it relies on the GSH redox cycle to resist injury by H_2O_2.

Identification of H_2O_2-consuming pathways. Although it was assumed that degradation of H_2O_2 was the primary defense of cells against it, the possibility was considered that different pathways might be involved in resistance to and degradation of H_2O_2. The rate of H_2O_2 consumption with or without prior exposure to BSO (to deplete GSH) and azide (to inhibit catalase) was therefore measured in five cell types. The results for four of them are displayed in Fig. 2. 17 h pretreatment with BSO had no effect on the cells' capacity to consume 5 mM H_2O_2 over a 3 h period. In contrast, azide inhibited H_2O_2 consumption by an average of 90%. Results with BSO plus azide were the same as with azide alone. Similar results were seen with B0467, a cell line whose LD_{50} for H_2O_2 was unaffected by azide (Table III). Thus, all five cell types tested appeared to utilize an azide-sensitive mechanism (probably catalase) almost exclusively to catabolize millimolar concentrations of extracellular H_2O_2.

Discussion

We conclude from this study that all nine human cell types tested were markedly resistant to lysis by H_2O_2 (with LD_{50}s in the 2–20 mM range), that under appropriate experimental conditions, resistance to H_2O_2 could be manifest in the absence

Table III. Effect of Inhibitors of Antioxidant Defenses on Human Cell Lysis by H_2O_2

Cell type	Control LD_{50}	Percent of control				
		BSO	BCNU	AT	Azide	Azide/BSO
	mM					
Erythrocytes	1.7±0.1*	‡	122±28	29±15	29±1	‡
SK-BR-2-III	3.3±0.7	67±4	70±9	86±8	48±6	70±24
Fibroblasts	4.7±0.8	5±1	10±6	68±26	44±9	4±2
SK-BR-1-III	5.2±1.3	121±21	36±4	54±18	44±11	78±21
HSB	6.0±0.8	87±14	112±52	80±10	72±11	54±4
Lymphocytes	9.3±0.3	137±36	125±19	93±15	23±3	37±17
B0467	9.4±1.3	140±20	74±25	63±14	77±13	85±14
SK-OV-3	19.4±0.9	74±16	66±27	26±10	24±10	23±13
CAMA-I	19.7±1.6	99±4	98±12	60±16	43±2	49±7

2 × 10^5 ^{51}Cr-labeled cells per ml in M-5% HS were incubated in the presence of reagent H_2O_2 (0.03–30 mM) for 3 h at 37°C in 5% CO_2/95% air. Specific ^{51}Cr release was measured and LD_{50} levels were calculated by interpolation. Before the assay, cells were treated with vehicle alone or where indicated, with 100 μg/ml BCNU for 10 min, 50 mM aminotriazole for 60 min in the presence of 0.44 U of glucose oxidase for the last 30 min, 0.2 mM BSO for 17 h. When used, AT and BSO also were present throughout the assay. Where indicated, 0.5 mM azide was present during the assay period only. Values represent the mean±SEM of triplicates in 2–10 experiments. * This value represents LD_{30} (mM H_2O_2). ‡ Indicates not tested.

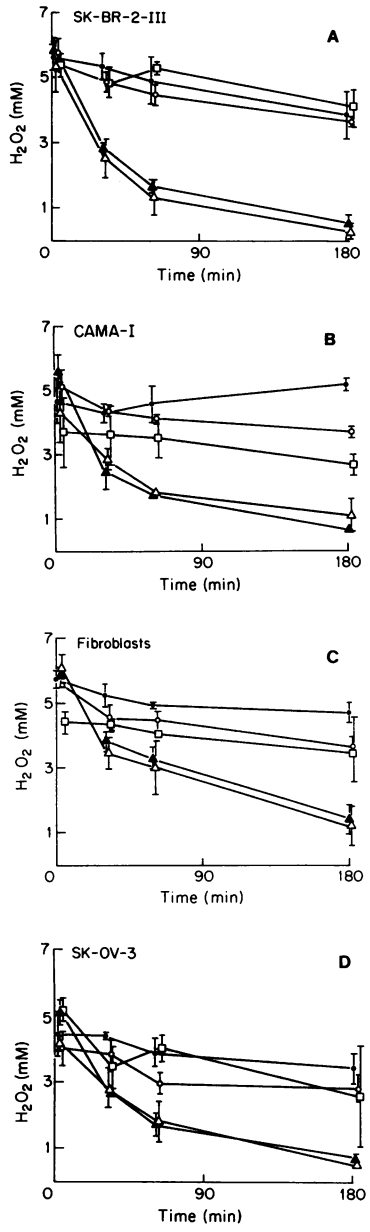

Figure 2. H_2O_2 consumption by human cells. 5 × 10^5 cells plated on 13-mm coverslips or 1 × 10^6 cells in 1 ml of M-5% HS were incubated with ~5 mM H_2O_2 at 37°C in 5% CO_2/95% air. At 0, 30, 60, and 180 min, H_2O_2 remaining in the medium was measured using an oxygen electrode. Cells treated with BSO were preincubated with 0.2 mM drug for 17 h before assay and then throughout the assay. Azide at 0.5 mM was present during the 180-min exposure of the cells to H_2O_2. Points are means±SEM of three to six experiments. (●), no cell control; (▲), cells + H_2O_2; (△), BSO-treated cells + H_2O_2; (○), azide treated cells + H_2O_2; (□), BSO + azide treated cells + H_2O_2.

of its detectable consumption, and that the specific activity or content of the primary known H_2O_2 scavenging systems (catalase and the GSH redox cycle) did not predict which of them, if either, played a major role in resistance to H_2O_2-mediated cytolysis in a given cell. These findings have implications for pathophysiologic and therapeutic concepts involving oxidative cellular injury and its pharmacologic control.

We focused on resistance to and degradation of H_2O_2 because preliminary studies identified H_2O_2 as the only enzymatically degradable O_2 reduction product capable of rapidly lysing the cells under study. The toxicity of H_2O_2 rather than O_2^- under these conditions agrees with prior studies on fibroblasts (26) and lymphocytes (27). Thus, our attention was directed away from measurement of tumor cell superoxide dismutase (28–34) and toward study of catalase and GSH redox cycle components and the consequences of their pharmacologic inhibition or depletion. However, it is possible that H_2O_2 lyses cells through interaction with components of the assay system, such as O_2^- or Fe^{++} (35) of cellular origin, resulting in the formation of $OH^\cdot$ or uncharacterized toxins in the vicinity of critical intracellular targets.

Measurement of the rate at which intact cells degrade H_2O_2 has apparently been reported for only one murine tumor (19), as well as for lymphocytes (27), granulocytes (36), and platelets (36). Our present study revealed that human cell degradation of H_2O_2 was rapid (mean initial rate, 84 nmol H_2O_2/min per mg cell protein with initial $[H_2O_2]$ = 5 mM) and almost completely inhibitable by 0.5 mM NaN_3, presumably reflecting the predominant role of catalase under these conditions. Yet, the rate of azide-inhibitable H_2O_2 consumption by a given cell type was not correlated with the specific activity of its catalase as measured in 0.2% Triton X-100 cell lysates (correlation coefficient, −0.71). This emphasizes that assays of enzymes in lysates may not closely predict their function in intact cells. The unlikely possibility exists that an azide-sensitive enzyme other than catalase was responsible for most of the H_2O_2 consumption.

More important, virtually complete inhibition of detectable H_2O_2 consumption did not sensitize the cells to destruction by all but millimolar quantities of H_2O_2. This demonstrates that resistance to the toxicity of H_2O_2 can have a biochemical basis distinct from catabolic pathways that perceptibly lower the extracellular concentration of H_2O_2. It is therefore clear that the cell types studied exhibited an inherent resistance to lysis by H_2O_2 even when their ability to consume H_2O_2 was impaired. In theory, the GSH redox cycle could function either to degrade H_2O_2 or to reduce lipid peroxides. The latter action would repair cellular injury rather than prevent it, and might be manifest as a resistance to peroxidative lysis out of proportion to consumption of peroxide. However, as noted below, a prominent role for the GSH redox cycle in resistance to H_2O_2-mediated cytolysis was shown in only one of the nine cell types studied.

In contrast to the predominance of an azide-sensitive mechanism (probably catalase) in degradation of millimolar H_2O_2 in all the human cell types tested, resistance of the cells to lysis by millimolar H_2O_2 during 3 h of exposure was attributable in part to the GSH redox cycle in two cell lines, catalase in seven cell lines, and neither in two others. These interpretations are based on the degree to which the cells were sensitized to H_2O_2 by pharmacologic agents directed against GSH, GR, or catalase. These patterns could not be predicted either from the LD_{50} of H_2O_2 for a given cell type nor from the specific activity or content of the enzymes and substrates involved. These results are consistent with those of Marklund et al. (30), who found no correlation between sensitivity to ionizing radiation or oxygen radical-producing drugs and levels of catalase, GPO, or superoxide dismutase in 46 normal and neoplastic cell types.

We do not understand the biochemical basis for resistance to millimolar H_2O_2 on the part of the human cell types studied here. We have not yet measured certain nonenzymatic antioxidants, such as ascorbate and tocopherol (37). It seems critical to identify the molecular targets whose oxidation by H_2O_2 leads to rapid cell death, and to measure the pool sizes and rates of regeneration of these molecules at rest and during oxidative stress. Another unknown is the role of the subcellular localization of antioxidant defense systems in relation to the critical targets of oxidative injury. Finally, the ^{51}Cr-release

assay used in this study reflects cell lysis. It is possible that H_2O_2 causes important but nonlytic forms of cellular damage at lower concentrations, as previously reported for platelets (36), PMNs (38), lymphocytes (39), natural killer cells (40), and endothelial cells (41).

In the mouse, we have found that 22 cell types are lysed within 3–4 h of exposure to H_2O_2 in the range of 3.73×10^{-5} M ($2.98–4.66 \times 10^{-5}$ M) (geometric mean, LD_{50}±SEM) (Fig. 3). For many of the murine tumors, lytic concentrations of H_2O_2 could be achieved rapidly by phagocytic leukocytes in a 1.4- to 4.5-fold excess over tumor cells, resulting in tumor cell death in vitro (9). Solid-phase glucose oxidase could inactivate some of these same tumors in vivo by generating H_2O_2 in the tumor bed, without harming the host (42). In contrast, the human cells studied here have LD_{50}s approximately 100 times higher (geometric mean±SEM = 3.55×10^{-3} M, $1.92–6.57 \times 10^{-3}$ M) (Fig. 3) with only one fibroblast line (FS4) exhibiting an LD_{50} in the same range as murine cells. Recently the amount of H_2O_2 generated by activated neutrophils has been quantified (43) and found to peak at 12.2 μM for 3×10^5 cells/ml. We think it unlikely that direct, lytic oxidative injury will be sustained by most of these human tumors under attack by leukocytes or enzymatic H_2O_2-generating systems. However, in this report we have only studied nine types of human cells, seven of them after in vitro passage, and do not know to what extent these observations may apply to other human cell types or to tumor masses in vivo. Several hematopoietic human tumor lines (CEM [44], Raji [45], and K562 [46]) have been reported to be susceptible to lysis by leukocyte-derived oxidants in other studies. In addition, an LD_{50} of 1.4 mM H_2O_2 has been reported for a murine sarcoma (47). Thus, the general distinction drawn here between large numbers of unselected murine and human cell types does not reflect an absolute species difference.

Indeed, it is of interest that certain normal human cell types seem to be much more sensitive to oxidative injury than the cells studied here. Weiss et al. (48) showed that human umbilical vein endothelial cells were sensitive to lysis by H_2O_2 released from stimulated neutrophils. The concentration of H_2O_2 generated by the neutrophils was 67 μM, 1–2 logs lower than the LD_{50}s of the human cells in this study. Harlan et al. (49) documented that micromolar H_2O_2 could lyse human endothelial cells and that the GSH redox cycle could protect these cells from a flux of H_2O_2 generated by glucose oxidase and glucose (50). Simon et al. (26) lysed human fibroblasts with enzymatically generated fluxes of H_2O_2 of 1.6–1.9 μM/min (26). Even in our study, where human fibroblasts were insensitive to fluxes of H_2O_2 attainable with glucose oxidase or xanthine oxidase, the cells were rendered up to 20-fold more sensitive to H_2O_2 by inhibition of the GSH redox cycle with BSO or BCNU. Thus, the possibility remains that endothelial cells or other components of tumor vasculature might be suitable targets for the localized delivery of oxidant stress to the tumor bed, in conjunction with pharmacologic inhibition of the GSH redox cycle (3, 4).

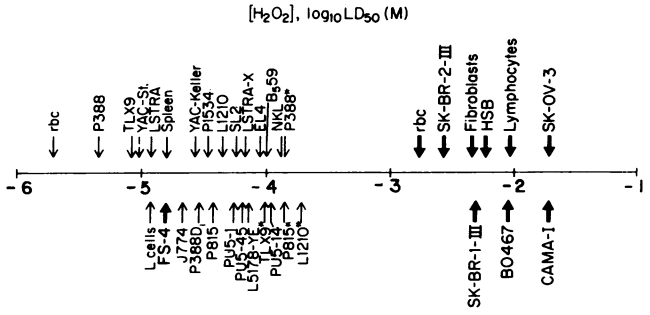

Figure 3. [H_2O_2], $\log_{10}$ LD_{50}(M). Values indicated are $\log_{10}$ of molar LD_{50}'s of H_2O_2 averaged for 105 experiments with 22 types of murine cells (→) and 10 types of human cells (➞). Results for FS-4 (a human fibroblast line) and 36 experiments with murine cells are from earlier reports (9, 51, 52) and have been averaged with results of 32 subsequent unpublished experiments. 18 additional experiments with murine cells were performed during the studies with human cells described in this report; these are presented separately and denoted (•). rbc, erythrocytes.

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