10-3-1986

The Effect of Glutathione Depletion by Diamide, Diethyl Maleate or Buthione Sulfoximine on the Surface Structure of Mouse L-Cells

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THE EFFECT OF GLUTATHIONE DEPLETION BY DIAMIDE, DIETHYL MALEATE OR BUTHIONE SULFOXIMINE ON THE SURFACE STRUCTURE OF MOUSE L-CELLS

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Abstract

Radiosensitizers that act by reducing glutathione levels have been studied by many workers as agents to enhance the killing of hypoxic tumour cells. In this paper we describe a scanning electron microscope (SEM) study of the surface morphology of mouse L-cells after exposure to three of these: diazenedicarboxylic acid bis N,N-dimethylamide (diamide), DL-buthionine-S,R-sulfoximine (BSO) and diethyl maleate (DEM). Diamide at 0.1 to 0.6 mMol/L for 10 min produced large blebs on the cell surface as seen in the SEM. Transmission electron microscope (TEM) images show that these are clear, featureless regions of cytoplasm. BSO treatment for 24 h at 0.5 to 2.0 mMol/L, by contrast, left the surface similar to control cells. DEM at 0.5 mMol/L for 2 h produces small blebs over the cell surface and a reduction in the number of microvilli. A combined 24 h BSO treatment with 2 h of DEM produced large blebs, which were similar in TEM micrographs to those produced by diamide. Thus, although all three sensitizers reduce glutathione levels, they have different effects on cellular morphology and hence such secondary effects may account for the different degrees of radiation sensitization seen with these agents.

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Introduction

Cellular thiols such as glutathione can protect against cell killing, mutagenicity or transformation produced by exposure to ionizing radiation or chemical carcinogens (Meister, 1983). Since cells cultured from patients with the disease 5-oxoprolinuria, characterized by a low intercellular glutathione concentration, are viable and show a reduced oxygen enhancement ratio after X-irradiation, thiol depletion has been suggested as a method to sensitize tumour cells to radiation therapy (Clark et al., 1984). Chemical agents which lower cellular thiol levels have been studied extensively alone or in combination with other sensitizers.

Three chemicals that have been widely used to reduce the levels of non-protein thiols, mainly glutathione, before irradiation are: Diazenedicarboxylic acid bis N,N-dimethylamide (diamide), which oxidizes glutathione; DL-buthionine-S,R-sulfoximine (BSO), a competitive inhibitor of glutamylcysteine, a key enzyme in the biosynthesis of glutathione; and diethyl maleate (DEM), a glutathione-depleting agent which lowers glutathione by DEM-glutathione binding catalyzed via glutathione-S-transferase as well as by spontaneous binding to glutathione.

Diamide has the longest history of the three compounds (Kosower et al., 1969). It is reported to decrease active Na^+-K^+ transport and to alter glucose and amino acid transport at the cell membrane (Szekely et al., 1982a; Leoncini and Maresca, 1983; Goldstein and Livingston, 1978; Chesney et al., 1985), to decrease actin binding at membrane sites (Spangenberg et al., 1984), to effect the rate of tubulin polymerization (Oliver et al., 1976), and to disrupt cellular morphology (Szekely et al., 1982a, 1982b; Power et al., 1977; Shelton et al., 1980). The radiosensitizing concentration of diamide is larger than that required to oxidize all of the cellular glutathione. This suggests that sensitization is partly by mechanisms other than glutathione depletion.

Although DEM has not been studied as extensively, there are suggestions that it may radiosensitize by mechanisms other than through

glutathione depletion (Biaglow et al., 1983). DEM has been reported to have many metabolic effects including inhibition of cell division, respiration and glycolysis (Webb, 1966). DEM's radiosensitizing effect may also be related to its high lipid solubility.

BSO, a competitive inhibitor of glutamyl-cysteine synthetase, depletes cells of glutathione as it is utilized in metabolism. Therefore, BSO decreases the glutathione concentration without affecting other sulfhydryls within the cell (Clark et al., 1984; Meister, 1983). However, BSO above 1 mMol/L is toxic to cells under prolonged culture conditions. BSO requires long, > 10 h, contact time to reduce glutathione levels and it may have, as yet undetected, side effects.

Even though all the consequences of thiol depletion on cellular functions have not been determined, it is clear that the perturbation in glutathione level is important in determining the response of cells to radiation in hypoxic and perhaps aerobic conditions. Studies of these sensitizers have concentrated on their chemical interactions to identify the mechanisms of radiosensitizations. Ultrastructural changes induced by exposure to the sensitizers have not been studied. Ultrastructure may influence the sensitization or give clues to the mode of action of the sensitizers.

In this paper, we describe a scanning electron microscopy (SEM) study of the surface morphology of mouse L-cells after exposure to diamide, BSO and DEM. Transmission electron microscopy (TEM) views of the exposed cells are also included to document the cellular ultrastructure.

Materials and Methods

Cell Culture

Mouse L-cell fibroblasts (from ATCC CCL 1, NCTC clone 929) were grown in 75 cm^2 flasks, in a dry, $37^{\circ}C$ incubator gassed with 98% air-2% CO_2. The cells were sub-cultured on a weekly basis, with cells being seeded into 25 mL fresh medium (1:1 mixture of Dulbecco's Basal Medium Eagle and Ham's Nutrient Mixture F-12, buffered with 0.020 Mol/L HEPES and 0.010 Mol/L NaHCO$_3$, adjusted to pH 7.4 and supplemented with 10% fetal bovine serum) at a concentration of 1 x 10^4 cells/mL.

Cells for experiments were trypsinized from an exponential growth phase culture by rinsing the monolayer twice with Ca++ and Mg++ free Earl's balanced salt (EBS), then once with 0.01% trypsin in Ca++ and Mg++ free EBS, and incubating for 8 to 10 min. The trypsinized cells were suspended in 10 mL of 1:1 Dulbecco's modified Eagle: Ham's F12 + 10% fetal bovine serum, counted on an electronic particle counter, then plated into either 75 cm^2 flasks at 5 x 10^5 cells/flask in 25 mL medium, or into 60 mm dishes containing 4 12-mm circular cover slips in 5 mL medium at 1.5 x 10^5 cells/dish. Cells in flasks were incubated in a dry, $37^{\circ}C$ incubator, gassed with 98% air-2% CO_2 for 2 days prior to addition of test compounds. Cells in

dishes were placed in a vented, plexiglass chamber, humidified by placing a water-saturated absorbent towel beneath the shelf within the chamber. The chamber was placed in the same incubator as the cells in flasks. The dishes were cultured for 24 to 28 h prior to addition of test compounds.

Scanning Electron Microscopy

Cells grown on 12-mm glass coverslips were rinsed quickly in Hank's buffer with 0.4 mol/L sucrose (HBS), then fixed in 4% glutaraldehyde in HBS (pH 7.4) for 45 min, followed by 2 washes in HBS. The coverslips with attached cells were passed through graded steps of distilled water: ethanol mixtures (2 x 10 min) then passed through a graded series of ethanol:amyl acetate mixtures (2 x 10 min), and finally critical-point dried using liquid CO_2 as the transitional fluid. The coverslips were attached to stubs, gold coated to ~ 20 nm in a Balzers Union Sputter coater, and viewed in a Hitachi S570 microscope operating at 10 or 15 kV with a 9 to 11 mm working distance and a 15° tilt.

Transmission Electron Microscopy

The sensitizer-treated cells were scraped from the flasks with a rubber policeman and pelleted. Two percent glutaraldehyde in Millonig's buffer (2.26% NaH$_2$PO$_4$.H$_2$O, 2.52% NaOH, pH 7.4) was added to the pellet. It was then cut into ~ 1 mm pieces and fixed for 1 h in 2% glutaraldehyde in Millonig's buffer. The samples were then washed twice for 2 min in Millonig's buffer followed by a 1-h fixation in 2% OsO$_4$ in Millonig's buffer at $4^{\circ}C$. After 2 additional buffer washes, the samples were dehydrated in a graded series of acetone:dis-tilled water mixture, with 2 10-min exposures at each step, then infiltrated for 1 h with a 50:50 mixture of acetone and Spurr's epoxy mixture (hard). Finally, the samples were passed through 2 changes of complete resin for 30 min each and then cured overnight at $60^{\circ}C$. Gold sections were cut with a diamond knife, stained with uranyl acetate and Reynold's lead citrate, and viewed in a Philips EM 300 electron microscope operated at 60 kV.

Sensitizer Treatment

Diazenedicarboxylic acid bis N,N-dimethyla-mide (Diamide) from Calbiochem (San Diego, CA) was dissolved in Dulbecco's phosphate buffered saline (PBS) at a concentration of 6 mMol/L as a stock solution. Diethyl maleate (DEM) from Sigma (St. Louis, MO) was dissolved at 50 mMol/L in EBS buffered with 10 mMol/L NaHCO$_3$ and 20 mMol/L HEPES plus 2.5% ethanol. DL-buthione-S,R-sulfo-ximine (BSO) from Chemalog (S. Plainfield, NJ) was dissolved in Dulbecco's PBS at 50 mMol/L. These stock solutions were added to the medium to give the treatment conditions.

Results

Scanning electron microscope images of mouse L-cells show large scale surface blebbing shortly after diamide addition. Cells, such as

those seen in Fig 1, show large surface blebs unlike those seen on normal cells in any part of the cell cycle. Other cells are covered with blebs, which are smaller (see Fig 2), though larger than those seen in untreated G_1 cells (Wetzel et al., 1978; Enlander et al.,[1] 1975). In the diamide treated sample, though, even the flattened cells show blebs. Untreated control cells are shown in Figs 3a to 3c.

In the transmission electron microscope, the blebbing induced by diamide is clearly visible. Organelle-free regions are seen pinching
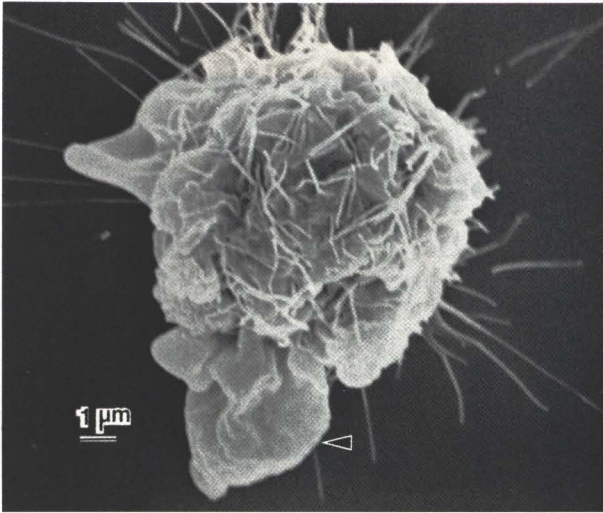

Fig 1. A SEM micrograph of a mouse L-cell treated for 10 min at 37°C with 0.4 mMol/L diamide in medium. An arrow points to the large bleb.
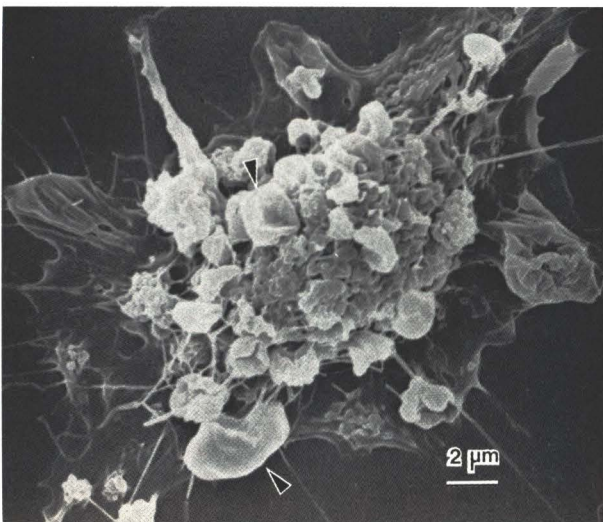

Fig 2. A SEM micrograph of a mouse L-cell treated with 0.2 mMol/L diamide in medium for 20 min at 37°C. Arrows point to large blebs.
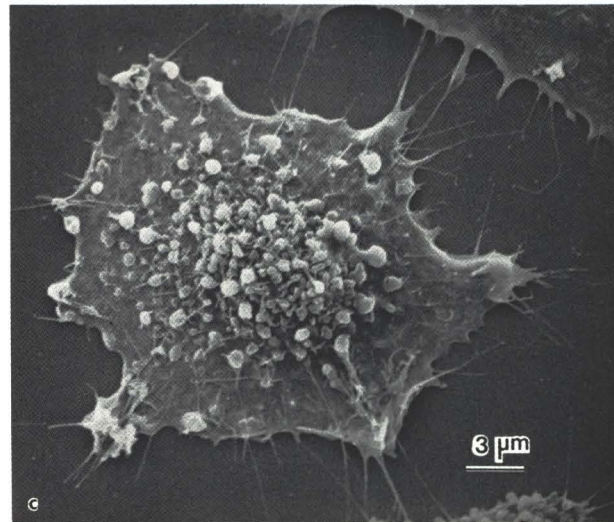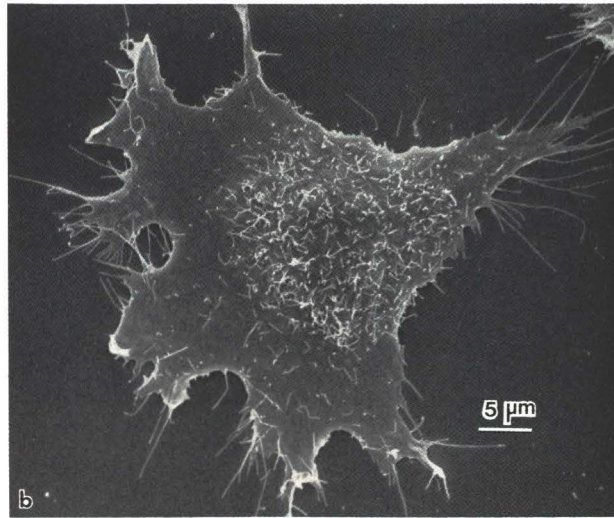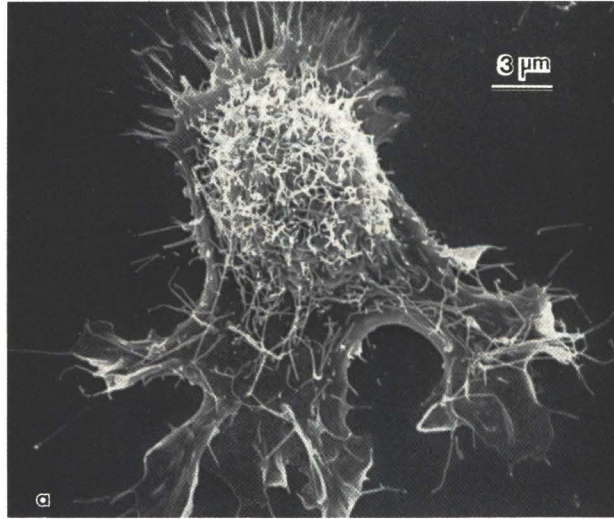

Fig 3. Three SEM micrographs of untreated control mouse L-cells. (a) partially rounded cell. (b) Flat cell. (c) Flat cell with small blebs.

off from the cell (see Fig 4). Bulbous organelle-free regions have been previously reported in diamide-treated cells (Power et al., 1977; Szekely et al., 1982b). The nucleus is invaginated and pushed to one side of the cell, vacuoles and swollen mitochondria collect at the edge of the organelle-free region which appears to pinch off from the cell. Mitochondrial particles are distinct and heavily stained in the diamide-treated cells. After exposure to 0.1 to 0.6 mMol/L diamide for 10 min, all the cells seen are affected. The ultrastructure does not change with longer exposures.

Since BSO reduces the level of non-protein sulfhydryls by blocking the enzyme pathway in

glutathione production, rather than by destroying the existing sulfhydryls within the cell, it must be added 10 to 24 h before radiation to produce the full sensitizing effect (Mitchell et al., 1983). It has been shown that the cell cycle is not affected by BSO at concentrations below 2 mMol/L that bring the glutathione levels down below 10% of the untreated CHO cells (Clark et al., 1984) and in C3H 10T1/2 cells (Hei et al., 1984). We also saw cells at various stages of mitosis in the BSO treated populations confirming that these L-cells can continue through the cycle. The SEM view of the L-cell surface after exposure to 0.5 mMol/L BSO for 20 h showed cells covered with microvilli (see Fig 5) which looked similar to untreated L-cells. The
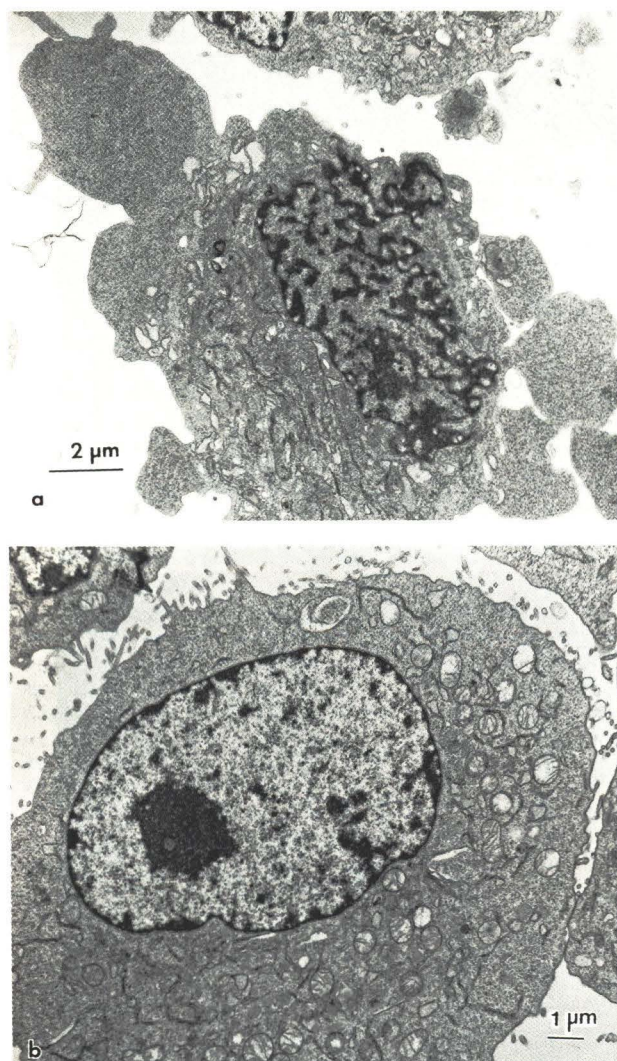

Fig 4. (a) A TEM micrograph of an L-cell exposed to 0.6 mMol/L diamide in medium for 20 min at 37°C. (b) A TEM micrograph of an untreated L-cell.
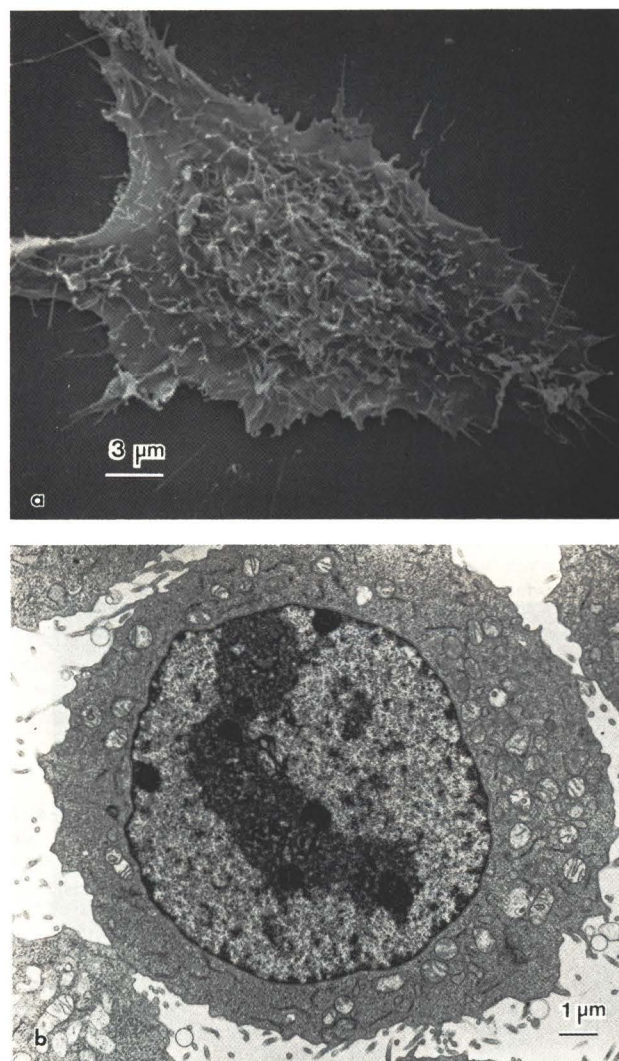
Fig 5. (a) A SEM micrograph of a mouse L-cell treated with 0.5 mMol/L BSO for 20 h at 37°C. (b) A TEM micrograph of an L-cell exposed to 2.0 mMol/L BSO for 21 h at 37°C.

morphology of BSO-treated cells seen in thin sections (Fig 6) show cells which are rounder than the controls and contain more ruffled portions of the plasma membrane. In other respects, they are identical to untreated cells.

In cells treated with DEM at 0.5 to 1.0 mMol/L the surface is usually covered with small blebs (see Fig 7), although occasionally a cell with a large bleb is seen. This is in contrast to the untreated cell (such as shown in Fig 3c) where only a small fraction of cells had blebs on their surface. The bleb covered surface after DEM treatment or less frequently on untreated cells is similar to that reported earlier for cells in early G$_1$ (Wetzel et al., 1978). However, since the L-cells were growing asynchronously in log phase and the DEM was present for

only 1 to 4 h, it could not have held the cells in that post-mitotic portion of the 24-h cycle. Thus, the blebbing is a direct result of DEM. In the TEM, the cytoplasm is comparable to the controls with the exception of the plasma membrane, which shows the small blebs (Fig 8). The cells are also rounded relative to the untreated controls.

In cells treated with BSO for 18 h, combined with exposure to DEM for the last 2 h, the appearance is similar to that seen after DEM
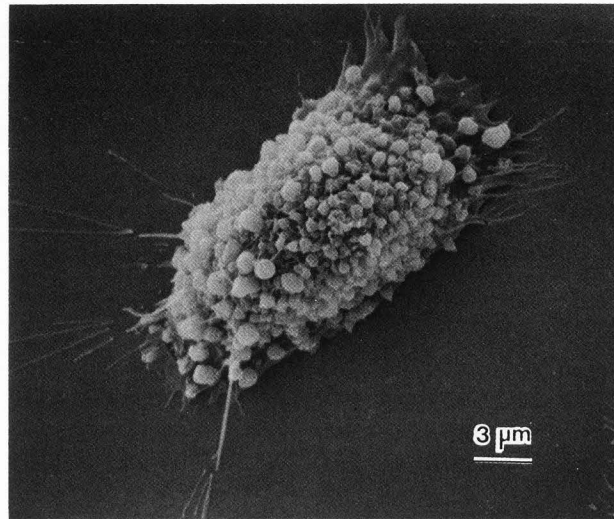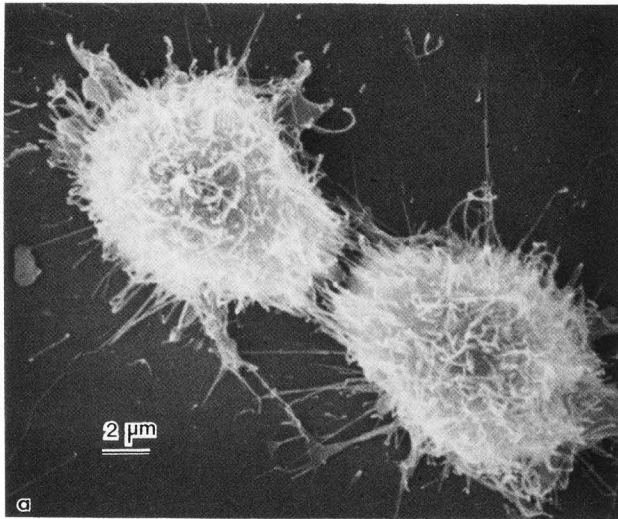

Fig 7. A SEM micrograph of a mouse L-cell exposed to 0.5 mMol/L DEM for 2 h at 37°C.
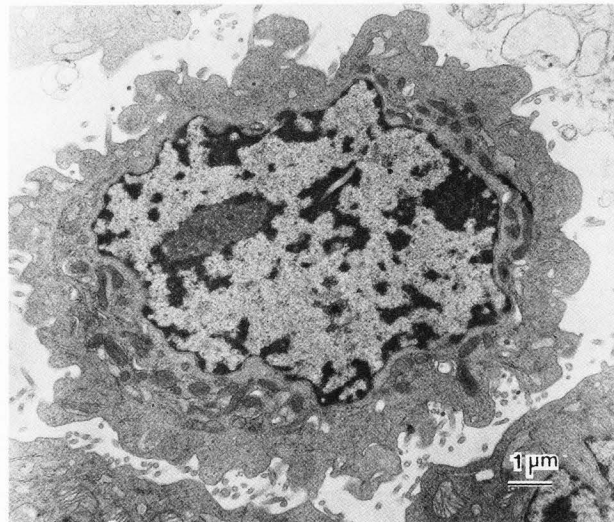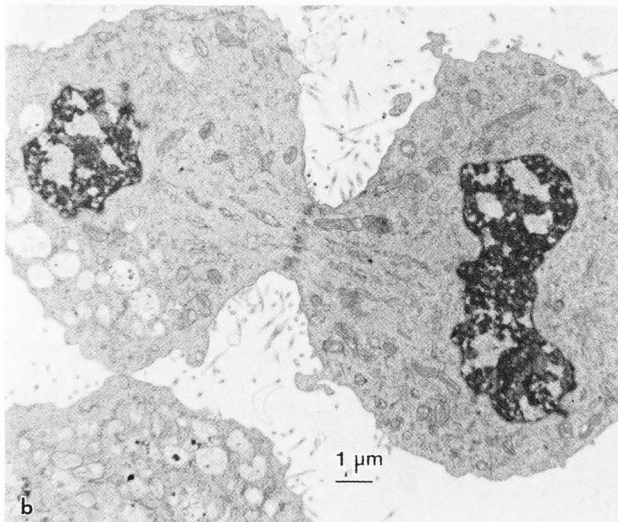

Fig 6. (a) A SEM micrograph of a dividing mouse L-cell seen after 20 h in 0.5 mMol/L BSO. (b) A TEM view of a dividing mouse L-cell which was exposed to 2.0 mMol/L BSO for 20 h.

Fig 8. A TEM electron micrograph of a mouse L-cell exposed to 1.0 mMol/L DEM for 3 h at 37°C.

Fig. 10. A TEM micrograph of a mouse L-cell exposed to 2.0 mMol/L BSO for 18 h followed by the addition of 1.0 mMol/L of DEM for 2 h. A large organelle-free region is visible (arrow).
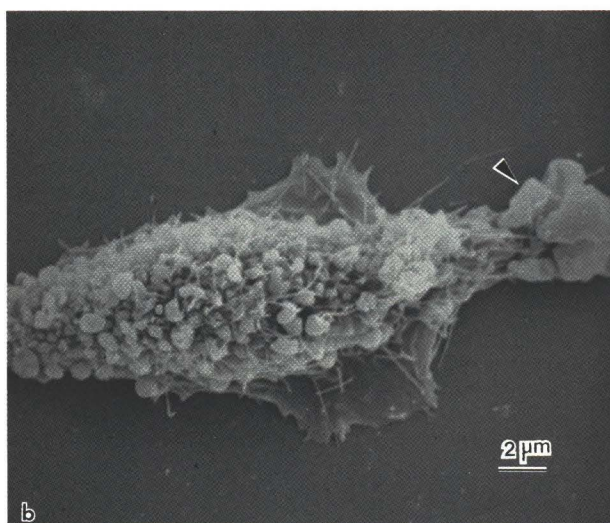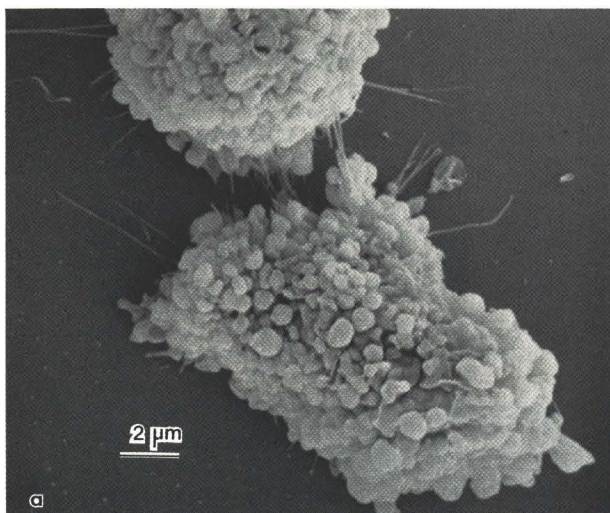

Fig 9. Mouse L-cells exposed to 2.0 mMol/L BSO for 18 h followed by the addition of 0.5 mMol/L of DEM for the final 2 h. (a) A Cell that has just completed mitosis is covered with small blebs. (b) An elongated cell with a large bleb (arrow).

Table 1

THE EFFECT OF THIOL-DEPLETING
RADIOSENSITIZERS ON CELLULAR MORPHOLOGY

	Large Blebs (%)	Small Blebs (%)	Smooth Surface (%)
Control	0	36	64
BSO	0	39	61
DEM	29	62	9
BSO + DEM	55	35	0
Diamide	100	0	0

alone; small blebs cover the cell surface (see Fig 9a). However, with the combined treatment, large blebs are formed at the leading edge of spreading cells (Fig 9a) or on elongated cells (see arrow Fig 9b), which are usually covered with microvilli alone. Thin sections of the cells exposed to the combined treatment show large organelle free regions, (Fig 10). In a few cells, microfilament bundles could be seen near the nucleus. This has the same appearance as seen in colcemid-treated cells.

A semi-quantitative measure of the morphological effect of the sensitizers was generated by counting the number of cells with blebs in SEM micrographs. Cells that had at least one large bleb as shown in Fig 1 or 2, were counted as having large blebs. Cells similar to those in Fig 3c or 7 were counted as having small blebs. Cells without blebs as seen in Figs 3a or 3b were also counted.

The percentage of cells in each condition is given in Table 1 for 2.0 mMol/L, BSO, 20 h; 0.5 mMol/L DEM, 2 h; 0.1 to 0.6 mMol/L diamide for 5 to 20 min . These results confirm the qualitative view seen from the micrographs: diamide and DEM plus BSO produce large blebs,

while BSO alone does not alter the cell surface from that of the control.

Discussion

The treatment times and concentrations of diamide, DEM and BSO were chosen to give maximum depletion of glutathione without producing a significant toxicity. Therefore, the blebbing and ultrastructural changes seen are due to treatment effects and not the result of generalized cell death. Small blebs are seen in untreated cultures of cells under normal growth conditions (Wetzel et al., 1978; Enlander et al., 1975). In both of these earlier studies, the frequency of cells with blebs depended upon their position within the cell cycle. Eighty-five percent of cells in telephase or early G_1, had blebs accounting for roughly half of the cells with blebs seen in the total population (Wetzel et al., 1978). Cells in other portions of mitosis and interphase cells had very few blebs. The large number of blebs we report after DEM or BSO + DEM treatment cannot be due to all cells being in telephase or early G_1, since BSO addition does not stop cells from cycling and the DEM exposure is too short. Thus, the altered cell surface reflects the influence of the chemical on the cell. Blebbing is not a feature of the normal mouse L-cell surface through most of the cell cycle (Price, 1970).

We have also shown that diamide by itself, or a combined BSO-DEM treatment, produced large scale blebbing on the L-cell surface. The other agents, BSO and DEM alone, did not produce large blebs. Thus, reduction of glutathione is not the cause of the ultrastructural changes seen with diamide, since, although BSO and DEM cause blebbing, it is different than that seen with diamide. It is only when BSO and DEM are used together to lower glutathione and non-protein sulfhydryls that small organelle-free regions are seen. BSO, which blocks glutathione, production by a specific enzyme binding, and lowers glutathione through metabolism, produces very little ultrastructural modification (see Figs 5 and 6).

The concentrations of the sensitizers used in these micrographs : 0.2 to 0.6 mMol/L for diamide, 0.5 to 2 mMol/L BSO and 0.5 to 1.0 mMol/L DEM, decrease the glutathione level to $\cong 0$ and the non-protein level to below 20% of the control value (Clark et al., 1984; Harris, 1979; Biaglow et al., 1983). Thus, all the sensitizers lowered the glutathione levels. However, different surface blebbing and changes in ultrastructure were seen after each treatment. BSO, which is the most benign of the thiol depletors, does not affect the cell cycle (Clark et al., 1984) at concentrations ($\leq$ 1 mMol/L) which are useful in radiosensitization. Although BSO reduces the glutathione level and sensitizes cells to radiation, it has little effect on ultrastructure as seen by SEM and TEM. The SEM and TEM micrographs show BSO-treated cells being very similar to controls. There is an increase in the number of small blebs on the cell surface, but to a lesser degree than with the other treatments. Since BSO addition sensitizes cells to radiation but does not alter the ultrastructure greatly, the two effects are not directly related.

DEM-treated cells are covered with small blebs and the mitochondria appear narrow, elongated and darkly stained. Some cells also have large blebs on their periphery. DEM is known to have a high lipid solubility and to inhibit respiration and glycolysis (Biaglow et al., 1983). Thus, interaction of DEM with membranes and membrane-protein links may explain the morphological effect and part of its radio-sensitization. Since DEM increases the sensitization seen with BSO alone (Biaglow et al., 1983; Mitchell et al., 1983), DEM sensitizes partially by a mechanism other than glutathione reduction. The fact that diamide, DEM and BSO are found to sensitize aerobic as well as hypoxic cells (Biaglow et al., 1983) also suggests that the direct effect of thiol depletion does not explain the total radiation sensitization. In fact, diamide has to be present at a concentration greater than that required to remove the total complement of glutathione. We have shown (Szekely et al., 1982a) that diamide addition decreases the activity of the Na^+-K^+ transport system. In view of the decreased K^+ uptake and the swelling seen in the cytoplasm, it is probable that a portion of the radiation sensitivity is due to an altered ionic milieu within the cells. Radiation studies have shown that cell survival is very sensitive to ion concentration (Raaphorst and Kruuv, 1977).

The blebbing seen after diamide, DEM or DEM + BSO exposure suggests that the membrane is affected by the chemicals. This may produce the blebbing seen in the SEM. The lytic effect of many thiol reagents is known to produce ion leakage in membranes (Deuticke et al., 1984). Erythrocytes are well studied: intermolecular cross-linking of membrane skeletal protein produces pores. Diamide also polymerizes the actin-binding protein and myosin (Caruso et al., 1984; Karlsson and Lindberg, 1985).

It has been suggested (Mitchell and Russo, 1983) that, since glutathione is known to provide protection from oxygen-induced free radical species and peroxides, that are normally produced at low levels as a result of oxygen metabolism, lowering glutathione levels will place the cell under oxidative stress. Peroxide formation is an important damaging event in irradiated biological membranes and hence may be one factor in the blebbing. Another mechanism of blebbing is suggested by experiments with N-ethylmaleimide-treated cells (Karlsson and Lindberg, 1985) which demonstrated that a decrease in thiols interferes with the formation of actin filament assemblies. A gradual de-rangement of peripheral microfilaments in the leading lamella was seen with a general depletion of microfilaments. Some DEM + BSO treated cells show anomalous clusters of microfilaments around the nucleus suggesting rearrangements of microfilaments. Microtubule

assembly is also inhibited by glutathione-reducing agents (Oliver et al., 1976).

In conclusion, although diamide, BSO and DEM all reduce glutathione and non-protein sulfhydryls, they do not have the same effect on the morphology of the treated cells. BSO has little effect at the concentrations used to sensitize cells to ionizing radiation, even after 24 h treatment. DEM exposure causes small blebs to appear on the surface of the cell, reduces the number of surface microvilli and causes elongation of mitochondria. Combined treatment with DEM and BSO enhances the effect of DEM alone: large blebs are located on the membrane, encompassing organelle-free regions. Diamide produces large organelle-free blebs that pinch off from the cytoplasm, an invaginated nucleus and mitochondria that contain darkly staining particles.

Acknowledgement

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Discussion with Reviewers

T.M. Seed: Is the degree of surface blebbing following diamide treatment proportional to the time of exposure or drug concentration?

Authors: In the study reported here, we selected the exposure time and diamide concentrations to correspond to a decrease in non-protein sulfhydryls of at least 40 % of control values as reported for CHO and V79 cells (Harris JW, Power JA (1973). Diamide: A new radiosensitizer for anoxic cells. Radiat. Res. 56, 97-109). At these levels of diamide all of the cells had large surface blebs when prepared for SEM. Thus at the diamide concentrations we used, 0.1 to 0.6 mMol/L, all of the L-cells had surface blebs by 5 min, the minimum exposure time.

J.R. Trevithick: Are blebs observed, which are similar to those observed when tissue culture cells are subjected to the inhibitor cytochalasin D, related to a reorganizaton of the actin in the cell cytoskeleton?

Authors: Although the oxidant diamide has been studied mainly for its effect of microtubules, it has also been shown to polymerize the actin-binding protein in platelets (Solum NO, Olsen TM (1985). Effects of diamide and dibucainee on platelet glycoprotein Ib, actin-binding protein and cytoskeleton. Biochim. Biophys. Acta 817, 249-260) and to increase the filamentous actin pool by 20-50% in HeLa cells (Blikstad I,

Carlsson (1982). On the dynamics of the microfilament system in HeLa cells. J. Cell Biol. 93, 122-128). In addition, electron micrographs of L-cells exposed to diamide for long periods of time (Szekely et al., 1982a) look very similar to cells exposed to cytochalasin D (Miranda AF, Godman GC, Deitch AD, Tanenbaum SW (1974). Actin of cytochalasin on cells of established lines. I. Early events. J. Cell Biol. 61, 41-500). However, we are not aware of any reports of DEM or BSO effects on actin filaments.