Promotion of murine B cell differentiation by 2-mercaptoethanol in contrast to glutathione

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ABSTRACT **The role of thiol compounds in B cell proliferation and differentiation was investigated with a stable, homogeneous population of an antigen-specific plasmablastoma, 2C3. This cell line expresses both membrane and secreted forms of immunoglobulin and is arrested at an intermediate stage of B cell development. Attempts to induce its differentiation into plasma cells using antigen, anti-idiotypic antibodies, or mitogens were unsuccessful. However, cultivation of 2C3 in the presence of 2-mercaptoethanol (5×10^{-5} M) changed its doubling time from 19.8 to 34.9 h. There was also a significant rise in intracellular glutathione and in immunoglobulin production, but little change in non-Ig protein secretion. In contrast, exposure of 2C3 to exogenous glutathione (5×10^{-3} M) reduced the doubling time to 11.0 h, with marked increases in proliferation. Moreover, there was no significant rise in either intracellular glutathione or immunoglobulin secretion. Distinct morphological differences were also apparent at the ultrastructural level. Thus, there is a dichotomy in the action of the two thiols. Although the effects of 2-mercaptoethanol could not be reversed, the two thiols together abrogated each other's effects, implying that their actions may be mediated through a common regulatory pathway.— Mattingly, M.; Chakrabarti, D.; Ghosh, S. K. Promotion of murine B cell differentiation by 2-mercaptoethanol in contrast to glutathione. *FASEB J.* 6: 3002–3007; 1992.**

Key Words: glutathione • 2-mercaptoethanol • differentiation • dichotomy • B cell

It is known that the differentiation of B cells into plasma cells involves precise regulatory events. One major event is the transition from expression of cell surface-associated immunoglobulin (mIg)[2] on B cells to production of secretory Ig (sIg) by the terminally differentiated plasma cells. The intermediate stage of this differentiation is marked by transient transformation into blast cells capable of expressing both the membrane and secreted forms of Ig molecules (1, 2). The mechanisms responsible for the differentiation-associated changes in B cell phenotype are not well understood.

There has been considerable interest recently in thiol compounds, particularly 2-mercaptoethanol (2-ME) and glutathione (GSH), which enhance the proliferative and differentiative responses of lymphocytes both in primary cultures and established cell lines (3–9). The mechanism of action of 2-ME has, in particular, been the focus of many reports suggesting that 2-ME exerts its effects primarily by increasing the intracellular levels of GSH and mixed disulfides (5–7). The intracellular GSH, a ubiquitous tripeptide, functions by activating enzymes, regulating microtubule formation, and altering intracellular redox potentials (8–11). Thus, 2-ME and GSH are considered to have complementary actions. Whether this reflects a common site of action remains to be elucidated. In this report, we demonstrate that there is a substantial dichotomy in the effects of these two compounds. Whereas 2-ME can freely enter inside a cell, an exogenously added GSH cannot (3, 8). As a result, the two thiols incorporated into the tissue culture medium exhibit contrasting effects on the proliferation and differentiation of a B cell line, 2C3 (2, 12–15).

2C3 cells represent a murine B-blastoma cell that expresses membrane-bound as well as secretory Igs. Both Igs are γ_1, κ, expressing the same unique clonotypic idiotype with known antigen specificity (2, 12–15). These B cell blasts behave as normal B cells in terms of mIg receptor-mediated endocytosis of antigen and mIg-specific antibodies (15). Furthermore, these cells, which are homogeneous and require no mitogenic stimuli to undergo proliferation, provide an interesting model system with which to elucidate differences in the mode of action of GSH and 2-ME in the absence of mitogenic stimuli. Previous studies have concentrated mostly on splenic T lymphocytes in the context of stimulation by a mitogen or lymphokines (3, 4, 8, 16, 17). The effects of thiols on B cell activation and proliferation have also been reported for heterogeneous splenic populations that contain T cells as well (3–6). These approaches precluded analyses of the differential effects of the thiols at various stages of B cell differentiation. In this report, we compare the effects of 2-ME with those of GSH on 2C3 cells that represent a homogeneous blast cell population.

MATERIALS AND METHODS

B Cell tumor model

The murine plasmablastoma 2C3 is an antigen-specific homogeneous population of a B cell hybridoma that expresses both membrane and secreted forms of anti-phthalate antibody (γ, κ) of unique idiotype. This cell line has been studied extensively and maintained in DMEM or RPMI 1640 containing 10% horse serum in 5% CO_2 at 37°C. It is continuously cultivated in vitro for no more than 2 months at a time, after which fresh culture is started from frozen

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[2]Abbreviations: 2-ME, 2-mercatoethanol; GSH, glutathione; GSSG, oxidized glutathione; Ig, immunoglobulin; Id, idiotype; TCA, trichloroacetic acid; mIg, membrane Ig; sIg, secretory Ig; BSA, bovine serum albumin fraction V; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid; OPD, *o*-phenylenediamine; PBS, phosphate-buffered saline; ISV, Ig-secretory vesicles; ELISA, enzyme-linked immunosorbent assay.

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cells. This cell line is thus maintained for 7 years without any discernible phenotypic changes (2, 12–15).

Media preparation

Tissue culture grade 2-ME was added to the RPMI 1640 or DMEM containing 10% horse serum at a final concentration of 5×10^{-5} M, unless otherwise specified. Medium containing glutathione was prepared by direct dissolution at a final concentration of 5×10^{-3} M unless specified otherwise, and the pH of the solution was adjusted to 7.2. The glutathione solution was stored for up to 7 days at −20°C.

Reagents

The hapten 4-aminophthalate was obtained from Pfaltz and Bauer, Waterbury, Conn. Bovine serum albumin fraction V (BSA), used for conjugation with the hapten, was from United States Biochemical Corporation, Cleveland, Ohio. *Escherichia coli* lipopolysaccharide, dimethyl sulfoxide, GSH, tissue culture grade 2-ME, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), yeast glutathione reductase, β-nicotinamide adenine dinucleotide phosphate (reduced form), and *o*-phenylenediamine (OPD) were purchased from Sigma Chemical, St. Louis, Mo.

Determination of intracellular glutathione/GSSG

This procedure was performed according to the method used by Suthanthiran et al. (8). Briefly, 2C3 cells were washed with phosphate-buffered saline (PBS) and lysed by freeze-thawing in sulfosalicylic acid at a final concentration of 4.3% containing 2 mM EDTA. The supernatants were then assayed for total glutathione by enzymatic recycling (18). The statistics program used for calculation was SPSS PC 3.1.

Determination of cell number

The number of viable cells was determined by actual counting under the microscope with a hemocytometer in the presence of 0.16% isotonic trypan blue. The cell doubling time was calculated according to the following formula. Doubling time = $\Delta t/P$, where Δt is the time elapsed during which cell growth was studied and P is the number of times the cell population doubled during the period from $t = 0$ to time t calculated from the formula; $P = \log G_t/G_0$ divided by log2, where G_t is the concentration of cells at time t and G_0 at time t_0.

Enzyme-linked immunosorbent assay (ELISA)

This procedure was performed on phthalate-bovine serum albumin as well as goat anti-mouse Ig coated 96-well ELISA plates (Falcon, Oxnard, Calif.), as described previously (14). Briefly, the tissue culture supernatants or cell lysates were applied onto the coated plates and incubated for 1 h, followed by blocking with 1% BSA. Subsequent steps included treatment with goat anti-mouse antibody-horseradish peroxidase conjugate (Fisher Scientific, Orangeburg, N.Y.), washing, and color development with *o*-phenylenediamine.

Biosynthetic radiolabeling of proteins

This method was adapted from our previous studies (12, 13). Briefly, 1×10^6 2C3 cells were plated in 200 μl of media depleted of leucine and lysine and preincubated for 30 min at 37°C in 5% CO_2. [^{3}H]Leucine and [^{3}H]lysine, at final concentrations of 5 μci/ml, then were both added to the plates containing depleted RPMI 1640 plus dialyzed horse serum. This was followed by incubation at 37°C for 9 h; thereafter the supernatants were collected and precipitated with either rabbit anti-2C3 antibody or 20% ice-cold trichloroacetic acid (TCA). Radioactivity in the precipitate was measured in a beta counter.

Electron microscopy

The cells were pelleted in microfuge tubes and embedded in Spurr resin. Sections about 800 ångströms thick were stained with uranyl acetate and lead citrate and examined under a Hitachi electron microscope, model 12A.

RESULTS AND DISCUSSION

Proliferation of 2C3 in the presence of antigen, anti-idiotypic antibodies, and mitogens

To determine whether 2C3 cells could be induced to differentiate into highly secretory plasma cells, the cells were exposed in vitro to different stimulatory substances for 24–72 h; the cellular responses were measured by counting the number of cells using trypan blue dye exclusion and by determining the concentration of 2C3 Ig secreted in the culture supernatant with ELISA. It was observed that neither phthalate-BSA, the 2C3-specific antigen (10 μg/ml), nor anti-idiotypic antibodies (both polyclonal and syngeneic monoclonal, 10 μg/ml) had any effect on the proliferative or secretory responses. Attempts to induce differentiation of 2C3 cells using mitogens such as 10 μg/ml LPS and 1% (v/v) DMSO were also unsuccessful. The lack of responsiveness of 2C3 to LPS suggests that this B cell blast is devoid of mitogen receptors.

Proliferation of 2C3 in the presence or absence of thiol compounds

In view of the reports that low molecular weight thiol compounds can enhance responses of lymphocytes to mitogenic stimuli (3, 4), and that 2-ME itself is capable of acting as a mitogen in vitro (19), it was of interest to determine if exposure to the thiols 2-ME and GSH would activate 2C3 cells. For this purpose, 2C3 cells were grown in the presence of 2-ME (5×10^{-5} M), GSH (5×10^{-3} M), and GSH + 2-ME (concentrations of both substances were kept the same as in assays with individual thiols). The cells were then harvested and counted microscopically at intervals of 3 h over a 24 h period and the doubling time was calculated for each treatment (**Table 1**). The data reveal that 2-ME at 5×10^{-5} M in culture medium significantly prolonged the doubling time, whereas extracellular GSH at a concentration of 5×10^{-3} M

TABLE 1. *Change in doubling time after thiol treatment*

Thiols[a]	Doubling time in hours[b]
None (control)	19.8 ± 5.7
GSH	11.0 ± 3.7
2-ME	34.9 ± 9.5
GSH + 2-ME	22.8 ± 7.6

[a] 2C3 cells were cultured at a density of 1×10^6 cells per well in 24-well tissue culture plates in TCM with or without the thiols. The experimental group contained 5×10^{-3} M of GSH, 5×10^{-5} M of 2-ME or both. [b] Cells were harvested and counted by trypan blue dye exclusion method at 6, 9, 12, and 24 h. The doubling time was calculated as described in Materials and Methods. Results are Mean ± SD of four separate experiments.

shortened the doubling time by almost half that for control cells cultured in the absence of the thiols. Addition of GSH and 2-ME simultaneously cancelled the effects exerted by the two thiols added individually and brought the doubling time close to that of the control value. These results clearly suggest that exogenously added GSH and 2-ME have contrasting effects on the growth and proliferation of 2C3 cells, which, however, can be moderated when both thiols are present.

Intracellular glutathione level

To determine whether the effects of exogenously added thiols on the doubling time of 2C3 correlate with the intracellular GSH concentration, levels of GSH were measured in 2C3 cells cultivated as described previously. The results in **Fig. 1** show that the intracellular GSH level of 2C3 in the presence of 2-ME (5×10^{-5} M) was significantly higher than that of untreated controls ($P < 0.05$). On the other hand, treatment of 2C3 with exogenous GSH did not raise intracellular GSH to the same extent. A somewhat similar observation was also made by others using heterogeneous splenic cells (3). However, unlike any previous observation, we noted that the intracellular GSH level of 2C3 cells grown in the presence of both GSH and 2-ME was not significantly different from that of untreated 2C3 cells. This suggests that extracellular GSH exerted a moderating effect on the intracellular GSH level augmented in presence of 2-ME.

Dose response effects of thiols on 2C3 cells

To investigate the effects of various concentrations of 2-ME and GSH on cell proliferation and Ig secretion, we cultivated 1×10^6 2C3 with different amounts of the thiols incorporated into the culture medium. The results shown in **Fig. 2** and **Fig. 3** indicate that for both cell proliferation and Ig secretion, optimum concentration of 2-ME is 5×10^{-5} M and that of GSH is 5×10^{-3} M.

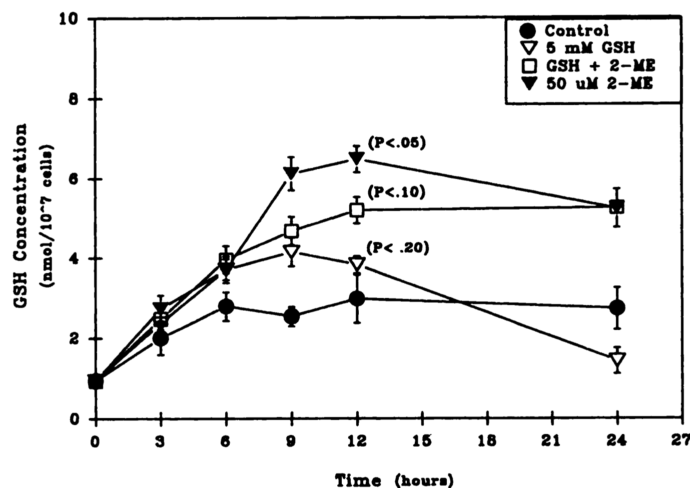

Figure 1. Intracellular glutathione level in 2C3 as a function of time. 2C3 cells (1×10^6) were grown in tissue culture medium containing 5×10^{-5} M 2-ME or 5×10^{-3} M GSH. Another group was set up containing 5×10^{-5} M 2-ME + 5×10^{-3} M GSH. The control consisted of 2C3 cells grown in medium without any thiol. The cells were harvested every 3 h for 24 h and checked for viability and cell counts. The cell lysates were then prepared and used for determination of total glutathione (GSH/GSSG) content by methods described in Materials and Methods. Data represent results of three independent experiments and expressed as mean ± SD.

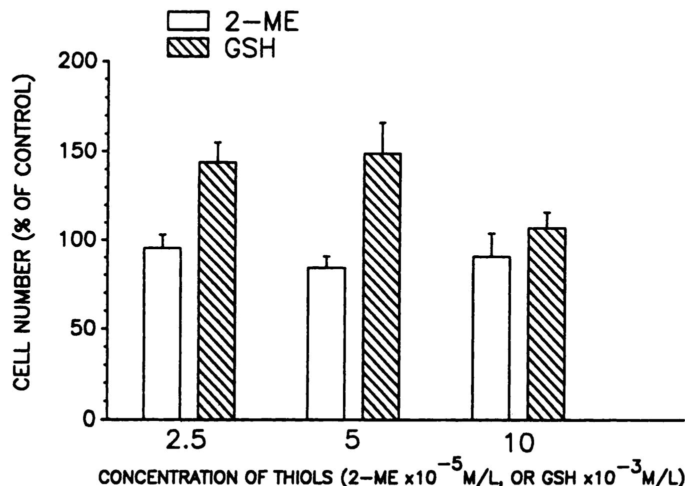

Figure 2. Effects of various doses of thiols on the growth of 2C3 cells. 2C3 cells (1×10^6) were grown in 24-well tissue culture plates containing different concentrations of 2-ME or GSH. The data are representative of two separate experiments and expressed as mean ± SD of triplicate.

Secretion of Ig and other proteins by 2C3 cells

To determine if differences in the proliferative responses of 2C3 observed in the presence of the two thiols were associated with any functional changes of 2C3, we measured the production and secretion of Ig by these cells using the ELISA technique. Results depicted in **Fig. 4** clearly show that treatment of 2C3 cells with 2-ME resulted in about a fivefold increase in the secretion of Ig, whereas neither GSH nor GSH plus 2-ME significantly affected Ig secretion above the control level.

To determine whether the production of non-Ig proteins is also differentially affected by the two thiols, we adopted a procedure described before that uses 2C3 cells (12). The relative amounts of radioactivity in proteins was determined in the precipitates obtained with either TCA or rabbit anti-2C3 Ig from the soluble supernatants of 2C3 culture grown in the presence of [^{3}H]leucine and [^{3}H]lysine. The results shown in **Fig. 5** confirm those of Fig. 4, and reveal further that most of the augmented secretion in 2C3 cells treated with 2-ME, but not in GSH or GSH plus 2-ME-treated cells, consisted of Ig. Thus, treatment with 2-ME, but not GSH, increased the production and secretion of Ig, a characteristic usually identified with plasma cells.

Morphological changes in presence of thiols

Consistent with the observations described here, discernible morphological changes were also noticed. Ig-secretory vesicles (ISV) normally associated with plasma cells (20) were seen mostly in 2-ME-treated 2C3 cells when viewed under a light microscope (data not shown). Furthermore, the electron micrographs of 2C3 cells exposed to GSH, 2-ME, or GSH plus 2-ME reveal that 2-ME-treated cells (**Fig. 6c**) exhibited more organized endoplasmic reticulum and conspicuous cytoplasm than any other groups (Fig. 6*a*, *b*, *d*). The treatment with GSH, on the other hand, produced multilobed nuclei with less prominent endoplasmic reticulum or an increase in cytoplasmic volume (Fig. 6*b*). Furthermore, in 2-ME-treated cells, mitochondria are less conspicuous both in number and size. This possibly reflects that a plasma cell, unlike an actively dividing cell, would have fewer require-

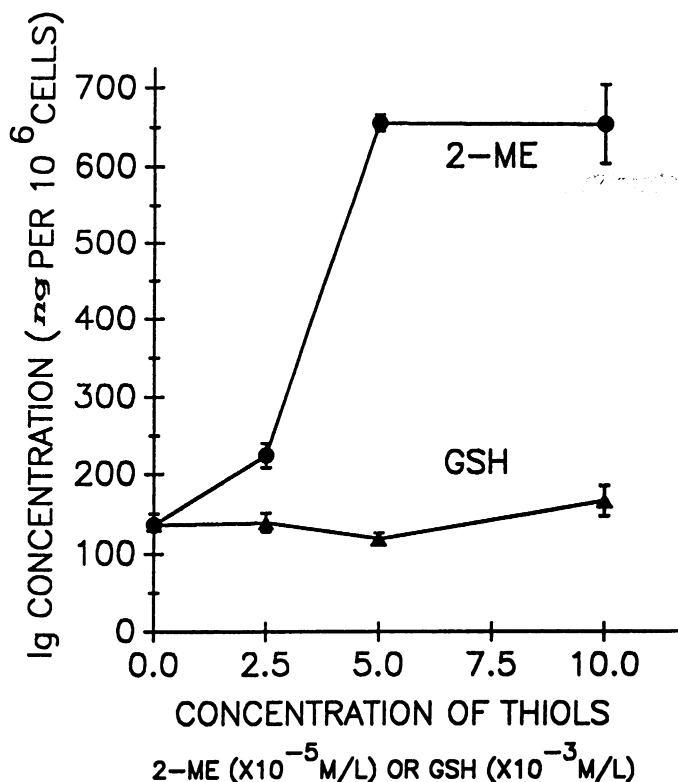

Figure 3. Effects of various doses of thiols on the level of Ig secreted by 2C3 cells. 2C3 cells (1×10^6) were grown in 24-well tissue culture plates containing different concentrations of 2-ME or GSH. The data are representative of two experiments and expressed as mean ± SD of triplicate.

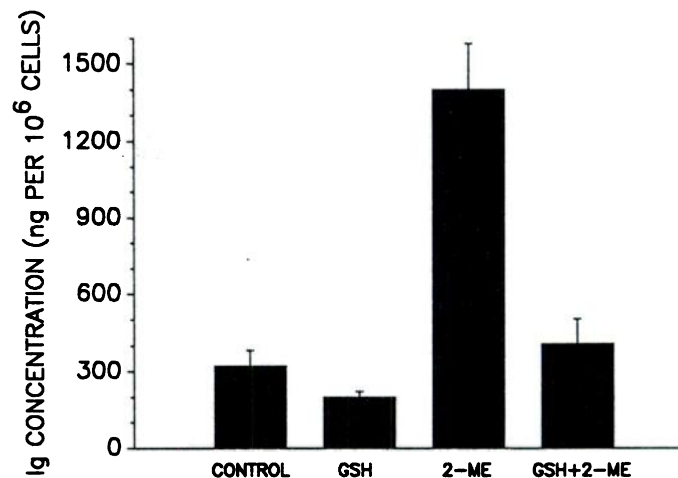

Figure 4. Concentration of secreted Ig in the supernatant of 2C3 cultivated with or without the thiols. 2C3 cells (1×10^6) were grown in 24-well tissue culture plates containing either 5×10^{-5} M 2-ME or 5×10^{-3} M GSH. Another group was set up containing 5×10^{-5} M 2-ME + 5×10^{-3} M GSH. The control consisted of 2C3 cells grown in the medium without any thiol. The cells were harvested after 9 h incubation, counted and subjected to centrifugation at 300 × *g* for 10 min. The supernatants were collected and tested for secreted Ig by ELISA as described in Materials and Methods. Data represent results of two independent experiments and expressed as mean ± SD.

ments for energy. However, in the presence of both GSH and 2-ME, the morphology of 2C3 resembled the untreated control cells, exhibiting euchromatic nucleus and little organized endoplasmic reticulum in the cytoplasm (Fig. 6*a*, *d*). This lends further support to our contention that although the two thiols may have diverse and unrelated effects on cell growth and differentiation, at least one major cellular event mediated by them involves a common regulatory pathway.

Reversibility of the effects of thiols

To ascertain if the changes in 2C3 cells brought about by exposure to thiols are reversible or not, 2C3 cells were exposed to 2-ME, GSH, or both for 9 h, followed by transfer into regular media without the thiols for an additional 9, 12, and 24 h. The levels of Ig secretion and the number of cell were determined as previously described. **Figure 7** demonstrates that the level of Ig secretion by 2C3 exposed to 2-ME did not revert to the control level even after removal of 2-ME. Similar experiments with GSH, however, registered no difference from the control values in either Ig secretion or the rate of proliferation (data not shown). Thus, the treatment with 2-ME seems to cause irreversible changes in the phenotype of 2C3 cells, as would be the case during differentiation of B cells into plasma cells.

In this study we have demonstrated that Ig production by a B cell line 2C3 can be markedly enhanced by incorporation of 2-ME but not GSH into the culture medium. The increased secretion of immunoglobulin is a specialized function of a differentiated B cell after its transformation into plasma cells, and it appears that 2-ME does increase this Ig-secretory function of 2C3 cells in the absence of mitogens. Furthermore, the effects of 2-ME but not of GSH are irreversible, and it is the treatment with 2-ME but not with exogenous GSH that leads to a significant rise in intracellular GSH. Contrary to this, the effects observed after exposure of 2C3 to exogenous GSH include greatly augmented cell doubling time and increased rate of proliferation. Interestingly, the simultaneous presence of 2-ME and GSH prevents

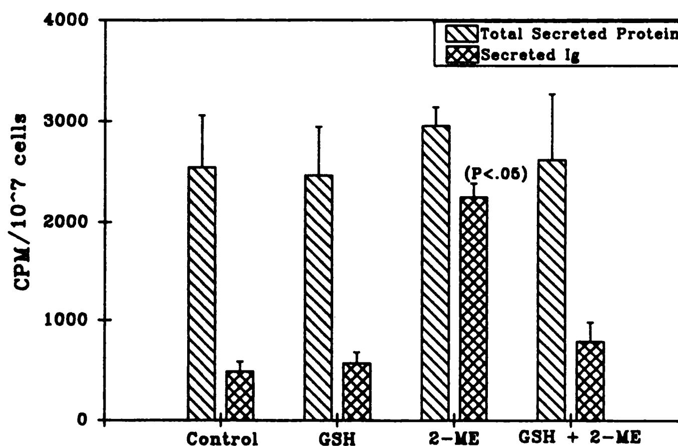

Figure 5. Determination of Ig and non-Ig proteins secreted by 2C3 cells. This was done as described in Materials and Methods, using supernatants collected after biosynthetic radiolabeling of 2C3. Cells (1×10^6) were suspended in 12-well tissue culture plates containing RPMI 1640 plus dialyzed 10% horse serum but free of leucine and lysine and preincubated for 30 min at 37°C in 5% CO_2. After incubation the cells were resuspended in the same medium containing 5 μc/ml of [^{3}H]leucine and [^{3}H]lysine and incubated as before for 9 h. Subsequently, aliquotes of the supernatants were precipitated with either rabbit anti-2C3 Ig antibody or ice-cold 20% TCA. Washed pellets were counted in a beta counter. Data represent results of three separate experiments. The significance level was determined by the analysis of variance.

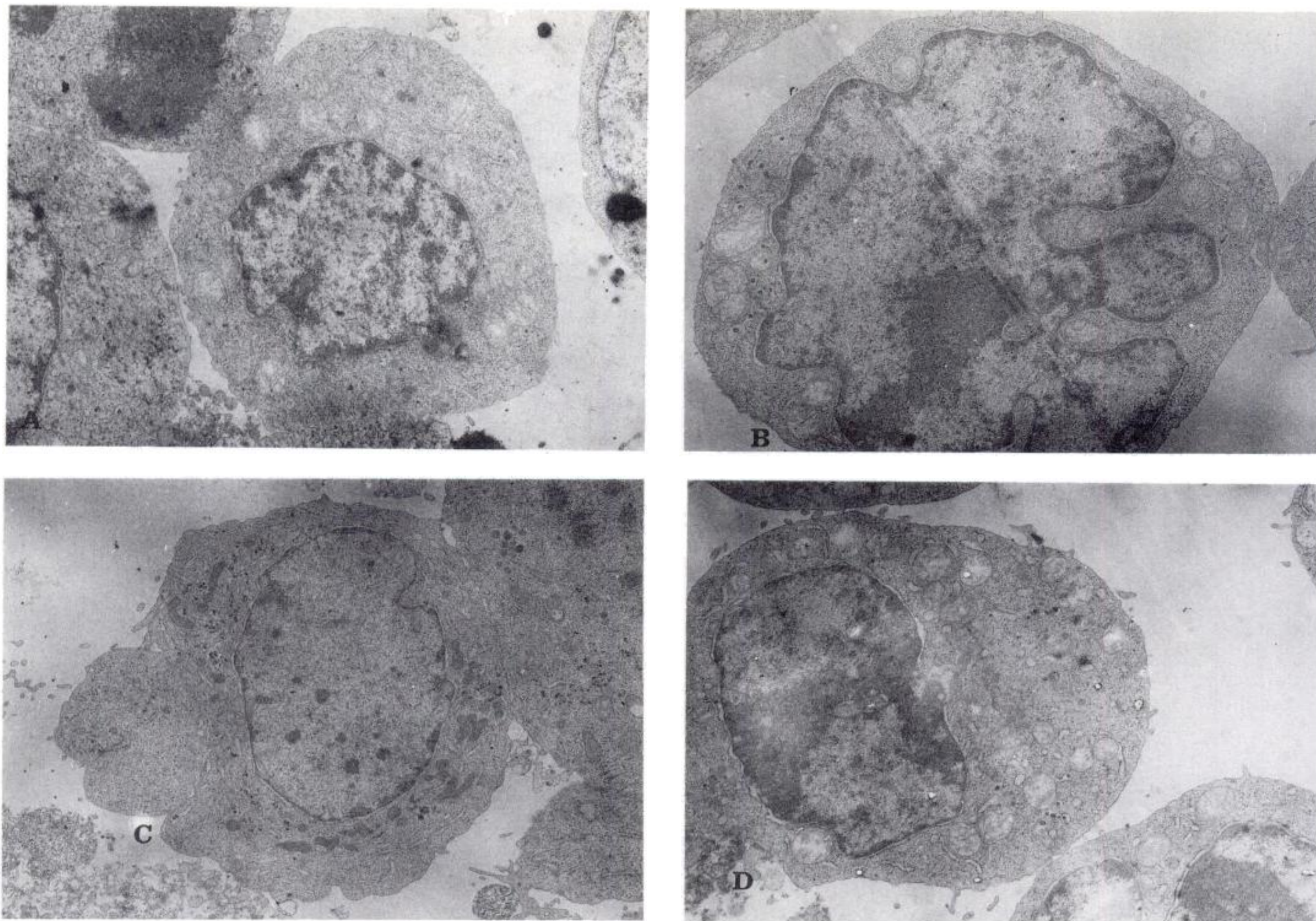

Figure 6. Electron micrographs of 2C3 cells grown in tissue culture medium without 2-ME or GSH (control) (*a*); in the presence of 5×10^{-3} M GSH (*b*); in the presence of 5×10^{-5} M 2-ME (*c*); and in the presence of 5×10^{-3} M GSH and 5×10^{-5} M 2-ME (*d*). *a, d*) 2C3 exhibits the morphology of a large lymphocyte with euchromatic nucleus and a cytoplasm containing little organized endoplasmic reticuluum (ER). *b, d*) 2C3 has contrasting features in both nucleus and cytoplasm. ER is prominent and organized (*c*) and nuclei is segmented (*b*). (Magnification × 13,000).

any cellular activation or increased secretion of Ig. The morphological studies reported here also suggest a dichotomy in the actions of GSH and 2-ME.

Observations similar to ours with regard to 2-ME have been reported by others (4, 5). Alberini et al. (7) reported increased secretion of IgM assembly intermediates by B-lymphomas after treatment with 2-ME and attributed the effects of 2-ME to modification of the redox gradient along the secretory pathway. In most other studies, its effects have been attributed to alterations in the intracellular GSH level, which can reverse inhibitory effects of oxidized glutathione and scavenge oxygen-derived radicals (3, 5, 6). How this increase in intracellular GSH level leads to enhanced secretory function and prolonged doubling time we observed with 2C3 is not currently understood. It is possible that 2-ME, acting through an elevated intracellular GSH level, alters intracellular protein sulfhydryls, and as a consequence induces the appropriate signal (or signals) for differentiation. The proliferative response observed in the presence of 2-ME possibly reflects the fact that 2C3 cells have not all been synchronized before exposure to 2-ME.

Exogenous GSH, on the other hand, cannot freely enter a cell (8). It exerts its effects primarily at the cell surface, possibly by reduction of cystine and as a source of cysteine moieties (8). Exogenous GSH is indeed of little consequence on the level of intracellular GSH for 2C3 cells. Cellular proliferation induced as a result of exposure to GSH is therefore largely a cell membrane-associated phenomenon. Recently it has been shown that GSH exerts regulatory effects on DNA synthesis in human T lymphocytes activated via their CD2 and CD3 molecules (8, 10). By modifying protein sulfhydryls at the cell surface, GSH may also bring about functional changes in receptor-ligand interaction, internalization, and degradation (9). Such alteration of surface proteins may initiate reparative processes and lead to cellular activation and increased proliferative response. Whether this is also the case for murine B cells remains to be established. There may be other explanations as well. Indeed, reports have suggested that GSH augments DNA synthesis by stimulating pentose phosphate pathway and purine nucleotide levels (21). In any case, the increased cellular activity follows active utilization or breakdown of GSH, and as a result the effect of exogenous GSH will depend on its sustained presence. This is probably the basis for reversibility of the effects of exogenous GSH observed with 2C3. We are currently investigating the underlying mechanism (or mechanisms).

The simultaneous presence of GSH and 2-ME reverses the effects exerted by the two thiols individually, as we have observed here, which suggests that their effects involve a cas-

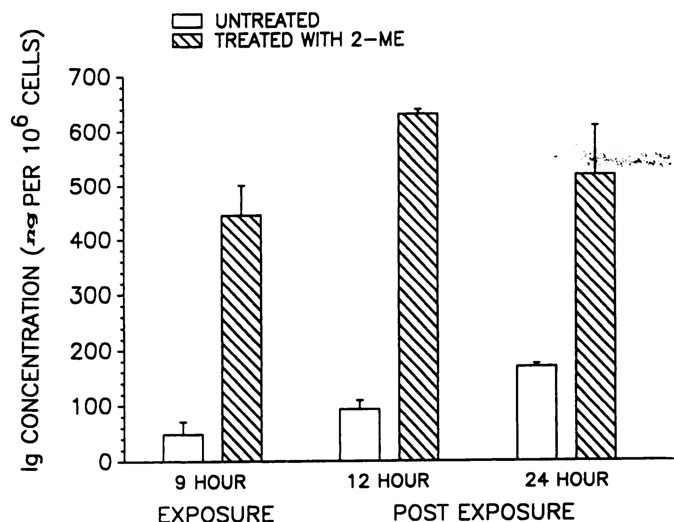

Figure 7. The effect of 2-ME is not reversible after initial exposure to 2-ME. 2C3 cells (1×10^6) were exposed to 5×10^{-5} 2-ME for 9 h. At the end of incubation, the cells were harvested and resuspended in tissue culture medium without 2-ME and reincubated for additional 9, 12, and 24 h. Supernatants from the first and second incubation periods were used to determine Ig concentration by ELISA. The control culture treated similarly but without 2-ME was also analyzed. Data are representative of two separate experiments and expressed as mean ± SD of triplicate.

cade of interdependent events mediated by one or more crucial sulfhydryl proteins or intermediary metabolic pathways. In other words, the proliferative signal generated as a result of GSH treatment and the differentiative signal induced in the presence of 2-ME are both most probably turned off by modulation of a common intermediate or pathway.

Thus, an important point emerges from the present study, namely, that the thiols 2-ME and exogenous GSH exert independent effects on overall functions of murine B lymphocytes. Because of the differences in the action of intra- and extracellular GSH, it will be worthwhile to investigate whether such a dichotomy is linked with the modifications of critical regulatory steps involved in the synthesis, assembly, and secretion of immunoglobulin. Indeed, from immunotherapeutic viewpoints, thiols may be useful in downregulating a neoplastic phenotype, which occasionally results in restoring normal phenotype. Experiments focusing on these aspects are currently in progress. FJ

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