Monochlorobimane (MCB) has been used by several investigators as a fluorescent label for quantifying glutathione (GSH) levels in human peripheral blood mononuclear cells (PBMC). This paper describes a biochemical evaluation of this approach. PBMC were incubated with MCB (10–100 μM) and the fluorescence in extracellular medium and cell lysates was measured. Nonlinear curves were obtained in both cases and no "plateau" was reached. The majority of the fluorescence was in the medium. Gel permeation (Sephadex G-25) of the lysate indicated a linear increase in protein–bimane adduct formation, reaching about 50% of the intracellular fluorescence after 1 h. Fractionation of the deproteinized samples with Sephadex G-10 showed that only about one-third of the "low-molecular-weight" fluorescence could be ascribed to GSH–bimane, in either the lysate or the medium. Furthermore, about 40% of the free GSH in lysates appeared unbound even after 1 h of incubation. These data are in line with our observation of an extremely low activity in PBMCs of glutathione S-transferase under the conditions employed. Our findings indicate that many variables influence the cellular fluorescence, including the presence of alternative metabolic pathways for MCB and the rapid excretion of GSH–bimane out of the cell. This lack of specificity limits the value of MCB as a GSH probe for PBMC and confirms earlier suggestions that a careful biochemical evaluation is a prerequisite for its application to any particular cell type. © 1994 Academic Press, Inc.

Glutathione (glutamylcysteinylglycine, GSH) is a major cellular anti-oxidant and, because it plays a central role in the maintenance of thiol redox status, is essential for the optimal functioning of numerous enzymes. In addition it is conjugated to many xenobiotics, catalyzed by the enzyme glutathione S-transferase (GST). GSH is therefore critical for cellular viability and is generally found in high concentrations (1–10 mM) in animal cells. Various pathological states have been associated with low GSH levels including malignancies (1,2), idiopathic pulmonary fibrosis (3), and the acquired immunodeficiency syndrome (4–8). GSH metabolism has been extensively reviewed (9,10).

Lymphocyte function is particularly dependent on GSH levels. GSH is known to influence lymphocyte growth and activation as well as their responsiveness to cytokines (11–13). A number of workers (7,8,14–16) have therefore attempted to measure GSH concentrations in subsets of peripheral blood mononuclear cells (PBMC) by flowcytometry. Obviously multiparameter flowcytometry is an attractive approach for this purpose, since the cell type can simultaneously be defined using a monoclonal antibody directed against the appropriate cell surface antigens. Several fluorescent probes have been used for flowcytometric GSH measurement, but in recent years monochlorobimane (MCB) has become the reagent of choice. MCB, itself nonfluorescent, is conjugated to GSH by glutathione S-transferase to yield a fluorescent adduct, as comprehensively reviewed in (17).

It is clear that there are certain prerequisites for such a probe. First, the reagent itself must be freely permeable through the cell membrane; second, it must be specific for GSH; and finally, the adduct must be retained within the cell. Although these conditions have, to some extent, been verified in certain cell types [for example hepatocytes (18), fibroblasts (19), and Chinese hamster ovary cells (20)], it remains uncertain in how far they hold true for PBMC. Here we report an investigation using biochemical fractionation procedures to evaluate
the validity of MCB as a fluorogenic reagent for quantification of GSH in this cell type.

MATERIALS AND METHODS

Subjects and Cell Isolation

Subjects were healthy volunteers (n = 4), age 30–60, who had used no medication in the previous 24 h. Blood (20 ml) was taken by venepuncture, and PBMCs were isolated immediately by density gradient centrifugation using standard techniques (21). After washing, the cells were resuspended in 2 ml phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin. Analysis was initiated within 15 min; meanwhile, the cells were kept on ice.

The total yield of PBMCs in the suspension ranged between 2 and 3 × 10^7 cells/ml, and differential counts indicated an average of about 80% lymphocytes and 20% monocytes. Contamination with polymorphonuclear leucocytes was less than 2%, and no significant contamination with erythrocytes was seen.

Fluorimetry and General Techniques

A 10 mM stock solution of monochlorobimane (Molecular Probes Inc., Eugene, OR) was prepared in ethanol, and aliquots were stored at −80°C in the dark.

In preliminary experiments, 0.5 × 10^6 PBMCs, suspended in PBS (pH 7.4; 163.9 mM Na⁺, 140.3 mM Cl⁻, 10.9 mM HPO₄²⁻, 1.8 mM H₂PO₄⁻), were brought to final concentrations in the range 10–100 µM MCB (total volume 1 ml). Tubes were incubated at 37°C, and the fluorescence was measured at intervals up to 60 min using a Perkin-Elmer LS-5 fluorimeter (excitation, 392 nm; emission, 480 nm; excitation slit, 5; emission slit, 20). Both controls without MCB and controls without cells were included. Exposure to light was avoided as much as possible throughout this work; the use of a small excitation slit contributes to minimizing photodecomposition.

In order to distinguish between fluorescent material released into the medium and that retained within the cells, tubes were centrifuged (400g, 5 min) immediately after incubation and the fluorescence of the supernatant was measured. The pellet was washed once with PBS and the cells resuspended in 1 ml PBS. Cells were disrupted by sonication at 0°C (3 × 10 sec, 10-µm peak probe amplitude) and the lysate was centrifuged (10,000g, 2 min). Fluorescence of the supernatant was measured as before.

The effect of the experimental manipulations on cell membrane integrity was checked with propidium iodide inclusion. Propidium iodide (Sigma, St. Louis, MO) was added after isolation of the cells, after washing the cells twice in PBS (400g, 5 min), and after incubation of the PBMC for 10, 30, or 60 min with 100 µM MCB (with or without washing the cells in PBS). Resuspended cells in 0.1% Nonin-P40 (BDH Chemicals LTD, Poole, UK) in PBS were used as a positive control. Propidium iodide inclusion measurements were carried out on a Coulter Elite Flow Cytometer.

The presence of an ATP-dependent GSH–bimane efflux pump was studied by preincubating samples for 15 min with 1 mM probenicid (Sigma) before the cells were incubated with MCB.

Gel Permeation Chromatography

Sephadex G-25 (fine grade; Pharmacia, Uppsala, Sweden) was employed to separate relatively low-molecular-weight MCB derivatives (i.e., GSH–bimane adduct and other fluorescent MCB metabolites) from protein–bimane adducts. Sephadex G-10 was used to distinguish the GSH–bimane adduct (Mᵣ 498) from possible hydrolytic or oxidation products of MCB (Mᵣ < 250) in deproteinized samples.

Columns (1 × 28 cm) were prepared and washed with PBS until fluorescence of the eluate was negligible (approximately 3 bed vol). The sample (200–500 µl) was brought onto the column and eluted with PBS at a flow rate of 1 ml/min. Fractions of 1 ml were collected and the fluorescence was measured as before.

Free ("Unbound") Glutathione Measurement

In order to quantify free GSH in the presence of preformed GSH–bimane adduct an enzymatic technique was used. PBMC (5 × 10^6 cells/ml) were incubated with 50 µM MCB for appropriate times (0–60 min). After pelleting, the cells were washed and lysed as before. An aliquot of the lysate (containing 0.1–1.0 nmol free GSH) was diluted to 970 µl in PBS, 20 µl of 1 mM MCB added, and the fluorescence was recorded until a stable signal was obtained. A 10-µl aliquot of a crude GST preparation was added (a mouse liver homogenate, 200 mg/ml, centrifuged at 30,000g for 30 min and dialyzed against several changes of PBS) and the recording continued. An abrupt increase in fluorescence (<1 min) was followed by a plateau which remained stable for several minutes. Blanks (minus GST, minus PBMC, minus bimane) and appropriate GSH standards were included with each batch of assays.

The glutathione concentration in the cytosolic fraction was also quantified on HPLC using the method of Fahey and Newton (22). A 200 × 3-mm Chromsep HPLC column with a Chromspher 5C18 cartridge (Chrompack, the Netherlands) was employed.

Measurement of Glutathione S-Transferase Activity

The reaction tube contained 860 µl PBS, 20 µl of 1 mM glutathione, and 20 µl of 1 mM MCB (prepared as a 1:10
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100 r

FIG. 1. Effect of incubation time on fluorescence at two different MCB concentrations. PBMCs (0.5 × 10⁶) were incubated with 10 µM (open symbols) or 100 µM (closed symbols) MCB up to 60 min. Broken lines indicate the corresponding blanks (without cells). Fluorescence expressed in arbitrary units. “Zero-time” blanks have been subtracted (samples containing 10 µM MCB, 11 units; 100 µM MCB, 98 units). Results were corrected for quenching. Values represent the mean of at least four independent experiments.

dilution of ethanolic stock in PBS). Fluorescence was recorded until a linear plot was obtained; the slight increase of fluorescence with time ("blank") presumably resulted from chemical reaction and/or photodecomposition of MCB by the exciting light.

The enzymatic reaction was initiated by the addition of 100 µl centrifugated PBMC lysate (equivalent to 2-3 × 10⁶ cells), bringing the total volume to 1 ml. The recording was continued until a linear plot was again established. After correction for the nonenzymatic blank, GST activity was calculated from the slope by reference to a GSH standard (see below). For comparative purposes, GST activity was also determined using 100-µl aliquots of a dilute mouse liver homogenate in place of the PBMC lysate.

The protein concentrations of lysates were measured by direct fluorescence (excitation, 280 nm; emission, 340 nm) of appropriate dilutions, using bovine serum albumin as standard. GST activities of all samples were expressed as nmol MCB adduct formed/min/mg protein.

RESULTS

Reaction of Intact Cells with MCB

The fluorescence of a suspension of PBMCs incubated with MCB increased rapidly with time. The curves were nonlinear (Fig. 1), and the fluorescence was still rising even after 1 h at 37°C. Experiments using different PBMCs concentrations showed that the fluorescence at any time point was proportional to cell number. As shown in Fig. 1, the rate of increase of the fluorescence was greater at higher MCB concentrations, although again the relationship was clearly nonlinear. The initial fluorescence of the cell-free controls was dependent on the MCB concentration but little increment was observed with time (Fig. 1). Minimal autofluorescence of the PBMC was noticed.

Centrifugation of the intact cells following incubation showed—surprisingly—that at all time points the majority of the fluorescent material was in the extracellular medium rather than in the cells. After 5 min at 37°C only 31% was found in the cells; after 60 min this value had fallen to 17%.

Experimental manipulations did not influence membrane integrity; propidium iodide inclusion did not exceed 3%, even after incubation with MCB for 60 min and washing the cells in PBS. More than 95% propidium iodide-positive cells resulted from resuspending the cells in Nonidet in PBS.

Preincubating cells with probenicid decreased fluorescence in the extracellular medium up to 30%.

Protein–Bimane Adduct Formation

Since it is well established that a nonenzymatic reaction may occur between cytosolic proteins and bimanes (23), we fractionated PBMC lysates using gel permeation on Sephadex G-25 following incubation of the intact cells with the reagent. Typical results are shown in Fig. 2 and Table 1. It appears that there is a linear increase in the absolute concentration of the protein–bimane adduct with time; after 1 h this material is responsible for about 50% of the total intracellular fluorescence.

FIG. 2. Typical elution profile of PBMC lysates on Sephadex G-25. PBMC (5 × 10⁶) were incubated with 50 µM MCB for 5 min (open symbols) or 30 min (closed symbols) and lysed as described in the text. Fluorescence was measured in the different fractions.
TABLE 1
Distribution of Fluorescence

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>5</th>
<th>15</th>
<th>30</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low molecular weight (%)</td>
<td>79</td>
<td>76</td>
<td>62</td>
<td>50</td>
</tr>
<tr>
<td>Protein–bimane (%)</td>
<td>21</td>
<td>24</td>
<td>38</td>
<td>50</td>
</tr>
</tbody>
</table>

Note. PBMCs lysates were fractionated using gel permeation on Sephadex G-25 following incubation of the intact cells (5 × 10⁶) with 50 μM MCB up to 60 min. Values represent the distribution of fluorescence between protein–bimane adduct and low-molecular-weight material at various times.

Samples of the incubation medium showed, as expected, very little protein–bimane adduct. This was less than 5% even after 1 h incubation. This small quantity is presumably the result of chemical reaction with extracellular protein and was not investigated further. The contribution of the blanks (cell-free control) to the fluorescence was 25 and 8% after incubation of 5 × 10⁶ PBMCs with 50 μM MCB for 5 or 30 min, respectively.

Sephadex G-10 Chromatography

The low-molecular-weight fluorescent material was further fractionated using Sephadex G-10. Samples of either lysate or extracellular medium were deproteinized with trichloroacetic acid (TCA), and the supernates were subjected to gel permeation as before. At all time points two distinct fluorescence peaks were present for both the medium and the lysate (Fig. 3): the earlier peak coeluted with the reaction product of GSH and MCB obtained using mouse liver GST. Less than half of the fluorescent material eluted at a volume compatible with a Mₐ of 498 (GSH–bimane adduct). The majority of the fluorescence appeared appreciably later as a distinct peak.

Free (Unbound) Glutathione Levels of PBMC

The enzymatic GSH assay described here proved to be exceedingly sensitive (detection limit of about 50 pmol) and reproducible (average differences for duplicates <5%). It is unaffected by the presence of preformed bimane adducts, and the use of liver GST presumably confers a high degree of specificity. Typical recordings are seen in Fig. 4.

Using this technique, free GSH levels following freshly isolated PBMC were found to be 1.7 ± 0.3 nmol/10⁶ cells (equivalent to 28.3 ± 5 nmol/mg protein) and are similar to GSH levels found by others (24, 25). Since experiments were in general complete within 2–3 h of cell isolation, we also measured GSH levels after maintaining the PBMC for 3 h on ice in order to examine possible spontaneous depletion. It appeared that the loss of GSH did not exceed 15% during this time. The accuracy of the enzymatic GSH assay was checked by an independent HPLC analysis. Both methods agreed to
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within 10% in the analysis of several lysates of fresh PBMC.

To determine the residual intracellular levels of free GSH (i.e., excluding preformed GSH–bimane adduct) we incubated intact PBMC with MCB for various times. A gradual depletion was seen, intracellular GSH levels reaching about 40% of their initial value after 1 h incubation. Complete "balance sheets" are now possible; these are illustrated in Figs. 5A (intracellular) and 5B (medium) and are summarized in Table 2. It is clear that the total GSH (intracellular free, intracellular adduct, and extracellular adduct) remains rather constant after 1 h incubation, confirming the validity of the fractionation procedures and excluding the possibility of ongoing GSH synthesis.

| TABLE 2

| Distribution of Total GSH (Free, Intracellular Bimane Adduct, and Extracellular Bimane Adduct) at Various Times before and after Incubation of 5 × 10⁶ PBMCs with 50 μM MCB |
|---|---|---|
| Time (min) | 0 | 5 | 60 |
| Intracellular free ("unbound") GSH | 7.5 | 5.2 | 3.1 |
| Intracellular GSH–bimane adduct | — | 0.4 | 0.5 |
| Extracellular GSH–bimane adduct | — | 0.7 | 3.3 |
| Total GSH | 7.5 | 6.3 | 6.9 |

Note. Values are nmol; note that 1 nmol is equivalent to 70 fluorescence units.

Glutathione S-Transferase Activity

The GST activity of PBMC was extremely low, even when using concentrated lysates (up to 3 × 10⁷ cells/ml), and despite the very sensitive fluorimetric assay, the slope barely exceeded that of the blank. In absolute terms, the activity of PBMC lysate was 0.08 ± 0.01 nmol adduct formed/min · mg protein (n = 3). By contrast, mouse liver yielded a value of 94 nmol adduct formed/min · mg protein, about 1000-fold higher.

DISCUSSION

The answer to our original question regarding the validity of flow cytometric data using MCB is clear; at least two of the three prerequisites do not hold for human PBMC. First, the specificity of MCB is poor. A flow cytometer registers the total fluorescence in the cell, and as shown in Fig. 5A, the GSH–bimane adduct contributes only a small part of this fluorescence, regardless of the incubation time. Second, it is apparent that the great majority of the GSH–bimane adduct is released from the cell and accumulates in the medium (Table 2).

The low-molecular-weight peak shown by gel permeation on Sephadex G-10 was only detectable in the presence of PBMCs and clearly is a major metabolic product of MCB. Indeed, the appearance of hydrolyzed fluorescent derivates has been reported following the incubation of monobromobimane with various biological materials (26). The observation that most of the GSH–bimane product leaves the cell is in accord with...
the presence of an ATP-dependent GSH S-conjugate export pump (27,28). ATP-dependent secretion of MCB–GSH adducts by rat fibroblasts was reported before and this efflux pump was inhibited by exposing the cells to probenicid (29). The present data demonstrate similar results; the accumulation of fluorescence in the extracellular medium was inhibited in the presence of probenicid.

Our present data seem to be in contrast with results obtained with studies of other cell types. For example, in the original study of MCB as a flowcytometric reagent, Rice et al. (20), using Chinese hamster ovary cells, stated that very little reaction with protein sulfhydrys was observed and that the only low-molecular-weight fluorescent derivative was the GSH–bimane adduct. The apparent discrepancy between these and our findings may be the extremely low GST activity of human PBMC. Clearly if the conjugation rate of MCB with GSH is several orders of magnitude lower then that in other cells, than the relative contribution of fluorescence due to decomposition or coupling of MCB to protein will be magnified accordingly. It should be noted that our values for GST activity are probably well below $V_{max}$, since the $K_m$ for MCB of the human isoenzymes is in excess of 200 $\mu$M (15). However, our purpose in this study was simply to establish—in pragmatic terms—the rate at which GSH can be derivatized under conditions generally employed. It is possible, although unlikely in our view, that higher MCB concentrations might yield a somewhat greater proportion of GSH–bimane adduct.

Previous work with lymphocytes (14) was done at comparable MCB concentrations; however, the labeling time was brief (10 min at 37°C plus an unspecified time on ice), the cells were pelleted (which will remove extracellular fluorescence), and proteins were removed by acid precipitation before analysis by reversed-phase HPLC. Thus in this earlier report only acid-soluble, intracellular fluorescent adducts were detected. The authors conclude that more than 99% of the MCB fluorescence is associated with the GSH peak (14), thereby validating MCB as a fluorescent reagent for measuring GSH levels in these cells. However, the other contributions to the fluorescence as reported in the present study were missed and the interpretation of data by application of MCB is therefore much more complex. The same considerations apply to a report (17) in which lymphocytes were GSH-depleted by pretreatment with dinitrochlorobenzene. It is indeed probable that GSH depletion reduces the formation of the acid-soluble adduct, but it does not validate the application of the flowcytometric GSH assay to PBMC.

Despite certain limitations which have already been pointed out by others (15,30,31) (in particular the incompleteness of GSH derivatization resulting from low GST activity), certain authors suggest that the use of MCB as a fluorescent probe may be of value for the measurement of “relative differences” in GSH levels of human PBMC (17). Our findings indicate that in addition to the GSH level many other variables influence the total cellular fluorescence obtained with monochlorobimane in human PBMC. These include GST activity, the decomposition rate of MCB to other fluorescent products, and the transport rate of the GSH–bimane adduct out of the cell. All of these variables may differ between cell subsets or may altered by disease or drugs and will further complicate the interpretation of data obtained when using MCB as a fluorescent label. The MCB GSH assay should be validated for each particular cell type and experimental condition.

REFERENCES