Immunology 1986 **57** 521–526

Inhibition of cell-mediated cytotoxicity by 2-cyclohexene-1-one: evidence for a role for glutathione and/or glutathione–protein interactions in cytolysis

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Accepted for publication 27 November 1985

SUMMARY

In order to explore the role of glutathione in cell-mediated cytotoxicity, we have examined the effect of the sulphydryl-reactive and glutathione-depleting agent 2-cyclohexene-1-one on antibody-dependent cellular cytotoxicity, spontaneous cell-mediated cytotoxicity, and cell-mediated lympholysis by human peripheral blood mononuclear cells. 2-Cyclohexene-1-one significantly inhibited ($P < 0{\cdot}001$) both antibody-dependent and spontaneous cell-mediated cytotoxicity using three different cell-line targets, at three different killer : target cell ratios (10 : 1, 25 : 1 and 50 : 1). Using K-562 cell-line targets, spontaneous cell-mediated cytotoxicity was inhibited by 2-cyclohexene-1-one with an ID_{50} of $0{\cdot}71 \times 10^{-4}$ M–$1{\cdot}48 \times 10^{-4}$ M, while antibody-dependent cellular cytotoxicity was less sensitive to inhibition, and required slightly higher concentrations of $1{\cdot}48 \times 10^{-4}$ M–$3{\cdot}98 \times 10^{-4}$ M to achieve 50% inhibition. Similar results were seen with human colon tumour cell-line and Chang liver cell-line cells as targets. Maximal inhibition occurred when 2-cyclohexene-1-one was added to the cytotoxicity assay 60 min prior to, at the start of, or within the first 60 min of a 4-hr assay; inhibition of cytotoxicity occurred with pretreatment of effector cells; and no inhibition of cytotoxicity was observed with pretreatment of target cells. Both the allogeneic mixed leucocyte reaction and cell-mediated lympholysis were also significantly inhibited ($P < 0{\cdot}001$) by 2-cyclohexene-1-one. These studies demonstrate that 2-cyclohexene-1-one is an effective inhibitor of cell-mediated cytotoxicity and suggest that glutathione, specific glutathione–protein interactions, or protein-bound sulphydryl groups are involved in allowing cells to carry out cytolysis.

INTRODUCTION

Cell-mediated cytotoxicity is a well-described immunological effector mechanism. Although the precise nature of the lytic molecules that mediate cytotoxicity remains to be determined, a number of the steps in the overall process have been characterized. The initial binding of the effector cell to the target requires recognition of determinants on the target cell by specific effector cell receptors (Goldfarb & Herberman, 1982; Trinchieri & Perussia, 1984). Subsequently, activation of the effector cell through a calcium-dependent process results in increased phospholipase A_2 activity (Targan & Newman, 1983). Target cell lysis, which is also calcium-dependent, is postulated to follow the exocytosis of lytic molecules from the secretory granules of the effector cell and their deposition on the plasma membrane of the target cell (Wright & Bonavida, 1982; Farram & Targan, 1983). At this point in the process, the effector cell is no longer necessary, and the calcium-independent lethal hit stage of cytotoxicity begins (Hiserodt, Britvan & Targan, 1983).

During the lethal hit stage, assembly and activation of lytic molecules lead to the formation of transmembrane channels on the target cell due to insertion or assembly of amphophilic macromolecules (Dourmashkin *et al.*, 1980; Henkart & Henkart, 1982). Dourmashkin *et al.* (1980) observed by electron microscopy that, during cell-mediated cytolysis, human peripheral blood leucocytes induced the formation of ring-like structures with an internal diameter of 15 nm on the target cell. Henkart & Henkart (1982) observed that the formation of ring structures was due to material (designated as cytolysin) that was released from the cytoplasmic granules of the effector cell. These investigations proposed that the ring structures are inserted into target-cell membranes similar to the insertion of terminal complement components in forming an aqueous pore (Henkart & Henkart, 1982; Henkart *et al.*, 1984). It has been proposed that NK cells (Podack & Dennert, 1983) and cytotoxic T cells

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; 2-CHX-1, 2-cyclohexene-1-one; CML, cell-mediated lympholysis; GSH, glutathione; MLR, mixed leucocyte reaction; MNC, mononuclear cells; PB, peripheral blood; SCMC, spontaneous cell-mediated cytotoxicity.

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(Dennert & Podack, 1983) effect lysis by a series of steps that include: (i) the migration of granules within the effector cell toward the target cell; (ii) the release of vesicles from the granules of the effector cell; (iii) the formation of tubular complexes which cause the target cell membrane lesions; (iv) the assembly of monomeric precursors (perforins) of the polymerized tubular complexes (polyperforins); and (v) the membrane insertion of the polyperforins, allowing the formation of transmembrane channels and target-cell lysis.

Glutathione (GSH) or GSH–protein interactions have been implicated as playing an important role in the activation of lymphocytes and polymorphonuclear leucocytes (Fischmann *et al.*, 1981; Wedner *et al.*, 1981; Hamilos & Wedner, 1985). These studies were performed by depleting intracellular levels of GSH using 2-cyclohexene-1-one (2-CHX-1), an agent that reacts with GSH by a thio-ether bond, resulting in a dose-dependent reduction of GSH levels, with no increase in the levels of oxidized GSH (Chasseaud, 1979). As 2-CHX-1 is effective without toxicity to MNC, we chose to examine its effects on cell-mediated cytotoxicity.

MATERIALS AND METHODS

Peripheral blood (PB) MNC were obtained from healthy normal human volunteers by Ficoll–Hypaque centrifugation according to the method of Boyum (1968). The MNC were harvested, then washed four times in M199 or RPMI-1640 assay medium (Gibco, Grand Island, NY); 10% heat-inactivated fetal bovine serum (Gibco) (M199 assay medium) or 5% heat-inactivated pooled human serum (1640 assay medium); 2 mM L-glutamine (Sigma Chemical Co., Louis, MO); 1% antibiotic-antimycotic solution (Gibco); 1 mM Hepes buffer (Sigma); and 5 mM $CaCl_2$ (M199 assay medium only).

Spontaneous cell-mediated cytotoxicity (SCMC) and antibody-dependent cellular cytotoxicity (ADCC) assays were performed as previously described (MacDermott *et al.*, 1980). In brief, isolated PB MNC and ^{51}chromium (New England Nuclear, Boston, MA; mCi/ml, 500 mCi/mg)-labelled K562 (myeloid cell line), human colon tumour (adenocarcinoma; Sloan Kettering Institute, New York), or Chang liver cell-line cells, were incubated at 50:1, 25:1 or 10:1 effector to target cell ratios in 96-well microtitre plates (total volume 150 μl). Cells plus media alone, cells plus media plus 2-CHX-1 (Aldrich Chemical, Milwaukee, WI), and cells plus antibody (rabbit anti-K562, anti-colon, or anti-Chang antibodies) alone, or cells plus antibody plus 2-CHX-1, were incubated at 37° in a humidified 5% CO_2–95% air incubator for either 4 hr or 18 hr, and then harvested using the Titertek Harvesting system (Skatron Inc, Sterling, VA). The amount of ^{51}chromium released (percentage lysis) by the targets into the supernatant (as counts per minute, c.p.m.) was determined using a Beckman Biogamma Counter. Using the average of triplicate release values, the percentage lysis was determined by the formula:

$$\% \text{ lysis} = \frac{\text{experimental c.p.m.} - \text{spontaneous c.p.m.}}{\text{maximal c.p.m.} - \text{spontaneous c.p.m.}} \times 100.$$

Maximal release was determined using 100 μl of 5% Triton-X 100.

Cell-mediated lympholysis (CML) was assessed as previously described by Sondell *et al.* (1975), using 5×10^4 effector cells/well from six healthy donors incubated individually with 2×10^5 irradiated (2600 rads) pooled stimulators, with and without 2-CHX-1 in RPMI-1640 assay medium, in triplicate, for 7 days at 37° in a 5% CO_2–95% air humidified incubator. Simultaneously, 25×10^6 non-irradiated MNC from each donor population were cultured in 15-ml loosely capped culture tubes containing 10 ml of assay medium. On day 7 these target cells were labelled with ^{51}chromium, washed in RPMI-1640 assay medium, and 5×10^3 cells were added to each well. The plates were centrifuged at 50 *g* for 5 min and incubated at 37° for 5 hr. After incubation, the plates were harvested as described above. Parallel plates were harvested for the mixed leucocyte reaction (MLR), with the plates being pulsed with 0·2 mCi of methyl-[^{3}H]thymidine (NEN, 80 Ci/mmol) after 5 days of incubation, and then incubated for an 18-hr period.

For examination of the parameters of inhibition, 2-CHX-1 was prepared in varying concentrations and added to the ADCC, SCMC, CML and MLR assays. ADCC and SCMC inhibition experiments were also performed by carrying out preincubation of either effector or target cells with 2-CHX-1 for

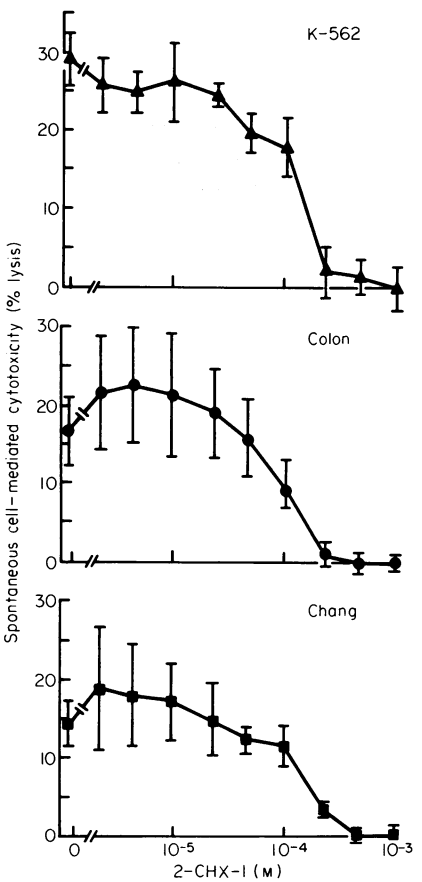

Figure 1. Effect of 2-cyclohexene-1-one on spontaneous cell-mediated cytotoxicity. Human peripheral blood mononuclear cells were incubated with the three different ^{51}Cr-labelled target cell lines shown (K-562, human colon tumour or Chang) at a killer to target ratio of 25:1. The assays were performed as described in the Materials and Methods with an incubation period of 18 hr. The results shown are the mean ± SEM for seven experiments, each performed in triplicate. Inhibition at the three highest concentrations was statistically significant ($P < 0{\cdot}001$). Similar results were obtained at 10:1 and 50:1 killer to target cell ratios (data not shown). The calculated ID_{50}s are given in Table 1.

varying lengths of time (1, 2, or 4 hr). The preincubated effector or target cells were washed three times and the assays then performed in the absence of added 2-CHX-1. Studies were also performed to assess the time at which the addition of 2-CHX-1 was most effective. The 2-CHX-1 was added to the cytotoxicity assays 60 min prior to beginning of the assay, at the beginning of the assay, and 60, 120 or 240 min after the beginning of a 4-hr cytotoxic assay. Statistical analysis was performed utilizing the Student's *t*-test and was confirmed using analysis of variance.

RESULTS

Figure 1 shows the effect of 2-CHX-1 on SCMC using normal human PB MNC and K-562, colon tumour, and Chang cells as targets, with a killer to target ratio of 25:1. The dose at which 2-CHX-1 caused 50% inhibition of cytotoxicity for SCMC ranged from 0·71 to $2·09 \times 10^{-4}$ M (Table 1). The effect on ADCC of 2-CHX-1 at ten different concentrations (Fig. 2) revealed marked inhibition ($P < 0·001$ at the two highest concentrations). The 2-CHX-1 ID_{50}s for ADCC were $1·48 \times 10^{-4}$ M–$3·98 \times 10^{-4}$ M (Table 1), indicating that SCMC was more sensitive to inhibition by 2-CHX-1 than ADCC. 2-CHX-1 was added at varying time-intervals, beginning before the SCMC or ADCC assay commenced, and throughout the 4-hr assay period (Fig. 3). All three concentrations of 2-CHX-1 examined (5×10^{-4} M, 1×10^{-3} and 5×10^{-3} M) resulted in marked inhibition of SCMC when added 60 min before the assay commenced, at the time the assay was plated, or 60 min after plating of the assay (Fig. 3a). When the same three concentrations of 2-CHX-1 were used at the same time-intervals in ADCC, moderate inhibition was observed with 5×10^{-4} M and 1×10^{-3} M 2-CHX-1, while only the highest concentration of 2-CHX-1 (5×10^{-3} M) produced marked inhibition when added before or at the time the assay was begun. Thus, SCMC revealed a greater sensitivity than ADCC to inhibition by 2-CHX-1, related to the time of addition of the drug to the assay and the drug concentration.

With K-562 cell-line cells as targets, preincubation of effector cells with 5×10^{-4} M 2-CHX-1, followed by washing of the effector cells, resulted in inhibition of both SCMC and ADCC (Table 2). SCMC was more sensitive to the effects of preincubation of effector cells with 2-CHX-1 than was ADCC (Table 2). The longer the preincubation period, the greater the degree of inhibition observed, and higher concentrations of 2-CHX-1 were more effective than lower concentrations (Table 2). Indeed, at low concentrations of 2-CHX-1 (5×10^{-5} M), SCMC was slightly inhibited, while ADCC was slightly augmented

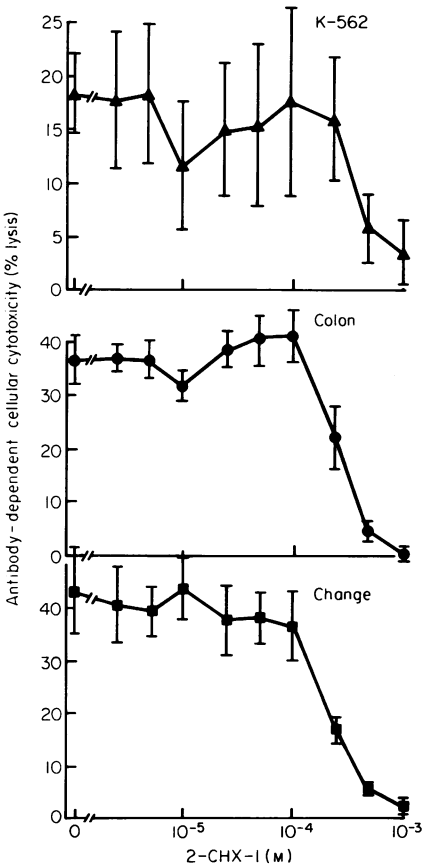

Figure 2. Effect of 2-cyclohexene-1-one on antibody-dependent cellular cytotoxicity. Human peripheral blood mononuclear cells were incubated with the three different ^{51}Cr-labelled cell lines (K-562, human colon tumour or Chang) at a killer to target ratio of 25:1 in the presence of appropriate antisera. The assays performed as described in the Materials and Methods with an incubation period of 18 hr. The results are the mean ± SEM for seven experiments, each performed in triplicate. Inhibition at the two highest concentrations was statistically significant ($P < 0·001$). ADCC values were derived after subtracting the SCMC values within each individual experiment. Similar results were obtained at 10:1 and 50:1 killer to target cell ratios. The calculated ID_{50}s are given in Table 1.

Table 1. Calculated ID_{50} values for the effect of 2-cyclohexene-1-one on cell-mediated cytotoxicity

Target cell	Killer:target ratio	ID_{50} ($\times 10^{-4}$)* SCMC	ADCC
K-562	50:1	1·31	1·48
	25:1	1·48	3·63
	10:1	0·71	3·98
Colon	50:1	0·82	2·82
	25:1	1·28	2·69
	10:1	1·82	2·00
Chang	50:1	1·55	1·95
	25:1	1·58	1·62
	10:1	2·09	2·82

* ID_{50} values for the effect of 2-cyclohexene-1-one (2-CHX-1) on spontaneous cell-mediated cytotoxicity (SCMC) and antibody-dependent cellular cytotoxicity (ADCC). The ID_{50}s were calculated as the concentration of 2-CHX-1 giving 50% inhibition of lysis. Each ID_{50} value was determined using the percentage inhibition curves for each target at ten concentrations of 2-CHX-1.

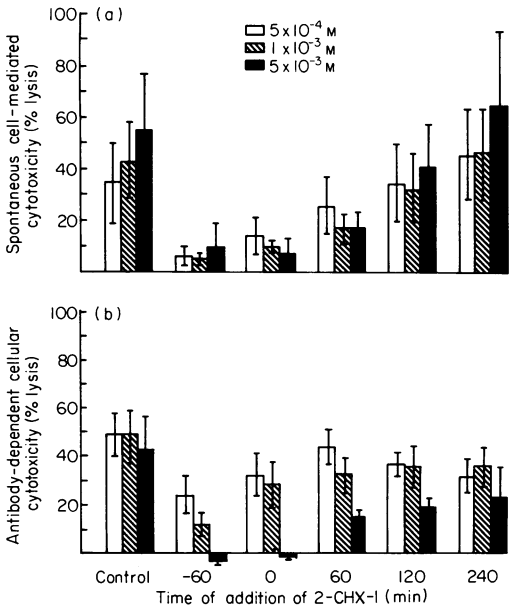

Figure 3. Effect of time of addition of 2-cyclohexene-1-one on inhibition of (a) spontaneous cell-mediated cytotoxicity (SCMC), or (b) antibody-dependent cellular cytotoxicity (ADCC). Human peripheral blood mononuclear cells were incubated with ^{51}Cr-labelled K-562 target cells at a 50:1 killer to target cell ratio for 4 hr with 2-CHX-1 at three different concentrations (5×10^{-4} M, 1×10^{-3} M and 5×10^{-3} M) added at various times (60 min before adding the target cells; at the start of the assay, and 60, 120, and 240 min after beginning the assay). Similar results were observed in 18-hr cytotoxicity assays.

Table 2. Effect of 2-cyclohexene-1-one preincubation of effector cells on SCMC and ADCC with K-562 target cells*

Preincubation time	Concentration of 2-CHX-1		
	No drug	5×10^{-5} M	5×10^{-4} M
SCMC			
4 hr	32·0±4·1†	20·0±5·4‡	0·9±0·5‡
2 hr		26·0±4·5‡	1·5±2·4‡
1 hr		24·2±3·7‡	14·7±1·8‡
ADCC§			
4 hr	22·9±6·9	27·9±6·9	0·1±1·0‡
2 hr		30·1±8·7	24·0±6·5
1 hr		36·4±7·6‡	18·0±2·7

* 18-hr assay, 50:1 killer:target ratio. Preincubation experiments were performed as described in the Materials and Methods. Similar results were obtained at 25:1 and 10:1 killer to target ratios with K-562 targets, and at all three killer:target ratios with Chang and colon tumour cell-line targets (data not shown).

† Mean±SEM percentage lysis for six experiments, each performed in triplicate.

‡ Statistically significant: $P < 0.05$ (compared with no drug).

§ SCMC subtracted from ADCC within each experiment.

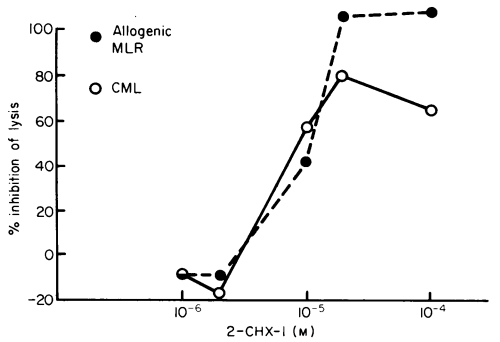

Figure 4. Percentage inhibition by 2-cyclohexene-1-one of the allogeneic mixed leukocyte reaction and cell-mediated lympholysis. Human peripheral blood mononuclear cells were sensitized with pooled allogeneic-irradiated mononuclear cells from six donors for 5 days. [^{3}H]Thymidine incorporation was used to assess allogeneic MLR over the last 18 hr and the results expressed as Δ c.p.m. Results shown for the MLR are based upon the mean Δ c.p.m. from six individuals, which was then used to calculate the percentage inhibition compared with control (no drug). For the determination of CML, lymphocytes from six individuals were sensitized and tested against ^{51}Cr-radiolabelled mononuclear target cells from six donors. The mean percentage cytotoxicity was then used to calculate percentage inhibition compared with control (no drug). Inhibition was significant ($P < 0.001$) at the two highest concentrations.

Table 3. Effect of 60-min 2-cyclohexene-1-one preincubation of K-562 target cells on SCMC and ADCC in a 4-hr cytotoxicity assay at a 50:1 killer to target cell ratio*

Drug concentration	SCMC	ADCC†
None	19·2±4·1 (6)‡	24·9±3·1 (6)
1×10^{-3} M	17·2±4·1 (5)	25·7±3·9 (5)
5×10^{-3} M	15·1±5·4 (5)	31·9±6·3 (5)§
1×10^{-2} M	15·4±4·3 (4)	35·9±4·3 (4)§

* Target cells were preincubated with 2-cyclohexene-1-one for 60 min, washed and added to ADCC or SCMC assays. Similar results were obtained in 18-hr assays; at 25:1 and 10:1 killer:target cell ratios with K-562 targets; and at all three killer:target cell ratios with Chang and colon tumour cell-line cells as targets (data not shown).

† The ADCC values derived after subtraction of SCMC values within each individual experiment.

‡ Mean±SEM percentage lysis for the number of experiments given in parentheses.

§ Significantly different from the no drug control ($P < 0.05$).

(Table 2). As shown in Table 3, there was no inhibition of either ADCC or SCMC when K-562 targets were preincubated with 2-CHX-1 at concentrations as high as 1×10^{-2} M. The separate effector cell and target cell preincubation studies indicate, therefore, that the inhibition caused by 2-CHX-1 is due to actions on the effector cells in the subsequent cytotoxicity assays. We next examined the effect of 2-CHX-1 on the allogeneic MLR and CML (Fig. 4). 2-CHX-1 was added at the initiation of the sensitization period for both assays, and resulted in inhibition of both the allogeneic MLR and CML with very similar dose inhibition curves (Fig. 4). Calculations based on the percentage inhibition curves yielded ID_{50}s of $1{\cdot}02 \times 10^{-5}$ M for the allogeneic MLR and $0{\cdot}81 \times 10^{-5}$ M for CML.

DISCUSSION

In the present study, we have observed that 2-CHX-1, which depletes intracellular glutathione (GSH) (Fischman *et al.*, 1981; Wedner *et al.*, 1981), is an effective inhibitor of cell-mediated cytotoxicity. SCMC was inhibited by 50% at concentrations of 2-CHX-1 ($0{\cdot}71 \times 10^{-4}$ M–$2{\cdot}09 \times 10^{-4}$ M) which in over 50 experiments have consistently reduced intracellular GSH levels to 30–40% of normal (Fischman *et al.*, 1981). Slightly higher concentrations of 2-CHX-1 ($1{\cdot}48 \times 10^{-4}$ M–$3{\cdot}98 \times 10^{-4}$ M) were needed for 50% inhibition of ADCC, and thus SCMC was more sensitive to inhibition by 2-CHX-1 than was ADCC. The ability of 2-CHX-1 to inhibit ADCC or SCMC was markedly reduced if added after 2 hr or more had passed in a 4-hr or 18-hr assay. Preincubation studies revealed that the effect of 2-CHX-1 was on the effector cells and not on the target cells. Finally, we found that 2-CHX-1 could inhibit both the allogeneic MLR and subsequent CML. Our data, therefore, suggest that normal intracellular GSH levels, protein-bound sulphydryl groups and/or GSH–protein interactions are necessary for effector cells to participate in cell-mediated cytotoxicity.

GSH is the most abundant non-protein sulphydryl-containing compound present in cells. The majority of GSH is located intracellularly and is free in the cytosol, although a portion is bound to protein in the form of GSH–protein mixed disulphides (Meister, 1973). The function of these GSH-containing proteins is not known; they may serve as a ready store of reduced GSH, since they can be cleaved by GSH reductase to yield both GSH and free protein sulphydryl groups. On the other hand, it has been suggested that there may be a regulatory role for GSH via disulphide interchange reactions (Meister, 1973; Isaacs & Binkley, 1977).

Although the step in lymphocyte activation that is blocked by 2-CHX-1 remains to be determined, there are several possibilities. A GSH protein–disulphide exchange reaction may be an early event allowing for transmission of signals to the cell for their activation. Recent data have demonstrated that cells pretreated with 2-CHX-1 fail to show a rise in cyclic AMP in response to lectin stimulation, although increases in cyclic AMP are seen in response to isoproteranol and prostaglandin E_1 (Wedner, 1984). This suggests that the 2-CHX-1-sensitive step may occur before stimulation of the adenylate cyclase catalytic unit. It is also possible that GSH is involved in maintenance of the integrity of the external plasma membrane, or in modulation of membrane receptors. Thus, although 2-CHX-1 is not toxic for cells, modulation of cell surface receptors could be involved in impairing cell-mediated cytotoxicity.

In the present study, a consistent differential effect of 2-CHX-1 on cytotoxicity was noted, with SCMC being more sensitive to GSH depletion than was ADCC. The differential effect was noted in: (i) the lower concentrations of 2-CHX-1 needed for 50% inhibition of SCMC (Table 1); (ii) the inhibition by lower 2-CHX-1 concentrations of SCMC but not ADCC in the time-course experiments (Fig. 3); and (iii) the greater inhibition of SCMC by preincubation of effector cells with 2-CHX-1 (Table 2). Because 2-CHX-1 may have effects in addition to its ability to deplete intracellular GSH, the differences in sensitivity to 2-CHX-1 inhibition of SCMC and ADCC may be the result of a series of complex biochemical processes which are differentially inhibited by 2-CHX-1. For example, a critical step in the sequence leading to lysis may be inhibited at low 2-CHX-1 concentrations, but not at higher concentrations. Indeed, preliminary data examining the enzyme GSH–protein mixed disulphide oxido-reductase suggest that it can be inhibited by 2-CHX-1 at low, but not high, concentrations (H. J. Wedner and P. Wilson, unpublished data). At present it is unclear whether the differential effects of 2-CHX-1 may relate to inhibition of the binding, triggering, cytotoxic factor release, or lytic molecule assembly steps in the cytotoxic process.

The inhibitory effect of 2-CHX-1 may be more complex than simple depletion of GSH. Recent studies in our laboratory (Hamilos & Wedner, 1985) have shown that agents such as buthionine sulphoxamine, which depletes intracellular GSH by interfering with its synthesis, and which does not effect GSH–protein interactions (Griffith & Meister, 1979), do not inhibit early events in lymphocyte activation. Indeed, the absolute level of GSH appears to be relatively unimportant for initiation of the activation response by mitogens, whereas the GSH level is critically important for propagation of the activation signal. Since the present study does not differentiate between signal transduction and propagation, we cannot say whether inhibition of GSH *per se*, or GSH–protein interactions, is the primary mechanism involved in 2-CHX-1-induced effects. Nevertheless, the availability of a non-toxic reagent that will interfere with destructive effector lymphocyte functions may eventually provide a new way of modulating events involved in the destruction of tissue grafts or mediation of autoimmune phenomena.

ACKNOWLEDGMENTS

This paper was published in part in abstract form in *Federation Proceedings*, **41,** 315, and was supported in part by UPHS grants AI-15322, AM-21474 and AI-18281.

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