Cell-mediated cytotoxicity by natural killer and killer cells, lipid peroxidation and glutathione

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Summary. In the course of spontaneous cell-mediated cytotoxicity (SCMC) and antibody-dependent cell-mediated cytotoxicity (ADCC) with human peripheral lymphocytes as effector cells, no lipid peroxidation occurred as measured by the production of ethane and thiobarbituric acid-reactive material. Furthermore, impairment of major cellular defense systems of target cells (K562 cells for SCMC, Chang liver cells for ADCC), by decreasing their glutathione content, had no effect on either lipid peroxidation or the cytotoxic response. These findings indicate that peroxidative damage is not a mechanism of NK and K cell-mediated cytotoxicity.
Key words. Natural killer cells; killer cells; lipid peroxidation; reduced glutathione; cytotoxicity.

Cytotoxic lymphocytes play a vital role in the immunological control of bacteria, viruses and tumor cells[1]. Contained in human peripheral blood are lymphocytes that possess natural killer (NK) cells, which exert a spontaneous cell-mediated cytotoxicity (SCMC) and killer (K) cells, which mediate antibody-dependent cell-mediated cytotoxicity (ADCC) against a variety of target cells[2,3]. Although cytotoxic reactions in general have been extensively investigated[1,4,5], mechanisms by which target cell damage occurs have not been entirely clarified[5].

Lymphocyte mediated-cytotoxicity may involve lysosomal enzymes in the course of cellular killing[1,6-8]. Also, the release of soluble factors, the lymphotoxins, which have cytostatic and/or cytolytic effects on certain cells, has been implicated[9]. It is unclear, however, whether or not reactive oxygen species are mediators of lymphocytotoxicity as is the case with granulocytes and macrophage-mediated ADCC[10-12]. With NK-cells, the release of superoxide ions (O_2^-) and hydrogen peroxide (H_2O_2) after target cell binding has been demonstrated[13,14]. The fact that both SCMC[15,16] and lymphocyte-mediated ADCC[17] are normal in patients with chronic granulomatous disease, however, argues strongly against this hypothesis as these patients do not possess the capability of producing reactive oxygen species. It has been shown that reactive oxygen species even repress natural killing in vitro[18]. On the other hand, the finding that hydroxyl radical scavengers inhibited SCMC favors the participation of this radical in NK-cell activity[19].

One of the mechanisms by which reactive oxygen intermediates could exert cellular toxicity is the peroxidation of membrane phospholipids (lipid peroxidation, LPO). This would be facilitated due to the fact that phospholipase A_2 is activated in the course of SCMC and ADCC[20-23]. Therefore, we were interested in investigating whether or not lipid peroxidation occurs in the course of cellular killing by NK and K-cells. Furthermore, we studied the effect of an impairment of the major defense systems against peroxidative damage by depleting glutathione (GSH) in the target cells of SCMC and ADCC.

Materials and methods. Preparation of lymphocytes. Mononuclear cells were isolated from peripheral blood of healthy human adult donors by the Ficoll-Paque technique as described previously[24]. Monocytes were removed from the suspension by adherence to the plastic of a tissue culture flask with overnight incubation at 37 °C. The lymphocytes were then poured off, centrifuged and washed twice with medium (RPMI 1640 medium, Flow Laboratories, Sydney, supplemented with 20 mmol/l Hepes, 24 mmol/l $NaHCO_3$, 0.2 mg/ml benzylpenicillin and 0.1 mg/ml streptomycin sulfate), before final suspension in medium plus 10% heat-inactivated fetal calf serum (HIFCS).

Target cells. For the SCMC assay, K562 cells (Queensland Institute of Medical Research) were cultured continuously in suspension. The cell suspension was centrifuged at 350 × g for 7 min, the cells washed twice followed by resuspension of the cell pellet to approximately 6×10^6 cells/2 ml in medium plus 10% HIFCS. This was incubated with 300 µCi of ^{51}Cr at 37 °C for 1 h, after which the suspension was centrifuged and the cells washed four times with fresh medium plus 10% HIFCS.

Chang liver cells (Commonwealth Serum Laboratories, Melbourne) were cultivated continuously in medium plus 10% HIFCS and harvested using 0.05% trypsin/0.02% EDTA (Flow Laboratories, Sydney). After washing, the cells were resuspended in fresh medium at 4×10^6 cells/5 ml and incubated with 300 µCi of ^{51}Cr (sodium chromate in 0.9% NaCl; Amersham) at 37 °C for 45 min with shaking at 60 oscillations/min. The labeled cells were then washed three times, resuspended, counted and diluted to 10^6 cells/ml. For the cytotoxicity assay, 1-ml aliquots were incubated at 25 °C for 60 min with either control rabbit or serum containing an antibody to human lung tissue (generously supplied by W. J. Halliday, University of Queensland). The Chang cells were then washed twice and resuspended in fresh medium plus 10% HIFCS. Control serum-treated Chang cells were used to measure any SCMC, while antibody-treated Chang cells served as targets for the ADCC assay. Actual ADCC could then be expressed after subtraction of any SCMC (this was never greater than 8% of cytotoxicity with antibody coated cells).

Cytotoxicity assay. Aliquots (0.8 ml) of medium or lymphocyte suspension (0.63×10^6 cells/ml) were added to sterile polycarbonate centrifuge tubes (Disposable Products, South Australia), followed by the target cells (0.4 ml) to give a final effector: target cell ratio of 50:1 for Chang and 25:1 for K562 cells. Tubes were then centrifuged at 200 × g for 1 min to establish cell-cell contact and incubated at 37 °C. After incubation for 2 h, tubes were centrifuged at 480 × g for 5 min. An aliquot of supernatant was then removed for the estimation of ^{51}Cr using an LKB mini gamma counter.

Tubes without lymphocytes were run concurrently for determination of spontaneous ^{51}Cr release. Cytotoxicity was calculated using the formula:

$$\text{Cytotoxicity} = \frac{\text{cpm}_{sup} - \text{cpm}_s}{\text{cpm}} \times 100\%$$

where cpm_{sup}, cpm_s and cpm are the supernatant, spontaneous release and total counts, respectively.

GSH depletion. To deplete intracellular glutathione, target cells were exposed at a concentration of 10^6 cells/ml to varying amounts of phorone (diisopropylidene acetone) added in 10 µl dimethyl sulfoxide (DMSO) per ml cell suspension. After incubation for 30 min at 37 °C with shaking at 60 oscillations/min, the cells were washed twice with medium, centrifuged and resuspended in medium and HIFCS. In experiments designed to determine GSH-content and cell viability after phorone-treatment, the cells were washed and resuspended in saline. An aliquot was taken to determine cell viability by means of the trypan blue-exclusion technique, while another aliquot of 500 µl was added to 500 µl of ice-cold 4% sulfosalicylic acid. Glutathione was determined using Ellman's reagent[25].

Assay of lipid peroxidation. To measure any possible lipid peroxidation, target cells were suspended to yield a concentration of 10^6 cells/ml, after which 0.4 ml were added to 0.8 ml of lymphocytes to yield a ratio of 1:50 for Chang liver cells and 1:25 for K562 cells. Incubations were carried out in gas tight flasks (12.5 ml reacti-flasks, Pierce Chemical Company, Illinois) at 37 °C for 2 h. At the end of the incubation period, gas samples were drawn

Table 1. Effect of phorone treatment on the glutathione content and the viability of Chang and K562 cells

Treatment[a]	Chang cells GSH (nmol/10^6 cells)	Viability (%)	K562 cells GSH (nmol/10^6 cells)	Viability (%)
None	42.3	100	10.2	100
DMSO (solvent control)	45.2	100	8.6	100
Phorone (0.72 µmol/ml)[b]	6.1	100	3.9	100
Phorone (1.81 µmol/ml)	7.0	100	4.7	99
Phorone (3.62 µmol/ml)	5.3	93	4.7	92
Phorone (5.43 µmol/ml)	7.0	97	4.7	84

[a]10^6 cells in 1 ml of RPMI 1640 medium were exposed to phorone dissolved in 10 µl DMSO (or 10 µl DMSO only for solvent controls) for 30 min. Subsequently, the cells were washed twice and GSH and cell viability were determined.
[b]Final concentration.

Table 2. Effect of glutathione depletion of target cells on lymphocyte-mediated ADCC and SCMC

Pretreatment of target cells	Lymphocytotoxicity (%)[a] ADCC (Chang liver cells)	SCMC (K562 cells)
None	39.6 ± 1.3	42.0 ± 0.8
DMSO (solvent controls)	38.5 ± 0.5	41.6 ± 1.5
Phorone (0.72 µmol/10^6 cells)	37.5 ± 2.1	38.1 ± 1.7
Phorone (1.81 µmol/10^6 cells)	37.0 ± 1.2	38.8 ± 2.6

[a]Values are means and their standard errors of five replicate samples.

Table 3. Production of thiobarbituric acid-reactive material and ethane as markers of lipid peroxidation in the course of lymphocyte-mediated cellular killing against Chang liver cells and K562 cells

Target cells	TBA-reactive material[a, b] (nmol/10^6 cells)	Ethane[a, b] (pmol/10^6 cells)	% Cytotoxicity[a, b]
Chang liver cells (control serum treated)	5.8 ± 0.3	9.8 ± 0.9	2.9 ± 0.8
Chang liver cells (antibody coated, ADCC)	5.8 ± 0.1	11.0 ± 0.6	85.9 ± 0.9
K562 cells (SCMC)	5.8 ± 0.2	8.6 ± 0.6	76.5 ± 1.4

[a]Values are means and their standard errors of quadruplicate samples.
[b]All parameters were determined 2 h after the start of the experiment.

Table 4. Production of thiobarbituric acid-reactive material and ethane as markers of lipid peroxidation in the course of lymphocyte-mediated cellular killing against glutathione-depleted Chang liver cells and K562 cells

Target cells[a]	TBA-reactive material[b, c] (µmol/10^6 cells)	Ethane[b, c] (pmol/10^6 cells)	% Cytotoxicity GSH-depleted target cells	Control target cells[d]
Chang liver cells (control serum treated)	4.7 ± 0.3	10.6 ± 0.7	3.0 ± 0.7	3.2 ± 1.1
Chang liver cells (antibody coated, ADCC)	5.2 ± 0.8	10.3 ± 0.6	49.2 ± 5.4	47.6 ± 5.8
K562 cells	5.0 ± 0.7	9.8 ± 0.7	33.3 ± 3.2	32.2 ± 3.3

[a]Target cells were exposed to 0.72 µmol phorone (in 10 µl DMSO) per 10^6 cells (in 1 ml) and washed twice before being employed.
[b]Values are means and their standard errors (X ± SEM) of three experiments using different batches of lymphocytes.
[c]All parameters were determined 2 h after the start of the experiment.
[d]Cytotoxicity against cells that were not pretreated with phorone were always run in parallel using the lymphocyte batches.

through a silicone-covered rubber septum and analyzed for their ethane content as described previously[26] using a Hewlett-Packard 5830A gas chromatograph equiped with a 3-ft column filled with Porapack Q 180-100 mesh and a flame-ionization detector.

An aliquot of the cell suspension (0.25 ml) was added to 0.5 ml of a 20% trichloroacetic acid solution, and the thiobarbituric acid-reactive material was determined according to published procedures[27].

Results. Treatment of Chang liver cells with 0.72 µmol/10^6 cells phorone led to a decrease of intracellular glutathione by 86%. With K562 cells, the same treatment resulted in a 62% depletion of cellular GSH (table 1). No further decrease in the content of GSH was seen upon treatment of either type of target cells with higher doses of phorone (table 1). At the two highest concentrations of 3.62 and 5.43 µmol/10^6 cells, however, cell viability was decreased in Chang cells and, more so, in K562 cells (table 1). Treatment of cells with the solvent for phorone, DMSO, had no or little effect on cellular glutathione concentration. Furthermore, GSH concentrations were found to remain at these diminished levels until they were used in the cytotoxicity assay.

Pretreatment of Chang liver cells with 0.72 or 1.81 µmol/10^6 cells phorone prior to antibody-coating and subjecting to killing by human peripheral lymphocytes had no effect on the extent of ADCC (table 2). Also, no enhancement of SCMC of control serum-treated Chang liver cells was seen upon depletion of GSH (data not shown). The same is true for the spontaneous cell-mediated cytotoxicity against K562 cells, which was not affected by depleting target cells of GSH (table 2).

In the course of both, SCMC and ADCC, virtually no lipid peroxidation was seen, despite the high cytotoxicity observed (table 3). The extent of the production of ethane and of thiobarbituric acid-reactive material as markers of lipid peroxidation did not differ from that seen with control serum treated Chang liver cells upon exposure to lymphocytes (table 3). In fact, the same amounts of ethane and TBA-reactive material were found when lymphocytes or target cells alone were incubated for 2 h at 37 °C (data not shown). The 1000-fold higher levels of TBA reactants as compared to ethane production is consistent with the known higher rate of formation of the former parameter of lipid peroxidation. After pretreatment of target cells with phorone to deplete cellular glutathione, impairing thereby the major defense systems against peroxidative damage, no sign of an enhanced lipid peroxidation was seen (table 4). Again, it was clear that GSH-depletion had no effect whatever on the lymphocytotoxicity (table 4).

Discussion. Glutathione is involved in several lines of defense against oxidative damage[28]. Thus, if this tripeptide is lacking, oxidative attack of cellular constituents is no longer controlled and can proceed more effectively. In fact, depletion of liver glutathione was shown to enhance NADPH-dependent lipid peroxidation both in vitro[26, 29] and in vivo[30]. Furthermore, GSH depletion sensitized tissue to lipid peroxidation induced by redox- cycling compounds, which produce reactive oxygen species in the course of their metabolism, in the presence of iron[31]. If oxygen free radicals were produced as mediators of lymphocytotoxicity, it would have been expected that target cells which were depleted of their cellular glutathione, would be more susceptible to cellular damage. However, this was not the case, indicating that oxidative damage might not have occurred. In fact, the evidence for oxygen radicals being produced in SCMC and ADCC is weak[12–16]. Recent results indicate that NK-sensitive target cells rapidly induce purified large granular lymphocytes to release a soluble factor that triggers a respiratory burst in monocytes[32]. Thus, the release of oxygen radicals by NK cells[12–14] might be a consequence of incomplete monocyte depletion in those experiments. In fact, the release of reactive oxygen species is a feature common to monocytes, macrophages and mast cells[10, 11, 33]. Recently, hydrogen peroxide was shown to be involved in mast cell-mediated tumor cell destruction[33]. The

involvement of hydroxyl radicals[19] at least, would have been an attractive hypothesis to explain the inhibition of cell-mediated cytotoxicity by ethanol[24], which is an efficient scavenger of hydroxyl radicals. Preliminary experiments done in our laboratory, however, gave no evidence for the production of O_2^-, the main precursor of all oxygen radicals (data not shown).

One of the major mechanisms through which an oxidative stress would lead to cellular damage is the peroxidation of polyunsaturated fatty acids in phospholipids of functional membranes[34]. In fact, the activation of phospholipase A_2 in peripheral blood mononuclear cells[20], and the inhibition of NK activity by inhibition of this enzyme[20,22], together with the findings of Carine and Hudig[23] that arachidonic acid is metabolized via the 5-lipoxygenase pathway in order to function in natural killing may suggest a peroxidative mechanism at least for SCMC. No signs of lipid peroxidation were observed, however, in the course of both SCMC and ADCC in our study. It cannot be fully excluded, however, that oxygen free radicals are produced locally leading to site-specific oxidative damage, including phospholipids, which is not measurable as an overall increase in lipid peroxidation. The fact that glutathione depletion had no effect on either cytotoxicity or lipid peroxidation, however, argues against this hypothesis. Thus, it seems that peroxidative damage is not a mechanism through which lymphocytes mediate their cytotoxicity.

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Enhancement of peritoneal macrophage activity by bovine gamma globulin in mice

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Summary. Mice treated with bovine gamma globulins showed an increased resistance to *Salmonella typhimurium* infection. This phenomenon seems to be bound to an increase of peritoneal macrophage phagocytic activity, as shown by the method of chemiluminescence, in experiements performed on peritoneal macrophages from mice treated with bovine gamma globulin.
Key words. Bovine gamma globulins; macrophages; chemiluminescence; phagocytic activity; *Salmonella typhimurium.*

Previous studies on macrophages have demonstrated that they can be activated by different agents: microorganisms, endotoxins, latex, culture-broth, peptones, plasmatic factors, etc. As indexes of this activation, microbicidal[1] and cytostatic[2] activity, induction of neutral proteinases[3], enhancement of spreading, phagocytosis and pinocytosis[4] and decrease of ectoenzymes[5] were studied. Moreover, secretion of enzymes, proteins, low molecular weight substances and specific factors were also demonstrated[6]. Furthermore, antigens seemed to be more immunogenic when challenged with stimulated rather than unstimulated macrophages. On the other hand, studies on peritoneal macrophages confirmed a different stimulation pattern depending on the various agents[7].

Bovine gamma globulins (BGG) are known as tolerance-inducing agents in several strains of mice; they influence both humoral and cellular immunological factors [8-11]. Amongst others, Lukic[9] clearly showed that differences in susceptibility to induction of tolerance were related to macrophage function in the induction phase of immunity.

Our previous finding that mice pretreated with bovine gamma