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SHORT COMMUNICATION

Protection by L-2-Oxothiazolidine-4-Carboxylic Acid of Hydrogen Peroxide-induced CD3ζ and CD16ζ Chain Down-regulation in Human Peripheral Blood Lymphocytes and Lymphokine-activated Killer Cells

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ABSTRACT. We investigated whether L-2-oxothiazolidine-4-carboxylic acid (OTC) [in the form of Procysteine®, kindly donated by Transcend Therapeutics] could protect peripheral blood lymphocytes (PBL) and lymphokine-activated killer (LAK) cells from CD3ζ and CD16ζ chain down-regulation induced by H_2O_2 produced by lipopolysaccharide (LPS)-activated autologous monocytes. OTC is known to enhance glutathione production in cells in which glutathione was depleted by reactive oxygen species. Our data showed that OTC induced a significant increase in CD3ζ and CD16ζ chain expression in peripheral blood lymphocytes and LAK cells, respectively, pretreated for 12 hr at 37°. Moreover, OTC significantly protected peripheral blood lymphocytes and LAK against decreased ζ chain expression induced by lipopolysaccharide-activated monocytes or the addition of H_2O_2 to the culture medium. Our experiments thus suggested that alterations in signal-transducing molecules, such as decreased CD3ζ and CD16ζ expression observed in cytotoxic T lymphocytes and LAK cells in response to oxidative stress, could be prevented by the use of OTC. BIOCHEM PHARMACOL **56**;5:657–662, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. ζ chain; hydrogen peroxide; L-2-oxothiazolidine-4-carboxylic acid; peripheral blood lymphocytes; macrophages; lymphokine-activated killer cells

Lymphocytes from tumor-bearing mice and cancer patients have been reported to exhibit decreased CD3ζ chain expression [1, 2]. Such a decrease has been correlated with reduced proliferative responses following antigenic challenge [3] and with reduced cytotoxic effector function [1]. Furthermore, a direct correlation has been established between decreased levels of CD3ζ expression on CTL¶ and tumor progression [4, 5]. H_2O_2 was demonstrated to reduce ζ chain expression in CTL; however, OTC was found to be an efficient scavenger of H_2O_2 production by increasing glutathione content in the cells. Therefore, we thought that OTC could prevent ζ-chain down-regulation induced by H_2O_2.

H_2O_2 derived from human macrophages isolated from metastatic lesions of malignant melanoma patients or from healthy donor activated monocytes can strongly inhibit the effector function of melanoma-specific CTL and their CD3ζ expression [6]. Under conditions of oxidative stress such as H_2O_2 production by macrophages, glutathione, a central component of the body's antioxidant defense mechanism, is often depleted and damage to cells from oxygen species can result. OTC was first described as a cysteine delivery system and glutathione enhancer by Williamson and Meister [7]. Extensive preclinical research has demonstrated that OTC increases glutathione levels in animal models where the latter was depleted following oxidative stress. In such models, treatment with glutathione esters was found to protect cells and tissues from damage [8–10]. Glutathione esters currently represent the only method of achieving supernormal levels of glutathione.

OTC is effective in enhancing glutathione levels because it is metabolized intracellularly to cysteine [11]. Oxoprolinase, an enzyme found in many plant and most animal cells, catalyzes the intracellular conversion of OTC to L-cysteine and carbon dioxide. In addition, treatment with OTC does not present the difficulties associated with cysteine admin-

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¶ *Abbreviations*: CTL, cytotoxic T lymphocytes; LAK, lymphokine-activated killer; LPS, lipopolysaccharides; OTC, L-2-oxothiazolidine-4-carboxylic acid; and PBL, peripheral blood lymphocytes.

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istration, such as high extracellular toxicity and limited cellular intake [12].

The present study was carried out to determine whether one of the commercially available forms of OTC, Procysteine® (kindly donated by Transcend Therapeutics), could increase the expression of CD3ζ on PBL and CD16ζ on LAK cells. We also determined whether the down-regulation of the two signal-transducing molecules, induced by LPS-activated monocytes (CD3ζ) or the addition of extraneous H_2O_2 (CD3ζ and CD16ζ), could be blocked by treatment with OTC.

MATERIALS AND METHODS

Preparation of Cells

Peripheral blood mononuclear cells were isolated from the whole blood of healthy patients by Ficoll-Hypaque (Pharmacia) gradient centrifugation. PBL were obtained from one aliquot by removal of plastic-adherent cells. Monocytes were isolated from a second aliquot by adherence (1.5 hr at 37°) to fetal calf serum-coated tissue culture dishes (Costar). Resultant monocyte cultures were 95% pure, as determined by mouse antihuman CD14 mAb (Serotec) staining and subsequent flow cytometric analysis. PBL were then cultured in AIM-V medium (Life Technologies). Activated monocytes were obtained by exposure to LPS (20 mg/mL; LPS from *Escherichia coli*, 055:B5, Sigma) for 30 min at 37°. The monocytes were then washed 3 times to remove LPS before 6-hr co-culture (37°) with autologous PBL (1×10^6 cells in AIM V), at monocyte-to-PBL ratios of 1:1, 1:2.5 and 1:5. LAK cells were obtained by incubation of PBL with 100 U/mL of interleukin-2 (R&D Systems) for 7 days. LAK cell purity was then determined to be between 95% and 99% by mouse antihuman CD16 mAb (Serotec) staining and subsequent flow-cytometric analysis.

Incubation with H_2O_2

PBL and LAK cells were cultured in serum-free AIM-V medium in the presence of 1×10^{-5} M H_2O_2 at 37° for 12 hr. H_2O_2 was then inactivated by the addition of 80 U/mL of catalase (Sigma).

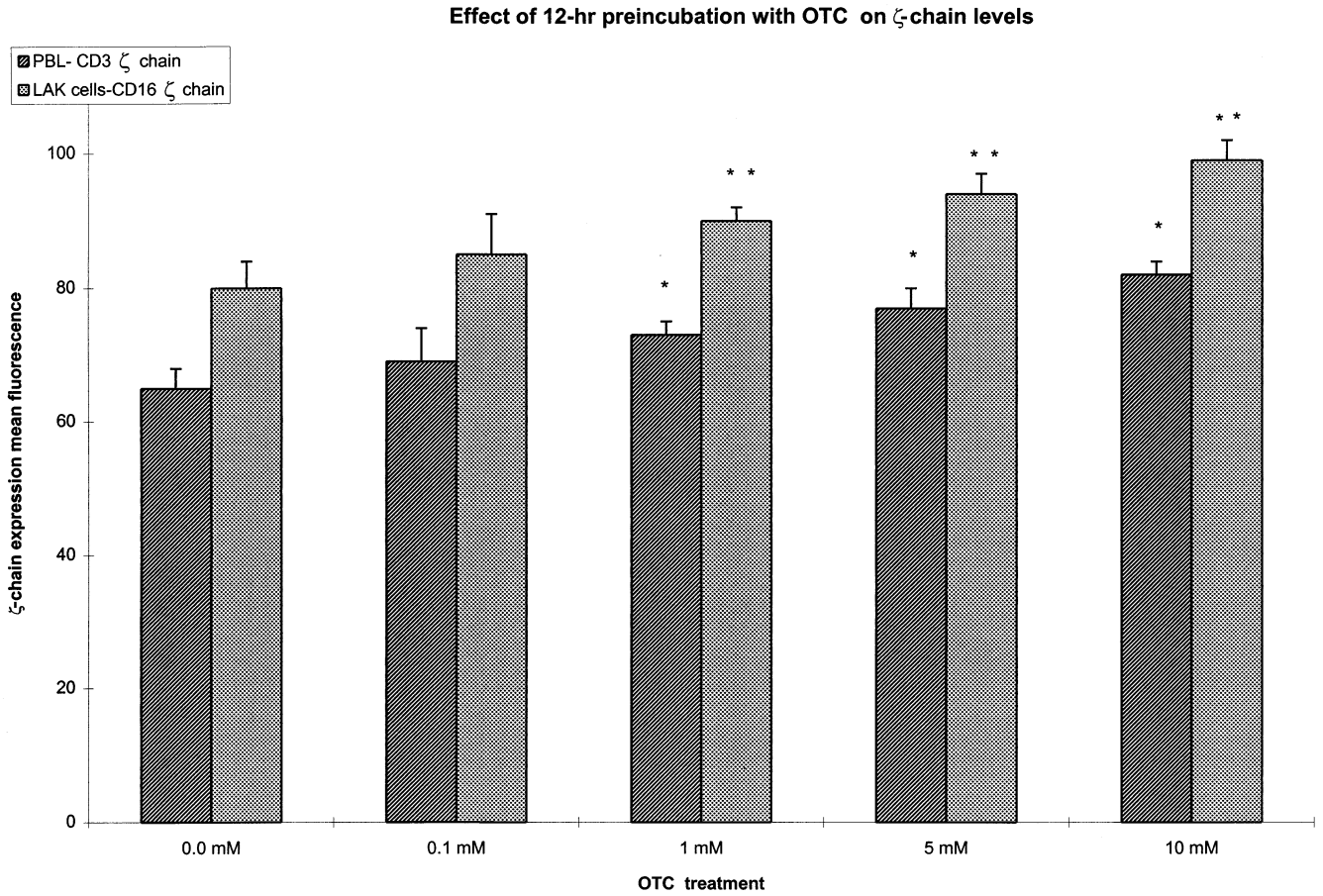

FIG. 1. Effect of 12-hr preincubation with OTC on CD3 (PBL) and CD16 (LAK cells) ζ-chain levels. OTC treatment is divided into five groups: 0.0 mM, 0.1 mM, 1 mM, 5 mM and 10 mM. Data are means ± SEM of five experiments. ⋆$P < 0.05$ vs 0.0 mM (PBL); ⋆⋆$P < 0.05$ vs 0.0 mM (LAK).

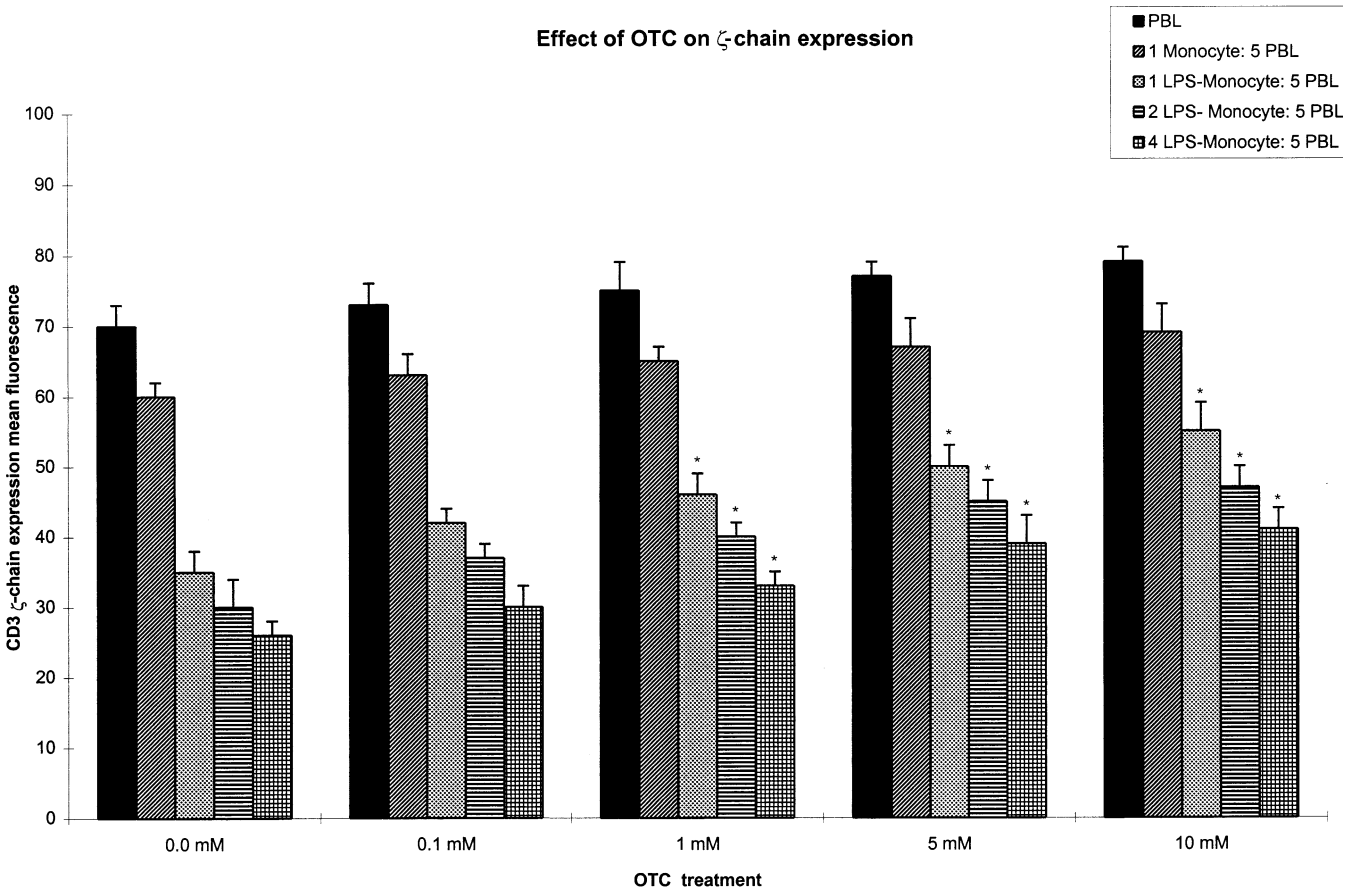

FIG. 2. Effect of OTC on CD3 (PBL) ζ-chain expression; PBL alone and with monocytes or LPS-activated monocytes (different cell concentrations). OTC treatment is divided into five groups: 0.0 mM, 0.1 mM, 1 mM, 5 mM and 10 mM. Data are means ± SEM of five experiments. ⋆$P < 0.05$ vs corresponding 0.0 mM.

OTC Treatment

Procysteine®, one of the commercially available forms of OTC, is 10–15% soluble w/v in an aqueous buffered solution when the pH is adjusted to 7. The solutions are then stable at 1–30° for 30 days. PBL and LAK cells were incubated with OTC concentrations of 0.1, 1, 5 and 10 mM for 12 hr at 37° prior to co-culture of PBL with autologous LPS-activated monocytes, incubation with H_2O_2, or direct determination of the effect of OTC on CD3ζ or CD16ζ chain levels. Control groups were incubated without OTC.

Flow-Cytometric Analysis

Expression of CD3ζ and CD16ζ was investigated using flow-cytometric analysis of permeabilized cells according to a previously described technique [5]. Briefly, PBL and LAK cells (2×10^5 cells/mL) were washed 3 times with Hank's balanced salt solution before being fixed with 0.5% formaldehyde (Sigma) in PBS for 20 min on ice and then permeabilized with 10 mg/mL of digitonin (Sigma) in PBS for 5 min on ice. Cells were then incubated with a saturating concentration of mouse antihuman ζ-chain mAb or IgG1 isotype control antibody (Serotec) before being stained with a rabbit antimouse-FITC antibody (Serotec). After blocking with normal mouse serum (Fluka), cells were double stained with mouse antihuman CD3 or CD16 phycoerythrin-conjugated monoclonal antibodies (Serotec) and subjected to flow cytometric analysis using a FACScan flow cytometer (Becton Dickinson). CD3 and CD16 cells were gated and measured for the expression of ζ molecules, expressed as mean fluorescence intensity.

Statistical Analysis

Statistical analysis was performed by ANOVA. Significance was assumed when $P < 0.05$.

RESULTS

Our data indicated that LAK cells inherently expressed more ζ chains than PBL (Fig. 1, 0.0 mM). Pretreatment of PBL and LAK cells with different OTC concentrations for 12 hr at 37° (Fig. 1) clearly induced an increase in CD3 and CD16 ζ-chain expression, respectively. The ζ-chain increase was directly correlated to OTC dosage and became significantly different from the untreated control at doses higher than 0.1 mM for LAK and PBL.

Monocytes intrinsically caused a slight reduction in

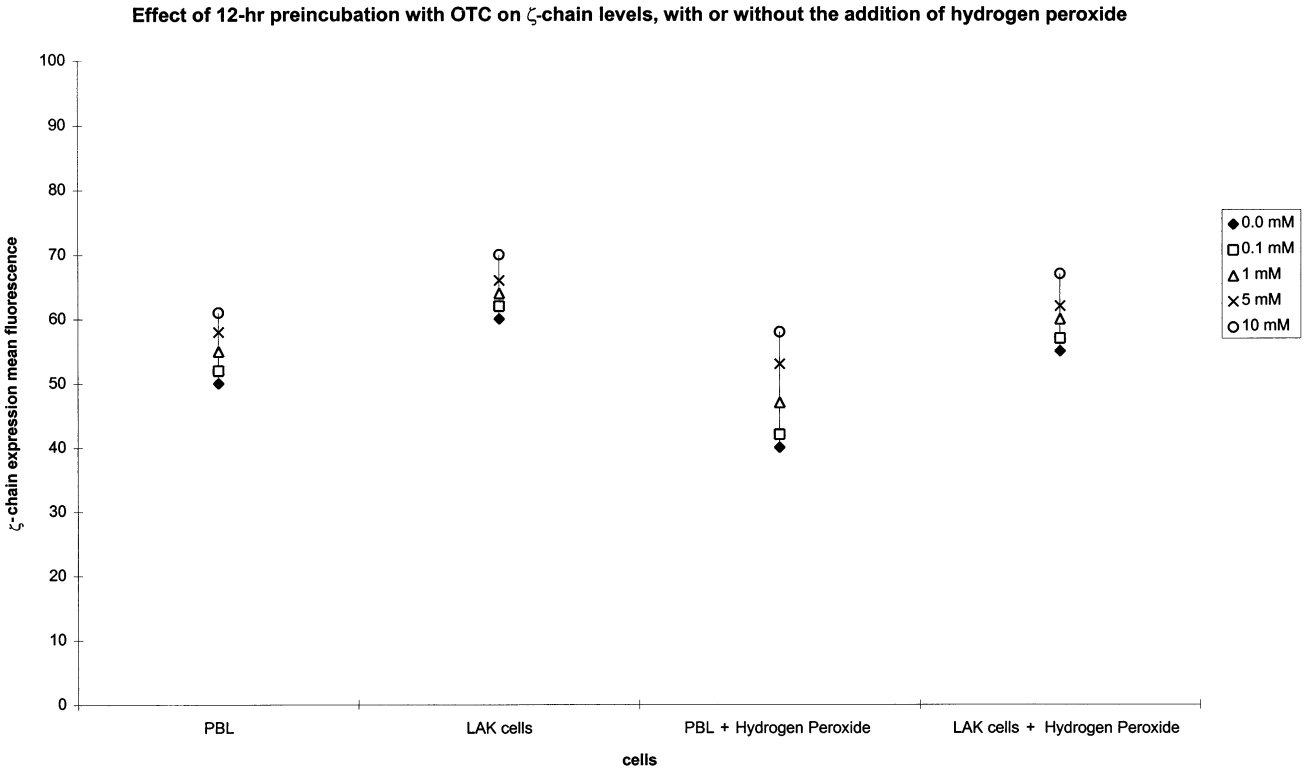

FIG. 3. Effect of 12-hr preincubation with OTC on CD3 (PBL) or CD16 (LAK cells) ζ-chain levels, with or without H_2O_2. OTC treatment was divided into five groups: 0.0 mM, 0.1 mM, 1 mM, 5 mM and 10 mM. A typical experiment (of the seven experiments performed) is reported.

CD3ζ chains expressed on PBL (Fig. 2, 0.0 mM). The inhibition more than doubled when the monocytes used for co-culture were previously activated with LPS for 6 hr at 37° and was directly correlated with the number of monocytes in culture. Pretreatment of exposed PBL with OTC for 12 hr at 37° inhibited the down-regulation induced by LPS-activated monocytes in a dose-dependent manner (Fig. 2). Although the intrinsic effect of nonactivated monocytes could not be reduced by OTC-pretreated PBL, highly significant differences with untreated controls were found when using OTC concentrations of 1, 5 and 10 mM.

To determine whether the effect of LPS-activated monocytes on PBL was indeed due to production of H_2O_2 by the former and subsequent depletion of glutathione, we directly added H_2O_2 (at a concentration of 10^{-5} M for 12 hr at 37°) to cultures of OTC-pretreated PBL. The data (Fig. 3 shows a typical example of seven experiments) indicated that H_20_2 impaired the expression of CD3ζ chains in PBL, and the effect was also observed with LAK cell CD16ζ chains. Again, it is noteworthy that LAK cells inherently expressed more CD16ζ than PBL expressed CD3ζ, and that OTC pretreatment inhibited the H_2O_2-induced down-regulation of ζ chains on both types of cells in a dose-dependent manner.

To demonstrate a specific role for H_2O_2 produced by stimulated monocytes for the loss of the ζ chain, we co-cultured PBL and LAK cells with autologous monocytes or incubated them with H_2O_2 for 12 hr prior to the assay, with or without catalase or 1 mM OTC (Table 1) (which was found to be the minimal concentration of OTC useful to maintain high ζ-chain levels: see Figs. 1 and 2). Following coincubation with LPS-stimulated monocytes, CD3ζ expression by PBL was significantly reduced, but such a reduction was abrogated by catalase (when maintained throughout the co-culture period) and by OTC (1 mM) treatment. Analogously, CD16ζ expression by LAK cells was preserved when catalase or OTC was used. Pretreatment of CTL and LAK cells with nontoxic concentrations (1×10^{-5} M) of H_2O_2, in which cells were more than 98% viable after treatment, also severely reduced CD3 and CD16ζ expression, an effect that could be abrogated by catalase as well as by 1 mM OTC.

DISCUSSION

Macrophages and monocytes are of central importance in host resistance to microorganisms through phagocytosis, secretion of reactive oxygen species, cytokine release, and antigen presentation. It has been shown that at certain stages of activation associated with H_2O_2 production, macrophages appear to be able to induce potent negative-feedback mechanisms that inhibit specific T-cell functions by altering T-cell receptor composition and suppressing natural killer activity [13].

Our experiments clearly indicate that LPS-activated monocytes are able to significantly inhibit one of the main

TABLE 1. Influence of catalase and OTC on ζ-chain expression

	Mean fluorescence intensity	
	CD3 ζ	CD16 ζ
PBL	69.2 ± 6.8	
LAK		74.2 ± 7.4
PBL + nonstimulated monocytes	60.1 ± 6.5	
LAK + nonstimulated monocytes		64.6 ± 6.5
PBL + LPS-stimulated monocytes	32.8 ± 2.5[a]	
LAK + LPS-stimulated monocytes		38.6 ± 0.3[a]
PBL + LPS-stimulated monocytes + 80 U/mL catalase	60.2 ± 6.3	
LAK + LPS-stimulated monocytes + 80 U/mL catalase		66.2 ± 5.6
PBL + LPS-stimulated monocytes + 1 mM OTC	57.6 ± 5.7	
LAK + LPS-stimulated monocytes + 1 mM OTC		63.5 ± 5.4
PBL + 80 U/mL catalase	60.1 ± 6.3	
LAK + 80 U/mL catalase		75.1 ± 0.3
PBL + 1×10^{-5} M H_2O_2	28.9 ± 2.4[b]	
LAK + 1×10^{-5} M H_2O_2		35.2 ± 0.3[b]
PBL + 1×10^{-5} M H_2O_2 + 1 mM OTC	49.4 ± 4.4	
LAK + 1×10^{-5} M H_2O_2 + 1 mM OTC		68.4 ± 0.5
PBL + 1×10^{-5} M H_2O_2 + 80 U/mL catalase	58.6 ± 5.3	
LAK + 1×10^{-5} M H_2O_2 + 80 U/mL catalase		70.2 ± 0.6

PBL and LAK cells were co-cultured with autologous monocytes or incubated with H_2O_2 for 12 hr prior to the assay, with or without catalase or 1 mM OTC. Data are the means ± SEM of five experiments.

[a] $P < 0.05$ versus PBL or LAK.

[b] $P < 0.05$ versus PBL (or LAK) + catalase.

signal-transducing molecules of lymphocytes, CD3ζ, and that the importance of the reduction is dependent on monocyte concentration (Fig. 2). Macrophages are known to deplete glutathione in cells via oxidative damage [14]. Glutathione plays an important role in protecting cells against the destructive effects of reactive oxygen intermediates and free radicals. H_2O_2 is known to modulate cellular functions, by inducing glutathione depletion, in many cells and also in lymphocytes [15].

It has previously been shown that depletion of glutathione greatly affects signal-transduction events in lymphocytes. Thus, it appears that one of the negative-feedback mechanisms exerted by macrophages on lymphocytes is inhibition of CD3 signal transduction via glutathione depletion. Our results (Fig. 2) support such a hypothesis. We found that ζ-chain down-regulation in PBL (CD3ζ) and LAK cells (CD16ζ) occurred in similar ways upon exposure to LPS-activated monocytes (Fig. 2) or 10^{-5} M H_2O_2 (Fig. 3), with or without catalase (Table 1). Such an observation indicates that it is indeed H_2O_2 production by macrophages that induces ζ-chain down-regulation in PBL and LAK cells.

The second aim of the study was to determine whether OTC, a compound converted to cysteine [7] by oxoprolinase [12] and one that thus enhances glutathione production in cells [11], could be used to control the observed inhibition of PBL and LAK cells by macrophages. The commercial form of OTC was used as pretreatment at concentrations ranging from 0.1 to 10 mM. We were able to demonstrate that OTC could markedly limit PBL CD3ζ down-regulation by macrophages, when used at 1-, 5- and 10-mM concentrations (Fig. 2). It was also clear that OTC could up-regulate ζ-chain expression in PBL and LAK cells (Fig. 1). Finally, it was apparent that the OTC concentration of 1 mM was sufficient to protect PBL and LAK cells from ζ-chain down-regulation induced by 10^{-5} M H_2O_2 (Fig. 3). Activated monocytes produce increased levels of reactive oxygen species (including H_2O_2) known to modulate cellular functions, including those of lymphocytes [13]. The presence of 80 U/mL of catalase during co-culture almost entirely abrogated the inhibitory effect of LPS-activated monocytes and H_2O_2 on CD3 and CD16ζ expression, as did co-culture with 1 mM OTC (Table 1). Catalase, a scavenger of H_2O_2, inhibits its biologic effects [16].

The observed up-regulation of signal-transducing molecules in PBL and LAK cells not exposed to reactive oxygen intermediates could be explained by the proposal that OTC, by enhancing the glutathione content of these healthy cells [17–21], may increase their metabolic rate (Fig. 1). Glutathione helps to maintain structural and functional viability during oxidant injury, in spite of endogenous production of reactive oxygen intermediates. Manipulation of glutathione metabolism, such as increasing it by treatment with OTC, may be a rewarding strategy in helping cells to counteract immunosuppression. Research aimed at developing drugs that counteract immune suppression should thus provide new and promising avenues for treatment, especially for immunotherapy.

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