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Effects of ifosfamide on immunocompetent effector cells

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Abstract We analyzed the effects of ifosfamide, a chemotherapeutic agent that is broadly used within anticancer therapy, on immunocompetent effector cell subpopulations. For our in vitro studies we used 4-hydroperoxyifosfamide (4-OOH-IF), which rapidly gives rise to 4-OH-IF, the activated form of ifosfamide. Activated cytotoxic T lymphocytes and natural killer (NK) cells were used because of their antitumor activity and their antiviral or antibacterial activity. Our study demonstrated three major findings. (1) The capacity of cytotoxic T cells to lyse their specific target cells was substantially reduced by 4-OH-IF treatment. This inhibition of the lytic activity could be correlated with a substantial depletion of the intracellular glutathione (GSH) levels. A rapid reconstitution of the depleted GSH levels and of the cytotoxic activity was achieved by incubation of the T cells with thiols such as mercaptoethanesulfonate (mesna). (2) In contrast to T cells the lytic activity of NK cells was not substantially affected by 4-OH-IF treatment; this increased resistance of NK cells against 4-OH-IF treatment could be explained by their higher initial GSH levels and by their higher rate of GSH synthesis. Furthermore, we demonstrated that (3) NK cells, but not T cells, have the capacity to take up cystine, the oxidized form of cysteine, from the medium. In conclusion we can state that NK cells are much more resistant to ifosfamide treatment compared to T cells with respect to intracellular GSH levels and cytotoxic activity.

Key words Ifosfamide · Cytotoxic T lymphocytes · Natural killer cells · Glutathione

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Introduction

The oxazaphosphorines cyclophosphamide and the isoform ifosfamide are the most frequently used antineoplastic agents in the therapy of solid tumors [17]. In vivo, ifosfamide is metabolically activated by hepatic mixed-function oxidases into 4-hydroxyifosfamide (4-OH-IF). Intracellularly, the activated form, 4-OH-IF, is decomposed to alkylating mustard, when acrolein is split off. For our in vitro investigations, we used the prodrug 4-hydroperoxyifosfamide (4-OOH-IF; kindly provided by ASTA-Medica, Frankfurt, Germany) which, in aqueous solutions, spontaneously gives rise to pharmacologically equivalent amounts of 4-OH-IF. It is known that the tripeptide glutathione (GSH) plays an important role in maintaining the cellular redox potential [10]. GSH irreversibly binds to the toxic metabolites of oxazaphosphorines like acrolein, which leads to a depletion of the GSH content in different cell types [1, 4]. Our previous results demonstrate that unstimulated human peripheral blood mononuclear cells (PBMC) are sensitive targets for an ifosfamide treatment because of this GSH modulation [3, 8]. The aim of this study was to analyze the effects of 4-OH-IF on the immunological functions of activated lymphocyte subpopulations ($CD3^-$ NK and $CD3^+$ CTL effector cells) and its influence on intracellular GSH levels.

Materials and methods

Treatments

Peripheral blood obtained from a healthy human volunteer was anticoagulated with Heparin Novo (Novo Nordisk Pharma GmBH, Mainz, Germany). Peripheral blood lymphocytes (PBL) were derived from monocyte-depleted PBMC, which were obtained after density gradient centrifugation on Ficoll Isopaque (Pharmacia Freiburg, Germany). The HLA types of donor and stimulator cells were determined according to an established method of Terasaki and McClelland [16] using different HLA antibodies and alloantisera; donor A: A2, BN60(40), B62(15), Bw6, Cw3, DR4, DR13(6), DQ6(1), DQ8(3), DR52, DR53; donor B: A24(9), A30(19), B7, B27, Bw4, Bw6, DR2, DQ1. PBL of donor A

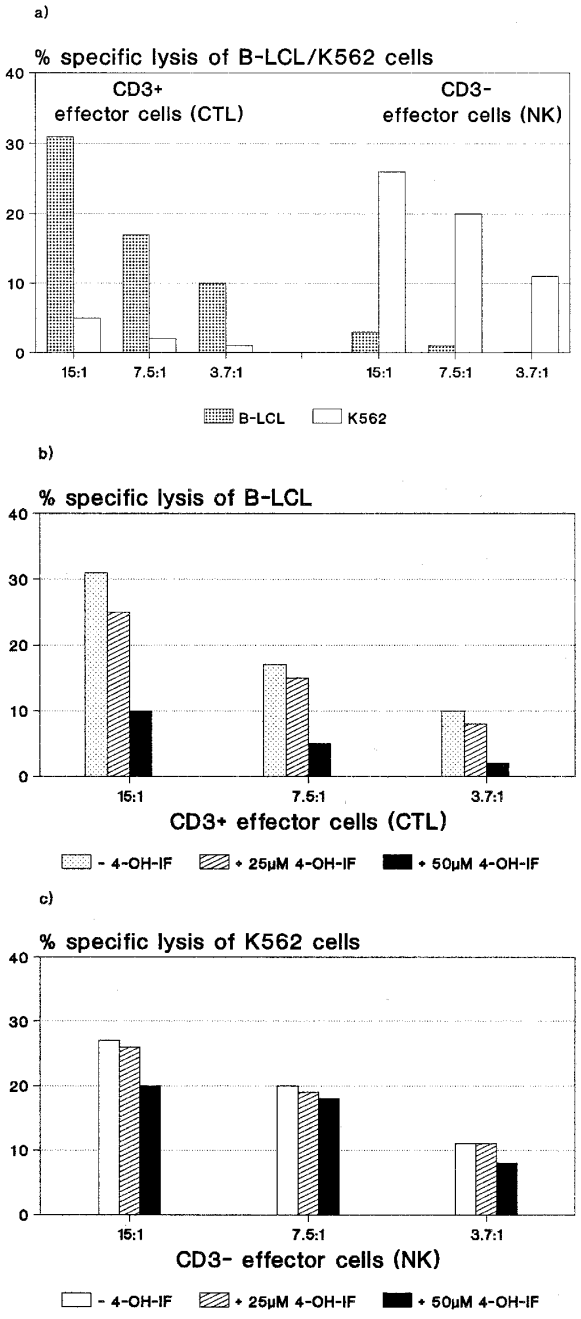

Fig. 1a–c The cytotoxic activity of separated $CD3^+$ cytotoxic T lymphocytes (*CTL*) and $CD3^-$ natural killer (*NK*) effector cells, either untreated or treated with 4-hydroxyifosfamide (4-OH-IF; 25, 50 µM for 1 h), was measured against chromium-labeled allogeneic B-LCL and K562 target cells. **a** The lysis of the allogeneic B-LCL was mediated only by the $CD3^+$ effector cell population, whereas the K562 target cells were lysed selectively by the $CD3^-$ effector cell population. **b** After ifosfamide treatment (50 µM 4-OH-IF) the capacity of $CD3^+$ CTL effector cells to lyse their allogeneic target cells B-LCL was reduced more than threefold. **c** In contrast, the capacity of $CD3^-$ effector cells to lyse K562 target cells was not affected significantly. The results are expressed as percentage specific cytotoxic activity at varyious E/T ratios ranging from 15 : 1 to 3.7 : 1. The data represent the mean values of three independent experiments; the SD of each data point was less than 10%

Table 1 The effects of 4-hydroxyifosfamide (*4-OH-IF*; 25, 50 µM for 1 h) or 4-OH-IF (50 µM) plus mercaptoethanesulfonate (mesna; 0.4 mM, 6 h) on glutathione levels in separated $CD3^+$ cytotoxic T lymphocytes and $CD3^-$ natural killer cells. SD of at least three independent experiments are given in parenthesis

Treatment	Glutathione level (nmol/mg protein)			
	Control	4-OH-IF 25 µM	4-OH-IF 50 µM	4-OH-IF + mesna 50 µM + 0.4 mM
$CD3^+$	33.6 (±7.4)	12.9 (±2.5)	6.34 (±0.7)	30.0 (±0.1)
$CD3^-$	41.0 (±1.2)	39.8 (±2.9)	27.8 (±1.8)	38.0 (±1.5)

were separated in a $CD3^+$ cytotoxic T lymphocyte (CTL) and a $CD3^-$ natural killer (NK) cell population by selection via adhesion and magnetic-bead separation [13, 11]. The purity of both cell fractions was more than 95% as determined by fluorescence-activated cell sorting (FACScan) using a CD3 (Dianova, Hamburg, Germany) monoclonal antibody (mAb) as a T cell marker and a CD16/56 (Becton-Dickinson, Heidelberg, Germany) mAb as a NK marker (data not shown). The $CD3^+$ fraction was stimulated using irradiated (20 Gy) allogeneic Epstein-Barr-virus-transformed B lymphoblastoid cell lines (B-LCL) derived of donor B and recombinant interleukin-2 (IL-2) (Euro Cetus, Frankfurt, Germany) at a final concentration of 100 IU/ml RPMI-1640 medium supplemented with 10% fetal calf serum, 6 mM L-glutamine and antibiotics for 5 days. The $CD3^-$ NK fraction was stimulated with IL-2 only. The separated IL-2-stimulated cell fractions were incubated at a final concentration of 0, 25, 50 and 100 µM 4-OH-IF at 37 °C for 1 h. The cells pretreated with 4-OH-IF (25 µM at 37 °C for 1 h) were incubated with 2-mercaptoethanesulfonate (mesna; ASTA-Medica, Frankfurt, Germany) at a final concentration of 0.4 mM for 6 h prior to an assay.

Determination of intracellular GSH

The total intracellular GSH (reduced and oxidized form) of the different effector cell populations, either untreated or treated with different concentrations of 4-OH-IF (25, 50, 100 µM for 1 h) and/or mesna (0.4 mM for 6 h), was quantified in relation to an internal standard by using the HPLC method described by Reed et al. [14].

^{51}Cr-release assay

The specificity of the $CD3^+$ and $CD3^-$ separated effector cells was monitored in a standard 4-h ^{51}Cr-release assay [7]. Allogeneic B-LCL and K562 cells, a human chronic myelogenous leukemia cell line (CCL 243, ATCC), were used as target cells. The percentage specific cytotoxic activity was calculated using the following formula: [(experimental release – spontaneous release)/(maximum release – spontaneous release)] × 100. The spontaneous release of both target cell lines was always less than 15%.

Measurement of [^{35}S]cysteine/cystine uptake

After two steps in uptake medium (McCoy's 5A, 10 mM HEPES without cystine, cysteine, methionine or glutathione) the uptake was started by adding 0.5 mM [^{35}S]cystine or [^{35}S]cysteine to the cell suspension. Aliquots of 0.05 ml were taken at different assay times and added a mineral oil/dibutyl phthalate (15/85 v/v) mixture. The radioactivity in the cell pellet was measured in a beta-counter (Beckman Instruments, Munich).

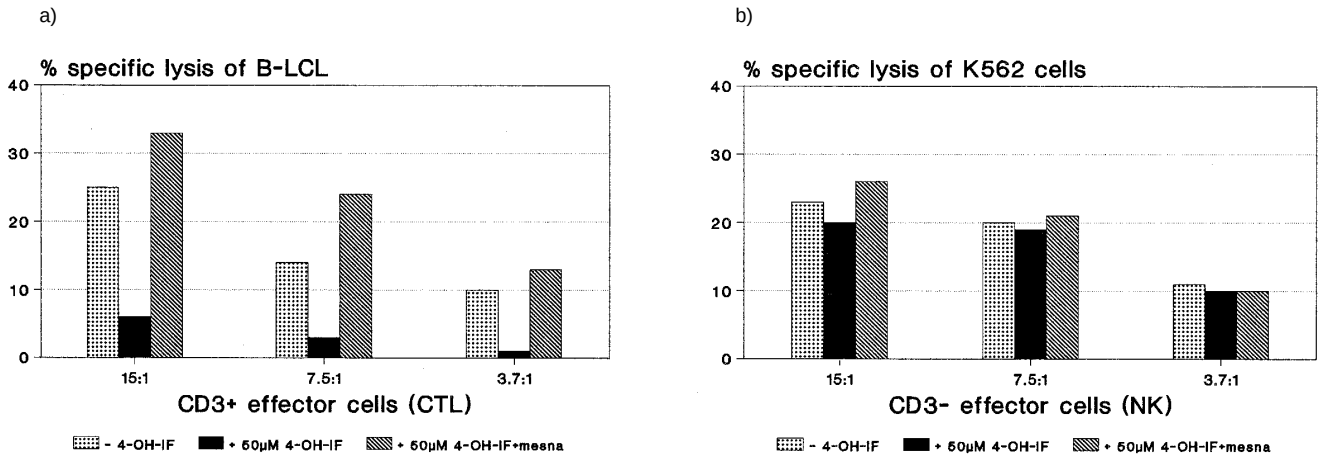

Fig. 2a, b The cytotoxic activity against allogeneic target cells (B-LCL) could be completely restored by incubation of ifosfamide-treated $CD3^+$ effector cells with mercaptoethanesulfonate (mesna; 50 µM 4-OH-IF plus 0.4 mM mesna). The cytotoxic activity of $CD3^-$ NK effector cells against K562 cells was not influenced by mesna treatment. The data represent the mean values of three independent experiments; the SD of each data point was less than 10%

Results

The effects of an ifosfamide treatment (0, 25, 50 µM for 1 h) on the cytotoxic activity of $CD3^+$ CTL and $CD3^-$ NK lymphocyte subsets was tested in a ^{51}Cr-release assay. The purity of both cell fractions was more than 95% as described in Materials and methods. Functionally the lysis of allogeneic B-LCL target cells was mediated selectively by the $CD3^+$ CTL population, whereas the lysis of the NK target cells K562 was mediated only by the $CD3^-$ NK cell population (Fig. 1a). After incubation of both effector cell populations separately with either 25 µM or 50 µM 4-OH-IF for 1 h the capacity of CTL and NK cells to lyse their specific target cells was significantly different. The lysis of the allogeneic B-LCL target cells that was mediated by CTL was reduced more than 3-fold after an incubation of the effector cells with 50 µM 4-OH-IF (Fig. 1b). In contrast, the lytic activity of NK cells against K562 target cells was not significantly influenced under these conditions (Fig. 1c). Only an incubation with 100 µM 4-OH-IF led to a 1.5-fold reduction of the cytotoxic activity of NK cells (data not shown). Concomitant with the loss of immunological functions, the intracellular GSH content of the 4-OH-IF-treated $CD3^+$ CTL effector cells that were used in this ^{51}Cr-release assay were found to be substantially reduced (about 2-fold when 25 µM 4-OH-IF was used and more than 5-fold with 50 µM 4-OH-IF), as shown in Table 1.

The GSH-depleted, ifosfamide-preincubated $CD3^+$ CTL effector cell population showed rapid restoration of the intracellular GSH contents to normal levels after incubation of the cells with 0.4 mM mesna for 6 h at 37 °C (Table 1). On the basis of these results, we addressed the question of whether mesna-treated $CD3^+$ CTL effector cells also showed a recovery in cytotoxic activity. The results derived from a ^{51}Cr-release assay are demonstrated in Fig. 2. A complete restoration of the cytotoxic activity of 4-OH-IF (50 µM)-pretreated $CD3^+$ CTL against allogeneic B-LCL was achieved by an incubation of the effector cells

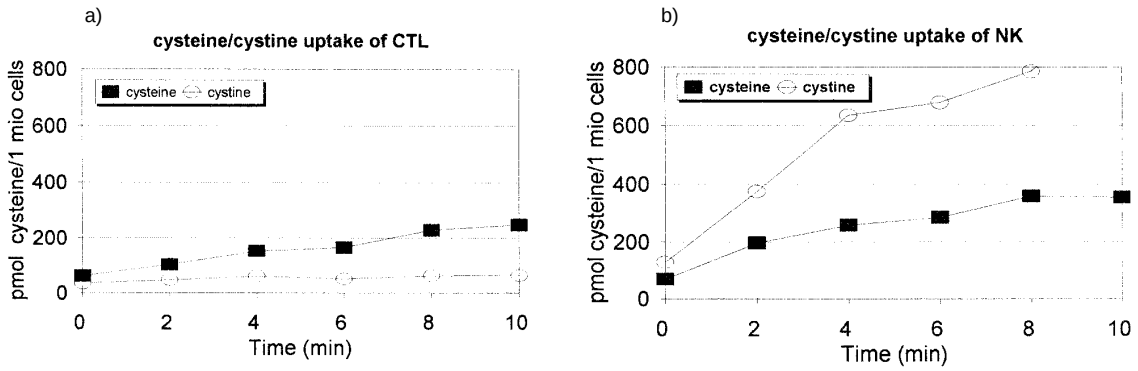

Fig. 3a, b Uptake of the amino acids cysteine and cystine in **(a)** $CD3^+$ CTL and **(b)** $CD3^-$ NK cells. The amino acid cysteine is taken up by CTL and NK cells. In contrast, the amino acid cystine is selectively taken up by NK cells but by CTL. Each data point represents the mean of four independent experiments; the SD was less than 15%

with mesna. The cytotoxic activity of $CD3^-$ NK effector cells (Fig. 2) and intracellular GSH levels (Table 1) was not enhanced by mesna treatment.

As demonstrated in Fig. 3, NK cells as well as CTL have a similar capacity to take up cysteine. However, in contrast to CTL, NK cells are able to take up cystine, the oxidised form of cysteine, very efficiently.

Discussion

Ifosfamide, which has been shown to be one of the most effective antineoplastic agents for the treatment of solid tumors, can induce GSH depletion in freshly isolated human PBL [6]. The aim of this study was to analyze the effects of an ifosfamide treatment on immunological functions and GSH levels of activated human effector lymphocytes. Roederer et al. showed that subsets of human PBMC differ substantially in mean GSH levels [15]; it has also been shown that GSH regulates IL-2 activity on cytotoxic lymphocytes [5]. We tested the cytotoxic activity of $CD3^+$ CTL and $CD3^-$ NK effector cell populations separately against allogeneic and NK target cells. The capacity of $CD3^+$ CTL to lyse their specific allogeneic target cells was substantially reduced by an ifosfamide treatment. This decrease of immunologically important functions of MHC-restricted effector CTL could be correlated with a substantial depletion of the intracellular GSH level in CTL. In contrast, the lytic activity and the intracellular GSH levels of $CD3^-$ NK cells were not significantly influenced up to 50 μM 4-OH-IF treatment. These results led us to the hypothesis that NK cells might have different mechanisms to maintain intracellular GSH levels. Our previous data demonstrate that NK cells have higher initial GSH levels and a 4-fold higher relative rate of GSH synthesis compared to CTL [12]. It is known that the rate-limiting step for the increase of GSH is the intracellular supply of cysteine, which can be imported into most mammalian cells for GSH synthesis [9, 2]. However, cysteine rapidly oxidizes to its disulfide form, cystine. With respect to the capacity to take up cysteine, CTL and NK cells showed no differences. However, NK cells differ significantly from CTL in their capacity to utilize cystine from the medium. The data presented here suggest that the different resistances to 4-OH-IF of CTL and NK could be based on different cystine-transport systems in the two cell types. Furthermore, we demonstrated that the reduced CTL function and GSH deficiency could be rescued by adding thiols, such as mesna, which provides cysteine to the cells by a disulfide-exchange reaction with the cystine in the medium.

Our findings may have further clinical implications for treatments that help to maintain normal GSH levels in $CD3^+$ CTL, may enhance immunocompetence and may help tumor patients to recover from illness during oxazaphosphorine therapy.

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