Thiols Decrease Human Interleukin (IL) 4 Production and IL-4-induced Immunoglobulin Synthesis

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Summary

N-Acetyl-L-cysteine (NAC) is an antioxidant precursor of intracellular glutathione (GSH), usually given in humans as a mucolytic agent. In vitro, NAC and GSH have been shown to act on T cells by increasing interleukin (IL) 2 production, synthesis and turnover of IL-2 receptors, proliferation, cytotoxic properties, and resistance to apoptosis. We report here that NAC and GSH decrease in a dose-dependent manner human IL-4 production by stimulated peripheral blood T cells and by T helper (Th) 0- and Th2-like T cell clones. This effect was associated with a decrease in IL-4 messenger RNA transcription. In contrast, NAC and GSH had no effect on interferon γ and increased IL-2 production and T cell proliferation. A functional consequence was the capacity of NAC and GSH to selectively decrease in a dose-dependent manner IL-4-induced immunoglobulin (Ig) E and IgG4 production by human peripheral blood mononuclear cells. Interestingly, NAC and GSH also acted directly on purified tonsilar B cells by decreasing the mature e messenger RNA, hence decreasing IgE production. In contrast, IgA and IgM production were not affected. At the same time, B cell proliferation was increased in a dose-dependent manner. Not all antioxidants tested but only SH-bearing molecules mimicked these properties. Finally, when given orally to mice, NAC decreased both IgE and IgG1 antibody responses to ovalbumin. These results demonstrate that NAC, GSH, and other thiols may control the production of both the Th2-derived cytokine IL-4 and IL-4-induced Ig in vitro and in vivo.

N-Acetyl-L-cysteine (NAC) is a thiol antioxidant usually used as a mucolytic drug (1), an antidote in acute poisoning (2), and proposed as an anti-HIV agent (3). NAC is also a precursor of intracellular glutathione (GSH) (4, 5). GSH is found in millimolar concentrations in most eukaryotic cells and plays important roles in several cellular functions (free radical scavenging, conjugation of toxic substances, and amino acid transport) (6). Several studies have indicated that NAC and GSH act on T cells by increasing resistance to oxidative stress-mediated apoptosis (7), cytotoxic properties (4, 8, 9), synthesis and turnover of IL-2 receptors, IL-2 production, and proliferation (8, 10). NAC and some other thiol antioxidants have been reported to regulate the activation of some transcription factors involved in IL-2 production; NAC decreases nuclear factor κB (NF-κB) (11) and increases activator protein 1 transcription factors (12).

T cell responses have been divided in two subclasses, Th1 and Th2, according to the profile of lymphokines produced, IFN-γ and IL-2 or IL-4, IL-5, and IL-13, respectively. A Th1 response may favor cell-mediated immunity, whereas a Th2 response provides help for humoral responses, including IgE and IgG4 (in humans) or IgG1 (in mouse) isotype switching. Some pathologies are associated with an abnormal predominant Th1 or Th2 response (13). Thus, numerous studies are currently aiming at modulating lymphokine production by T cells. In view of the effects of NAC on T cells, we have evaluated whether NAC could selectively regulate Th1- or Th2-derived cytokine production. We report here that NAC and GSH decreased IL-4 production by stimulated T cells and also acted on B cells to selectively decrease IgE and IgG4 production. Both these in vitro effects on T and B cells were associated with an action at the transcriptional level on IL-4 and IgE mes-
senger RNA (mRNA). In a mouse model, oral administration of NAC also resulted in a decrease of IgE and IgG1 responses to ovalbumin.

Materials and Methods

Compounds Tested. NAC, S-methyl cysteine (SMC), reduced GSH and oxidized GSH (GS-GS), captopril, d-penicillamine, t-methionine, t-carnitine, ascorbic acid, superoxide dismutase (SOD), and catalase were all purchased from Sigma Chemical Co. (St Louis, MO). Dithiothreitol (DTT) was purchased from Aldrich (Buchs, Switzerland). All the compounds were soluble in culture medium, and the pH was adjusted to 7.4 before use.

Isolation of Peripheral Blood T Cells. Characterization of T Cell Clones, and Isolation of Tonsillar B Cells. PBMC were isolated from heparinized venous blood from healthy subjects by Ficoll–Hypaque (Pharmacia, Uppsala, Sweden), and T cells were purified by rosetting with SRBC. The purity of T cells, determined by flow cytometry on a FACStar™ Plus cell sorter (Becton Dickinson, Embdengeim, Belgium) using FITC-labeled mouse anti-human CD3 mAb (Becton Dickinson), was >95%. The generation and characterization of the human CD4+ Th0 and Th2-like T cell clones (TCC) have been previously described (14, 15). T cells were cultured in complete medium consisting of RPMI 1640 (GIBCO BRL, Basel, Switzerland) supplemented with 10% heat-inactivated human AB+ serum (CTS, Annemasse, France), 2 mM glutamine, 20 mM sodium pyruvate (Sigma Chemical Co.), 50 µg/ml streptomycin, and 50 U/ml penicillin (GIBCO BRL).

Human B cells were isolated from Ficoll-separated tonsillar mononuclear cells by a two-step negative selection procedure using sheep cell rosetting and magnetic bead depletion (Dynal, Oslo, Norway) to remove T cells. B cell purity was routinely >98% as determined by FACStar™ analysis using FITC-labeled mouse anti-human CD19 and anti-CD21 mAbs (Becton Dickinson) and FITC-labeled mouse anti-human CD4 mAb (Becton Dickinson, Oxford, UK; Biosys, Compiegne, France). A total of 107 cells were incubated with 0.5, 1, or 2 g/liter NAC adjusted to pH 7.5 and changed every 2 d. At different times after immunization, levels of total IgG and ovalbumin-specific IgE, IgG1, IgG2a, and IgM were measured using affinity-purified peroxidase-labeled Ab (Jackson ImmunoResearch Laboratories, West Grove, PA). Sera were diluted at 1:4 for specific IgE, 1:100 for specific IgG1, IgG2a, IgG2b, and IgM, and 1:100,000 for total IgG quantification.

Statistical Analysis. Statistical analysis was performed using Student’s t test.

Results and Discussion

Since NAC and GSH have been shown to increase proliferation and IL-2 production by T cells (7, 8, 10), we investigated whether they may modulate Th1/Th2 lymphokine production by human T cells. Stimulation of peripheral blood T cells by PMA plus ionomycin or of TCC by anti-CD3 mAb or Con A was set up in the presence or absence of NAC or GSH. The cultures were set up in the absence or presence of different concentrations of thiols. After 14 d, cell-free supernatants were collected and stored in aliquots at −70°C until tested for IgE, IgG4, IgM, and IgA by ELISA as described (17). In kinetic experiments, NAC was added to stimulated B cells either at the beginning of the culture or at day 4 or 7.

Proliferation Assays. Tonsillar B cells (2 × 10⁶/200 µl per well), T cells, and TCC (2 × 10⁶/200 µl per well) were cultured in 96-well cell culture plates and stimulated in triplicate for either 3 d with IL-4 plus anti-CD40 mAb for B cells, or 48 h with PMA plus ionomycin or Con A for purified T cells and TCC, respectively, with or without different concentrations of the thiols tested. Cells were then pulsed for 6 h with [3H]thymidine (Amersham International, Little Chalfont, UK) before measurement of radioactivity. Results are expressed in counts per minute.

Molecular Analysis of IL-4 and IgE Transcripts by Northern Blot. Tonsillar B cells were incubated for 8 d alone or in the presence of IL-4, IL-4 plus anti-CD40 mAb, or IL-4 plus anti-CD40 mAb plus 10 mM NAC. T cells were cultured for 6 h with PMA plus ionomycin with or without 10 mM NAC. Total RNA was isolated by extraction with RNazol (Biotecx Laboratories Inc., Houston, TX) according to the manufacturer’s instructions, subjected to denaturing agarose electrophoresis (1.5 µg total RNA per lane), transferred to a nylon+ membrane, and fixed by UV irradiation. Hybridizations with cRNA probes complementary to Cε mRNA and IL-4 mRNA were performed as described on B and T cells, respectively (18).

Flow Cytometric Analysis. After overnight incubation of purified human tonsillar B cells (2 × 10⁶/200 µl per well) with 200 U/ml IL-4 alone or in the presence of 0.5–20 mM NAC, the surface phenotype was performed by flow cytometry with FITC-labeled mouse anti-human CD19 and anti-CD21 mAbs (Becton Dickinson and Immunotech, Marseille, France, respectively) as described (17). The mouse anti-human CD40 mAb (Serotec) was detected using a FITC-labeled goat anti-mouse Ab (Bioart, Meudon, France). Control mouse IgG subclass Ab were all purchased from Becton Dickinson.

Modulation of Ovalbumin-induced IgE Production in Mice Treated with NAC. Female 6–8-wk-old C57BL/6J bm mice (Biological Research Laboratories, Fullsindorf, Switzerland) were injected intraperitoneally with 1 µg ovalbumin (Sigma Chemical Co.; grade IV) and 2 mg aluminum hydroxide (Serva, Heidelberg, Germany) to favor specific IgE response (19). Mice drank water containing 0.5, 1, or 2 g/liter NAC adjusted to pH 7.5 and changed every 2 d. At different times after immunization, levels of total IgG and ovalbumin-specific IgE, IgG1, IgG2b, and IgM Ab were measured in sera by ELISA. Specific IgG1, IgG2b, and IgM were quantified using affinity-purified peroxidase-labeled Ab (The Binding Site, Birmingham, UK; Biosys, Compiegne, France). Specific IgE was evaluated using a rat anti-mouse IgE mAb (20) revealed by a mouse peroxidase-labeled anti-rat IgG Ab (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Sera were diluted at 1:4 for specific IgE, 1:100 for specific IgG1, IgG2a, IgG2b, and IgM, and 1:100,000 for total IgG quantification.
of 0.5–20 mM NAC (Fig. 1). Results showed that 0.5–20 mM NAC decreased in a dose-dependent manner IL-4 production by purified T cells (Fig. 1a) and by Th0- and Th2-like TCC (Fig. 1, b and c, respectively) stimulated for 16 h. In each case, a significant decrease was detected with 1 mM (P < 0.05) and was maximal with 20 mM NAC. Similar observations were made with three other TCC (two Th0 and one Th2 like) and with purified T cells stimulated with anti-CD3 plus anti-CD28 mAbs (data not shown). The decrease of IL-4 production was not due to a toxic effect of NAC as assessed by trypan blue and propidium iodide staining of T cells stimulated in the presence of 20 mM NAC (data not shown). Moreover, in agreement with others (7, 8, 10), we found that 0.5–20 mM NAC increased in a dose-dependent manner the proliferation of stimulated T cells and TCC (Fig. 1d). GSH has been shown to increase IL-4 internalization and degradation (21). Nevertheless, the decrease of IL-4 production induced by NAC was associated with a decrease of IL-4 mRNA transcription (Fig. 2). These data, reinforced by the fact that IL-4 was quantified in the 16-h supernatants, allow us to exclude the possibility that the decrease of IL-4 production could result only from its consumption by proliferative T cells. NAC and DTT have been shown to regulate the activation of some transcription factors: They decrease NFkB and increase activator protein 1 expression (11, 12). One can speculate that thiols may affect the activation of transcription factors belonging to the NFkB family and implicated in the transcription of IL-4 (such as nuclear factor of activated T cells) (22). Further experiments are required to test this hypothesis. In parallel to the decrease of IL-4 production, IL-2 production was significantly increased by NAC up to 5 mM (Fig. 1, a and b), as reported by others (10), whereas IFN-γ production was not affected whatever the time (Fig. 1, a and b). NAC did not affect IL-5 production by T cells and Th0 TCC (Fig. 1, a and b) but decreased IL-5 production by the Th2-like TCC (Fig. 1c). This decrease in IL-5 could be a consequence of the NAC-induced IL-4 reduction, as

![Figure 1. NAC decreases IL-4 production and increases the proliferation of stimulated T cells. Purified T cells (a) and Th0 TCC (TT7) (b) were stimulated, respectively, with PMA plus ionomycin or Con A in the presence or absence of 0.5–20 mM NAC. (a) IL-2 (×10) (△), IL-4 (×0.03) (○), and IFN-γ (□) were quantified in the 16-h supernatants by ELISA, and IL-5 (○) in the 48-h supernatants. (b) Lymphokines were quantified in the 16-h cell-free supernatants. (c) A Th2-like TCC (POD) was stimulated with Con A (top) or with anti-CD3 mAb (bottom) with or without 0.5–20 mM NAC. (d) Purified T cells (□) and a Th2-like TCC (POD) (○) were incubated, respectively, with PMA plus ionomycin or Con A in the presence of 0.5–20 mM NAC for 48 h and then pulsed for 6 h with [3H]thymidine. Results of five experiments ± SEM are presented.](image)
previously reported (23). These data indicate that NAC, while increasing T cell proliferation, preferentially decreases IL-4, and under some circumstances IL-5, but has no effect on IFN-γ.

A key function of IL-4 is its ability to induce IgE and IgG4 production by human B cells (24, 25). IgE production by B cells also requires a physical interaction with T cells (26) or basophils and mast cells (27), involving a number of surface molecules including CD40 (28) and CD21 (29). Based on the observation that NAC was able to decrease IL-4 production by T cells, we tested if NAC would also inhibit IL-4-induced IgE and IgG4 production by PBMC. NAC decreased both IgE and IgG4 production in a dose-dependent manner (IC50 = 1 mM) (P < 0.05) (data not shown). Even the superinduction of IgE and IgG4 brought about by addition of anti-CD40 mAb was decreased in a dose-dependent manner by NAC (IC50 = 1 and 2 mM, respectively) (Fig. 3 a). A significant decrease of IgE and IgG4 was observed with 0.5 mM NAC (P < 0.05) and was maximal with 20 mM. In contrast, IgA and IgM isotypes were not affected (Fig. 3 b). Interestingly, NAC added to highly purified B cells stimulated with anti-CD40 mAb plus IL-4 also decreased to a higher extent IgE (IC50 = 0.3 mM) and IgG4 (IC50 = 0.8 mM), but not IgA and IgM (Fig. 3, c and d), while increasing B cell proliferation (Fig. 4 a). As observed for T cells, NAC was not toxic for B cells as assessed by trypan blue and propidium staining (data not shown). Addition of NAC to IL-4 plus anti-CD40 mAb-stimulated B cells at days 0, 4, and 7 revealed that the strongest inhibitory effect of NAC on IgE synthesis was observed when NAC was added at day 0. However, addition of NAC at day 4 or 7 still led to a significant reduction in the synthesis of IgE (IC50 = 1 and 6 mM, respectively) (Fig. 4 b). Thus, one can speculate that NAC may have therapeutic applications even on an ongoing IgE response.

To investigate the potential mechanism of action of NAC on IgE synthesis, the expressions of IgE transcripts and some key cell surface molecules, CD21 and CD40, were analyzed. The mature e mRNA transcript was decreased by NAC in B cells stimulated with IL-4 plus anti-CD40 mAb. Conversely, the sterile e mRNA transcript was increased (Fig. 5 a). NAC appears to be the first type of inhibitor of IgE that can selectively decrease the mature e transcript. The other
reported IgE inhibitors, including IFN-γ, IFN-α (30), and TGF-β (18), inhibited both the mature and sterile ε transcripts. These results indicate that NAC can act downstream of the IL-4-induced sterile ε transcript by decreasing the generation of the mature ε mRNA. Finally, the expression of CD21 and CD40 was decreased in a dose-dependent manner by treatment of purified B cells stimulated with IL-4 in the presence of NAC (Fig. 5 b). The expression of CD21 was totally inhibited with 20 mM NAC, whereas the expression of CD40 was only partly reduced (50% of decrease). The expression of other B cell markers, such as CD19 (Fig. 5 b), CD11a, and CD20 (not shown), was unaffected. In fact, CD40–CD40 ligand interaction is essential for the switching process that takes place during the first 2 and 3 d of the B cell assays (Lecoanet-Henchoz, S., unpublished results). The partial decrease of CD40 expression induced by NAC cannot account for the total inhibition of the mature ε mRNA expression. First, NAC still promotes B cell proliferation, although CD40 expression is decreased. Second, the ability of NAC added at day 7 to decrease IgE production cannot be explained only by a decrease of CD40. The NAC-induced disappearance of CD21 expression could be responsible since the CD23–CD21 interaction favors the generation of IgE-producing B cells (31). Taken together, these effects of NAC on IL-4 production, mature ε mRNA transcription, and the expression of surface molecules involved in IgE regulation could at least partly explain the decrease of human IgE production in vitro.

Figure 4. NAC increases tonsillar B cell proliferation while decreasing IgE production in a time-dependent manner. (a) Tonsillar B cells were stimulated with IL-4 plus anti-CD40 mAb with or without 0.1–20 mM NAC for 3 d and then pulsed for 6 h with [3H]thymidine. (b) Tonsillar B cells were stimulated with IL-4 plus anti-CD40 mAb and 1–20 mM NAC added either at the beginning (△), at day 4 (○), or day 7 (□) of the culture. IgE was quantified in the 14-d supernatants. a and b present data from one (out of three) representative experiment.

Figure 5. NAC decreases mature ε mRNA, CD21, and CD40 expression on human tonsillar B cells. (a) Purified tonsillar B cells were incubated for 8 d alone or in the presence of IL-4, IL-4 plus anti-CD40 mAb, or IL-4 plus anti-CD40 mAb plus 10 mM NAC. RNA was isolated and used for Northern blot analysis using probes complementary to εε. The lefthand upper panel shows an autoradiograph from a 12-h exposure (alone ± IL-4). The righthand upper panel shows a 1-h exposure (IL-4 + αCD40 ± NAC). As a control, the Northern blot nylon membrane was stained with methylene blue (bottom). (b) After overnight incubation of tonsillar B cells with IL-4 alone or in the presence of different concentrations of NAC, the expression of CD19 (△), CD21 (○), and CD40 (□) was evaluated by cytometry. Results from one (out of three) representative experiment are expressed in percentage of decrease of expression.
These in vitro findings were extended to an in vivo mouse model. Mice were treated with ovalbumin to induce an IgE response and were given NAC dissolved in drinking water. 1 wk after immunization, both ovalbumin-specific IgE and IgG1 (the mouse isotype equivalent to human IgG4) responses were highly reduced (68 and 78% inhibition at 1 g/liter of NAC, respectively) compared with untreated animals (Fig. 6). A significant decrease was also observed using 0.5 g/liter of NAC but not with lower concentrations (data not shown). 2 wk after immunization, the inhibitory effect of NAC on IgE and IgG1 responses was still observed (30 and 32% decrease, respectively). The synthesis of the other Ig isotypes was not significantly affected, in agreement with the in vitro findings. On the basis of the volume of water drunk in 1 d, the quantity of NAC swallowed by a mouse could be estimated at 50 mg/kg per day, comparable with 9 and 300 mg/kg per day given in humans as a mucolytic or as an antidote in poisoning, respectively. This suggests that NAC acts in a range of doses that are already used in human therapeutics. These results suggest that in vivo NAC selectively down-regulates the production of antigen-specific IgE and IgG1 Ab, the two isotypes regulated by mouse IL-4 (32).

To investigate if antioxidants, in addition to NAC, similarly reduce IL-4 and IgE production, we tested the compounds listed in Table 1. The antioxidants without an SH group (such as l-carnitine, catalase, ascorbic acid, and SOD) and the molecules without a free thiol (such as methionine, SMC, and GS-SG) had no activity. It appears that free SH groups are critical for molecules to decrease IL-4 and IgE production. This was the case for NAC, GSH, DTT, and also for captopril and d-penicillamine, two drugs used in humans. In agreement with others, we found that GSH increased IL-2 production by T cells (data not shown) and T cell proliferation (Table 1) (7, 8, 10). Whereas GSH, captopril, and d-penicillamine used from 0.5 to 20 mM were as potent as NAC in decreasing IL-4 and IgE synthesis, the dithiol DTT was the most efficient (IC50 = 0.5 and 0.2 mM, respectively).

NAC and GSH are able to increase IL-2 production and use T cell proliferation, and to decrease T cell apoptosis (7, 8, 10). Because of its ability to restore intra- and extracellular GSH levels and to decrease HIV replication (33, 34), NAC has been proposed in the treatment of HIV infection (3, 5). It has been suggested that, during AIDS, a switch of the T cell cytokine pattern from a Th1 to a Th2 type could be associated with a poor prognosis (35). Moreover, IL-4-deficient mice have been shown to be resistant to retrovirus-induced immunodeficiency syndrome (36), and it has been very recently reported that NAC induced a decrease of IL-4 production by HIV-infected cells (37). We report here that NAC decreases synthesis of the Th2-derived cytokine IL-4 and IL-4-induced Ig production while increasing B and T cell proliferation. Experiments are in progress to define the molecular target(s) of thiols, as they may constitute new therapeutic molecules to treat Th2-mediated diseases such as allergic disorders and AIDS.

Figure 6. Oral administration of NAC decreases specific IgE and IgG1 Ab responses in ovalbumin-immunized mice. C57BL/6J bm mice injected intraperitoneally with ovalbumin drank water containing (+) or not (−) 1 g/liter NAC. 8 d after immunization, IgE, IgG1, IgG2a, IgG2b, and IgM Abs directed against ovalbumin and polyclonal IgG were measured. Results are expressed in OD values. Results from one set of experiments with nine mice per group are presented.
Table 1. Thiol-containing Compounds Selectively Decrease IL-4 and IgE/IgG4 Production

| Compound                  | Decrease in IL-4 production | Decrease in IFN-γ production | Increase of proliferation | Selective decrease of IgE/IgG4 production in vitro |
|---------------------------|----------------------------|-----------------------------|--------------------------|-----------------------------------------------|
| NAC                       | +                          | -                           | +                        | +                                             |
| GSH                       | +                          | -                           | +                        | +                                             |
| DTT                       | +                          | -                           | +                        | +                                             |
| Captopril                 | +                          | -                           | +                        | +                                             |
| D-penicillamine           | +                          | -                           | +                        | +                                             |
| L-carnitine               | -                          | -                           | Not tested               | -                                             |
| Catalase                  | -                          | -                           | Not tested               | -                                             |
| Ascorbic acid             | -                          | -                           | -                        | -                                             |
| SOD                       | -                          | -                           | -                        | -                                             |
| SMC                       | -                          | -                           | -                        | -                                             |
| Methionine                | -                          | -                           | -                        | -                                             |
| GS-SG                     | -                          | -                           | -                        | -                                             |

Summary of the effects of NAC and other compounds on lymphokine production, proliferation, and IgE/IgG4 production by human lymphocytes. Three sets of compounds were tested: (a) antioxidants with a free thiol group (0.5–20 mM, NAC; 0.5–20 mM, reduced GSH; 0.1–2 mM, DTT; 0.5–20 mM, captopril; and 0.5–20 mM, d-penicillamine); (b) antioxidants without a free thiol group (0.5–20 mM, l-carnitine; 50–400 U/ml, catalase; 10–100 μM, ascorbic acid; 10–400 U/ml, SOD); and (c) S-substituted compounds (SMC, 0.1–20 mM; methionine, 0.1–20 mM; GS-SG, 0.1–10 mM). Purified peripheral blood T cells were stimulated with PMA plus ionomycin in the presence or absence of the compounds tested. Proliferation assays were then performed as described in Materials and Methods, and lymphokines were measured by ELISA in the 24- and 72-h cell-free supernatants. PBL synthesis of IL-4-dependent Ig was evaluated using the in vitro assay described in Materials and Methods in the presence or absence of the compounds tested.

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