Analysis of the mechanisms of human cytotoxic T lymphocyte response inhibition by NO

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Abstract

NO is a potent cellular mediator which has been shown to modulate several immune mechanisms. Using human T lymphocytes as responder cells in a primary mixed lymphocyte reaction, we demonstrated that, at the initiation of the culture, exogenously provided NO via sodium nitroprusside, in non-toxic concentrations, inhibited both allogeneic proliferative and primary cytotoxic responses in a dose-dependent manner. In contrast, it had no effect on the cytotoxic activity of established human TCRαβ and TCRγδ cytotoxic T lymphocyte (CTL) clones. The NO inhibitory effect on primary cytotoxic T cell response correlates with inhibition of T cell blastogenesis. Furthermore, under our stimulation conditions, NO induced an inhibition of IL-2 production, an alteration of IL-2Rα expression, and a down-regulation of NF-AT translocation in CD4+ and CD8+ allostimulated T cells. Furthermore, we demonstrate that the inhibition of allospecific CTL activity by the NO donor was at least in part related to an inhibition of granzyme B and Fas ligand transcription as revealed respectively by RNase protection and RT-PCR analysis. These results suggest that NO may function to fine tune human CD3+ T cell activation and subsequent CTL generation.

Introduction

NO, a short-lived molecule, has been identified as a potent biological mediator. It is synthesized from the oxidation of the terminal guanido nitrogen atom of L-arginine by a family of NADPH-dependent enzymes, the NO synthases (NOS) (1–3). In addition to the existence of two constitutive isoforms (neuronal NOS and endothelial NOS), a third isoform, the inducible NOS (iNOS), can be elicited in different cell types by various stimuli such as bacteria, microbial products, cytokines and immunological stimuli (4,5). The iNOS isoform is normally associated with immune effector cells such as macrophages, which can be induced to express iNOS as part of their cytotoxic repertoire (4). Induction of iNOS has been reported in human cells, including macrophages (6), neutrophils (7) and NK cells (8).

The NO functional repertoire includes inflammation and antimicrobial defense (9). While direct evidence for the critical biological function of iNOS has been provided by a strain of mice with disrupted iNOS genes (10), other reports indicated the NO association with several important immunopathologies including rheumatoid arthritis, diabetes, systemic lupus erythematosus and septic shock (11). NO has been shown to modulate several immune mechanisms (12–15). It plays a role in the induction of non-specific immunity, and the antimicrobial response to a variety of extracellular parasites and some tumor cells (16). In this respect, iNOS-derived NO can regulate T cell proliferation, cytokine production, apoptosis and signaling activity in vivo and in vitro (17). The diverse biological effects of NO are known to result from its interaction...
with target proteins that contain metal or thiols strategically located in either allosteric or active sites (18). These target proteins include ion channels, transporters, enzymes, G proteins, transcription factors and molecules involved in the apoptotic process (19–21).

We have reported earlier that NO may play a role in the control of cell-mediated cytotoxicity restricted [cytotoxic T lymphocytes (CTL)] or not (NK) by the MHC. CTL play a major role in the protection and recovery from certain viral, bacterial and parasitic infections. These cells mediate allograft rejection and play a pivotal role in the immune surveillance against tumors (22,23). Evidence has been provided indicating that the differentiation of these cells is regulated by heterogeneous combination of signals and by a complex network of cytokines acting either independently or synergistically (24). Several mechanisms have been proposed to account for CTL cytotoxicity. Exocytosis of granules containing perforin and serine esterases (granzymes) is believed to be the major mediator of the cellular cytotoxicity exhibited by CTL (25). Additional studies demonstrated a granule-independent killing pathway involving Fas ligand (FasL) (26). The interaction of FasL with its receptor is believed to promote the deletion of potentially harmful, damaged or unnecessary cells during the immune response.

Although there is increasing evidence that NO is involved in immune function, its effect on the alteration of human T cell activation and the subsequent antigen-specific T cell response generation is not quite clear and remains to be established. We have therefore attempted to further investigate the mechanism by which NO induces the alteration of CTL response. In the present study, we demonstrate that the immunosuppressive effect of NO on human allospecific CTL generation involves at least an alteration of T cell activation and the subsequent cytotoxic gene transcription.

**Methods**

**Chemicals**

Sodium nitroprusside (SNP) was purchased from Sigma (St Louis, MO).

**Cells**

Peripheral blood mononuclear cells (PBMC), obtained from batch leukopheresis of normal adult volunteers (Banque du sang, Hôpital St Louis, Paris, France), were isolated using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. T cells were purified by Percoll (Pharmacia) gradient centrifugation (>95% CD3+), as previously described (27). E418 and LAZ 509, two lymphoblastoid B cell lines, were cultured in complete medium [DMEM medium (Biochrom, Berlin, Germany); 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine] supplemented with 15% heat-inactivated FCS. The T cells clones were cultured in DMEM-F12 complete medium (DMEM-F12 (Biochrom); 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine).

**Mixed lymphocyte reactions (MLR)**

MLRs were performed by incubating 10⁶ T cells or 10⁶ PBMC with 2.5 × 10⁵ irradiated (80 Gy) stimulating E418 or LAZ 509 cells in complete medium supplemented with 15% heat-inactivated human serum (J. Boy Institute, Reims, France) for 6 days at 37°C in 5% CO₂.

**Cytotoxicity assay**

Cells were harvested from cultures and resuspended in medium. Serial dilutions of effector cells were distributed in triplicates (0.1 ml/well) in round-bottom microtiter plates. Targets cells were labeled with 200 μCi [5¹Cr]Na₂CrO₄ (5 μCi/ml, sp. act. 57 μCi/mmol; Amersham, Little Chalfont, UK) for 1 h at 37°C, followed by 3 washes. Cytotoxicity assays were performed at different E:T ratios as described (28). Plates were incubated for 4 h at 37°C, 5% CO₂. Supernatants were collected and 5¹Cr release was measured in a Kontron counter (Packard, Meriden, CT).

Spontaneous and maximum release was determined by incubating target cells in medium alone and in a 0.5 N HCl solution respectively. The percentage of specific lysis was calculated as follows: [(experimental 5¹Cr release ± spontaneous 5¹Cr release)/(maximum 5¹Cr release – spontaneous 5¹Cr release)] × 100.

**Flow cytometry analysis**

Side versus forward scatter (SSC/FSC) plot analysis was used to measure the proportion of blast cells in the total cell population. Blasts cells are defined as lymphocytes with high SSC and high FSC, whereas unstimulated cells have low SSC and low FSC. For each experimental point 20,000 cells were analyzed. (flow rate 500 cells/s). All analysis were performed using a FACScan with CellQuest 3.2 software (Becton Dickinson, Mountain View, CA).

As previously described (29), cell surface markers were detected by direct and indirect immunofluorescence labeling using the following mAbs: CD25 (IL2-Rα, ACT-1, IgG1) and isotype-matched control from Dako (Glostrup, Denmark). Anti-CD122 (IL2-Rβ, CF1, IgG1) mAb was obtained from Immunotech (Marseille, France). FITC-labeled goat anti-mouse Fab fragment was purchased from Jackson ImmunoResearch (West Grove, PA). Rat anti-human IL-2Rγ mAb (TUGh4, IgG2b) has been described (30). This mAb does not recognize the IL-2-binding site of the IL-2Rγ chain. Rat isotype-matched control (R2b00) was purchased from Caltag (Richmond, CA) and FITC-labeled mouse anti-rat Ig was obtained from Jackson ImmunoResearch. Acquisitions were performed in a logarithmic scale. Data were reported on a linear scale for the representation and the analysis data. Statistical analysis was performed using Student’s t-test.

**Fluorescence microscopy**

Cells were harvested after 6 days of stimulation, suspended in 1 × PBS and cytocentrifuged onto slides at 700 r.p.m. for 2 min (Cytospin; Shandon, Pittsburgh, PA). They were fixed in paraformaldehyde 2% (Sigma) for 10 min, rinsed and hydrated 5 min in 1 × PBS, then permeabilized with 0.1% Triton X-100 (Sigma) for 8 min and washed in 1 × PBS. The indirect immunofluorescence procedure was performed in the
 Supernatants from MLR were tested for IL-2, IFN-γ production after 72 h of culture. Human IFN-γ, IL-2 and TNF-α concentrations in MLR supernatants were determined using ELISA immunoassays (Immunotech, Marseille, France).

Cytokine assays

Supernatants from MLR were tested for IL-2, IFN-γ and tumor necrosis factor (TNF)-α production after 72 h of culture. Human IFN-γ, IL-2 and TNF-α concentrations in MLR supernatants were determined using ELISA immunoassays (Immunotech, Marseille, France).

RNase protection assay

Cells were harvested 5 days after beginning of the stimulation. Total RNA was extracted from the cells according to the method of Chomczynski and Sacchi (31). Granzyme and perforin RNA were analyzed by RNase protection assay using the Riboquant multiprobe kit (PharMingen) according to the manufacturer’s protocol. Briefly, 10 μg of total cellular RNA was hybridized overnight to a 32P-labeled RNA probe that had been synthesized from hApo-4 template set. After digestion of the free probe and single-strand RNA the protected double-strand RNAs were purified and resolved on a 5% polyacrylamide gel. Transcripts levels were quantified by autoradiography and densiometric analysis was performed using Photocapt version 97-04 for Windows and Bioprofil Bio1D Windows application version 99-04. RNA loading was estimated by measuring the intensities of the protected fragments of the housekeeping genes L32 and GAPDH. For quantification, backgrounds were subtracted from specific bands and those values were divided by the intensity of the L32 housekeeping transcript band.

Semi-quantitative RT-PCR

Analysis of FasL mRNA expression was performed as previously described (32). T lymphocytes were harvested from MLR at day 5 and incubated for 3 h with LAZ 509 cells (T cells:LAZ 509 cell ratio of 4:1). Total RNA was isolated using TRizol reagent (Life Technologies, Gaithersburg, MD) and reverse transcribed using a cDNA synthesis kit (Superscript; Life Technologies). Various amounts of cDNA were amplified in PCR using the following primers: FasL: 5′-primer-ATCCAtTTTCTgCTCCTCCACCTACAgAagGA, 3′-primer-ATA-gAAATTCTgACCAAgAgAgTCACGATACgTgAC; β-actin: 5′- primer-TcgTCgAACAgggCCTCCggCATgTgC, 3′-primer-TTTgC gAgAggCtggAgAgC. PCR conditions were as follows: 94°C for 90 s (94°C for 15 s, 55°C for 30 s, 72°C for 50 s) for 20 cycles for β-actin or 28 cycles for FasL on a Perkin Elmer apparatus. After amplification, PCR products were separated by electrophoresis on 1% agarose gel containing ethidium bromide (0.01%) and visualized under UV light illumination. Quantification was performed using the same protocol as used for Northern blot analysis.

Results

Differential effect of NO donor on primary CTL response and established CTL clones

To test whether exogenous NO plays a role in the regulation of human CTL generation, enriched T cells (CD4+ and CD8+) were stimulated with allogeneic irradiated LAZ 509 lymphoblastoid B cell line in the absence or presence of SNP, used at non-toxic concentrations (10–50 μM) added at the initiation of the primary MLR cultures. The non-toxicity of SNP was evaluated by measuring the incidence of T cell apoptosis in the MLR by Annexin V-propidium iodide and showed no significant modification in the presence or absence of SNP (data not shown). After 6 days of culture, effector cells were assessed for their lytic activity against LAZ 509 stimulating target in a 4-h 51Cr-release assay. The results illustrated in Fig. 1 demonstrated that SNP added at the indicated concentrations resulted in an inhibition of CTL activity in a dose-dependent manner with a maximum inhibition observed at 50 μM. Our results consistently showed that the inhibitory effect of SNP occurred at all E:T ratios. Similar effects were observed using distinct NO donor compounds (NOC-18, Calbiochem, San Diego, CA) (data not shown).

In order to further investigate the effect of NO donor on CTL activity, the effect of SNP on alloreactive CTL clones activity was examined. We used two alloreactive CTL clones (TCR αβ and TCR γδ) previously established (33), both specific for the E418 lymphoblastoid B cell line. The effector cells were incubated in the presence of various doses of SNP (10–50 μM). After the incubation period, their lytic activity towards the E418-specific target was measured. As shown in Fig. 2, the...
cytotoxic activity was measured in a 4-h 51Cr-release assay. Data concentration of 10±50
absence or in the presence of the NO donor, SNP, at a TCR
a
NO donor-induced down-regulation of IL-2R expression on
incubation of both CTL clones with SNP did not reduce target
cell lysis induced by both clones. These data suggest that
differentiated CTL are no longer susceptible to the NO
inhibitory effect and that the inhibitory activity of NO is likely
to target early activation signals.

In order to further examine the basis of SNP-induced inhibition
of T cell function, experiments were performed to assess
whether NO interferes with T cell activation. For this purpose,
we have examined the effect of NO donor on IL-2R expression
as a marker of early T cell activation and on T cell
blastogenesis. The data depicted in Fig. 3(A) show that SNP
dramatically inhibited the blastogenesis of T cells upon
allostimulation. Data in Fig. 3(B) demonstrated that 6-day
MLR alloactivated T cells express IL-2Rα. Addition of SNP to
the culture resulted in a significant decrease in IL-2Rα chain
expression as revealed by immunofluorescence analysis. In
contrast, SNP had no effect on β and γ chains of IL2-R.

Differential effect of NO donor on IL-2, IFN-γ and TNF-α
production following T cell activation
To determine whether the inhibitory effect of SNP on
allospecific CTL generation could be attributed to a decrease
in cytokine production, crucial for the development of
cytotoxic and proliferative responses, culture supernatants
were collected at day 3 of stimulation, previously found to be
optimal for cytokine production under these experimental
conditions (34,35).

IL-2, IFN-γ and TNF-α assays were performed using
ELISA. As shown in Fig. 4(A), addition of SNP induced a
significant inhibition of IL-2 (70% inhibition at the highest
SNP concentration). Only a marginal effect (20% inhibition)
on IFN-γ production was observed (Fig. 4B). However, as
shown in Fig. 4(C), addition of SNP to MLR culture resulted
in a slight increase in TNF-α production (30%). Although
the amount of cytokines measured in the supernatant
reflects a balance between production by both CD4+ and CD8+ T cells and use by these cells, the results
suggest a differential effect of NO on cytokine production
by alloactivated human T lymphocytes.

Down-regulation of NF-AT translocation in activated T
lymphocytes in the presence of the NO donor
NF-AT plays an important role in the control of T cell activation
and differentiation. We therefore asked whether the inhibitory
effect of NO in our experimental model interferes with the
translocation of NF-AT from the cytoplasm to the nucleus.
Using fluorescence microscopy, the data depicted in Fig. 5
demonstrate that in resting T lymphocytes NF-AT remains in
the cytoplasm (Fig. 5A), alloactivation of CD3+ T cells resulted
in the translocation of NF-AT from the cytoplasm to the nucleus
(Fig. 5B). In the presence of NO (10–20 μM), this phenomenon
was greatly altered (Fig. 5C and D).

Inhibition of granzyme B and FasL transcription in
alloreactive CTL by the NO donor
To get an insight into the molecular basis of the inhibitory
action of SNP on CTL differentiation, we examined its interfer-
ence with the acquisition of allospecific CTL lytic potential.
RNase protection assay analysis was performed with RNA
extracted from T cells cultured with stimulator cells for 6 days
in the absence or presence of SNP (10–50 μM). As depicted in
Fig 6(A), in the absence of SNP, the accumulation of granzyme
B mRNA was pronounced in control culture. Addition of SNP
resulted in an inhibition, in a dose-dependent manner, of
granzyme B mRNA accumulation (Fig. 6A) and had only a
marginal effect on perforin gene transcription. It is also shown
in Fig. 6(A) that the transcription of granzyme H, an analogue
of granzyme B with chemotrypsin-like activity (36), was also
greatly altered by the NO donor.

Semi-quantitative PCR was used to investigate the effect of
the NO donor on FasL transcription in alloactivated CD3+ T
cells. Data depicted in Fig. 6(B) indicate that FasL mRNA
expression was significantly inhibited in the presence of SNP,
as compared to control culture, in a dose-dependant manner
with maximal inhibition at 50 μM (57 ± 8% inhibition).
Discussion

The aim of this study was to examine the effect of NO, an important modulator of T cell function (12), on the development of human alloreactive CTL response. Our data indicate that exogenous NO was effective in inhibiting the development of allogeneic cytotoxic response, suggesting a role of NO as an important regulator of human antigen-specific CTL response. This is compatible with the reported regulatory effect of this mediator on the in vivo antitumor response (37), on the alloantigen-induced activation (38), on the in vivo allograft response and with the potential role of iNOS in the control of the histological grade of rejection in a rat model (39).

Although the diverse biological effects of NO are known to result from its interaction with target proteins including transcription factors, the functional consequences of NO on human CD3+ T cell activation and function are not yet clearly established and remain controversial. We therefore attempted, in the course of these studies, to examine the possible mechanisms by which NO may interfere with CTL generation and subsequent cytolytic activity. We first have shown that NO did not influence T cell survival in our model (data not shown). However, it interferes with T cell activation and has a dual effect on resting versus committed T cells. While it selectively inhibited CTL generation from resting T cells, it had no effect on the lytic activity of established T cell clones. This suggests that NO may play a role during the differentiation process or inductive phase of CTL response affecting the differentiation of CTL precursors in mature effectors. Kinetics analysis based on the daily addition of SNP revealed that its inhibitory effect progressively decreased and was no longer detectable after 24 h of culture (data not shown). In addition, while the present study indicates that NO down-regulates IL-2Rα on resting cells, it has been reported that this mediator had no effect on phytohemagglutinin-stimulated T lymphocytes (40). Because IL-2Rβ and IL-2Rγ expression were not down-regulated, it is conceivable that the inhibition of IL-2Rα may result in the alteration of high-affinity IL-2 receptor which is a critical component in T cell proliferation under low IL-2 concentrations observed in the presence of the NO donor. These findings are consistent with other
reports indicating that iNOS-deficient mice developed an enhanced Th1 response following infections and antigenic stimulation (41). It is likely that through inhibition of IL-2 production presumably by both CD4+ and CD8+ T cells, NO may serve as a self-regulatory molecule preventing the over-expansion of Th1 cells. In this context, it was suggested that NO could be modulating the signaling cascade initiated by IL-12 in the differentiation of precursor cells to the Th1 pathway (42). This is unlikely in our experimental system in which macrophages were depleted and IL-12 was not produced (43). Moreover, it should be noted that besides inhibiting IL-2 production and altering IL-2 receptor expression, NO was found to regulate rodent T cell activation via reversible disruption of the JAK3/STATS signaling pathway, inducing T cell anergy (44). Previous studies have effectively shown that tyrosine nitrosylation prevented tyrosine phosphorylation of this pathway (45). In the present studies, we demonstrated that NO treatment resulted in an impairment of NF-AT translocation in activated CD3+ T cells. This factor is a major player in the control of T cell activation and differentiation, and, in all likelihood, also of the cell cycle and apoptosis of T lymphocytes (46).

It is admitted that NO is mainly produced by human monocytes and antigen-presenting cells (47,48). In this respect, increased NO production by antigen-presenting dendritic cells has been reported to be responsible for low allogeneic MLR (49). It is tempting to speculate that the inhibitory function of macrophages on T cell activation, generally associated with prostaglandin E2 release (50), also involves endogenous production of NO. Indeed, the activation of macrophages requires an inductive signal (IFN-γ) from T lymphoblasts which in combination with TNF-α triggers NO production, thus shutting off further T cell activation. Evidence has also been provided indicating that macrophage-derived NO can inhibit lymphocyte responses in vivo (37) and that treatment of mice with N^G-L-MMA during anti-tumor vaccination increased the subsequent anti-tumor response (51). It is noteworthy that NO is produced by human tumor cells and may therefore provide tumors with a subtle mechanism to paralyze the immune T cell function and to promote their growth. Furthermore, NO was recently found to play a critical role in the immunomodulatory effects induced by IL-12. While NO low levels were required for IL-12-mediated immunostimulation, IL-12-mediated immunosuppression was associated with very high NO production (51,52). In the same context, Hibbs et al. previously established that some of the inflammatory cytokines that are released during IL-2 therapy, such as IFN-γ and TNF-α, are potent activation signals for an L-arginine-dependent cytotoxic pathway in macrophages and endothelial cells (53,54). It has become clear that growth of solid tumors is regulated by interactions of endothelial cells of the tumor vasculature, tumor-infiltrating immune cells such as T cells, NK cells, macrophages and the tumor cells themselves. Most of these cellular components have been shown to generate NO which functions as an intercellular and/or intracellular signal (55–57), suggesting that this mediator is an essential component of the tumor microenvironment and may play a role in the conflict between the tumor and the immune system of the host (28). Therefore, understanding the cytokine repertoire during T cell activation and its interplay with NO may be critical to the regulation of clonal expansion and differentiation of antigen-reactive cells.

To gain further insight into the molecular basis of the NO-induced inhibitory effect on CTL induction, we have examined the effect of NO on the expression of some essential components of cell-mediated cytotoxicity. Our data point to an inhibitory role for NO in the differentiation of CTL precursors into lytically active effectors by a mechanism mainly involving a decrease in granzyme B transcription, which is a crucial event of the CTL lytic pathway. Recently, it has been reported that granzyme B can directly process DFF45/ICAD in target
cells, suggesting that this pathway is physiologically essential for CTL-induced target cell death (58). It should be noted that the inhibition of CTL generation correlated with a specific inhibition of granzyme B transcription, but not the perforin gene, confirming that the regulation of these genes is under distinct mechanisms (43). It should be noted that NO treatment resulted also in down-regulation of granzyme H, while having no effect on granzyme A. Importantly, we demonstrated in the course of these studies that NO had a negative regulatory effect on FasL transcription in alloreactive CTL which is involved in the alternate pathway of cell-mediated cytotoxicity (32). As exposure to NO has been shown to affect specific signal-transduction pathways in T lymphocytes such as p21\(^{ras}\) (59) and transcription factors (60,61), and based on our findings indicating a NO inhibitory effect on NF-AT translocation, one could speculate on the
involvement of these signals in modulation of granzyme and FasL expression. Evidence has been provided indicating the existence of NF-AT-responsive sites in the human granzyme B (62) and FasL promoter (63), thus establishing a link between NF-AT activation and FasL transcription (64,65).

Taken together, our data support the notion that in humans, NO is more than just a cytotoxic molecule but may exert a substantial immunoregulatory activity on CD3+ T cell function and play a role in the maintenance of immunologic homeostasis by virtue of its role in T cell suppression. Therefore, a better understanding of the molecular mechanism of how NO inhibits T cell activation may help to predict the complex functions of this factor during the development of the T cell response in normal and pathological situations.

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Abbreviations

PBMC peripheral blood mononuclear cell
CTL cytotoxic T lymphocyte
FasL Fas ligand
iNOS inducible NOS
MLR mixed lymphocyte reaction
NOS NO synthase
SNP sodium nitroprusside
TNF tumor necrosis factor

References


