The stilbene disulfonic acid DIDS stimulates the production of TNF-α in human lymphocytes

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Exposure of human peripheral blood mononuclear cells (PBMC) to the stilbene derivative DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid) (60 μM and above) significantly increased the release of tumor necrosis factor-α (TNF-α), as determined by TNF-α activity in the incubation media. When the TNF-α message was analyzed in PBMC by a reverse transcription/polymerase chain reaction (RT/PCR)-based procedure, it was found that incubation with DIDS (60 μM) was followed by a time-dependent accumulation of TNF-α mRNA. Measurements of intracellular pH showed that the presence of increasing concentrations of DIDS resulted in a progressive intracellular alkalinization of PBMC. It is suggested that the known DIDS effect of inhibiting transmembrane anion exchange, i.e., chloride/bicarbonate exchange, might play a role in the stimulation of TNF-α production by PBMC exposed to DIDS. © 1992 Academic Press, Inc.

Tumor necrosis factor-α (TNF-α) is a cytokine produced by peripheral blood mononuclear cells (PBMC) and other cell types (1), that possesses a wide range of immune, inflammatory and cell regulatory properties (2). Thus, TNF-α is an important mediator of septic shock, possesses antitumoral and antiviral activities, and exhibits diverse metabolic effects (2). Its production is known to be stimulated by bacterial products such as endotoxin and lipopolysaccharide (LPS), as well as a diversity of agents like phorbol esters, mitogens, growth factors and lymphokines (1,2). Several studies have demonstrated that activation of PBMC is associated to intracellular alkalinization, and the mechanism undergoing the alkalinization is believed to be a stimulation of Na⁺/H⁺ exchange (see ref. 3 for review). In addition to Na⁺/H⁺ exchange, there is evidence for the presence of electroneutral Na⁺-independent Cl⁻/HCO₃⁻ exchange in the plasma membrane of PBMC (4). The activity of this transport system is markedly accelerated by increasing intracellular pH (pHi), and is inhibited by stilbene derivatives (4). At present, it has not been established whether the Na⁺-dependent form of anion exchange plays also a role in the regulation of pHi in PBMC (3,5). Here, we have explored in PBMC the effects of the stilbene derivative 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) on TNF-α production. Our finding of an stimulating effect may allow further studies to elucidate possible signals that control the production of TNF-α in lymphocytes.
MATERIALS AND METHODS

Lymphoprep was purchased from Nycomed Pharma AS, Oslo, Norway; DIDS, LPS, dimethyl sulfoxide (DMSO), and actinomycin D were from Sigma; RPMI 1640 and M-MuLV reverse transcriptase were from Gibco-BRL; anti-TNF-α antibody, random hexamers (primers), RNase inhibitor, dNTP and restriction endonucleases were obtained from Boehringer Mannheim, FRG; oligonucleotides were synthesized by Scandinavian Gene Synthesis, Köping, Sweden; multiprime labeling kit and [α-32P]-dCTP were from Amersham, Buckinghamshire, UK; Taq DNA polymerase was from Perkin-Elmer Cetus, and fluorescent dye 2,7-bis(carboxyethyl)-5,6-carboxy-fluorescein (BCECF) from Molecular Probes, Junction City, OR.

Stimulation of TNF-α activity. For isolation of PBMC, 20 ml of venous blood were collected from healthy human donors, diluted (1:2) with 0.9% NaCl, and overlaid on a Lymphoprep of density 1.077 g/ml. After centrifugation (600 x g, 30 min), the mononuclear cell layer was harvested at the interphase and washed twice in 0.9% NaCl. PBMC, resuspended in RPMI 1640 with penicillin-streptomycin (100 U and 100 µg/ml, respectively), supplemented also with 1% fetal calf serum (5×10⁶ cells/ml), were incubated (24 h) in the presence of DIDS (0, 30, 60, 120 and 240 µM). As a positive control, incubation in the presence of LPS (20 pg/ml) was carried out. The incubation media were collected after centrifugation (<500 x g) and stored at -40°C until their analyses for TNF-α activity, which were performed by a cell lytic assay as described (6). Briefly, mouse L-929 fibroblast cells were grown in a 96-well flat-bottomed plate at 50,000 cells per well in test-medium (RPMI 1640 with penicillin-streptomycin, containing also 10% fetal calf serum and 1 µg/ml of actinomycin D) and incubated with serially diluted test samples in a humidified air at 37°C with 5% CO₂. After 18 h, test samples were removed, the plates were washed with PBS, and residual cells were stained with a 0.5% solution of crystal violet in methanol/water (1:4, vol/vol). Absorbance at 540 nm was measured in a Titertek Multiskan ELISA reader and cell lysis was calculated considering that the optical density of L-929 cells incubated with test-medium alone represented 0% lysis. The TNF-α cytotoxicity titer, expressed as units/ml, is defined as the reciprocal of the dilution of test sample that causes 50% destruction of actinomycin D treated L929 cells. Direct addition of DIDS (from 30 µM to 240 µM) to L-929 cells showed that DIDS had not influenced cell viability. The specificity of the bioassay was assessed by neutralizing the TNF-α activity of selected samples (100 µl) with 100 µl of anti-TNF-α antibody (for 2 h at 37°C) prior to their addition to L-929 cells.

Analysis of TNF-α mRNA. Total RNA from PBMC exposed either to DIDS (60 µM) or LPS (20 µg/ml) for different periods of time, was isolated by the guanidinium-thiocyanate/phenol/chloroform method (7), and processed for mRNA analysis essentially as described (8). 1 µg of total RNA was reverse-transcribed with M-MuLV reverse transcriptase (100 units, 60 min at 37°C) in 10 µl volume of PCR buffer (20 mM Tris-Cl pH 8.3, 2.5 mM MgCl₂, 50 mM KCl and 0.01% gelatin) supplemented with 5 mM dithiothreitol, 1 mM dNTP, RNase inhibitor (10 units), and random hexamers (100 ng). cDNA synthesis proceeded was stopped by heat (95°C for 5 min), being finally quick-chilled on ice. The whole cDNA product was PCR-amplified using 2 units of Taq polymerase (30 cycles, 94°C for 1 min, 55°C for 1 min and 72°C for 2 min each cycle) in 50 µl of PCR buffer, containing also 0.2 mM dNTP, 50 µCi/ml of 32P-labeled dCTP, and corresponding primers (40 ng each). Blank reactions with no RNA were carried out in all experiments. Also, PCR-amplification of a fragment of β-actin cDNA was used as internal control for each sample. Oligonucleotides (5’ → 3’) d(GTCAAGATC ATCTTCTCGAACC) and d(CAGATAGATGGGCTCATACC) were the upstream and downstream primers, respectively, used to amplify a 360 base-pair (bp) fragment from human TNF-α cDNA (nucleotides 314-673, ref. 9). d(TCTACAAATGAGCCTGCTG) and d(GTGGT AGGATCCCTCATGAGG) were the primers used to amplify a 314 bp fragment from human β-actin cDNA (that is located between nucleotides 1319-2079 in the reported human β-actin gene sequence, cf. ref. 10). After PCRs, 8 µl aliquots of the PCR reactions were electrophoresed in a 2% agarose gel. bands were visualized by ethidium bromide staining, and excised (equal size bands) for determination of radioactivities. Values were corrected for background radioactivity using blank samples with no RNA. TNF-α mRNA values were further normalized to those of β-actin mRNA, and were given as their ratio (TNF-α cpm/β-actin cpm). Validation experiments of PCR assays using known amounts of total RNA (0.5, 1, 2, and 4 µg) were carried out. Resultant cpm-values corresponding to either TNF-α or β-actin mRNA had linearity with respect to input of total RNA within the used range. The identity of the PCR products from TNF-α cDNA amplification was assessed through endonuclease treatments. BglI and PvuII yielded the predicted restriction fragments, while EcoRI (no restriction site for this endonuclease would be present in the expected region) could not digest the amplified PCR product. An additional
assessment was also obtained by Southern blot analysis, using a 32P-labeled PstI/BamHI fragment (1.6 kb-long) of TNF-α cDNA (cf. ref. 9) as hybridization probe.

Measurement of pHi. Cell pHi was measured using the pH sensitive fluorescent dye BCECF, incorporated on its ester form. PBMC, resuspended in a neutral medium containing 118 mM NaCl, 20 mM NaHCO3, 2 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 10 mM glucose and 15 mM MOPS-Tris (pH 7.4), were incubated with BCECF (5 μM) for 30 min at 37°C. After washing (twice with neutral medium), PBMC were finally resuspended in neutral medium (1.5x10⁶ cells/ml) containing serial concentrations of DIDS for incubation (at different times, i.e. 30, 60, and 90 min). Finally, fluorescence was determined at 37°C, using a Flow Cytometer FACStarPLUS (Becton Dickinson, San Jose, CA) set at 488 and 530 nm for excitation and emission, respectively. A pH-calibration curve of BCECF-loaded cells was previously performed using the nigericine technique (11). Since DIDS was dissolved in DMSO (1 μl/ml), the effect of DMSO on pHi was also tested.

Statistical analysis. Results are presented as mean ± SE. Statistical analyses of the results were made using the Friedman and the Wilcoxon tests.

RESULTS AND DISCUSSION

In this study we have investigated the effect of DIDS on the production of TNF-α by PBMC. It was found that DIDS stimulated the release of TNF-α in a dose-dependent manner (Fig. 1). At a concentration of 60 μM (and above), this compound caused a significant increase in the TNF-α release from PBMC, as compared to PBMC incubated in the absence of DIDS (31.00 ± 5.21 vs 2.00 ± 1.31, U/ml, n=7, p<0.001; Fig. 2). Apparently, this effect was not secondary to cell death, since trypan blue exclusion showed that DIDS did not affect PBMC viability (>95 % after 24 h incubation with 30-240 μM DIDS). TNF-α release from PBMC was estimated through a bioassay based on the ability of the culture medium to induce cytotoxicity on mouse L-929 fibroblasts. This cytotoxicity assay, although more variable than immunoassays, measures the cytokine as a bioactive product, whereas immunoassay may recognize inactive products.

![Fig. 1](image-url)
FIG. 2. Effects of DIDS and LPS on TNF-α production. PBMC were incubated (24 h) either with DIDS (60 µM), LPS (20 µg/ml), or a combination of these two compounds (the same concentration each as alone). Incubation media were collected for determination of TNF-α activity through a cell lytic bioassay (cf. Materials and Methods); n=7.

(12,13). Other cytokines might exert cytotoxic effects in this assay (12), but this does not appear to be the case in our study, since the cytotoxic effect of culture media from DIDS-stimulated PBMC was found to be neutralized (>80%) by incubation with an anti-TNF-α monoclonal antibody prior to the assay.

In order to elucidate if DIDS could modify the cellular levels of TNF-α mRNA, total RNA from PBMC stimulated with DIDS (60 µM) for different periods of time, was analyzed by RT-PCR. It was observed that DIDS induced an accumulation of TNF-α mRNA, which was maximal after 3 h-stimulation (Fig. 3). Further studies are needed to investigate whether the

FIG. 3. Effects of DIDS on TNF-α mRNA accumulation in PBMC. Cells were incubated either with DIDS (60 µM) for different times or with LPS (20 µg/ml) for 3 h. TNF-α mRNA was analyzed by RT-PCR (cf. Materials and Methods). Values of cpm corresponding to TNF-α mRNA were normalized to those obtained for β-actin mRNA. One typical experiment out of four is shown.
accumulation of TNF-α mRNA in the presence of DIDS is due to a diminished degradation of this particular message or to an enhanced transcription of the TNF-α gene. Previous reports have shown that TNF-α mRNA is constitutively expressed in human macrophages (14), human leukocytes (15) and some tumor cell lines (16), although these unstimulated cells did not release TNF-α protein into the medium, suggesting that TNF-α expression might be controlled at a translational level. In our study, the observed TNF-α mRNA accumulation in PBMC exposed to DIDS indicates that DIDS-stimulation is exerted at a pretranslational level. However, it is possible that DIDS influences TNF-α production also at a translational level, as reported with other stimuli in human monocytes (17).

Changes in pH are thought to represent an important step in the transduction of a variety of signals (18). Cellular responses to mitogens, growth factors and lymphokines appear to be associated with cytosolic alkalinization (3,19-21), seemingly caused by stimulation of the amiloride-sensitive Na+/H+ exchanger (21). In fact, there are data suggesting that TNF-α gene expression might be stimulated by a cell alkalinization secondary to activation of Na+/H+ exchanger. For instance, mitogens and phorbol esters, which are potent inducers of TNF-α (15,17,22), have been reported to activate Na⁺/H⁺ antiporter in lymphocytes and macrophages with resulting cell alkalinization (23). Also protein kinase C, which plays an important role in signal transduction for production of TNF-α (24), is known to activate Na⁺/H⁺ antiporter (25). Anti-CD3 antibody, calcium inophore A23187, and α₂-adrenergic receptor agonists, which appear to up-regulate TNF-α gene expression (22,26), are able to activate Na⁺/H⁺ exchanger as well (27-29). Moreover, PGE₂ and dibutyryl cAMP, that down-regulate TNF-α gene expression (30,31), seem to inhibit Na⁺/H⁺ antiporter activity (32). In addition to Na⁺/H⁺ antiporter, the stilbene-sensitive Na⁺-independent Cl⁻/HCO₃⁻ anion exchanger is another transport system involved in the regulation of pH in PBMC (4). This anion exchanger translocates Cl⁻ and HCO₃⁻ across the cell membrane, and because the inward gradient for Cl⁻ usually exceeds that for HCO₃⁻, the direction of net transport is Cl⁻ influx and HCO₃⁻ efflux, leading to intracellular acidification (4). Thus, inhibition of the Cl⁻/HCO₃⁻ anion exchanger in PBMC might be followed by cell alkalinization. To investigate this possibility, the effect of DIDS on pH was analyzed in PBMC from healthy donors. Basal pH in PBMC incubated in neutral medium, was found to range between 7.35 and 7.45 (n=12), in accordance with values reported by others (33). The pH was alkalinized in a concentration-dependent manner in PBMC exposed during 30 min to increasing concentrations of DIDS (Fig. 4). At a concentration of 60 μM, this compound significantly increased pH in PBMC, as compared to controls with PBMC incubated in the absence of DIDS (pH of 7.59±0.06 vs 7.43±0.03, n=6, p<0.01). A less potent effect was observed after 60 min exposure, and no changes in the pH were observed when PBMC were exposed to DIDS during 90 min (data not shown). DIDS effect on pH was specific, since exposure (30 min) to increasing concentrations of the solvent alone (DMSO), did not produce any alteration in pH (Fig. 4). It is possible that the stimulatory effect of DIDS on TNF-α production is linked to its ability to increase pH via inhibition of the Cl⁻/HCO₃⁻ anion exchanger. In accordance with this view of a role of pH is our finding that exposure of PBMC to LPS (20 μg/ml, 30 min), which is known to activate Na⁺/H⁺ exchange, increased both pH (7.58±0.04 vs 7.40±0.02 in the controls, n=6, p<0.01) and TNF-α mRNA levels (Fig. 3). In
an attempt to further clarify the hypothetical pH role for the accumulation of TNF-α mRNA, we incubated PBMC (for 3 h) in a test-medium (20 mM NaCO3, 118 mM NaCl, 2 mM KCl, 1 mM MgCl2, 1 mM CaCl2, and 10 mM glucose) buffered at different pH (with either 20 mM MES-Tris for pH 7.2 and 7.4, or 20 mM MOPS-Tris for pH 7.6 and 7.8). Under these conditions no changes were observed in the levels of TNF-α message. But in the present test-medium (buffered at pH 7.4), even DIDS was unable to increase the TNF-α mRNA levels, in contrast to its stimulatory effect in the supplemented RPMI 1640 medium.

It should be kept in mind that, in different cell types, DIDS has been described to possess other effects in addition to those derived from its binding-mediated inhibition of Cl- /HCO3- anion exchanger(s). Thus, for example, DIDS specifically binds to CD4 and blocks multiple CD4-dependent events associated with acute and established HIV-1 infections (34). Therefore, it is probable that the intracellular signalling mechanisms responsible for TNF-α gene expression are rather complex, and processes other than changes in the pH may also play a role. This is suggested by our results with DIDS and LPS used together (60 μM and 20 μg/ml, respectively), which appeared to exert an additive stimulating effect on TNF-α production (Fig. 2), while the alkalinizing effect observed with DIDS (pHi of 7.59±0.06 vs 7.43±0.03, n=6, p<0.01) was not furtherly increased by the simultaneous exposure to LPS (pHi of 7.57±0.02, n=6). The hypothesis of a complex mechanism becomes strengthened if one considers recent findings somehow related suggesting that LPS induces TNF-α production in HL60 cells via activation of phospholipase A2 and that the level of this induction is regulated by the activity of 5-lipoxygenase and cyclooxygenase pathways (35).

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