Taurine chloramine, a product of activated neutrophils, inhibits in vitro the generation of nitric oxide and other macrophage inflammatory mediators

Janusz Marcinkiewicz, Agnieszka Grabowska, Joanna Bereta,* and Teresa Stelmaszynska

*Department of Immunology, Jagiellonian University Medical College and *Institute of Molecular Biology of Jagiellonian University, Cracow, Poland*

Abstract: Taurine (Tau) is an exceptionally abundant free amino acid in the cytosol of inflammatory cells and especially in neutrophils. Taurine protects cells from self-destruction during processes that generate oxidants. The major function of Tau in leukocytes is to trap chlorinated oxidants (HOCl). Taurine reacts with HOCl to produce the long-lived compound taurine chloramine (TauCl). Previously, we have shown that other products of the neutrophil chlorinating system are able to modify functions of macrophages. In this study, we investigated in vitro the influence of TauCl on the generation of inflammatory mediators by activated macrophages. We have found that TauCl inhibited the generation of nitric oxide, prostaglandin E_2, tumor necrosis factor α, and interleukin-6, but TauCl slightly enhanced the release of IL-1α. The formation of nitrites by interferon-γ–activated macrophages was inhibited by TauCl in a dose-dependent manner. Taurine chloramine also reduced the level of inducible nitric oxide synthase (iNOS) mRNA in macrophages, in a similar concentration-dependent manner. Although our experiments do not exclude a direct effect of TauCl on enzymatic activity of iNOS, the inhibition of iNOS expression seems to be the major mechanism responsible for suppression of NO formation. Finally, we discuss the biological role of TauCl in vivo. We suggest that at the site of inflammation TauCl works as a specific signaling molecule of activated neutrophils that coordinates the generation of inflammatory mediators in macrophages. *J. Leukoc. Biol.* 58: 667-674; 1995.

Key Words: *taurine · nitric oxide · neutrophils · macrophages · tumor necrosis factor α · interleukin-1α · interleukin-6 · prostaglandin E_2*

INTRODUCTION

Neutrophils (polymorphonuclear cells, PMNs) and macrophages (Mϕs) are two types of professional phagocytes responsible for killing and/or destroying the antigen. PMNs are responsible for acute inflammation, whereas macrophages participate in both acute and chronic inflammation. Activated phagocytes generate a number of proinflammatory and toxic substances such as free radicals, cytokines, and eicosanoids [1]. Some of them are produced in both types of cells. Macrophages and PMNs share some killing molecules (products of the respiratory burst, nitric oxide, tumor necrosis factor α). However, the generation of nitric oxide seems to be crucial for macrophage cytotoxic and inflammatory function [2, 3]. Chlorination, on the other hand, seems to be the most important "killing system" in PMNs [4, 5].

The chlorination takes place in the phagosomal vacuoles of PMNs as well as extracellularly. This process is catalyzed by myeloperoxidase, the enzyme specific for neutrophils, eosinophils, and monocytes but not for macrophages [6]. The chlorination results from oxidation of chloride ions by H_2O_2 to hypochlorous acid (HOCl). This very toxic and reactive compound chlorinates many species including proteins and free amino acids [5]. The precise mechanisms of chlorination of proteins are still unknown. However, it was previously shown that they lead to the oxidation of amino groups to the carbonyl and carboxyl moieties via chloramines [4].

Taurine, 2-aminoethanesulfonate (Tau), was reported to be the most abundant intracellular free amino acid. Taurine is present at unusually high concentrations (10–30 mM) in leukocytes, especially in neutrophils [7–9]. Taurine seems to have a dual biological role. It has been proposed that Tau is a scavenger for chlorinated oxidants. The reaction of Tau with HOCl leads to the formation of long-lived taurine chloramine (TauCl). This process protects cells from the cytotoxic and cytolytic action of hypochlorous acid. Taurine chloramine itself, however, may exert prolonged oxidative and chlorinating effects long after the initiation of the res-

---

Abbreviations: EIA, enzyme immunoassay; FCS, fetal calf serum; IFN-γ, interferon-γ; IL-4, interleukin-4; iNOS, inducible nitric oxide synthase; L-NMMA, N^G-monomethyl-L-arginine; LPS, lipopolysaccharide; MME, murine brain microvascular endothelial cell; Mϕ, macrophage; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PGE_2, prostaglandin E_2; PMN, polymorphonuclear cell; SSC, standard saline citrate; Tau, taurine; TNF-α, tumor necrosis factor α.

Reprint requests: J. Marcinkiewicz, Department of Immunology, Jagiellonian University Medical College, Czysta 18, 31-121 Cracow, Poland.

Received May 8, 1995; revised August 14, 1995; accepted September 18, 1995.

piratory burst and at some distance from the cell of origin [4, 7].

Neutrophils are followed and scavenged by macrophages at local inflammatory sites, and macrophages seem to be the major target cells for granulocyte-derived biologically active agents including chloramines. We previously reported that chlorinated proteins are more immunogenic than native ones and chlorination enhances the ability of macrophages to process/present the antigens [10, 11]. We also found that after chlorination, gram-positive bacteria lost their ability to induce the production of NO and TNF-α by macrophages during phagocytosis [12]. These results suggest that PMNs may play a role in down-regulating the inflammatory and cytotoxic functions of macrophages.

Nitric oxide is one of the strongest defense molecules of macrophages and is responsible for the destruction of microorganisms and tumor cells (protective functions) [2, 3]. Nitric oxide, on the other hand, contributes to the suppression of lymphocyte proliferation, hypotension in sepsis, and destruction of cells in autoimmune diseases (pathological functions) [13]. Moreover, NO can react with superoxide anion (O_2^-) to form peroxynitrite ($ONOO^-$), an extremely powerful oxidant, which is a tissue-damaging agent [2, 14]. Thus, the potential toxicity of NO makes it important to understand the regulation of its production. Various agents such as transforming growth factor-β (TGF-β), IL-4, IL-10, and platelet-derived growth factor (PDGF) have all been reported to suppress the induction of NO release from macrophages [14–16].

The aim of this study was to test the hypothesis that taurine chloramine, a product of activated neutrophils, is able to regulate the generation of nitric oxide and other inflammatory mediators released by macrophages.

MATERIALS AND METHODS

Mice

Inbred CBA/J male mice from the breeding unit, Department of Immunology, Cracow, Poland, were used between 6 and 8 weeks of age.

Cells

Isolation of macrophages: Peritoneal mouse macrophages were induced by intraperitoneal injection of 2 ml of thioglycollate medium (Difco, Detroit, MI). Cells were collected 5 days later by washing out the peritoneal cavity with 5 ml of DPBS containing 5 U heparin/ml (Polfa, Warsaw, Poland). Cells were centrifuged and red blood cells were lysed by osmotic shock using distilled water; osmolarity was restored by adding 2× concentrated PBS. Cells were purified by adherence to tissue culture plastic plates for 2 h (37°C, 5% CO_2). After removal of nonadherent cells, the resultant monolayer contained >95% macrophages [17].

Maintenance of cells

Murine J774.2 macrophages were a generous gift from Prof. Sir John Vane (William Harvey Research Institute, London). The cells were routinely propagated in RPMI 1640 medium with 5% fetal calf serum (FCS) (both Gibco, Grand Island, NY) in a 5% CO_2 atmosphere by serial biweekly passages. For experiments cells were centrifuged and resuspended in RPMI 1640 at a concentration of 1×10^6/ml. Murine brain microvascular endothelial cells (MMEs) were a gift from Dr. Robert Auerbach (University of Wisconsin, Madison). They were grown in a medium consisted of Dulbecco's modified Eagle's medium (DMEM; Life Technologies Ltd., Paisley, UK), 20% FCS, endothelial cell growth supplement (30 μg/ml; Sigma, St. Louis, MO), 2 mM glutamine, and antibiotics.

Cell culture. Activation of the cells

Peritoneal mouse macrophages (Mϕ-Tio) and J774.2 macrophages were cultured in flat-bottom 24-well plates (Falcon, New Haven, CT) at a concentration of 1×10^6/ml/well in RPMI 1640 supplemented with 5% FCS, glutamine (2 mM) (Sigma), and gentamicin (50 mg/l) (Irvine Scientific, Santa Ana, CA) at 37°C in an atmosphere of 5% CO_2. The following stimulators were used: murine recombinant interferon-γ (rIFN-γ; Sigma) in a dose of 20 U/ml, human rTNF-α (Genzyme, Cambridge, MA) in a dose of 25 ng/ml, and lipopolysaccharide (LPS; *E. coli*, 0.111:B4 Sigma) in a dose of 1 μg/ml. Activated cells were incubated with either taurine or taurine chloramine (0.1–0.6 mM) if not otherwise stated. After 24 h supernatants were collected and frozen for measurement of nitrite, TNF-α, IL-1α, IL-6, and PGE_2.

MME cells were cultured in 96-well flat-bottom plates (Nunc, Roskilde, Denmark) in 100 μl of culture medium. At 2–3 days after cells reached confluence (1.6×10^4 cells/well), the medium was replaced with 100 μl of RPMI 1640 with 2% FCS. Cells were stimulated with human rTNF-α (100 U/ml) and murine rIFN-γ (100 U/ml). We have previously demonstrated that MME cells, in response to inflammatory cytokines, undergo activation characterized, in part, by expression of inducible nitric oxide synthase (iNOS). However, IFN-γ alone was not able to induce iNOS expression in MME cells. The expression of iNOS in MME cells requires a dual signal, which can be provided by a combination of TNF and IFN-γ [18]. Taurine and taurine chloramine were used as described above. Nitrite concentration was determined in supernatants after 24 h of incubation (37°C, 5% CO_2).

Synthesis of chloramines

Taurine, ethanolamine, serine, and phosphoethanolamine were obtained from Sigma. *N*-Monochlorotaurine, *N*-monochloroethanolamine, and *N*-monochloroserine were prepared by dropwise additions of 5 ml of 2 mM NaOCl (Sigma) solution in 0.06 M phosphate buffer (pH 7.4–7.5) with vigorous stirring to 5 ml of 29 mM amine solution in the same buffer. The concentration of *N*-monochloro derivatives was determined spectophotometrically assuming $\varepsilon_{252\ nm} = 415\ M^{-1}\ cm^{-1}$.

N-Dichlorophosphoethanolamine was the main product of NaOCl treatment of phosphoethanolamine and it was determined assuming $\varepsilon_{300\ nm} = 300\ M^{-1}\ cm^{-1}$ [19, 20]. Chloramines were used immediately after preparation. Stock solution of taurine chloramine was kept at 4°C for a maximum period of 2 weeks before use.

Measurement of cell viability

Cells were cultured in 96-well plates for 24 h with or without taurine chloramine. Cell respiration, an indicator of cell viability, was assessed by the mitochondria-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) to formazan [21]. Cells were incubated with MTT (500 μg/ml) for 3 h at 37°C. Culture medium was removed by aspiration and the cells were solubilized in a 10% sodium dodecyl sulfate (SDS) solution (Sigma). The extent of reduction of MTT to formazan within cells was quantitated by measurement of optical density at 550 nm (OD_{550}).

Cell adhesion assay

Cells were labeled with PKH2 Green cell linker (Sigma) according to the recommended protocol and resuspended in RPMI + 5% FCS. The cells (3×10^4/well) were placed in aliquots in flat-bottom microtiter plates (Falcon) either untreated or previously coated by overnight incubation with human serum fibronectin (Sigma) at 10 μg/ml in PBS and washed twice with PBS before use. Cells were incubated with or without taurine chloramine for 24 h at 37°C in 5% CO_2. Cells were counted in each well, after washing in prewarmed RPMI medium to remove nonadherent cells,

CCD video camera (COHU-4910) and images were imported into the NIH-Image program (Wayne Rasband, public domain) using a digital translation card (LG-3, Scion). Nine images were taken in a 3 × 3 grid from each well covering 34% of the surface area of the well. Two-dimensional rolling ball background subtraction and a 5 × 5 smoothing convolution were performed on each image. A cell-counting algorithm was used to enumerate the number of cells per image and per well. Data are presented as the mean and standard deviation of adherent cell number per well (Dr. Mike Binqks from the Department of Immunology UCL London, personal communication).

Nitrite (NO_2^-) determination

Nitric oxide (NO), quantified by the accumulation of nitrite (as a stable end product), was determined by a microplate assay [22]. Briefly, 100-μl samples were removed from the supernatants and incubated with an equal volume of Griess reagent (1% sulfanilamide, 0.1% *N*-1-naphthylenediamine dihydrochloride, 2.5% H_3PO_4) at room temperature for 10 min. The absorbance at 540 nm was determined with a microplate reader. Nitrite concentration was calculated from a sodium nitrite standard curve. The lower limit of detection of the assay was 2 μM nitrite. The standard curve was obtained in the presence of 0.6 mM TauCl. Taurine chloramine did not interfere with the assay.

RNA preparation and Northern blot analysis

Cells were cultured in 94-mm tissue culture plates (3×10^7 cells/plate). After 16 h of incubation of cells with the indicated factors, total RNA was prepared using the phenol extraction method [23]. RNA samples (5 μg) were separated electrophoretically in 1% agarose gel under denaturating conditions [24]. RNA was then capillary transferred to Hybond-N membranes (Amersham, UK) according to the manufacturer's instruction. The blots were baked at 80°C for 2 h, prehybridized overnight, and hybridized to cDNA probes specific for the murine macrophage iNOS (a gift from Drs. Q.W. Xie and C. Nathan) and for 18S RNA. The probes were labeled by the Random Primers DNA Labeling System (Amersham, UK). Hybridizations were performed at 68°C for 24 h. Nonspecifically bound radioactivity was removed by washing the blots in 2× standard saline citrate (SSC) at room temperature, followed by two subsequent washes in 2× SSC, 0.1% sodium dodecyl sulfate (SDS) at 68°C for 30 min each. The blots were then subjected to autoradiography using intensifying screens.

Cytokine assays. Bioassays

Levels of TNF-α and IL-6 in the supernatants were measured using the specific indicator cell lines and testing cell viability with the mitochondrial indicator dye MTT, as published previously [21]. Mean optical density value for triplicate cultures were converted to U/ml by fitting experimental titrations to the standard curves. Mouse recombinant TNF-α (Genzyme) and mouse rIL-6 (Sigma) were used as standards, respectively. IL-6 bioactivity was analyzed by measuring growth of the B9 hybridoma cell line, which is IL-6 dependent [25]. The 5×10^3 B9 cells were cultured with tested supernatants for 72 h in a total volume of 200 μl. Viability was determined by MTT staining. The TNF-α level was measured using the L-929 fibroblast cytotoxicity assay [25]. Briefly, L-929 cells were placed in flat-bottom 96-well microtiter plates at a density of 2×10^4/well into 100 μl of RPMI 1640 supplemented with 2% FCS. After 24 h, 100 μl of tested samples, supplemented with actinomycin D (1 μg/ml), was added to L-929 cultures and incubated for 16 h at 37°C in 5% CO_2. The cells were stained subsequently for 3.5 h with MTT.

TABLE 1. Influence of Taurine Chloramine on Adhesion of the Cells[a]

Macrophages (Mf-Tio) incubated with	Adherent cell number per well	
	A plates precosted with fibronectin	B untreated plates
— (control)	8522 ± 1449	3647 ± 54
Taurine, 0.6 mM	9549 ± 474	3189 ± 656
Taurine chloramine, 0.6 mM	8962 ± 1432	3083 ± 123

[a]Labeled macrophages at a concentration of 3 ´ 104 cells/well were incubated with or without taurine chloramine either on plates precoated with fibronectin (A) or on untreated plates (B). After 24 h cells were washed out and the number of adherent cells was counted using a digital fluorescent videomicroscopy system (for details see Materials and Methods). Data are presented as the mean ± SEM of adherent cell number per well.

TABLE 2. Influence of Taurine Chloramine on iNOS-Dependent Generation of Nitric Oxide

Taurine chloramine[a] [mM]	NO_2^- (μM) released by		
	Mϕ-Tio[b]	J774.2 cells[b]	MME cells[c]
— (control)	82 ± 19[d]	79 ± 8.6	72 ± 5.8
0.6	*2 ± 1.2	*2 ± 0.7	—
0.5	—	—	*2 ± 1
0.4	*5 ± 3.4	*7 ± 1.2	*3 ± 0.8
0.3	*26 ± 10	*13 ± 3.6	*5 ± 1.2
0.2	**38 ± 8	**31 ± 5.2	*15 ± 2
0.1	63 ± 13	71 ± 11	68 ± 4

[a]Taurine monochloramine was added to cell culture at different doses simultaneously with activator.

[b]Cells were stimulated with IFN-γ (20 U/ml). NO_2^- level was determined in supernatants collected after 24 h. The level of NO_2^- in nonstimulated cells was below 4 μM.

[c]Cells stimulated with IFN-γ (100 U/ml) and TNF-α (100 U/ml).

[d]Each value is the mean ± SEM of 12 (Mϕ-Tio), 7 (J774.2 cells), and 3 (MME cells) independent experiments (*$P < .05$, **$P < .01$).

Immunoassays

TNF-α was measured using an ELISA kit for mouse tumor necrosis factor-α (Genzyme). Interleukin-1α was measured using a mouse interleukin-1α ELISA kit (Genzyme). Prostaglandin E_2 was determined by using a prostaglandin E_2 enzyme immunoassay (EIA) system (Amersham, UK).

Specific inhibition of NO synthesis

The specific inhibitor of NO synthase N^G-monomethyl-L-arginine (L-NMMA) (Calbiochem, La Jolla, CA) was used at a concentration of 100 μM for inhibition of NO production.

Statistical analysis

Results are expressed as mean ± SEM. Statistical significance was determined by Student's *t*-test and the differences were regarded as significant for $P < .05$.

RESULTS

Influence of taurine chloramine on viability and adhesion of the cells

In preliminary experiments, we tested the cytotoxic effect of TauCl on peritoneal macrophages (Mϕ-Tio) and J774.2 and MME cells. TauCl at a concentration of 1 mM and higher was cytotoxic. The viability of all cell types cultured with TauCl in the range 0.03–0.6 mM was not significantly different from that of untreated cells (data not shown). In

TABLE 3. Effect of Chloramines on Nitric Oxide Production by Macrophages

Mϕ-Tio + IFN-γ[a] incubated with	Nitrates (% of control)[b] 0.1 mM	0.3 mM	0.6 mM
— (control)	100	100	100
Taurine	98 ± 3	95 ± 2	97 + 7
Taurine monochloramine	83 ± 15	*33 ± 4	*2 + 2
Taurine dichloramine	92 + 16	*23 ± 18	*1 + 0.8
Monochloroethanolamine	79 ± 10	*19 + 8	*3 + 2
Dichlorophosphoethanolamine	91 ± 13	**43 + 10	*7 + 6
Serine monochloraminec	104 ± 14	78 + 16	*12 + 4
Serine monochloramined	73 + 19	*15 + 11	*2 + 1.8
Sodium hypochlorite (NaOCl)	**68 + 11	*22 + 7	*3 + 1.6

[a]Macrophages (Mϕ-Tio) activated with IFN-γ (20 Y/ml) (control) and incubated with taurine or different chloramines. After 24 h supernatants were collected and tested for NO_2^- concentration. Nonstimulated cells released about 2 μM NO_2^-.

[b]Results are shown as percentage of NO_2^- (μM) level in control group (Mϕ-Tio stimulated with IFN-γ). Data are the means ± SEM of three separate experiments (*P < .05, **P < .01).

[c]Serine cholramine kept at 4°C foor 24 h.

[d]Serine chloramine used immediatley after preparation.

further experiments, we used only noncytotoxic concentrations of TauCl (0.03–0.6 mM).

Because the production of cell-derived mediators may require adherence [1], we tested the influence of taurine chloramine on adhesion of the cells. As shown in **Table 1**, treatment of peritoneal macrophages (Mϕ-Tio) with taurine chloramine (0.6 mM) did not cause cells to detach.

Taurine chloramine inhibits iNOS-dependent generation of nitric oxide

As shown in previous reports [2, 12], exposure to IFN-γ resulted in generation of NO_2^- (as a stable end product of NO oxidation) by peritoneal macrophages (82 ± 19 μM) and the J774.2 macrophage cell line (79 ± 9 μM) (**Table 2**). Stimulation of MME cells with IFN-γ and TNF-α also leads to high nitrite accumulation (72 ± 6 μM). The release of NO_2^- from nonactivated cells was negligible (0–2 μM). The generation of NO was dependent on an inducible form of nitric oxide synthase (iNOS) in all cell types tested. As shown in Table 2, taurine chloramine inhibited the release of NO_2^- in a dose-dependent manner. The activity of TauCl was similar in all cell types tested. The highest concentration of TauCl (0.6 mM) completely abrogated the generation of NO (the inhibition by 98%). Taurine chloramine (0.1 mM) only slightly inhibited NO release (inhibition by 8–22%, depending on the cell type). Further experiments were performed using peritoneal macrophages (Mϕ-Tio).

Influence of different chloramines on NO generation

To determine whether the inhibition of NO release by TauCl is specific for taurine derivatives, we have tested chloramines of different amino acids. Native taurine (**Table 3**), as well as other native amino acids (data not shown), had no influence on NO generation. In contrast, chloramines such as taurine monochloramine, taurine dichloramine, *N*-monochloroethanolamine, and *N*-dichlorophosphoethanolamine, as well as NaOCl, all inhibited nitric oxide release in a similar, dose-dependent manner (Table 3). However, serine chloramine (SerCl) was active only when used immediately after preparation (0.3 mM SerCl, inhibition of NO generation by 85%). Serine chloramine lost its inhibitory properties after staying in a solution for 24 h (inhibition of NO generation by 22%). This effect correlated with decomposition of SerCl as monitored by spectrophotometric analysis (data not shown). Taurine monochloramine and taurine dichloramine similarly affected NO release. Because in vivo chlorination of taurine results mainly in monochloramine formation [8], we tested taurine monochloramine, further referred to as chloramine (TauCl), as the representative of taurine chloramines in our study.

Taurine chloramine inhibits the generation of NO by macrophages stimulated with different agents

Peritoneal macrophages were activated in vitro by either LPS, IFN-γ, TNF-α, or a combination of IFN-γ and TNF-α. Activated macrophages where cultured either alone or in the presence of different concentrations of TauCl. As shown

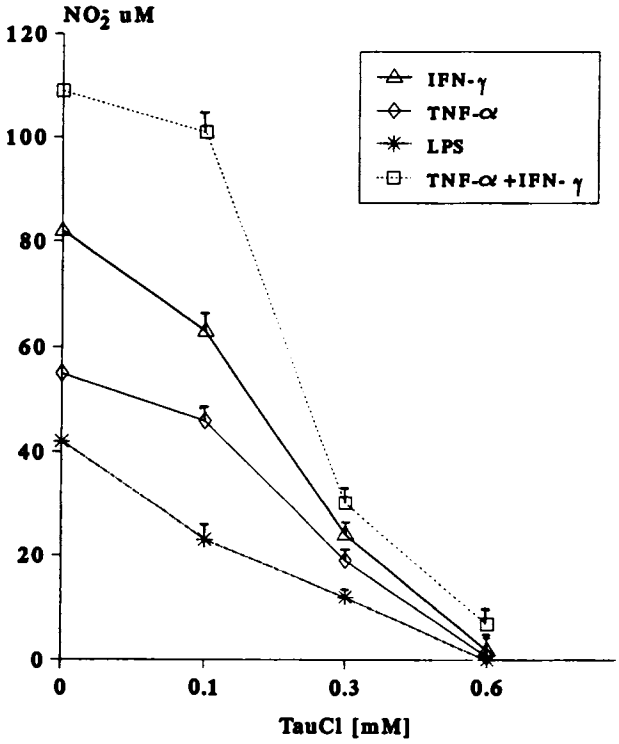

Fig. 1. Taurine chloramine inhibits the generation of NO by macrophages stimulated with different agents. Macrophages (Mϕ-Tio) were activated with IFN-γ (20 U/ml), TNF-α (25 ng/ml), LPS (1 μg/ml), or IFN-γ (20 U/ml) + TNF-α (25 ng/ml). Taurine chloramine was added to the culture at a concentration of 0.1, 0.3, or 0.6 mM. NO_2^- concentration was estimated in supernatants after 24 h. The level of NO_2^- in nonstimulated cells was 0–4 μM. Each value is the mean ± SEM of four separate experiments.

in **Figure 1**, taurine chloramine inhibited the generation of NO following all types of stimulation.

Time course of inhibition of NO generation by TauCl

To determine whether the inhibition of NO_2^- accumulation by TauCl results from inhibition of iNOS expression or from inhibition of its enzymatic activity, we compared the time dependence of the TauCl effect with that of L-NMMA, a specific inhibitor of NOS enzymatic activity [26]. Taurine chloramine or L-NMMA was added to macrophages at different times after activation with IFN-γ. As shown in **Figure 2**, L-NMMA inhibited almost completely NO_2^- accumulation in the culture medium when added simultaneously with IFN-γ or within 6 h after stimulation (time required for induction of iNOS expression and synthesis of a significant amount of the enzyme). In contrast, TauCl revealed its full inhibitory effect only when added to the cells simultaneously with IFN-γ or within 2 h after the stimulation with IFN-γ (inhibition by 94%). Taurine chloramine added 8 h after the activation inhibited NO_2^- accumulation by 42%, and when added after 22 h (2 h before supernatant collection) it did not affect the level of nitrites.

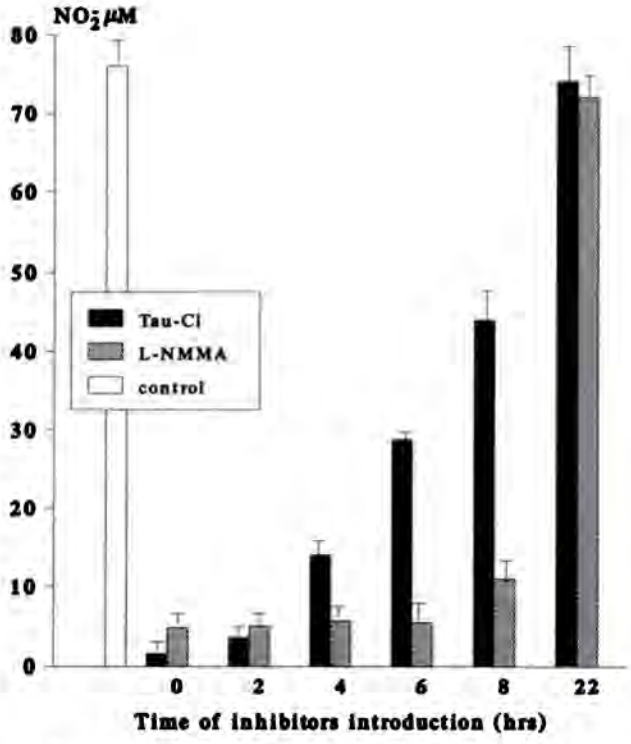

Fig. 2. Time course of inhibition of NO production by taurine chloramine and L-NMMA. Macrophages (Mϕ-Tio) were activated with IFN-γ (20 U/ml) (control). TauCl (0.6 mM) or L-NMMA (100 μM) was added to the cell culture at different times after addition of IFN-γ. At 24 h after IFN-γ addition, supernatants were collected from all cultures and tested for NO_2^- concentration. The level of NO_2^- in macrophages exposed only to medium was approximately 4 μM. The figure shows the results (mean ± SEM) of one of three separate experiments.

In addition, we have shown that taurine chloramine did not change significantly the production of NO by IFN-γ–pretreated macrophages when incubated for a further 24 h after washing out the stimulator (**Fig. 3**). In contrast to taurine chloramine, L-NMMA, an inhibitor of iNOS, almost completely blocked NO generation. These data indicate that TauCl is active early in the IFN-γ–dependent pathway of iNOS generation. The results did not confirm the data that suggest a direct effect of taurine chloramine on NO synthase activity [27]. We also investigated how long the macrophages had to be preincubated with TauCl to reveal its suppressive activity. Taurine chloramine was washed out from the cells before stimulation with IFN-γ. Incubation of cells with TauCl (0.6 mM) for 5 min did not affect the generation of NO, whereas incubation for 20–40 min inhibited the generation of NO by 65% (NO_2^- 26 μM versus 74 μM in the control) (data not shown).

Inhibition of mRNA of iNOS by taurine chloramine

Northern blot analysis demonstrated that the inhibition of nitrite accumulation in culture medium of IFN-γ–stimulated macrophages by taurine chloramine is accompanied by a reduced level of iNOS mRNA. Incubation of murine macrophages with IFN-γ significantly increased the iNOS mRNA level. However, when the cells were incubated with IFN-γ in the presence of taurine chloramine, the IFN-γ–mediated increase was strongly diminished. The effect of TauCl was dose dependent (**Fig. 4** top). Taurine did not inhibit the iNOS mRNA level. The inhibitory effect of TauCl on cytokine-induced iNOS mRNA expression was also observed in MME cells (Fig. 4 bottom).

Influence of TauCl on the production of cytokines and PGE_2 by macrophages

Thioglycollate-induced peritoneal macrophages (Mϕ-Tio), when activated with IFN-γ, release a variety of mediators involved in inflammation, such as nitric oxide, PGE_2, IL-1α, IL-6, and TNF-α [1]. Nonactivated Mϕ-Tio do not generate NO, but they release substantial amounts of cytokines and PGE_2 (**Table 4**).

Taurine did not influence the production of these mediators. As shown in Table 4, the release of TNF-α, IL-6, and PGE_2 was inhibited by TauCl in a dose-dependent manner. The highest nontoxic concentration of TauCl (0.6 mM) inhibited release of TNF-α by 50%, IL-6 by 80%, and PGE_2 by 82%. The release of IL-1α was not inhibited by TauCl. In some experiments we even observed an enhancement of IL-1α production in the presence of taurine chloramine (Table 4). Taurine chloramine alone, in the absence of the stimulating agent IFN-γ, did not change the release of NO, TNF-α, IL-6, and IL-1α. On the contrary, the release of PGE_2 from nonstimulated macrophages was inhibited by TauCl in a dose-dependent manner (data not shown).

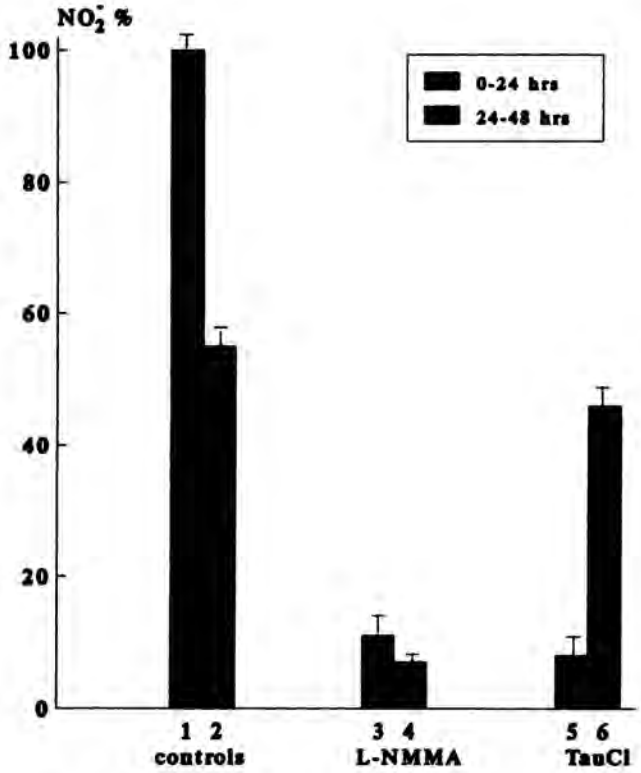

Fig. 3. Influence of TauCl on NO release by macrophages after washing out of IFN-γ. Macrophages (Mϕ-Tio) were stimulated with IFN-γ (20 U/ml) (groups 1–6). L-NMMA (group 3), and TauCl (0.6 mM) (group 5) were added at the time of activation. After 24 h supernatants were removed and tested for NO_2^- accumulation (0–24 h). Macrophages from groups 2, 4, and 6 were washed out and cultured with either the medium alone (2) or L-NMMA (4) or TauCl (6) for a further 24 h. The accumulation of nitrites throughout this additional period of incubation (24–48 h) was measured. Results are expressed as percentage of NO_2^- release (66 μM) by macrophages stimulated with IFN-γ (group 1). The level of nitrites in nonstimulated macrophages was < 4 μM. Group 6 versus group 2, $P > .1$.

DISCUSSION

Taurine is exceptionally abundant in the cytosol of inflammatory cells and especially in neutrophils, where it represents over 70% of the free amino acid pool [8, 9]. It is commonly accepted that Tau functions in biological systems as a general detoxifier. The major function of Tau in leukocytes is to trap chlorinated oxidants (HOCl). Thereby, taurine may specifically protect cells from self-destruction during processes that generate oxidants [7, 8]. Taurine may protect not only neutrophils that produce oxidants but also bystander cells such as platelets, lymphocytes, and macrophages [8]. In contrast to the role of Tau, the biological role of TauCl is not well defined. It is reasonable to ask whether taurine chloramine acts on macrophages, the major partner cells of neutrophils, at the site of inflammation. Park et al. [27] have shown that TauCl inhibited the production of nitric oxide and TNF-α by RAW 264.7 cells.

In this study we investigated the influence of TauCl on the generation of inflammatory mediators by activated macrophages. Previously, we showed that the products of the neutrophil chlorinating system were able to modify macrophage functions [10–12]. The present results indicated that one of them, taurine chloramine, strongly inhibited NO generation in activated peritoneal macrophages (Mϕ-Tio), J774.2 cells, and MME cells. In all tested cells the generation of NO depends on the inducible form of NO synthase (iNOS) [2, 18, 26]. Generation of NO by macrophages, engaged in both protective and destructive reactions, is regulated by a variety of endogenous factors such as TGF-β, IL-4, and LPS [14, 15]. Mechanisms by which iNOS is negatively regulated have so far been analyzed only in macrophages treated with TGF-β. TGF-β destabilizes iNOS mRNA, decreases its translation, and increases the degradation of iNOS protein [16]. TGF-β regulates NO generation in an autocrine manner.

We have shown that TauCl reduces the level of iNOS mRNA in a dose-dependent manner closely correlated with the inhibition of nitrite accumulation. These data, together with the finding that the strongest inhibitory effect of TauCl was observed when TauCl was added simultaneously with

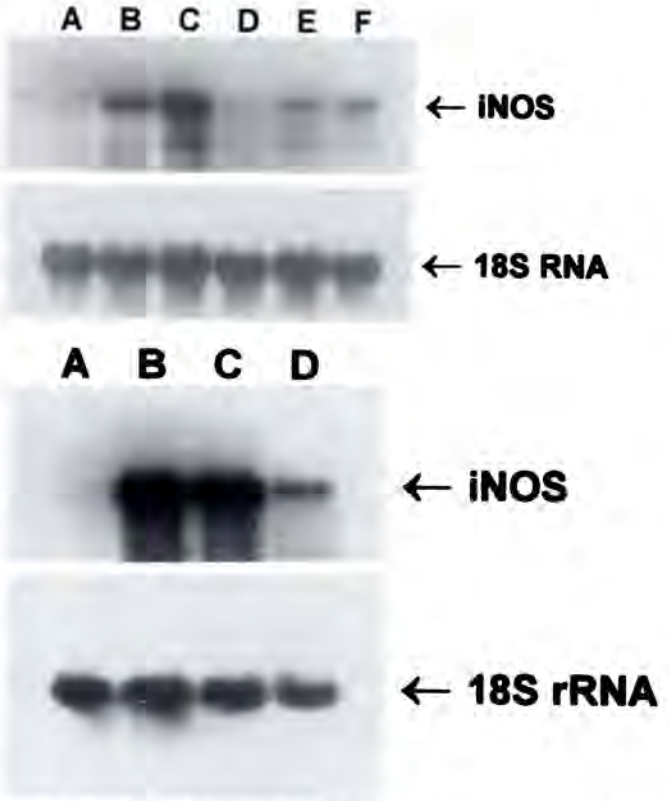

Fig. 4. Northern blot analysis of iNOS mRNA in murine peritoneal macrophages and in MME cells. (Top) Peritoneal macrophages were incubated for 16 h with medium alone (lane A), with IFN-γ (lane B), with IFN-γ in the presence of taurine (0.6 mM) (lane C), or with IFN-γ in the presence of taurine chloramine at a concentration of 0.6 mM (lane D), 0.3 mM (lane E), or 0.1 mM (lane F). Equal loading of wells with RNA samples was verified by hybridization with 18S rRNA-specific cDNA probe. (Bottom) MME cells were incubated for 16 h with medium alone (lane A), with IFN-γ and TNF-α (lane B), or with IFN-γ and TNF-α in the presence of taurine chloramine at a concentration of 0.2 mM (lane C) or 0.4 mM (lane D).

TABLE 4. Influence of Taurine Chloramine on Release of PGE2 and Cytokines

Group	PGE_2(pg/ml)	IL-1α (pg/ml	IL-6 (pg/ml)	TNF (U/ml	TNF (ng/ml)
Control[a]	97 ± 10[b]	71 ± 28	6 ± 2	8 ± 6[c]	3.4 ± 0.5[d]
IFN-γ	410 ± 14	137 ± 60	87 ± 9	88 ± 12	6.7 ± 1.1
IFN-γ + Tau (0.6 mM)	380 ± 12	141 ± 16	95 ± 6	91 ± 16	6.1 ± 0.9
IFN-γ + TauCl (0.6 mM)	*77 ± 15	220 ± 86	*17 ± 2	**45 ± 9	**3.2 ± 0.3
IFN-γ + TauCl (0.3 mM)	**132 ± 19	196 ± 75	**54 ± 17	**58 ± 19	5.1 ± 2.0
IFN-γ + TauCl (0.1 mM)	396 ± 16	160 ± 24	96 ± 7	105 ± 24	6.9 ± 1.6

[a]Nonstimulated macrophages (Mϕ-Tio) were used as a control group. Macrophages were activated with IFN-γ (20 U/ml) and incubated with taurine or taurine chloramine for 24 h. The levels of cytokines and PGE_2 were measured in supernatants.

[b]Mean ± SEM of one of four separate experiments.

[c]TNF-α measured using the L-929 cell line.

[d]TNF-α measured by immunoassay.

IFN-γ, suggested that the transcription of the iNOS gene was inhibited. These data are in agreement with a previous study of the influence of TauCl on iNOS induced in RAW 264.7 cells [27]. We cannot exclude the possibility that TauCl also decreases the stability of iNOS mRNA or also affects the process of translation.

Inhibition of message induction could result from inhibition of the interaction of stimulators with their receptors on macrophages, blocking the signal transduction pathway(s), or inhibiting the process of transcription. Taurine chloramine does not seem to interact directly with any particular stimulator of macrophages, as it inhibited NO generation by macrophages activated with a variety of different stimuli such as IFN-γ, LPS, and TNF-α. Furthermore, taurine chloramine had the same effect on NO generation whether it was added to macrophages simultaneously with IFN-γ or 2 h later. Finally, in RAW 264.7 cells, preincubation of activators (LPS, IFN-γ) with TauCl alone only slightly diminished the activity of the activator [27]. TauCl is transported into macrophages and other cells via its own specific receptor [27]. Thus, it is unlikely that the inhibition of macrophage function by TauCl is exerted at the level of the receptors for macrophage activators.

According to previous reports [27], taurine chloramine might also inhibit the enzymatic activity of NOS. However, our time course experiments comparing the action of taurine chloramine with that of NOS inhibitor showed significant differences in the effects of the two agents. Moreover, because TauCl did not affect the release of nitrites from IFN-γ–pretreated macrophages (in which the stimulator was washed out), it is unlikely that it might directly inhibit NO synthase activity. These findings, together with iNOS mRNA analysis, suggested that the major mechanism responsible for inhibition of NO production by TauCl was the decrease in iNOS synthesis rather than inhibition of the enzymatic activity of iNOS.

To determine whether the regulation of NO generation may be achieved in vitro by other chloramines, we have tested two forms of TauCl: taurine monochloramine and taurine dichloramine. In addition, we used chloramines of amino acids that are components of mammalian plasma membranes: serine, ethanolamine, and phosphoethanolamine. All chloramines inhibited the generation of NO in vitro in a dose-dependent manner. These results are at variance with the experiments of Park et al. on RAW 264.7 cells [27]. These authors concluded that the effect was specific for taurine chloramine because serine chloramine did not have such an effect. In our hands, serine chloramine, when used immediately after preparation, suppressed Mϕ-Tio activity as effectively as TauCl did, and we believed that, in vitro, any active chloramines can influence NO generation, because of their oxidizing and chlorinating properties. They are able to react with purine and pyrimidine nucleotides as well as with proteins, leading to the inactivation of target species [4, 28]. However, despite the strong activity of different chloramines in vitro, only taurine monochloramine may be significant in vivo. In contrast to the majority of the chlorinated alpha amino acids, taurine chloramine has a half-life that is exceptionally long [8]. Thus, both the high concentration of taurine and the stability of TauCl strengthen the potential impact of this chlorinated amine in vivo [7, 8].

Along with the strong inhibitory activity of TauCl on NO generation, we have shown the inhibition of PGE_2, TNF-α, and IL-6, all inflammatory mediators elicited by distinct signaling mechanisms. Endogenous TNF-α is considered to be an obligatory factor for iNOS induction [29]. However, addition of exogenous TNF-α did not restore NO generation in the presence of TauCl, indicating that the suppressor activity of TauCl is not merely an indirect effect of inhibiting TNF-α production. The effects of TauCl were not simply indiscriminate inhibition of macrophage function, because the production of IL-1α was not changed and in some experiments an enhancement was observed. The mechanism of TauCl-dependent inhibition of PGE_2 and cytokine generation remains unclear. TauCl either interferes directly with each particular pathway or, in addition, breaks the positive feedback loop in which NO, PGE_2, and cytokines are involved. It has been reported that under some conditions nitric oxide may enhance the activity of cyclooxygenase [30] and may increase the production of inflammatory cytokines by macrophages [31].

The results presented in this paper are in keeping with our hypothesis that the chlorinating system of neutrophils

plays a dual biological role [32]. During phagocytosis, chlorination of antigens in neutrophils is responsible for their degradation (killing system). It also serves as the regulatory system that modulates proinflammatory (cytotoxic) mechanisms in macrophages [12]. Several mechanisms, beside chlorination, contribute to the modulation of macrophage functions by PMNs [33, 34].

We suggest that NO and TNF-α are induced in macrophages when live and/or toxic antigen has to be destroyed. But when antigen is already inactivated by neutrophils, the additional macrophage killing system (NO, TNF-α) does not need to be triggered. Under these conditions TauCl therefore acts as a neutrophil-derived signaling molecule that is responsible for the down-regulation of production of inflammatory mediators by macrophages and neutrophils.

ACKNOWLEDGMENTS

This work was supported by grants 6P-207-063-06, and 6P-203-033-05 from the Committee of Scientific Research (Warsaw, Poland).

REFERENCES

1. Rappolee, D.A., Werb, Z. (1988) Secretory products of phagocytes. *Curr. Opin. Immunol.* **1**, 47–52.
2. Nussler, A.K., Billiar, T.R. (1993) Inflammation, immunoregulation, and inducible nitric oxide synthase. *J. Leukoc. Biol.* **54**, 171–178.
3. Nathan, C.F., Hibbs, J.B.J. (1991) Role of nitric oxide synthesis in macrophages antimicrobial activity. *Curr. Opin. Immunol.* **3**, 65–69.
4. Zgliczynski, J.M., Stelmaszynska, T. (1988) The respiratory burst of neutrophilic granulocytes and its influence on infected tissues. In *The Respiratory Burst and Its Physiological Significance* (A.J. Sbarra and R.R. Strauss, eds) Plenum, New York, 315–347.
5. Klebanoff, S.J., Hamon, C.B. (1972) Role of myeloperoxidase mediated anti-microbial systems in intact leucocytes. *J. Reticuloendothel. Soc.* **12**, 170–175.
6. Thomas, E.L. (1979) Myeloperoxidase, hydrogen peroxide, chloride antimicrobial system: effect of exogenous amines on antibacterial action against *Escherichia coli*. *Infect. Immun.* **25**, 110–114.
7. Learn, D.B., Fried, W.A., Thomas, E.L. (1990) Taurine and hypotaurine content of human leucocytes. *J. Leukoc. Biol.* **48**, 174–182.
8. Wright, Ch.E., Tallan, H.H., Lin, Y.Y. (1986) Taurine: biological update. *Annu. Rev. Biochem.* **55**, 427–453.
9. Fukkuda, K., Hirai, Y., Yoshida, H., Hakajima, T., Usii, T. (1982) Free amino acids content of lymphocytes and granulocytes compared. *Clin. Chem.* **28**, 1758–1761.
10. Marcinkiewicz, J., Chain, B.M., Olszowska, E., Olszowski, S., Zgliczynski, J.M. (1991) Enhancement of immunogenic properties of ovalbumin as a result of its chlorination. *Int. J. Biochem.* **23**, 1393–1395.
11. Marcinkiewicz, J., Olszowska, E., Olszowski, S., Zgliczynski, J.M. (1992) Enhancement of trinitrophenyl-specific humoral response as the result of carrier chlorination. *Immunology* **76**, 385–388.
12. Marcinkiewicz, J., Czajkowska, B., Grabowska, A., Kasprowicz, A., Kociszewska, B. (1994) Differential effects of chlorination of bacteria on their capacity to generate NO, TNF alfa and IL-6 in macrophages. *Immunology* **83**, 611–616.
13. Kolb, H., Kolb-Bachofen, V. (1992) A pathogenic factor in autoimmunity: nitric oxide. *Immunol. Today* **13**, 157–161.
14. Xie, Q., Nathan, C. (1994) The high-output nitric oxide pathway: role and regulation. *J. Leukoc. Biol.* **56**, 576–582.
15. Bogdan, Ch., Vodovotz, Y., Paik, J., Xie, Q., Nathan, C. (1994) Mechanism of suppression of nitric oxide synthase expression by interleukin-4 in primary mouse macrophages. *J. Leukoc. Biol.* **55**, 227–233.
16. Vodovotz, Y., Bogdan, Ch., Paik, J., Xie, Q., Nathan, C. (1993) Mechanisms of suppression of macrophage nitric oxide release by transforming growth factor beta. *J. Exp. Med.* **178**, 605–613.
17. Marcinkiewicz, J., Pater, M., Grabowska, A. (1994) An improved experimental model for the study of in vitro release of nitric oxide by murine peritoneal macrophages. *Arch. Immunol. Ther. Exp.* **42**, 95–99.
18. Bereta, M., Bereta, J., Cohen, S., Cohen, M.C. (1992) Low density lipoprotein inhibits accumulation of nitrites in murine brain endothelial cell cultures. *Biochem. Biophys. Res. Commun.* **186**, 315–320.
19. Thomas, E.L., Grisham, M.B., Jefferson, M.M. (1986) Preparation and characterization of chloramines. *Methods Enzymol.* **132**, 569–571.
20. Zgliczynski, M.J., Stelmaszynska, T., Domanski, J., Ostrowski, W. (1971) Chloramines as intermediates of oxidation reaction of amino acids by myeloperoxidase. *Biochim. Biophys. Acta* **253**, 419–424.
21. Tada, H., Shiho, O., Kuroshima, K., Koyama, M., Tsukamoto, K. (1986) An improved colorometric assay for interleukin 2. *J. Immunol. Methods* **93**, 157–160.
22. Ding, A.H., Nathan, C.F., Stuehr, D.J. (1988) Release of reactive nitrogen intermediates from mouse peritoneal macrophages: comparison of activating cytokines and evidence for independent production. *J. Immunol.* **141**, 2407–2412.
23. Rose-John, S., Dietrich, A., Marks, F. (1988) Molecular cloning of mouse protein C (PCK) cDNA from Swiss 3T3 fibroblast. *Gene* **74**, 465–471.
24. Sambrook, J., Fritsh, E.F., Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
25. Marcinkiewicz, M. (1991) In vitro cytokine release by activated murine peritoneal macrophages: role of prostaglandins in the differential regulation of tumor necrosis factor alpha, interleukin 1, interleukin 6. *Cytokine* **3**, 327–332.
26. Moncada, S., Higgs, A. (1993) The L-arginine–nitric oxide pathway. *N. Engl. J. Med.* **329**, 2002–2012.
27. Park, E., Quinn, M.R., Wright, Ch.E., Schuller-Levis, G. (1993) Taurine chloramine inhibits the synthesis of nitric oxide and the release of tumor necrosis factor in activated RAW 264.7 cells. *J. Leukoc. Biol.* **54**, 119–124.
28. Davies, J.M., Horowitz, D.A., Davies, K.J. (1994) Inhibition of collagenase activity by *N*-chlorotaurine, a product of activated neutrophils. *Arthritis Rheum.* **37**, 424–427.
29. Drapier, J.C., Wietzerbin, J., Hibbs, J.B. (1988) Interferon-γ and tumor necrosis factor induce the L-arginine–dependent cytotoxic effector mechanism in murine macrophages. *Eur. J. Immunol.* **18**, 1587–1591.
30. Salvemini, D., Misko, T.P., Masferrer, J., Seibert, K., Currie, M.G., Needleman, P. (1993) Nitric oxide activates cyclooxygenase enzymes. *Proc. Natl. Acad. Sci. USA* **90**, 7240–7244.
31. Marcinkiewicz, J., Grabowska, A., Chain, B. (1995) Nitric oxide up-regulates the release of inflammatory mediators by mouse macrophages. *Eur. J. Immunol.* **25**, 947–951.
32. Marcinkiewicz, J., Zgliczynski, J.M. (1993) Granulocyte macrophage interactions in the induction of immune response. In *Biologie prospective* (M.M. Galteau, G. Siest, and J. Henny, eds) Libbey Eurotext, Paris, 269–272.
33. Lloyd, A.R., Oppenheim, J.J. (1992) Poly's lament: the neglected role of polymorphonuclear neutrophil in the afferent limb on the immune response. *Immunol. Today* **13**, 169–172.
34. Yui, S., Sasaki, T., Yamazaki, M. (1993) Augmentation and suppression of TNF release from macrophages by inflammatory polymorphonuclear leukocytes. *Microbiol. Immunol.* **37**, 801–808.