Free Rad. Res., Vol. 28, pp. 179–191
Reprints available directly from the publisher
Photocopying permitted by license only

Nitric Oxide Production in Human Macrophagic Cells Phagocytizing Opsonized Zymosan: Direct Characterization by Measurement of the Luminol Dependent Chemiluminescence

ANTOINE GROSS[a,*], NATHALIE DUGAS[b], SANDRA SPIESSER[a], IOANIS VOULDOUKIS[c], CHANTAL DAMAIS[c], JEAN PIERRE KOLB[d], BERNARD DUGAS[e] and JACQUES DORNAND[a,†]

[a]*INSERM U431, Université de Montpellier II, 34095, Montpellier,* [b]*Laboratoire "Virus, Neurone et Immunité", IFR du Kremlin Bicètre, 94276 Kremlin Bicêtre,* [c]*INSERM U313, Hôpital la Pitié Salpétrière, 75013 Paris, France,* [d]*INSERM U365, Institut Curie, 75005 Paris and* [e]*FRACTALES BIOTECH, Hopital St Joseph, 75014, Paris*

Accepted by Prof. B. Halliwell

(Received 29 September 1997; In revised form 10 November 1997)

When differentiated into mature macrophages by the combination of all-*trans* retinoic acid and 1,25-dihydroxyvitamin D_3, the human promonocytic cell lines U937 and THP-1 expressed inducible nitric oxide synthase (iNOS) transcripts. During their differentiation, the cells acquired the capacity to produce not only superoxide anion ($O_2^{\cdot-}$) but also nitric oxide ($^{\cdot}NO$) in response to IgG (or IgE)-opsonized zymosan. The inhibitors of the iNOS pathway, aminoguanidine and N^G-monomethyl-L-arginine (L-NMMA), suppressed the production of $^{\cdot}NO$ and enhanced the steady-state concentration of $O_2^{\cdot-}$ determined. Conversely, superoxide dismutase (SOD) scavenged the $O_2^{\cdot-}$ released and increased the $^{\cdot}NO$-derived nitrite concentration detected. These data suggested a possible interaction between $O_2^{\cdot-}$ and $^{\cdot}NO$. In differentiated U937 (or THP-1) cells, IgG or IgE-opsonized zymosan induced a strong time-dependent luminol-dependent chemiluminescence (LDCL), which was abrogated by SOD and partially inhibited by aminoguanidine or L-NMMA. Since the iNOS inhibitors did not directly scavenge $O_2^{\cdot-}$, LDCL determination in the presence or absence of SOD and/or iNOS inhibitors demonstrated a concomitant production of $O_2^{\cdot-}$ and $^{\cdot}NO$. These radicals induced the formation of a $^{\cdot}NO$-derived product(s), probably peroxynitrite ($ONOO^-$), which was required to elicit maximal LDCL. Finally, LDCL measurement provided a convenient tool to characterize iNOS triggering and demonstrated an interaction between NADPH oxidase and iNOS products in human macrophagic cells phagocytizing opsonized-zymosan. These findings show that in activated macrophages, iNOS activity can be involved in LDCL and support the debated hypothesis of iNOS participation to the microbicidal activity of human macrophages.

Keywords: Nitric oxide, inducible nitric oxide synthase, superoxide, peroxynitrite, human macrophagic cells, promonocytic cell differenciation, macrophage activation, luminol chemiluminescence

* Corresponding author. Tel.: 33 (0) 467144244. Fax: 33 (0) 467143338.
† Present address: INSERM U431, Université de Montpellier II, CC100, Place Eugène Bataillon, 34095 Montpellier, France.

INTRODUCTION

Once activated during phagocytosis of parasites, monocytic phagocytes produce large amounts of reactive oxygen species which involves an activated NADPH-oxidase able to catalyze the one electron-reduction of molecular oxygen to superoxide anion ($O_2^{\bullet -}$).[1] Activated monocytic phagocytes also produce hydrogen peroxide and hypochlorous acid which mediate the antimicrobicidal activity of the cells and which, under defined physiopathological situations may induce molecular and cellular toxicity.

It is now clearly demonstrated that murine macrophages can produce $^{\bullet}NO$, and that iNOS is a component of the antimicrobial activity of the cells.[2–8] In humans, the situation is less clear. Despite some contradictory results,[9] it was reported that in very particular conditions activated human macrophages and lymphocytes also produce nitric oxide ($^{\bullet}NO$) through the activation of the inducible NO synthase (iNOS) pathway.[10–16]

In a cell-free system, $^{\bullet}NO$ and $O_2^{\bullet -}$ react together to produce the highly pro-oxidant molecule peroxynitrite ($ONOO^-$), and this molecule induces a potent luminol-dependent chemiluminescence (LDCL).[17] Such a mechanism also occurs in biological systems: in PMA-stimulated human polymorphonuclear leukocytes[18] and in murine macrophages activated in the presence of immune complexes,[19] iNOS is stimulated during the activation process and contributes to the cell-generated LDCL. $ONOO^-$ is suggested to be the mediator of the phenomenon.

We recently demonstrated that a functional iNOS could be induced in the human promonocytic cell line U937 after its treatment with retinoic acid (RA) and vitamin D3 (VD3).[20] The expression of this enzyme correlated with the terminal differentiation of U937 in macrophage-like cells capable of adherence, cytokine production, phagocytosis of opsonized zymosan or of bacteria, $O_2^{\bullet -}$ production and antimicrobial activity.[20–22] Herein, we report that $^{\bullet}NO$ was produced in human differentiated macrophagic cells activated by IgG (or IgE)-opsonized zymosan which, according to our previous results, also produced $O_2^{\bullet -}$.[20–22] Taking advantage of the measurement of LDCL, we confirmed and extended these data, and demonstrated that products of the catalytic activity of both iNOS and NADPH oxidase were required to elicit a maximal luminol-dependent light emission during phagocytosis of opsonized zymosan. We established that $^{\bullet}NO$ and $O_2^{\bullet -}$ were concomitantly produced and suggested that the potent oxidant $ONOO^-$ might participate in the luminol-dependent light emission. We concluded that in human macrophages phagocytizing opsonized zymosan, besides other reactive oxygen species, $^{\bullet}NO$ was one of the components of the oxidative burst of the cells characterized by LDCL, and that measurement of LDCL was an indicator of iNOS activation. The involvement of iNOS in macrophage microbicidal functions through the synthesis of deleterious $^{\bullet}NO$-derived products is discussed.

MATERIALS AND METHODS

Reagents

All *trans*-Retinoic acid (RA), Superoxide Dismutase from bovine erythrocytes (SOD), Hypoxanthine, Xanthine Oxidase (XO), amino-guanidine, zymosan (Zy) and luminol were purchased from Sigma (St Louis, MO, USA), L-NMMA from Calbiochem (La, Jolla, CA). 1,25-Dihydroxy-vitamin D_3 (VD3) was kindly provided by Dr D. Lando (Roussel-Uclaf, Romainville, France).

Cell Culture and Differentiation

THP-1 and U937 leukemia cells were cultured in Iscove medium containing 1 mM of L-arginine and supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 10% FCS. These cells were plated at a cell concentration of 2×10^5 cells/ml in the same medium and incubated for 4

days at 37°C under a 95% air-5% CO_2 humid atmosphere in the presence or in the absence of 0.1 μM RA and/or 0.1 μM VD3, these concentrations being already described as optimal in this system.[21] Culture medium was shown to be endotoxin-free, as assessed by the limulus amoebocyte lysate assay (E-toxate, Sigma). Cell viability was assessed by trypan blue exclusion staining after each culture period.

Cell Activation

10^6 differentiated cells in one ml Iscove medium 10% FCS were pretreated or not at 37°C for 15 min with or without different additives as indicated in legend. Then, 50 μl of a 1 mg/ml suspension of zymosan opsonized either with normal human serum (CNTS, Paris, France) (Normal serum Ops Zy) or with an hyper IgE serum (Pharmacia France, Les Ulis, France) (Hyper IgE serum Ops Zy) was added to the cell suspensions which were cultured for further 48 h.

Nitrite and Nitrate Analysis

To assess the steady-state amount of ·NO produced, the cell supernatants were assayed for accumulation of the stable end product of ·NO, NO_2^-, measured using the Griess reaction as previously described.[13] The standard curve was done with $NaNO_2$ diluted in Iscove medium. Optical densities (O.D.) were measured at 550 nm using an autoreader (Dynatech Laboratories Inc, Alexandria, VA). In some experiments, the nitrate accumulated in cell supernatants was reduced to nitrite before nitrite determination. Supernatants were treated for 30 min with nitrate reductase (0.1 U/ml, Boehringer Mannheim) in the presence of 100 μM NADPH and 10 μM FAD. The remaining NADPH was then oxidized with lactate dehydrogenase (10 U/ml, Boehringer) in the presence of 10 mM sodium pyruvate, and the nitrite concentration measured.

Superoxide Determination

The production of $O_2^{\cdot-}$ was measured by reduction of ferricytochrome c according to the technique of Pick,[1] the specificity of the reaction being assessed by its inhibition by SOD. Control and differentiated U937 cells (2×10^5 cells in 200 μl RPMI 1640 without phenol red) were dispensed in 96-well microtitration plate and incubated for 2 h at 37°C in the presence of the different additives and 160 μM ferricytochrome c (Sigma) in the absence or presence of 300 U/ml of SOD. The absorbance was recorded at 550 nm and the steady-state concentration of $O_2^{\cdot-}$ released was calculated.

Luminol-Dependent Chemiluminescence (LDCL)

To measure oxidative burst activity, LDCL was monitored over a fixed period in a luminometer (Lumicon, Hamliton, Switzerland) as previously described.[20–21] Briefly, 5×10^5 cells in 500 μl RPMI medium without phenol red, pre-treated or not with different compounds were transfered in a counting tube filled with 500 μl luminol (10^{-4} M in PBS). The suspension was prewarmed at 37°C, before addition of 50 μl of a 1 mg/ml suspension of Normal serum Ops Zy or Hyper IgE serum Ops Zy. Light emission was then measured for 30 s with 2 min intervals during 40 min. In some experiments, LDCL was induced by the Xanthine/Xanthine Oxidase (X/Xo) system. The chemiluminescence reactions were carried out in 0.05 M carbonate buffer pH 8.5, containing luminol and 1.5 U/ml Xo. The reaction was iniated by addition of 0.25 mM hypoxanthine[23] and light emission was measured for 15 s with 30 s intervals during 15 min.

Analysis of iNOS mRNA Expression by Reverse Transcriptase-polymerase Chain Reaction (RT-PCR)

Total RNA from U937 or THP-1 cells treated or not with RA and/or VD3 were extracted with

Trizol (Gibco BRL Life Technologies), as described by the manufacturer.[22] The reverse transcription reaction was performed at 42°C for 90 min on 20 μg total RNA, using the Murine Moloney-Leukemia-Virus Reverse Transcriptase (M-MLV-RT, Gibco BRL Life Technologies), oligo-dT (12–18 oligo-dT, Gibco BRL Life Technologies) in the presence of 1 μCi ^{32}P-dCTP (ICN, Orsay, France), to allow a quantitation of synthesized cDNA.

1 to 4 ng of each cDNA was amplified using 2.5 U Gold Star Polymerase (Eurogentec, Seraing, Belgium) and 1 μM specific primers,[22] defined as follows:

protein	primers	amplicon length (bp)
β2-microglobulin	5′-primer: 5′-CCA-GCA-GAG-AAT-GGA-AAG-TC-3′,	
	3′-primer: 5′-GAT-GCT-GCT-TAC-ATG-TCT-CG-3′,	268
iNOS	5′-primer: 5′-TCC-GAG-GCA-AAC-AGC-ACA-TTC-A-3′,	
	3′-primer: 5′-GGG-TTG-GGG-GTG-TGG-TGA-TGT	464

The number of cycles (17 to 35) and cDNA concentrations were adjusted for each pair of primers, amplification of β_2-microglobulin was used as a control. PCR products were run on 1.2% agarose gels supplemented with ethidium bromide and their size was evaluated using m.w. standards (123 bp-ladder, Gibco BRL Life Tech-nologies). In experiments concerning iNOS, the PCR amplicons were analyzed by Southern blot: after agarose gels, the PCR products were blotted onto a Hybon N nylon membrane, hybridized with a ^{32}P-labelled hepatic iNOS cDNA specific probe (iNOS Hep clone 3D, 2.1 kb fragment from base 1757 to 3119, gift from Dr A. Nüssler (Ulm, Germany)) as previously[20] and analyzed on a Phosphorimager (Molecular Dynamics, Inc.).

RESULTS

Expression of the Inducible Form of Nitric Oxide Synthase in Macrophage-Like Cells

As already reported[20–22, 24], treatment of the promonocytic cell line U937 cells with a combination of 10^{-7} M RA and 10^{-7} M VD3 induced their differentiation into macrophage-like cells. In a concomitant manner, the inducible form of NOS was expressed: experimental results in Figure 1 show that iNOS transcripts which were not constitutively expressed in U937, were observed after a four-day RA/VD3 treatment of the cells. On the contrary, non differentiated THP-1 cells, that are more mature than U937 cells, displayed a spontaneous expression of iNOS mRNA (Figure 1). The RA/VD3 treatment of the THP-1 cells which also promoted their terminal differentiation into functional macrophage-like cells did not significantly modify the expression of the iNOS transcripts. In both experiments, iNOS mRNAs were analyzed by RT-PCR, the assays being normalized by measuring β_2-microglobulin mRNAs: a) the amplified fragments displayed the expected size, b) after blotting, they hybridized with a ^{32}P-labelled hepatocyte iNOS cDNA specific probe, c) the cloning and sequencing of the PCR products indicated that the amplified fragment was 100% homologous to the corresponding fragment of the hepatocyte iNOS [20, and data not shown].

Production of Oxidative Metabolites in Macrophage-Like Cells Activated by Opsonized Zymosan

The differentiation of U937 (or THP-1 cells) by RA/VD3 resulted in an increased cell surface expression of various classes of Fc receptors for immunoglobulins, including the Fcγ receptors for IgG and the low affinity receptor for IgE (FcεRII/CD23).[20,21] In differentiated cells, triggering of such receptors by Normal serum Ops Zy or Hyper IgE serum Ops Zy induced not only the production

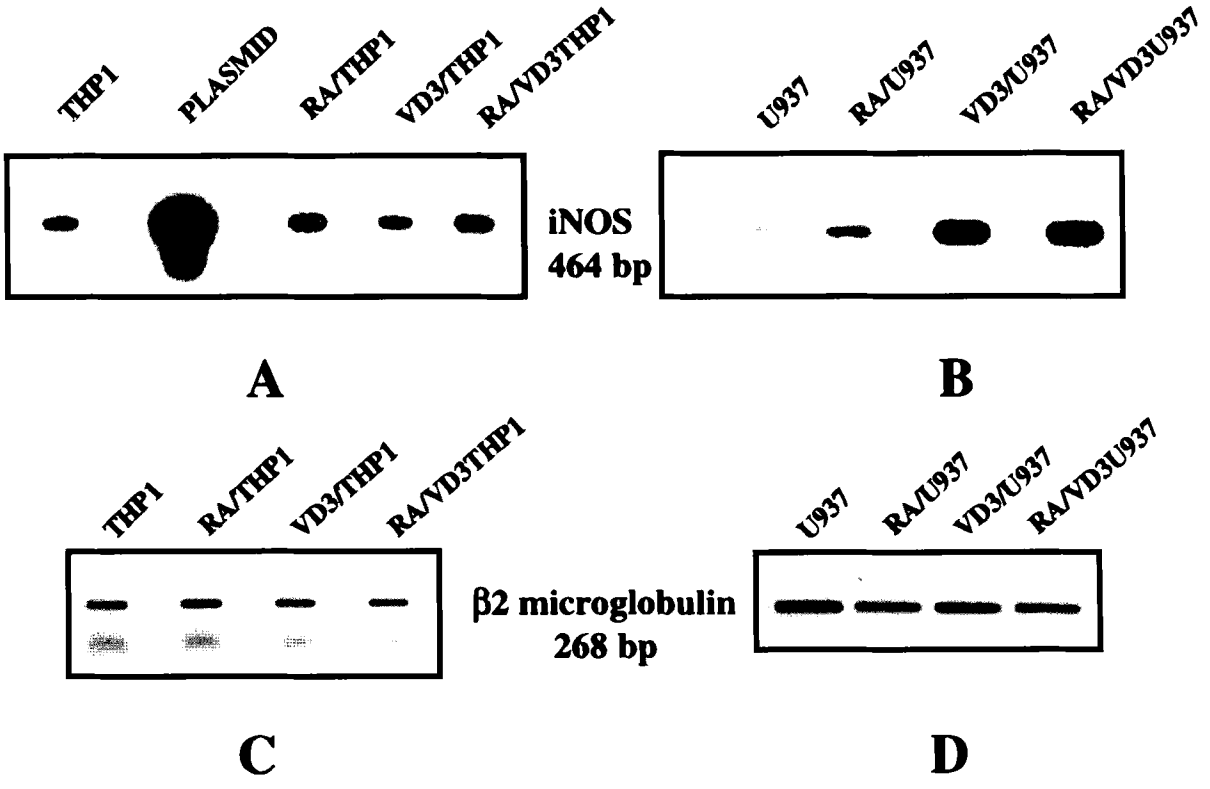

FIGURE 1 iNOS gene expression in U937 and THP-1 cells induced or not with RA and/or VD3. mRNAs were extracted from cells cultured for 4 days in the presence or absence of RA and/or VD3. iNOS (or β_2-microglobulin) transcripts were analyzed by RT-PCR. The PCR products corresponding to iNOS were obtained with 3ng mRNA-derived cDNA and 35 PCR sequential cycles (for β_2 microglobulin, 1ng cDNA and 17 PCR sequential cycles were used). The iNOS primers-derived products were separated on agarose gel, blotted onto a nylon membrane, hybridized with the [^{32}P]-labelled hepatocyte iNOS specific cDNA probe and analyzed on a Phosphorimager (Figures A and B). The plasmid in which the hepatocyte iNOS cDNA was inserted was introduced as a control in PCR reaction A. Figures C and D represent an inverted view of PCR products obtained with the β_2-microglobulin primers, after ethidium bromide staining. Data are from one experiment representative of three.

of $O_2^{\bullet-}$, but also that of nitrogen derivatives evaluated by nitrite accumulation in cell supernatants during 48 hours. On the contrary, experiments performed in parallel showed that both types of opsonized zymosan were totally inefficient in non differentiated cells (data not shown). The responses of RA/VD3-treated U937 cells are shown in Figure 2A/2B and Table I. In the presence of the iNOS inhibitor aminoguanidine, the production of nitrogen derivatives was inhibited. This inhibitory effect was dose-dependent (not shown) with a maximal effect at 1.5mM aminoguanidine. L-NMMA, another iNOS inhibitor exerted a similar inhibitory effect as aminoguanidine but at higher concentrations (2.5mM instead of 1.5mM) (Table I). These results established that nitrite was produced through iNOS activation and •NO formation. Surprisingly, we observed that the iNOS inhibitors enhanced the steady-state concentration of $O_2^{\bullet-}$ measured in the supernatants of the activated cells (Figure 2A). As expected, SOD dose-dependently scavenged the $O_2^{\bullet-}$ produced by zymosan-treated cells, the maximal effect occurring at 30 U/ml. In parallel, it could be observed that SOD significantly enhanced the zymosan-induced concentration of nitrite directly measured by the Griess reaction (Figure 2B, Table I), while in the same experiments, it lowered the zymosan-induced production of nitrate measured after nitrate reduction (Table I).

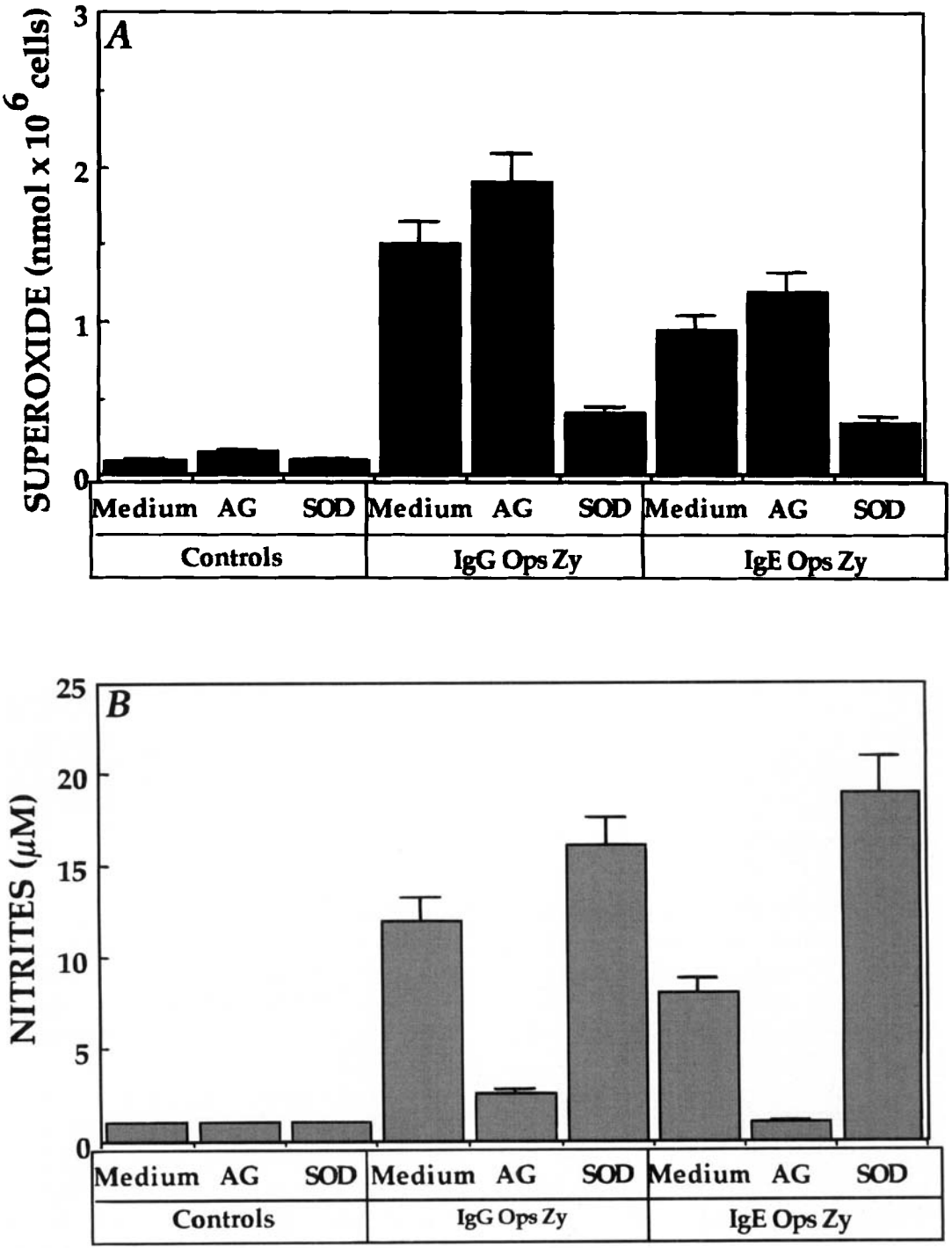

FIGURE 2 Production of oxidative metabolites in RA/VD3-treated U937 cells activated by Normal serum Ops Zy or Hyper IgE serum Ops Zy. U937 cells treated for 4 days in the presence of RA and VD3 were cultured for further 48h at 37°C at 10^6cells/ml in Iscove medium supplemented with 10%FCS (or for 2h in PBS supplemented with ferricytochrome c) in the presence or absence of 50 μg/ml zymosan opsonized with a normal serum or an hyper IgE serum. When indicated, 1.5 mM aminoguanidine (AG) or 30 U/mL SOD were added to the cell culture 10 min before zymosan. The nitrite concentrations were evaluated in the different cell supernatants 48h after the addition of opsonized zymosan. In parallel experiments, superoxide production was measured for two hours with ferricytochrome c in cells (10^6/ml RPMI without phenol red) which received the same additives. The data are the mean ± SEM of 3 different experiments.

TABLE I Nitrate and nitrate production by RA/VD-treated U937 cells activated by opsonized zymosan

CELL TREATMENT	$NO2^-$ (μM)	$(NO2^- + NO3)^-$ μM
None	2.5±0.5	5.4±0.8
L-NMMA	0.7±0.1	4.0±0.1
SOD	1.9±0.2	4.3±0.6
IgG Ops Zy	9.4±0.9	20.4±0.9
IgG Ops Zy + L-NMMA	0.7±0.3	6.5±0.6
IgG Ops Zy + SOD	13.1±0.7	16.7±0.8
IgE Ops Zy	10.5±0.6	23.0±0.6
IgE Ops Zy + L-NMMA	1.8±0.4	5.4±0.4
IgE Ops Zy + SOD	15.9±0.7	18.7±0.6

U937 cells treated for 4 days in the presence RA and/or VD3 were cultured for further 48h at 37°C at 10^6 cells/ml in Iscove medium supplemented with 10%FCS in the presence or absence of 50μg/ml zymosan opsonized with a normal serum or an hyper IgE serum. When indicated, 2.5 mM L-NMMA or 30 U/ml SOD were added to the cell culture 10 min before Ops Zy. The nitrite and (nitrite + nitrate) concentrations were evaluated in the different cell supernatants 48h after addition of opsonized zymosan. Data are the mean +/− SEM of 4 experiments.

Similar data were obtained in RA/VD3-differentiated THP-1 cells (not shown).

Relative Contribution of ·NO and $O_2^{\cdot-}$ to the LDCL of RA/VD-treated U937 (or THP-1) Cells Treated with Opsonized Zymosan

Upon addition of Normal serum Ops Zy, the luminol-dependent chemiluminescence of RA/VD3- treated U937 cells (Figure 3A) (or differentiated THP-1 cells, Figure 3B) increased 30–50 times over its initial level in a time-dependent way whereas non differentiated cells gave no response (not shown and[21–24]). The maximal effect was reached after 8–10 min. When cells were pretreated for 10 min with aminoguanidine before Normal serum Ops Zy addition, the iNOS inhibitor strongly diminished the LDCL observed, both rate and peak intensities were affected. The inhibitory effect of aminoguanine was dose-dependent (Figure 4), the maximal decrease of light emission (40–50%) being found for inhibitor concentrations close to 1.5 mM. The LCDL elicited in differentiated cells by Normal serum Ops Zy was also diminished by L-NMMA (Figure 3); in these experiments the maximal inhibition occured at 2.5 mM L-NMMA (Figure 4).

As previously found for the measurement of $O_2^{\cdot-}$ steady-state concentration (Figure 1), SOD strongly inhibited by LDCL of macrophage-like cells treated by Normal serum Ops Zy (Figure 3). Maximal inhibition of LDCL (80–85%) was observed in the presence of 30 U/ml SOD. When combined together, SOD and aminoguanidine completely abrogated the LDCL of the activated cells. Data similar to those of Figure 3 were obtained when Hyper IgE serum Ops Zy substituted for Normal serum Ops Zy.

Effect of iNOS Inhibitors on the Luminol Chemiluminescence Induced in a Cell-Free System

The Xanthine/Xanthine oxidase (X/Xo) system is an efficient producer of $O_2^{\cdot-}$. It was reporter that at concentrations up to 1 mM, L-NMMA did not exert a scavenger effect on $O_2^{\cdot-}$ production by the X/Xo system.[18] We repeated this experiment, and in addition we observed that aminoguanine was also unable to affect the X/Xo-induced steady-state concentration of $O_2^{\cdot-}$ produced (data not shown). In certain experimental conditions, the X/Xo cell-free system also induced luminol chemiluminescence.[23] We observed that the X/Xo-dependent chemiluminescence was neither affected by aminoguanidine nor by L-NMMA, while it was inhibited by 30 U/ml SOD (Figure 5).

DISCUSSION

Upon their differentiation through the monocyte/macrophage pathway, the human promonocytic cells U937 and THP-1 acquire the capacity to phagocytize, to produce $O_2^{\cdot-}$ and to kill parasites.[20–22,24] Here, we show that the differentiated cells which expressed iNOS mRNAs have also acquired the ability to produce. ·NO through iNOS activation when they phagocytized Normal serum Ops Zy or Hyper IgE

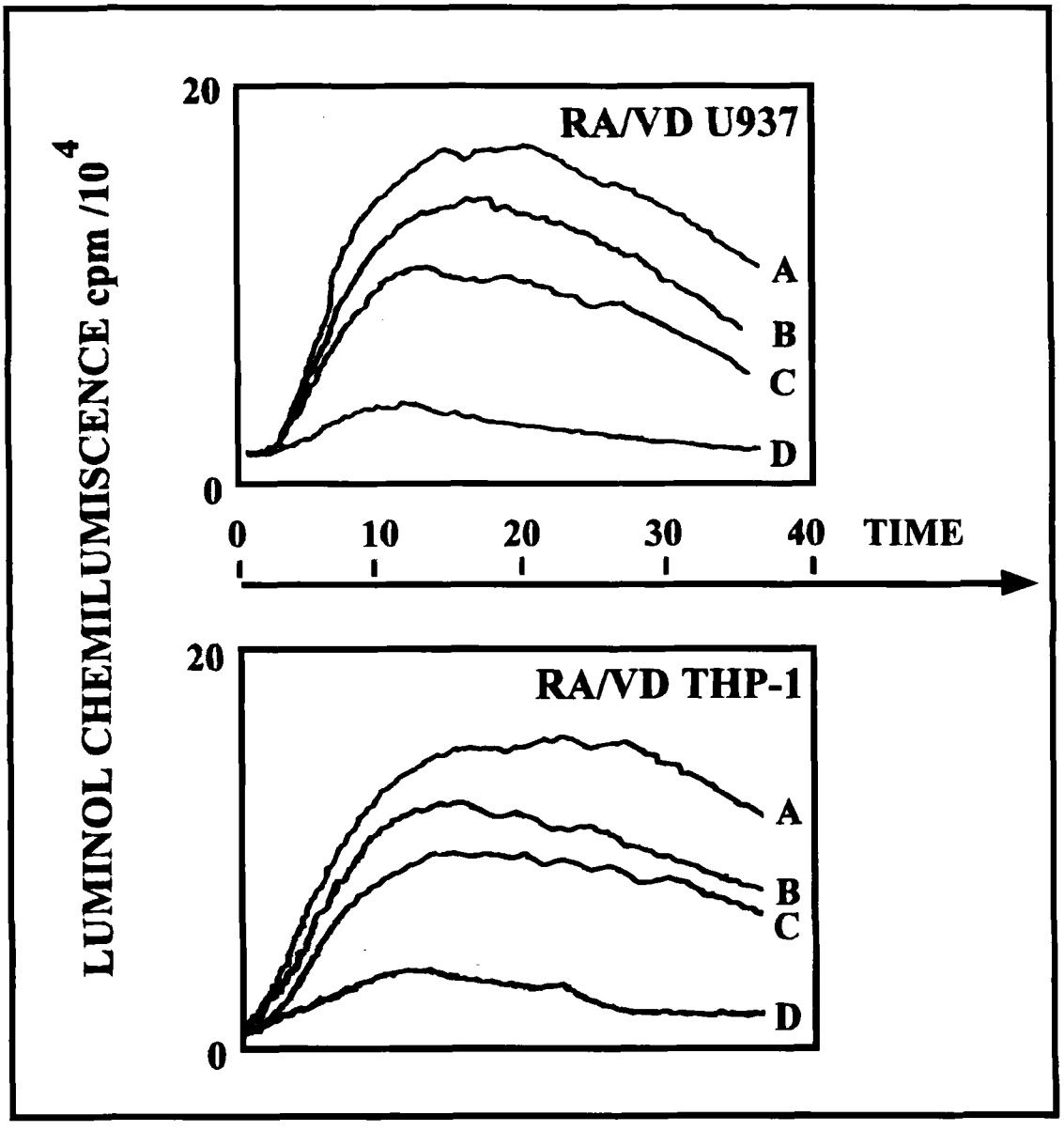

FIGURE 3 Kinetics of the luminol dependent chemiluminescence of RA/VD3-treated U937 or THP-1 cells with Normal serum Ops Zy. The chemiluminescence induced in RA/VD3-treated cells by Normal serum Ops Zy. was analyzed as described in Materials and Methods **(A)**. Effect of 2.5 mM L-NMMA **(B)**, 1.5 mM aminoguanidine **(C)** and 30U/ml SOD **(D)**. The cells (2.5×10^5/ml) were incubated at 37°C with iNOS inhibitors 15 min before addition of Normal serum Ops Zy, SOD was added just before the stimulus. Reading were started 1 min before the addition of the stimulus (50 μg/ml Ops Zym) and continued for up to 40 min. Luminol final concentration was 3 mM. Data are from one experiment representative of more than 10.

serum Ops Zy, i.e. after ligation of Fc receptors expressed on mature cells. This result demonstrates for the first time an opsonized zymosan-induced iNOS activation in human macrophagic cells, and is in line with a recent report showing $^{\bullet}NO$ production upon Fcγ receptor-triggering in murine macrophages.[25] The fact that in our experiments, SOD enhanced the concentration of

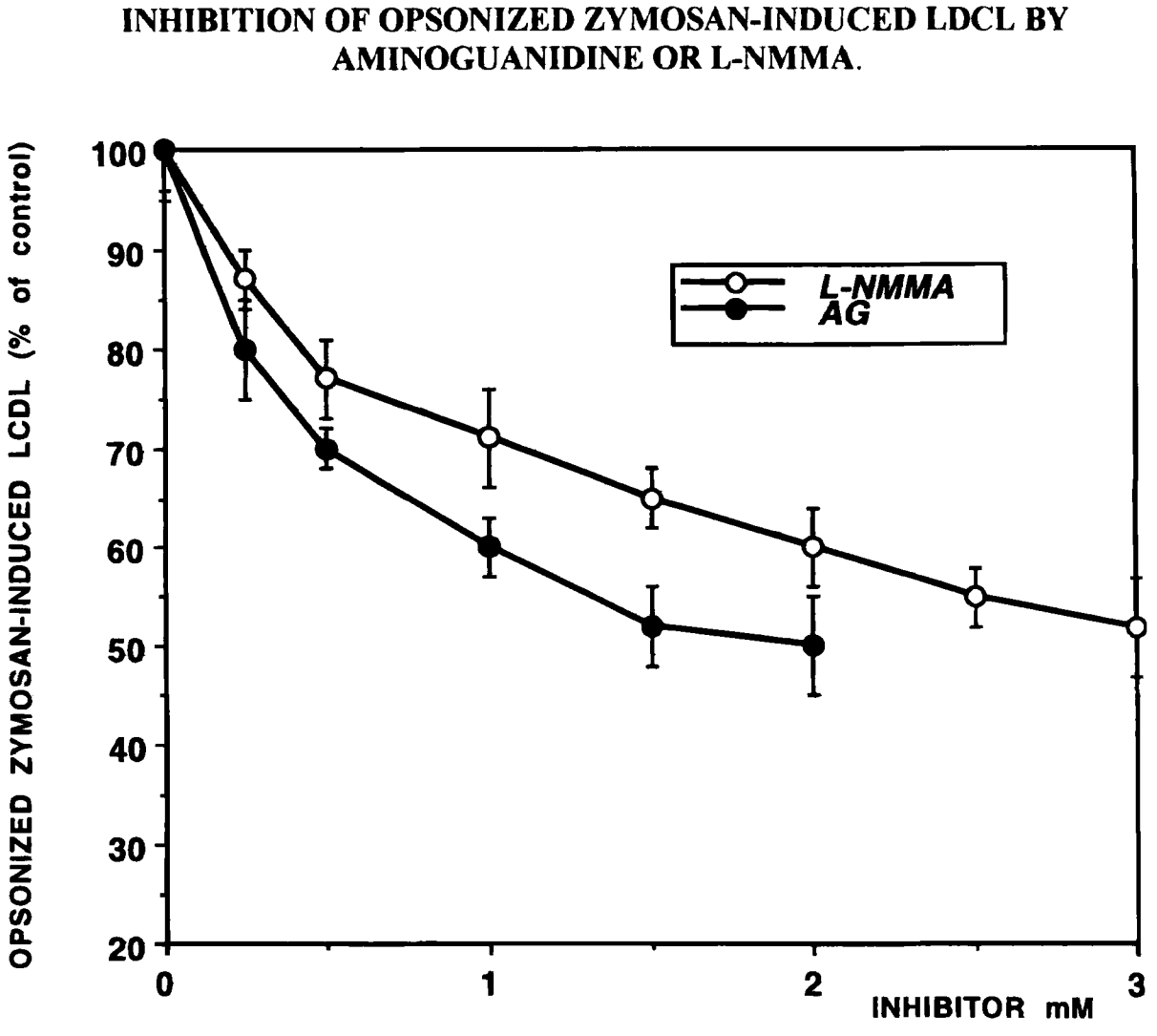

FIGURE 4 Effect of aminoguanidine and L-NMMA on LDCL induced by Normal serum Ops Zy in RA/VD3-treated U937 cells. RA/VD3-treated U937 cells ($2.5\,10^5$/ml) were incubated at 37 °C for 15 min with or without different concentrations of iNOS inhibitors in the presence of luminol (final concentration 50 mM). 50mg/ml of Ops Zym was then added to the cell suspension and the chemiluminescence was monitored during 45 min as described in Figure 3. The integral counts were evaluated. Results are mean ±SEM of 5 different experiments and expressed as % of control value in the absence of inhibitors.

nitrite accumulated in the Ops Zy-activated cell supernatants, and aminoguanidine or L-NMMA increased the steady-state concentration of $O_2^{\cdot-}$ measured, led us to suspect an interaction between the 'NO and $O_2^{\cdot-}$ released.

Ops Zy-activated cells induced a potent LDCL,[20–22] which was not only dramatically suppressed by SOD, but also partially inhibited (up to 50 %) by aminoguanidine or L-NMMA. As the iNOS inhibitors did not directly scavenge $O_2^{\cdot-}$ in either a cell-free system[18] or in activated cells, and did not affect the X/Xo-induced LDCL, the data show that the Ops Zy-triggered LDCL involved the generation of 'NO. This phenomenon finally demonstrated an activation of iNOS in the first minutes of Ops Zy phagocytosis, as

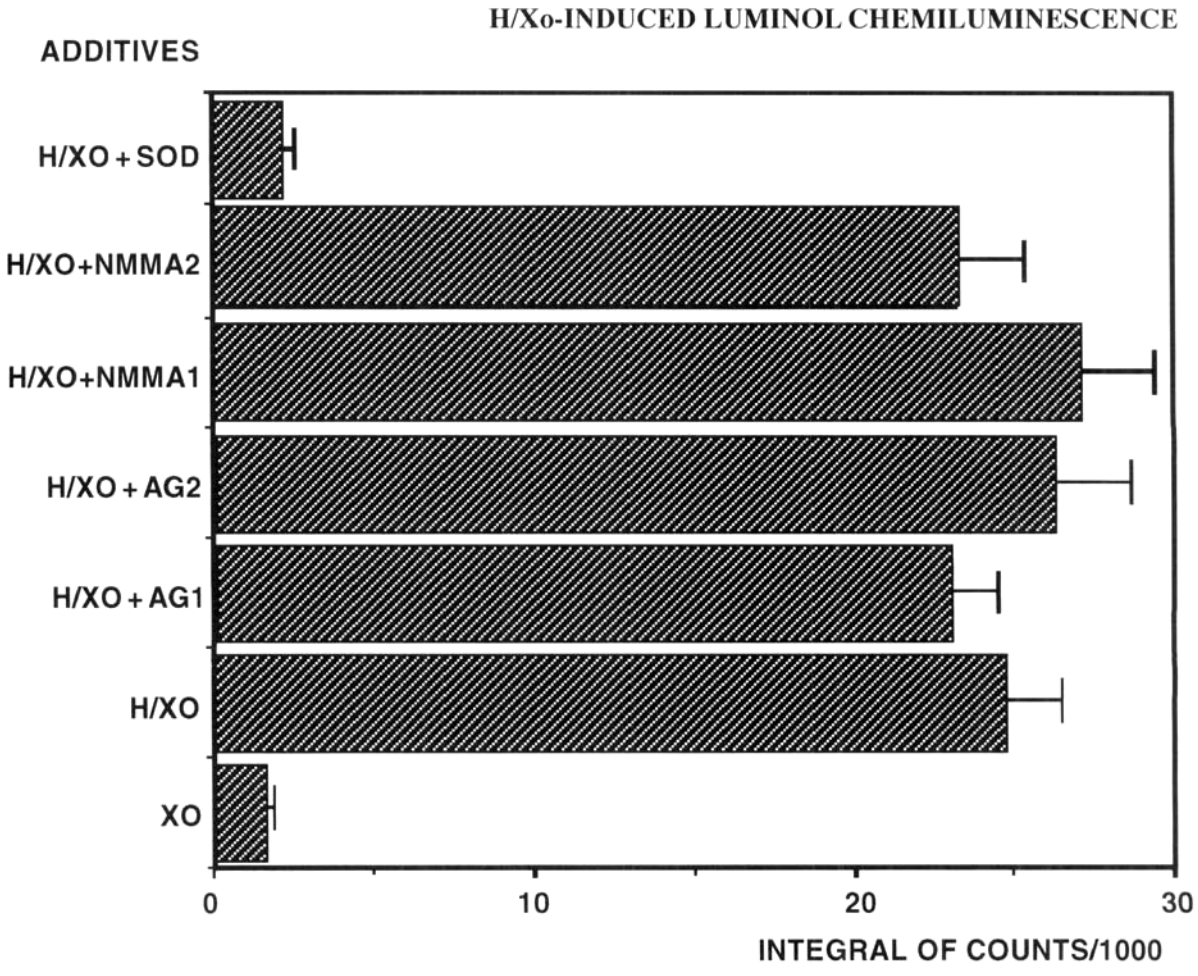

FIGURE 5 Effect of aminoguanidine, L-NMMA and SOD on the luminol chemiluminescence induced in the Xanthine/Xanthine oxidase system. At time 0, 0.25 mM hypoxanthine was added (H/Xo) or not (Xo) to a reaction mixture which contained 1.50 U/ml Xo and 50 mM luminol in 0.05 M carbonate buffer pH 8.5 at 37°C in the presence or absence of different additives: 1 mM (AG1) or 2 mM (AG2) aminoguanidine, 1.5 mM (NMMA1) or 3 mM (NMMA2) L-NMMA. The luminol chemiluminescence was then monitored for 15 min. Results are expressed in integral counts. Data are from one experiment representative of three in which luminescence measurements were performed in triplicates.

well as NADPH oxidase stimulation. As shown with SOD and/or iNOS inhibitors, iNOS activation was not sufficient by itself to induce LDCL. In fact, $^{\bullet}NO$ exacerbated the SOD-dependent luminol response pointing to the formation of $^{\bullet}NO$ byproduct which contributed to luminol chemiluminescence. The reactive anion $ONOO^-$ might be this byproduct: when $^{\bullet}NO$ and $O_2^{\bullet-}$-generating reactions were simultaneously present in a cell-free system, they promoted a LDCL higher than the $O_2^{\bullet-}$-generating reaction alone: the $ONOO^-$ formed interacts with luminol to produce an instable luminol intermediate and a potent emission of light,[17] moreover SOD inhibits LDCL in such cell-free systems.[26] Other findings are also in line with $ONOO^-$ formation: 1) The guanidine inhibitors of iNOS are also scavengers of $ONOO^-$.[27] Thus, aminoguanidine could have affected the Ops Zy-induced LDCL at several levels, which should explain why it is a

better inhibitor of LDCL than L-NMMA. 2) In supernatants of cells activated by Ops Zy in the presence of SOD, the concentration of $NO_2^{\cdot-}$ increased, probably because there was no formation of $ONOO^-$ and more ·NO available. On the contrary, the concentration of $NO_3^{\cdot-}$ which directly came from $ONOO^-$, decreased (Table I). A participation of $ONOO^-$ in LDCL has been postulated in other biological systems: in PMA-activated Kupffer cells,[26] in endothelial cells,[28] in PMA-activated human PMN,[18] and recently in IFNγ-treated rodent macrophages activated by immune complexes.[19,29]

Due to the scavenging property of SOD, NADPH oxidase is considered to be responsible for the LDCL induced by Ops Zy-activated phagocytes. Our present data on macrophagic cells and those of others on PMN[18] disagree with this proposal. The ·NO-derived product being a critical mediator of cell-derived luminol chemiluminescence, the inhibitory effect of SOD depends both on inhibition of its formation by preventing the reaction of $O_2^{\cdot-}$ with ·NO, and on inhibition of $O_2^{\cdot-}$-mediated oxidation of luminol radical.

Recently, it was observed that lucigenin, which has frequently been utilized for detection of $O_2^{\cdot-}$, was reduced univalently by intracellular enzymes and that the resultant lucigenin cation radical autoxidized generating $O_2^{\cdot-}$.[30–31] This established that lucigenin cannot be used to measure $O_2^{\cdot-}$ formation. In parallel, our data focus attention on cells where the intracellular enzymes, NADPH oxidase and iNOS are simultaneously activated, SOD-dependent LDCL is not a reliable measure of the sole NADPH oxidase activity, iNOS and NADPH oxidase inhibitors can help to correctly analyze the phenomenon. Indeed, LDCL determination in the presence or absence of SOD and/or iNOS inhibitors showed: 1) that Ops Zy triggered iNOS activity and ·NO production in human mature macrophages, 2) that iNOS was directly and rapidly stimulated, the kinetics could not be observed by measurement of nitrite (or L-citrulline) in the supernatants of cells activated for several hours 3) that in human macrophages, when iNOS and NADPH oxidase were concomitantly activated, their products ·NO and $O_2^{\cdot-}$ interacted together to generate a ·NO-mediated intermediate, possibly $ONOO^-$ LDCL determination appears in fact as a convenient assay to demonstrate the triggering of iNOS activity in mature macrophagic cells and gives complementary information to experiments measuring the steady state concentrations of $O_2^{\cdot-}$ and ·NO produced.

In macrophages, ·NO release has been proposed as a host antimicrobial effector system, displaying activity against fungi, bacteria, and parasites.[2–8] However, most of the published data concern rodents. Despite a controversy on the subject,[9] it was claimed that reactive nitrogen intermediates were produced in infections of human macrophages with mycobacteria[32] and human immunodeficiency virus[33] and that ·NO participated to the killing of *Leishmania major* induced after ligation of the FcεRII/CD23 surface antigen.[13] Moreover, the nitration of ingested bacteria during their phagocytosis by cytokine-treated human neutrophils, which might result from the synthesis of $ONOO^-$[34] or from other ·NO-derived nitrating species,[35] demonstrated a production of ·NO, deleterious for the parasite in a human phagocyte.

Finally, our data support the possibility of ·NO-derived microbicidal activity in human genuine specialized macrophages in a well-defined stage of differentiation. They showed the concomitant release of ·NO and $O_2^{\cdot-}$ after Fc receptor ligation, an event occuring during the phagocytosis of opsonized parasite and established an interaction between these radicals leading to the formation of a reactive metabolite which might be $ONOO^-$, a highly bactericidal anion.[36] In addition, in activated phagocytes, ·NO production is a process which must be taken into account in LDCL measurement, and LDCL is an indicator of oxidant production which must be cautiously considered.

Acknowledgements

This work has been supported by grants from INSERM, IREB (Institut de Recherche sur les Boissons). AG was supported by a fellowship from ARC.

References

[1] Pick, E. (1986). Microassays for superoxide and hydrogen peroxide production and nitroblue tetrazolium reduction using an enzyme immunoassay microplate reader. *Methods in Enzymology*, **132**, 407–421.

[2] Granger, D., Hibbs, J. J., Perfect, J. and Durack, D. (1988). Specific amino acid (L-arginine) requirement for the microbiostatic activity of murine macrophages. *Journal of Clinical Investigation*, **81**, 1129–1136.

[3] Denis, M. (1991). Interferon γ-treated murine macrophage inhibit growth of tubercle bacilli via the generation of reactive nitrogen intermediates. *Cellular Immunology*, **132**, 150–157.

[4] James, S. and Glaven, J. (1990). Macrophage cytotoxicity against schistosomula of *Schistosoma mansoni* involves arginine-dependent production of reactive nitrogen intermediates. *Journal of Immunology*, **143**, 4208–4212.

[5] Adams, H., Hibbs, J. J. Jr., Taintor, R. R. and Krahenbuhl, J. L. (1990). Microbiostatic effect of murine activated macrophages for toxoplasma gondii. Role for synthesis of inorganic nitrogen oxides from L-arginine. *Journal of Immunology*, **144**, 2725–2729.

[6] Liew, F. Y., Millott, S., Parkinson, C., Palmer, R. and Moncada, S. (1990). Macrophage killing of Leishmania parasite in vivo is mediated by nitric oxide from L-arginine. *Journal of Immunology*, **144**, 4794–4797.

[7] Moncada, S., Palmer, R. M. J. and Higgs, E. A. (1991). Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacological Review*, **143**, 109–142.

[8] Liew, F. Y. and Cox, F. E. (1991). Nonspecific defence mechanism: the role of nitric oxide. *Immunology Today*, **6**, A17–A21.

[9] Scheemann, M., Schoedon, G., Hofer, S., Blau, N., Guerrero, L. and Schaffner, A. (1993). Nitric oxide synthase is not a component of the antimicrobial armature of human mononuclear phagocytes. *Journal of Infectious Disease*, **167**, 1358–1363.

[10] Mannick, J. B., Asano, K., Izumi, E. K. and Stamler, J. S. (1994). Nitric oxide produced by B lymphocytes inhibits apoptosis and Epstein Barr virus reactivation. *Cell*, **79**, 1137–1146.

[11] Ouaaz, F., Sola, B., Issaly, F., Kolb, J. P., Davi, F., Mentz, F., Arock, M., Paul-Eugène, N., Körner, M., Dugas, B., Debré, P. and Mossalayi, M. D. (1994). Growth arrest and terminal differentiation of leukemic myelomonocytic cells induced through litigation of the CD23 surface antigen. *Blood*, **84**, 3095–3104.

[12] Paul-Eugène, N., Kolb, J. P., Sarfati, M., Arock, M., Ouaaz, F., Debré, P., Mossalayi, M. D. and Dugas, B. (1995). Ligation of CD23 activates soluble guanylate cyclase in human monocytes via an L-arginine-dependent mechanism. *Journal of Leukocyte. Biology*, **57**, 160–167.

[13] Vouldoukis, I., Riveros-Moreno, V., Dugas, B., Ouaaz, F., Bécherel, P., Debré, P., Moncada, S. and Mossalayi, M. D. (1995). The killing of *Leishmania major* by human macrophages is mediated by nitric oxide induced after ligation of the FcεRII/CD23 surface antigen. *Proceedings of the National Academy of Science of the USA*, **92**, 7804–7808.

[14] Dugas, B., Debré, P. and Moncada, S. (1996). Nitric oxide a vital poison inside the immune and inflammatory network *Research in Immunology*, **146**, 664–670.

[15] Paul-Eugène, N., Mossalayi, M., Sarfati, M., Yamaoka, K., Aubry, J. P., Bonnefoy, J. Y., Dougas, B. and Kolb, J. P. (1995). Evidence for a role of FcεRII/CD23 in the IL-4-induced nitric oxide production by normal human mononuclear phagocytes. *Cellular Immunology*, **163**, 314–318.

[16] Lopez-Moratalla, N., Gonzalez, A., Aymerich, M. S., Lopez-Zabalza, M. H., Pio, R., de Castro, P. and Santiago, E. (1997). Monocyte inducible nitric oxide synthase in multiple sclerosis: regulatory role of nitric oxide. *Nitric Oxide*, **1**, 95–104.

[17] Radi, R., Cosgrove, T., Beckman, J. S. and Freeman, B. A. (1993). Peroxynitrite-induced luminol chemiluminescence. *Biochemical Journal*, **290**, 51–57.

[18] Catz, S. D., Carreras, M. C. and Poderoso, J. J. (1995). Nitric oxide synthase inhibitors decrease human polymorphonuclear leukocyte luminol-dependant chemiluminescence. *Free Radical Biology and Medicine*, **19**, 741–748.

[19] Muto, S., Sumi, K., Inage, Y., Matsumoto, K. and Yuki, H. (1996). Nitric oxide and immune complexes are involved in the induction of novel luminol chemiluminescence in cytotoxic macrophages. *Biological and Pharmacological Bulletin*, **19**, 1521–1523.

[20] Dugas, N., Mossalayi, M. D., Calenda, A., Leotard, A., Becherel, P., Mentz, F., Ouaz, F., Arock, M., Debré, P., Dornand, J. and Dugas, B. (1996). Role of nitric oxide in the antitumoral effect of retinoic acid and 1,25-dihydrovitamin D3 on human promocytic leukemic cells. *Blood*, **88**, 3528–3534.

[21] Defacque, H., Dornand, J., Commes, T., Cabane, S., Sevilla, C. and Marti, J. (1994). Different combinations of retinoids and vitamin D3 analogs efficiently promote growth inhibition and differentiation of myelomonocytic leukemia cell lines. *Journal of Pharmacological and Experimental Therapeutics*, **271**, 193–196.

[22] Caron, E., Gross, A., Liautard, J. P. and Dornand, J. (1996). *Brucella* species release a specific, protease-sensitive inhibitor of TNF-α expression active on human macrophage-like cells. *Journal of Immunology*, **257**, 2885–2893.

[23] Radi, R., Rubbio, H., Thomson, L. and Prodanov, E. (1990). Luminol chemiluminescence using xanthine and hypoxanthine as xanthine oxidase substrates. *Free Radical Biology and Medicine*, **8**, 121–126.

[24] Taimi, M., Defacque, H., Commes, T., Favero, J., Caron, E., Marti, J. J. and Dornand, J. (1993). Effect of retinoic acid and vitamin D on the expression of interleukin-1β, tumor necrosis factor α and interleukin-6 in the human monocytic cell line U937. *Immunology*, **79**, 229–235.

[25] Mozaffarian, N., Berman, J. W. and Casadevall, A. (1995). Immune complexes increase nitric oxide production by interferon-γ-stimulated murine macrophage-like J774 A. 1 cells. *Journal of Leukocyte Biology*, **57**, 657–662.

[26] Wang, J. F., Komarov, P., Sies, H. and de Groot, H. (1991). Contribution of nitric oxide synthase to luminol-dependent chemiluminescence generate by phorbol-ester-activated Kupffer cells. *Biochemical Journal*, **279**, 311–314.

[27] Szabo, C., Ferrer-Sueta, G., Zingarelli, B., Southan, G. J. and Radi, R. (1997). Mercaptoethylguanidine and guanidine inhibitors of nitric-oxide synthase react with peroxynitrite and protect against peroxinitrite-induced oxidative damage. *Journal of Biological Chemistry*, **272**, 9030–9036.

[28] Saran, M., Michel, C. and Bors, W. (1990). Reaction of •NO with $O_2^{\bullet-}$. Implications for the action of endothelium-derived relaxing factor(EDRF). *Free Radical Research Communication*, **10**, 221–226.

[29] Wang, J. F., Komarov, P. and de Groot, H. (1993). Luminol chemiluminescence in rat macrophages and granulocytes: The role of •NO, $O_2^{\bullet-}$/H2O2 and HOC1. *Archives of Biochemistry and Biophysics*, **304**, 189–196.

[30] Liochev, S. I. and Fridovich, I. (1997). Lucigenin (Bis-*N*-methylacridinium) as a mediator of superoxide anion production. *Archives of Biochemistry and Biophysics*, **337**, 115–120.

[31] Liochev, S. I. and Fridovich, I. (1997). Lucigenin luminescence as a measure of intracellular superoxide dismutase activity in *Escherichia coli*. *Proceedings of the National Academy of Sciences of the USA*, **94**, 2891–2896.

[32] Dumarey, C. H., Labrousse, V., Rastogi, N., Vargaftig, B. B. and Bachelet, M. (1994). Selective Mycobacterium avium-induced production of nitric oxide by human monocyte-derived macrophages. *Journal of Leukocyte Biology*, **56**, 36–40.

[33] Bukrinsky, M., Nottel, H. S. L. M., Schmidtmayerova, H., Dubrovsky, L., Flanagan, C., Mullins, M. E., Lipton, S. A. and Gendelman, H. E. (1995). Regulation of nitric oxide synthase activity in human immunodeficiency virus type 1 (HIV-1)-infected monocytes: implications for HIV-associated neurological disease. *Journal of Experimental Medicine*, **181**, 735–745.

[34] Evans, T. J., Buttery, L. D. K., Carpenter, A., Springall, D. R., Polak, J. M. and Cohen, J. (1996). Cytokine-treated human neutropils contain indicible nitric oxidase that produces nitration of ingested bacteria. *Proceedings of the National Academy of Sciences of the USA*, **93**, 9553–9558.

[35] Eiserich, J. P., Cross, C. E., Jones, A. D., Halliwell, B. and van der Vliet, A. (1996). Formation of nitrating and chlorinating species by reaction of nitrite with hypochlorous acid. *Journal of Biological Chemistry*, **271**, 19199–19208.

[36] Zhu, L., Gunn, C. and Beckman, J. S. (1992). Bactericidal activity of peroxynitrite. *Archives of Biochemistry and Biophysics*, **298**, 452–457.