

# Cryptic O<sub>2</sub><sup>-</sup>-generating NADPH oxidase in dendritic cells

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## Summary

All the components of the O<sub>2</sub><sup>-</sup>-generating NADPH oxidase typically found in neutrophils, namely a membrane-bound low potential flavocytochrome *b* and oxidase activation factors of cytosolic origin, are immunodetectable in murine dendritic cells (DCs). However, in contrast to neutrophils, DCs challenged with phorbol myristate acetate (PMA) can barely mount a significant respiratory burst. Nevertheless, DCs generate a substantial amount of O<sub>2</sub><sup>-</sup> in the presence of PMA following preincubation with pro-inflammatory ligands such as lipopolysaccharide and pansorbin, and to a lesser extent with anti-CD40 or polyinosinic polycytidylic acid. We found that the virtual lack of the oxidase response to PMA alone is specifically controlled in DCs. Through the use of homologous and heterologous cell-free systems of oxidase activation, we showed the following: (1) a NADPH oxidase inhibitory factor is located in DC membranes; it exerts its effect on oxidase activation and not on

the activated oxidase. (2) The inhibition is relieved by pretreatment of DC membranes with β-octylglucoside (β-OG). (3) The β-OG-extracted inhibitory factor prevents the activation of neutrophil oxidase. (4) The inhibitory activity is lost after treatment of DC membranes with proteinase K or heating, which points to the protein nature of the inhibitory factor. Overall, these data indicate that the O<sub>2</sub><sup>-</sup>-generating oxidase in DCs is cryptic, owing to the presence of a membrane-bound inhibitor of protein nature that prevents oxidase activation. The inhibition is relieved under specific conditions, including a prolonged contact of DCs with pro-inflammatory ligands from microbial origin, allowing a substantial production of O<sub>2</sub><sup>-</sup>, which may contribute to the response of DCs to a microbial exposure.

Key words: Dendritic cell, NADPH oxidase, ROS, Superoxide anion

## Introduction

Dendritic cells (DCs) are professional antigen-presenting cells (reviewed by Banchereau and Steinman, 1998; Mellman and Steinman, 2001; Turnbull and MacPherson, 2001; Rosenzweig et al., 2002; Zitvogel, 2002). Their morphological and functional properties depend on their maturation state. Immature DCs present in non-lymphoid tissues have the capacity to undergo intensive endocytic activity. They can detect, engulf and process soluble and particulate antigens, including bacteria and fungi (Svensson et al., 1997; Chen et al., 1998; Garrett et al., 2000; Gildea et al., 2001). Internalized antigens are converted to short peptides by proteolysis in endosomes and phagolysosomes. The resulting peptides are loaded mainly on the major histocompatibility complex II (MHC II). At the onset of maturation, DCs migrate to secondary lymphoid tissues, where they trigger a specific T-cell response; dendrites appear on the cell surface and DCs lose the capacity to phagocytose antigens, whereas they acquire the capacity to present MHC-peptide complexes to naive T cells that become activated. DCs can also interact with B cells (Dubois et al., 1999) and natural killer cells (Gerosa et al., 2002). They can also present self-antigens and anergise autoreactive T cells (Steinman and Nussenzweig, 2002). The

high capacity of immature DCs to endocytose foreign antigens resembles that of neutrophils and macrophages. Since the latter cells have the capacity to produce large amounts of the superoxide anion O<sub>2</sub><sup>-</sup> and derived microbicidal reactive oxygen species (ROS) through the activation of an NADPH oxidase complex (for reviews, see Dinauer, 1993; De Leo and Quinn, 1996; Vignais, 2002), we wondered whether DCs contained a similar oxidase complex. The neutrophil and macrophage NADPH oxidase comprises a membrane-bound flavocytochrome *b*, that is the catalytic core of the enzyme, and associated proteins of cytosolic origin, the function of which is to activate the flavocytochrome *b*. The membrane-bound flavocytochrome *b* consists of two subunits, a small protein of 22 kDa, p22phox (phox for phagocyte oxidase) and a large glycoprotein of 90-100 kDa, gp91phox, also referred as Nox2 in the recent literature. The Nox2 subunit contains the redox carriers, one FAD and two low potential hemes *b*, needed for the transfer of electrons from NADPH to O<sub>2</sub>. The flavocytochrome *b*-associated cytosolic proteins are a small G protein Rac and a triad of phox proteins of 47 kDa, 67 kDa and 40 kDa, termed p47phox, p67phox and p40phox, respectively (reviewed by Vignais, 2002).

During the course of an investigation on the comparative

distribution of the NADPH oxidase components in different types of leukocyte cells, we found that murine DCs are equipped with all the components of the oxidase complex. Whereas DCs were found to express p47phox, p67phox, p40phox, p22phox and the deglycosylated form of Nox2, O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> production was barely detectable under conditions known to be effective in the case of neutrophils. Here, we demonstrate the presence in DC membranes of an inhibitory factor that is responsible for the lack of NADPH oxidase activation, and provide evidence for the relief of the oxidase inhibition after a few hours contact with microbial pro-inflammatory ligands, e.g. lipopolysaccharide (LPS) and pansorbin (PSB). Anti-CD40, an antibody that mimics a contact with T cells, and polyinosinic polycytidylic acid (poly I:C), an analogue of double-stranded RNA (dsRNA), could replace the pro-inflammatory ligands although the oxidase response was less acute.

## Materials and Methods

### Reagents

Arachidonic acid, cytochrome *c*, diisopropylfluorophosphate, homovanillic acid (4-hydroxy-3-methoxyphenyl acetic acid; HVA), horseradish peroxidase (HRP) type II, phorbol 12-myristate 13-acetate (PMA), superoxide dismutase (SOD), lipopolysaccharide (LPS), pansorbin (PSB), nitroblue tetrazolium (NBT) and phenosafranin were from Sigma-Aldrich (Saint Quentin Fallavier, France). Arachidonic acid was dissolved in ethanol and stored under N<sub>2</sub> at -80°C until used.  $\beta$ -octylglucoside ( $\beta$ -OG) was from Alexis (Coger, Paris, France). NADPH, leupeptin and guanosine-5'-O-(3-thiotriphosphate) (GTP $\gamma$ S) were from Roche (Meylan, France). Hydrogen peroxide 30% (v/v) and the fluorSave<sup>TM</sup> reagent were from Calbiochem (La Jolla, CA). Protein A-horseradish peroxidase conjugate was from Biorad (Ivry sur Seine, France). Cy3 donkey anti-rabbit IgGs were from the Jackson Immunoresearch laboratories (West Grove, PA). The enhanced chemiluminescence (ECL) detection kit and polyinosinic polycytidylic acid (poly I:C) were from Amersham Pharmacia Biotech (Orsay, France). Nitrocellulose membranes were from Schleicher & Schuell (Ecquevilly, France).

### Antibodies

Polyclonal antisera directed against the C-terminal sequences of both human gp91phox (anti-gp91phox) and p22phox (anti-p22phox) were obtained from G. Brandolin (CEA-Grenoble, France) and used as previously described (Doussière et al., 1993). Preparation of polyclonal antibodies directed against the full length p40phox protein (anti-p40phox), the C-terminal 18 amino acid residues of p47phox (anti-p47Cter) and the full length p67phox protein (anti-p67phox) were reported previously (Fuchs et al., 1997). Monoclonal anti-CD40 and anti-MHC II antibodies were from Pharmingen (San Diego, CA).

### Culture of dendritic cells from splenic progenitors

DCs derived from BALB/c murine splenic progenitors were grown in an Iscove DMEM medium supplemented with 10% fetal calf serum (FCS), GM-CSF and Flt-3 ligand (Flt-3L) as previously described (Berthier et al., 2000; Martinon-Ego et al., 2001). On day 12, the cell population consisted of approximately 60% immature DCs, 30% mature DCs and 10% macrophages as assessed by flow cytometry analysis with anti-MHC II antibodies. Macrophage contaminants were removed by plastic adhesion for 1 hour in fresh culture medium, immediately before harvesting the cells for biochemical investigations. The contaminating macrophages in the final DC preparation were estimated to be less than 1%.

### DC isolation from mouse spleen (sDCs)

In some experiments, sDCs were directly isolated from BALB/c mouse spleen. In others, to increase the yield of sDCs, peritoneal injections in BALB/c mice were performed daily for nine consecutive days with 10  $\mu$ g of recombinant human Flt-3L (human Chinese Hamster Ovary cell derived, kindly provided by Immunex, Seattle, WA). In both cases, sDCs were purified as described (Maldonado-Lopez et al., 2001; Rizzitelli et al., 2002). The spleen tissue was treated with collagenase in Ca<sup>2+</sup>/Mg<sup>2+</sup>-free medium to dissociate the cells. The low density cells enriched in DCs were separated by centrifugation on a Nycodenz gradient (Nycodem, Oslo, Norway) and withdrawn. They were incubated with biotinylated anti-CD11c antibodies and streptavidin magnetic microbeads (Miltenyi Biotec, Paris, France), and then purified using the MACS isolation kit. The CD11c<sup>+</sup> cell population contained 95-97% sDCs; most of them (>95%) were in an immature state.

### Preparation of cytosolic and membrane fractions

DCs were washed with PBS pH 7.4 that consisted of 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, supplemented with 10  $\mu$ g/ml leupeptin and 1 mM diisopropylfluorophosphate. The cell suspension at a concentration of 10<sup>8</sup> cells/ml was placed on ice, and the cells were disrupted by sonication four times for 30 seconds at 10 W output. After a 5 minute centrifugation at 10,000 g, the supernatant fraction was withdrawn and centrifuged at 300,000 g for 30 minutes at 4°C in a TL-100 centrifuge. The supernatant was referred to as cytosol, and the membranes were resuspended in an equal volume of PBS. A purified membrane fraction of DCs was prepared as follows. After sonication, the cell particles were subfractionated by a 30 minute centrifugation at 150,000 g on a sucrose gradient consisting of two sucrose layers of 15% and 40% (w/v) in 50 mM Tris-HCl pH 7.4 supplemented with 1 mM EDTA. The cytosolic fraction corresponded to the supernatant fluid. The membrane fraction was collected at the 15-40% sucrose interface, diluted 2.5 times with PBS and pelleted by centrifugation for 30 minutes at 300,000 g in a TL-100 rotor. Membrane and cytosolic fractions from neutrophils were prepared as described (Doussière et al., 1996). Protein concentration was assayed with the bicinchoninic acid reagent (Pierce, Bezons, France) in the presence of 0.1% sodium dodecyl sulfate (SDS), using bovine serum albumin as standard.

### KCl extract and $\beta$ -OG extract of DC membranes

The KCl extract of DC membranes was prepared as follows. DC membranes (2 mg protein) in 1.5 ml of 0.5 M KCl were subjected to ultrasonic irradiation at 10 W output at 4°C for 1 minute, with one second interruption every second. Membranes were sedimented by centrifugation at 300,000 g for 20 minutes in a TL-100 rotor. The supernatant referred to as KCl extract was dialyzed overnight against 10 mM phosphate buffer (pH 7.2) and 1.5 mM NaCl (phosphate/NaCl medium), and then concentrated by freeze-drying to a volume of 110  $\mu$ l. The  $\beta$ -OG extract of DC membranes was prepared as follows. DC membranes (2 mg protein) were incubated with  $\beta$ -OG (1% final concentration) in a volume of 240  $\mu$ l for 15 minutes at 0°C. The mixture was supplemented with 0.5 M KCl (final volume 3.4 ml) to lower the concentration of  $\beta$ -OG to about 0.07%, and centrifuged at 300,000 g for 20 minutes in the TL-100 rotor. The supernatant referred to as  $\beta$ -OG extract was dialyzed overnight against the phosphate/NaCl medium, and then concentrated to a volume of 110  $\mu$ l.

### Immunoprecipitation assays and immunoblotting

The p67phox, p47phox and p40phox proteins were immunoprecipitated using 30  $\mu$ l of anti-p67phox antiserum, as described (Fuchs et al., 1997; Grandvaux et al., 2001). To reveal the  $\alpha$  and  $\beta$  subunits of DC flavocytochrome *b*<sub>558</sub>, two antibodies directed

to the C terminal regions of the two corresponding subunits of the human protein were used (Doussière et al., 1993). The immunocomplexes, as well as 100 µg of cytosolic or membrane proteins (see above), were solubilized in Laemmli's depolymerisation buffer, loaded on a 12% polyacrylamide gel, subjected to SDS-PAGE and then electrotransferred to a nitrocellulose membrane. For protein immunodetection, the membrane was blocked for 1 hour at room temperature in PBS supplemented with 0.1% Tween 20 (T-PBS) and 5% nonfat dried milk. After three washes with T-PBS, the nitrocellulose was probed for 1 hour with the antibodies at the following dilutions: anti-Nox2 1/500, anti-p22phox 1/500, anti-p67phox 1/750, anti-p47phox 1/2,500, anti-p40phox 1/1,000. The primary antibodies were then detected with the protein A-horseradish conjugate, using ECL.

#### RNA isolation and RT-PCR

Total cellular RNA was extracted from DCs ( $5 \times 10^6$  cells) using the RNeasy Protect Mini kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instruction, and reversely transcribed using the Superscript cDNA synthesis kit from Gibco BRL (Pontoise, France). The cDNA was amplified by PCR on a LightCycler System (Roche) (Gallagher et al., 2001), using two oligonucleotide primers derived from the murine Nox2 cDNA sequence, namely 5'-<sup>1</sup>ATGGGG-AACTGGGCTGTGAATGAA<sup>24-3'</sup> and 5'-<sup>271</sup>TGAGGTTCCTGTC-CAGTTGTCTTCG<sup>295-3'</sup>. PCR products were separated by electrophoresis on a 1.6% agarose gel, stained with ethidium bromide and visualized by UV illumination. Product specificity was determined by sequencing the amplified fragments excised from the gel.

#### Assay of O<sub>2</sub><sup>-</sup> production by DCs

In some experiments, generation of O<sub>2</sub><sup>-</sup> by PMA-stimulated DCs was assayed by measurement of the rate of reduction of cytochrome *c* inhibitable by SOD. DCs ( $5 \times 10^6$  cells) were suspended in 1 ml of PBS/20 mM glucose/1 mM MgCl<sub>2</sub>/200 µM cytochrome *c*, in a photometric cuvette at 37°C, and cytochrome *c* reduction was measured at 550 nm. PMA was added at the final concentration of 1 µg/ml. To assess the specific oxidase activity, 40 µg/ml SOD was added to quench O<sub>2</sub><sup>-</sup> and the residual reduction of cytochrome *c* was recorded. The rate of SOD-sensitive production of O<sub>2</sub><sup>-</sup> was calculated from the difference between the two slopes. In most of the cases, more than 95% of the reduction of cytochrome *c* was inhibited by SOD, indicating that reduction of cytochrome *c* was essentially due to O<sub>2</sub><sup>-</sup>.

In other experiments, DCs were preincubated with the pro-inflammatory ligands LPS (1 µg/ml) or PSB (20 µg/ml) at 37°C overnight in Petri dishes; anti-CD40 (1 µg/ml) and poly I:C (50 µg/ml) were also used. Each Petri dish contained 10<sup>6</sup> cells suspended in 10 ml of Iscove DMEM medium supplemented with 10% FCS, GM-CSF and Flt-3L (Berthier et al., 2000; Martinon-Ego et al., 2001). After preincubation, the cells from each dish were pelleted by low speed centrifugation and washed twice with PBS supplemented with 40 mM glucose (PBS-glucose). They were finally resuspended in 200 µl of PBS-glucose and transferred to the wells of a microtiter plate for assay of O<sub>2</sub><sup>-</sup> production upon activation with PMA (final concentration of 1 µg/ml). Production of O<sub>2</sub><sup>-</sup> was assayed by reduction of NBT (100 µM). Incubation was carried out at 37°C and lasted for 3 hours. Reduction of NBT to the red purple formazan was monitored at 550 nm using ELISA plate reader. For microscopic examination, cytopspins were prepared from 40 µl aliquots of the cell suspensions supplemented with 60 µl PBS.

#### Assay of O<sub>2</sub><sup>-</sup> production by DC membranes in the cell-free system

The assay consisted of an activation step followed by measurement of the elicited oxidase activity. It was performed in a 96-well microtiter

plate. Each well was filled with 50 µl of PBS containing the indicated amounts of cytosolic and membrane proteins, 0.5 mM ATP, 10 µM GTPγS and 2.5 mM MgSO<sub>4</sub>. Arachidonic acid in ethanol was distributed in the wells in amounts ranging from 5 to 40 nmol/well. When indicated, KCN was added at the final concentration of 1 mM. The plate was shaken using a Labsystems integrated EIA Management System (iEMS) for 10 minutes at 25°C. Then, cytochrome *c* and NADPH in 175 µl PBS were added (final concentrations 150 µM and 300 µM, respectively). Cytochrome *c* reduction was recorded at 550 nm for 3 minutes. Parallel cell-free assays were carried out with membrane and cytosol from bovine neutrophils.

In some experiments, NADPH oxidase activation was carried out in the presence of β-OG. After a 5 minute incubation of β-OG with the membrane fraction from either neutrophils or DCs at room temperature, as described in the text, neutrophil cytosol, ATP, GTPγS, KCN and MgSO<sub>4</sub> in PBS were added (see above), resulting in a final β-OG concentration lower than the critical micellar concentration (CMC). Aliquots of this suspension were withdrawn and distributed in the wells of the plate, followed by addition of arachidonic acid at increasing concentrations. Incubation lasted for 10 minutes, followed by addition of cytochrome *c* and NADPH in PBS.

#### Assay of H<sub>2</sub>O<sub>2</sub> production

Production of hydrogen peroxide by DCs was measured using HVA and HRP in a 2 ml assay adapted from a previously described method (Baggiolini et al., 1986).

#### Confocal microscopy

Murine DCs were diluted to  $2 \times 10^6$ /ml and incubated for 10 minutes at room temperature and 20 minutes at 37°C on poly-L-lysine-coated lamellas. After three washes with PBS, cells were fixed with 3% paraformaldehyde for 10 minutes and permeabilised with 0.05% saponin in PBS supplemented with 0.2% BSA. They were first incubated with appropriate polyclonal antibodies, as indicated in the text, and stained with Cy3 donkey anti-rabbit IgGs. The coverslips were then mounted in FluorSave<sup>TM</sup> reagent. Analysis was performed using a confocal laser microscope (TCS-SP2, Leica, Heidelberg, Germany), equipped with an helium/neon laser. Excitation was at 543 nm and emission at 570 nm.

#### Spectrophotometric experiments

Difference optical spectra (dithionite reduced minus oxidized) of whole DCs and DC membranes were recorded with a double-beam Perkin-Elmer 557 spectrophotometer. For measurement of heme *b* oxidation-reduction potentials, spectra were recorded with a Hewlett-Packard 8452A diode array spectrophotometer. DC membranes recovered from the sucrose gradient were suspended in 50 mM Hepes pH 7.0 at the concentration of 2.1 mg protein/ml. The suspension was clarified by addition of 0.2% Triton X-100, and placed in a 1-cm-wide cuvette that was sealed with a gas-tight rubber stopper in which three needles were inserted. One of the needles was used for flushing argon, the other for gas evacuation and the third to inject microliter volumes of appropriate solutions. The argon pressure in the cuvette was maintained at a slightly higher value than the atmospheric pressure to ensure anaerobiosis. The stopper was equipped with an axle-bearing for the passage of a plastic stick. The helicoidal-shaped extremity of the stick plunged in the cuvette medium. The rotation of the stick driven by a motor was used to make the membrane suspension homogeneous. Since the purpose of the potentiometric titration was to detect and quantitate the presence of a low potential heme *b* similar to that present in neutrophil flavocytochrome *b*, phenosafranin, a dye with a mid-point potential of -252 mV at pH 7.0, was chosen as potential indicator (Light et al., 1981). Moreover, oxidized phenosafranin has a maximal absorbance at 525 nm, far from the Soret

peak of heme *b*. After a 4 minute equilibration with argon, phenosafranin was added to the membrane suspension (final concentration 2  $\mu$ M), and the potentiometric titration started with the sequential additions of 1  $\mu$ l aliquots of a freshly prepared solution of 5 mM sodium dithionite in 50 mM HEPES pH 7.0. After each addition, the optical spectrum was recorded. Reduction of heme *b* was assessed by the increase of the Soret peak at 426 nm. The increase of the peak at 444 nm was used to monitor the reduction of hemes *aa*<sub>3</sub> (due presumably to contaminating mitochondrial membranes). Reduction of phenosafranin corresponded to a decrease of the peak at 525 nm. When reduction of heme *b* reached a maximal value, the back reaction was started by a controlled delivery of air into the cuvette medium. The heme *b* potential was calculated by plotting the log of the ratio of oxidized heme *b* to reduced heme *b* at various potential values derived from the phenosafranin spectrum.

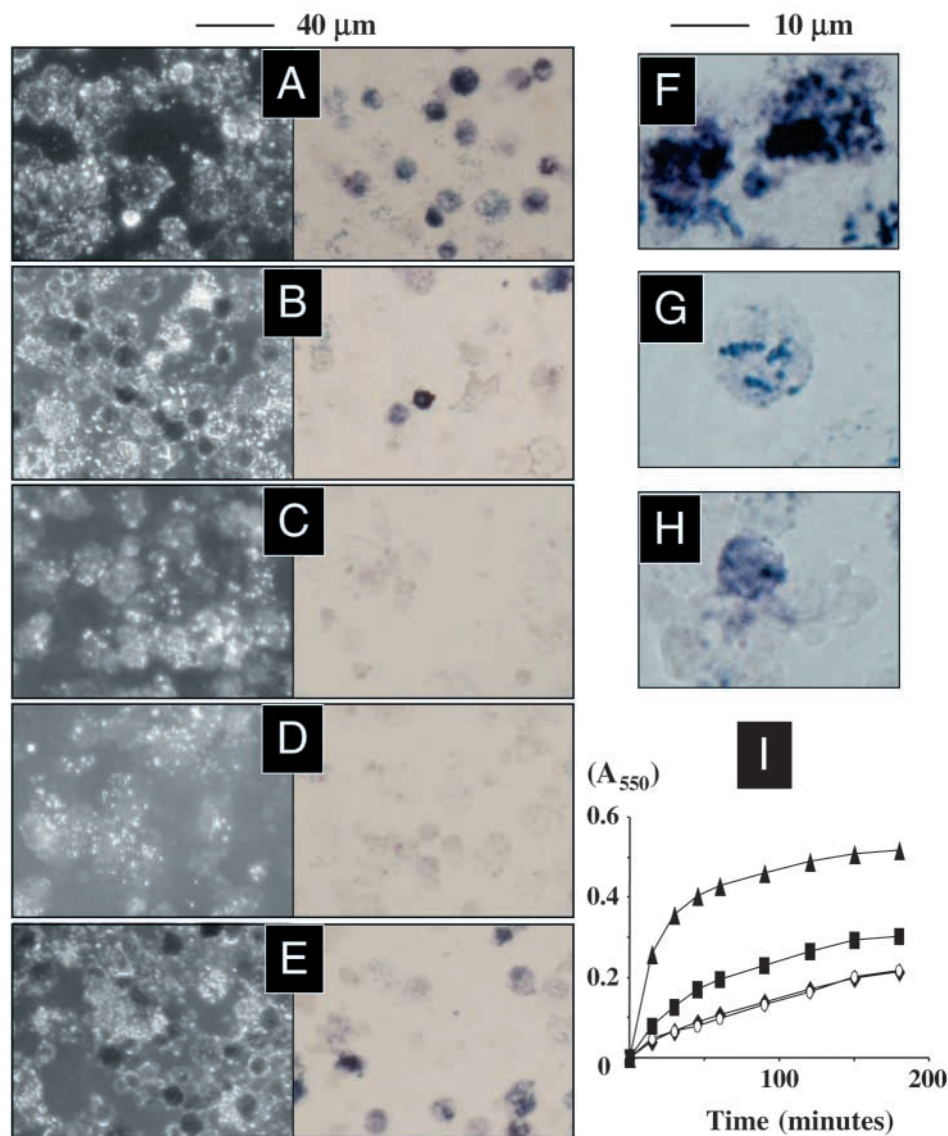
## Results

### Generation of reactive oxygen species by DCs requires pretreatment with pro-inflammatory ligands

NBT was chosen as a probe to screen by microscopic

examination the capability of DCs to generate  $O_2^-$  in response to different treatments. PMA, a protein kinase C (PKC) activator that is known as an efficient elicitor of the NADPH oxidase activity of resting neutrophils, was used to activate the production of  $O_2^-$  by DCs. After 3 hours of contact with PMA, a few cells were found to be filled with reduced NBT (Fig. 1B). In as much as DCs are involved in the host defence against microbial aggressions, we wondered whether a contact with pro-inflammatory ligands from microbial origin, for example LPS or PSB, could activate signalling pathways implicated in the production of  $O_2^-$ . Indeed, microscopic examination of DCs exposed overnight to LPS followed by a 3 hour incubation with PMA and NBT showed an intense accumulation of reduced NBT within the cells, indicating a substantial production of  $O_2^-$  (Fig. 1A). This situation contrasted with the lower accumulation of reduced NBT particles in DCs treated with PMA alone (Fig. 1B) or with the paucity of reduced NBT particles in DCs treated overnight with LPS alone (Fig. 1C) and the near absence of reduced NBT in control cells (Fig. 1D). Similar results were obtained when LPS was replaced by

PSB (not shown). All the above experiments were conducted with cultured DCs. To ascertain whether the elicited oxidase activity by cultured DCs had a physiological meaning, complementary experiments were carried out with DCs directly isolated from mouse spleen (sDCs). The PMA-dependent accumulation of reduced NBT in sDCs pretreated with LPS was confirmed (Fig. 1E). In the absence of pretreatment with LPS, however, there was no significant reduction of NBT by sDCs upon addition of PMA. The



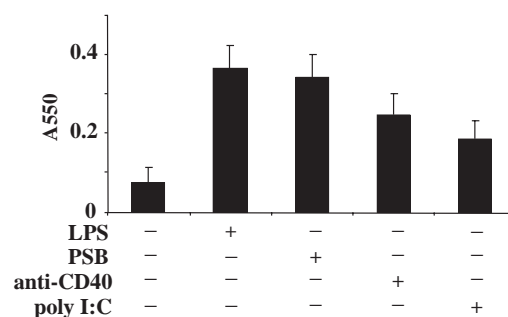
**Fig. 1.** Effect of preincubation with LPS on the reduction of NBT by DCs challenged with PMA. General views of DCs (A-D) or sDCs (E). Left, phase contrast images; right, NBT staining. (A,E) Preincubation with LPS, followed by incubation with PMA. (B) No LPS in the preincubation medium, incubation with PMA. (C) Preincubation with LPS, no PMA in the incubation medium. (D) Control cells, no LPS in the preincubation medium, no PMA in the incubation medium. Micrographs F, G and H correspond to high magnification images of selected cells from plates A, B and E, respectively. (I) Time course of NBT reduction within DCs. Preincubation with LPS, followed by incubation with PMA (▲). Other conditions: no preincubation with LPS, PMA in the incubation medium (■); Preincubation with LPS, no PMA in the incubation medium (◆); Control, no preincubation with LPS, no PMA in the incubation medium (○). The results are representative of five separate experiments.

difference between the response of DCs and sDCs to PMA alone might be related to the presence of numerous mature cells (>30%) in DCs and their virtual absence in sDCs. High magnification (Fig. 1F,G) allowed the detection of qualitative differences in the distribution of reduced NBT particles accumulated within PMA-treated DCs, depending on preincubation with LPS. In the absence of LPS, the reduced NBT particles were localized in a small percentage of cells, and assembled in structures of different conformations that might reflect different states of activation. In some cells, the NBT particles were essentially at the periphery. In others, they appeared to populate microtrabecular structures, among which vesicular profiles could be individualized. In DCs treated first with LPS and then with PMA, reduced NBT particles accumulated in such a large amount that details in their distribution and topography were hardly discernible. Large patches of reduced NBT were also observable in sDCs first treated by LPS and then by PMA (Fig. 1H).

The time course of NBT reduction in DCs subjected to the above-mentioned conditions was determined spectrophotometrically at 550 nm (Fig. 1I). The slow and steady increase of absorbance observed with control DCs (absence of LPS and PMA) and also with DCs pretreated with LPS, not followed by addition of PMA, did not correlate with accumulation of reduced NBT in the cells as detected under the microscope. This absorbance increase was therefore not related to an oxidase activity. A different situation occurred with DCs challenged with PMA or with DCs first treated with LPS and then challenged with PMA. In contrast with control DCs, the kinetics were biphasic, characterized by a rapid increase in absorbance during the first half an hour followed by a slow decrease to reach a plateau after 2-3 hours. The rise in absorbance by DCs treated with PMA or LPS plus PMA was clearly related to the accumulation of reduced NBT in DCs found under the microscope (Fig. 1A,B). After deduction of the value of absorbance in control DCs, the overnight contact of DCs with LPS was found to enhance at least four times the oxidase activity of DCs challenged with PMA alone. Thus, LPS acts as a primer in the oxidative response of DCs to PMA. Similar kinetics were obtained when LPS was replaced by PSB in the preincubation medium (not shown). To achieve an effective priming effect, the contact of DCs with the pro-inflammatory ligands should last for at least 10-12 hours. The length of the preincubation period required to obtain noticeable priming suggested that protein synthesis is necessary in the oxidative response to PMA. An alternative explanation is the slow relief of an inhibited state of the NADPH oxidase under the control of a signaling system triggered by LPS.

To test the specificity of the priming effect, the pro-inflammatory ligands LPS and PSB were replaced by the anti-CD40 antibody and the oligonucleotide poly I:C. CD40 triggering of DCs by anti-CD40 mimics the interaction of DCs with T cells (Caux et al., 1994). Poly I:C, a dsRNA analogue, mimics the recognition of viral pathogens by DCs and induces the synthesis of pro-inflammatory cytokines (Cella et al., 1999; Verdijk et al., 1999). Although significant, the PMA-dependent reduction of NBT was less acute than with LPS or PSB (Fig. 2).

It is noteworthy that reduced NBT is essentially detected within DCs, not outside the cells. The near absence of secretion of  $O_2^-$  outside DCs was confirmed by using the  $O_2^-$ -dependent reduction of cytochrome *c*. Upon addition of PMA (1  $\mu$ g/ml),



**Fig. 2.** Effect of an overnight contact of DCs with different agonists (LPS, PSB, anti-CD40, poly I:C) on the increase in the PMA-dependent NBT reduction. The absorbance values are those obtained at the plateau. Three to five separate experiments were carried out to check the effect of the different stimuli. Data are means  $\pm$  s.d.

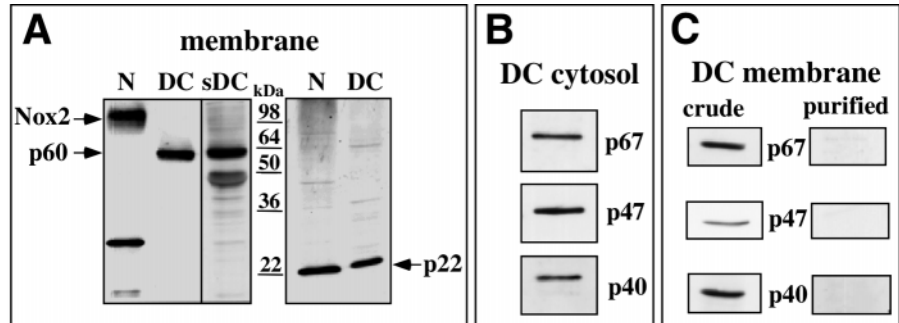
the rate of  $O_2^-$  production by DCs at 37°C was very low ( $\leq 0.1$  to 0.6 nmol/minute/ $10^6$  cells, 9 assays). Interestingly, whereas the production of  $O_2^-$  in PMA-activated DCs was equal to or less than 0.1 nmol  $O_2^-$ /minute/ $10^6$  cells, that of LPS-pretreated DCs was at least five times higher, which is consistent with the large accumulation of reduced NBT within LPS-pretreated DCs (Fig. 1A). The production of  $H_2O_2$ , a direct derivative of  $O_2^-$ , on a period of 20 minutes in the presence of 1 mM aminotriazole, an inhibitor of catalase, was in the same range as that of  $O_2^-$ . Comparison of these values with those reported in the literature for neutrophils and B cells showed that the apparent capacity of activated DCs to secrete  $O_2^-$  and  $H_2O_2$  is <3% of that of human neutrophils (Yagisawa et al., 1996) and of the same order as those found in activated B cells (Cohen-Tanugi et al., 1991).

#### Characterization and subcellular localization of the components of the $O_2^-$ -generating NADPH oxidase in DCs

The presence of phox proteins in DCs was analyzed by immunoblotting of cytosolic and membrane fractions. As illustrated in Fig. 3A,B, murine DCs contain the typical components of the NADPH oxidase complex detected by antibodies directed to human Nox2, p22phox, p67phox, p47phox and p40phox. The apparent molecular masses of the DC phox proteins probed with the antibodies were similar to those of reference bovine neutrophils, with the exception of the protein detected with anti-Nox2 antibodies. This protein migrated in SDS-PAGE with an apparent molecular mass of 60 kDa instead of 90-100 kDa, typical of human and bovine neutrophils; it is referred to here as p60. p60 was also found in membranes of DCs directly purified from mouse spleen (sDCs in Fig. 3A). A membrane protein of the same size (58 kDa) was recently identified as the large subunit of the membrane-bound flavocytochrome *b<sub>558</sub>* in murine neutrophils, the low molecular mass of murine Nox2 being explained by a defect of glycosylation (Björqvinsdóttir et al., 1996). Confirmation of the identity of the Nox2 component expressed by murine DCs was carried out by reverse transcription of RNA from DCs followed by PCR amplification. Primers derived from the murine Nox2 cDNA sequence were used. Following electrophoresis, a predominant cDNA band was detected in the

**Fig. 3.** Immunodetection of the protein components of the  $O_2^-$ -generating NADPH oxidase in membrane and cytosolic fractions from murine DCs and bovine neutrophils.  $5 \times 10^6$  cell equivalents of DC and neutrophil membrane and cytosol fractions were subjected to SDS-PAGE and then to western blot, using antibodies directed to the two subunits, Nox2 and p22phox, of the membrane-bound flavocytochrome  $b_{558}$  and to the cytosolic factors of oxidase activation, p67phox, p47phox and p40phox.

(A) Immunoblots of murine DC membranes, sDC membranes and bovine neutrophil (N) membranes revealed with anti-Nox2 and anti-p22phox antibodies. In this experiment, sDC was obtained from spleens of mice pretreated with Flt-3L. Molecular mass markers (kDa) are indicated. (B) Immunoblots of murine DC cytosol revealed with anti-p67phox, anti-p47phox and anti-p40phox antibodies. (C) Immunoblots of DC membranes showing that p67phox, p47phox and p40phox are detected in crude DC membranes, but not in DC membranes purified on sucrose gradient.



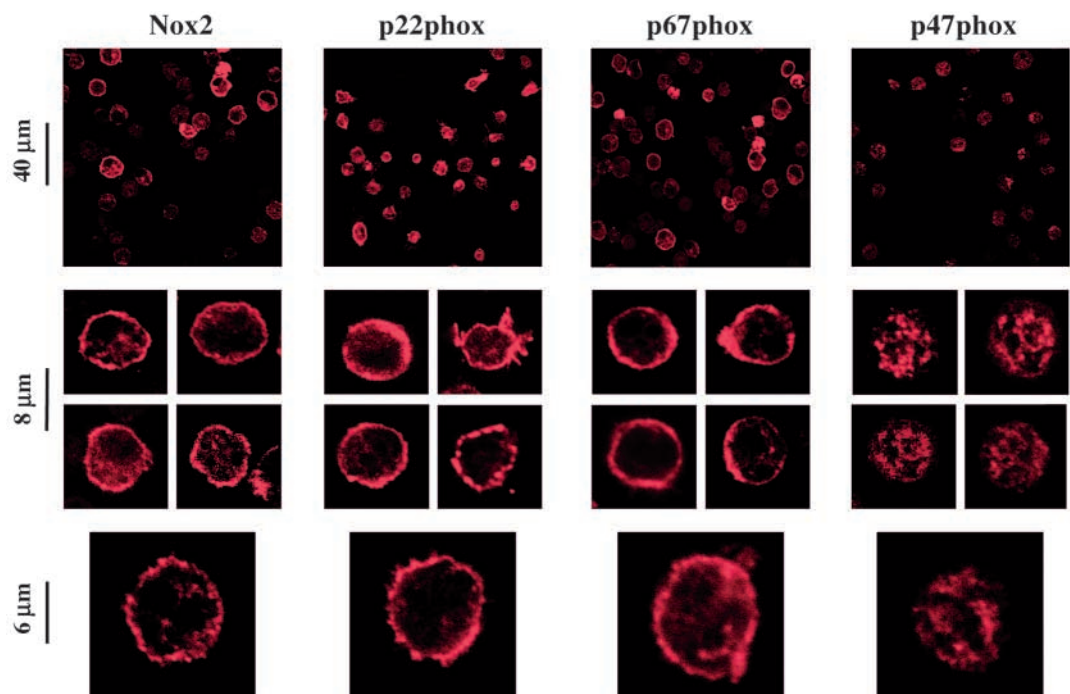
gel; the band was excised and sequenced. Sequencing demonstrated its identity with the 1-295 base fragment of Nox2 found in murine macrophages (Björgvinsdóttir et al., 1996). The cDNA cloning data were consistent with the immunological data showing that DC p60 is immunoreactive to human Nox2 antibodies. Comparative western blots carried out with LPS-primed DCs and unprimed DCs did not reveal any significant difference in the amounts of p60 and the cytosolic factors of oxidase activation (data not shown); an increased synthesis of these proteins in LPS-primed DCs is therefore unlikely.

In neutrophils p67phox, p47phox and p40phox are associated to form a complex in the cytosol (Someya et al., 1993). A similar complex was recovered from DC cytosol by immunoprecipitation, using p67phox antibodies (data not shown). Curiously, a significant amount of the cytosolic phox proteins, more particularly p67phox and p40phox, was associated with crude DC membranes. This association

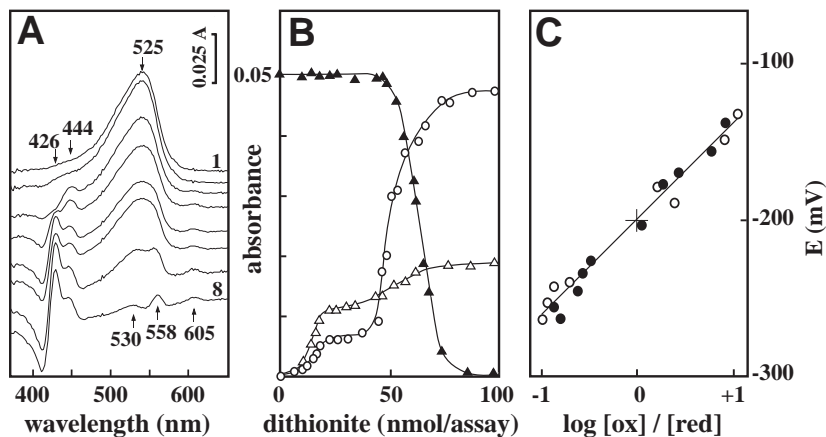
disappeared when DC membranes were purified by centrifugation on sucrose gradient (Fig. 3C), suggesting a loose binding of cytosolic phox proteins to the membrane. Another strategic component of the NADPH oxidase, namely Rac, has been detected in DCs, the two effective isoforms Rac1 and Rac2 being present at comparable levels (Garrett et al., 2000).

The subcellular localization of the NADPH oxidase components in DCs was assessed by confocal microscopy. A predominantly peripheral fluorescence was seen with antibodies directed to Nox2 and p22phox (Fig. 4). Immunodetection of p67phox close to the cell membrane was in agreement with the results of the western blotting experiment reported above. Clusters of p47phox were observed predominantly in the cytosol.

**Evidence for a low potential heme *b* in DC membranes**  
The difference optical spectrum of DC membranes illustrated



**Fig. 4.** Subcellular localization of the  $O_2^-$ -generating NADPH oxidase in DCs examined by confocal immunofluorescence microscopy. Cells were immunostained for different components of the NADPH oxidase using specific antibodies, followed by Cy3 donkey anti-rabbit antibodies (see Materials and Methods). Controls without specific antibodies were performed and found to be negative (not shown).



**Fig. 5.** Titration of a low potential heme *b* in DC membranes. (A) Effect of sodium dithionite on the optical spectrum of DC membranes in the presence of phenosafranin as redox indicator. Conditions of titration are described in Materials and Methods. Selected traces (1 to 8) illustrate the effect of the dithionite-dependent reduction on the increase of the peaks of heme *b* at 426 nm and heme *a*<sub>3</sub> at 444 nm, and on the decrease of the peak of phenosafranin at 525 nm. (B) Relationship between the spectral modifications of phenosafranin (525 nm) ▲, heme *b* (426 nm) ○ and heme *a*<sub>3</sub> (444 nm) △ upon reduction by sodium dithionite. (C) Nernst plot of the redox titration of the low potential heme *b* in DC

membranes. The ox/red ratio of the low potential heme *b* was calculated from the second wave of absorbance increase (at high concentration of sodium dithionite) at 426 nm. Forward titration (○) and backward titration (●).

in Fig. 5A, trace 8 (sodium dithionite reduced minus oxidized) exhibited peaks at 605 nm, 558 nm, 530 nm, 444 nm and 426 nm. By comparison with the difference spectrum of heart mitochondria (not shown), the peaks at 605 nm and 444 nm were ascribed to heme *aa*<sub>3</sub>, a specific mitochondrial redox carrier, which indicated the presence of submitochondrial particles in the DC membrane fraction. Other peaks at 558 nm, 530 nm and 426 nm (Soret band) denoted the presence of heme *b*. The sizes of the reduced peaks at 426 nm (heme *b*) and at 444 nm (heme *a*<sub>3</sub>) led us to conclude that the molar ratio of heme *b* to heme *a*<sub>3</sub> was significantly higher in DC membranes than in heart mitochondria, and to infer that DC membranes were likely to harbour, in addition to mitochondrial heme *b*, another heme *b* species. This heme *b* could be the prosthetic group of the 60 kDa protein, p60, detected in DC membranes with anti-Nox2 antibodies. It remained to be found whether it was a low potential heme *b* similar to that present in the neutrophil Nox2 (Cross et al., 1981; Cross et al., 1984).

To measure the redox potential of heme *b* in DC membranes, we used as redox indicator phenosafranin, which has a redox potential ( $E_{m,7}$ ) of  $-252$  mV, close to that of the neutrophil heme *b* ( $E_{m,7} = -245$  mV) (Cross et al., 1981). After each addition of sodium dithionite to the anaerobic suspension of DC membranes, the spectrum was recorded between 370 nm and 650 nm (Fig. 5A). Reduction of heme *b* and heme *aa*<sub>3</sub> was determined by measuring the increase in absorbance at 426 nm and 444 nm, respectively. Reduction of phenosafranin was followed by the decrease in absorbance at 525 nm. At low concentrations of sodium dithionite, a first wave of simultaneous reduction of heme *b* (426 nm) and heme *aa*<sub>3</sub> (444 nm) was recorded, unaccompanied by any modification of the phenosafranin spectrum (Fig. 5B). Above a threshold concentration of sodium dithionite, the heme *b* reduction increased abruptly in parallel with the reduction of phenosafranin. The second wave of heme *b* reduction reflected the titration of a heme *b* species with a redox potential in the same range as that of phenosafranin, whereas the first wave of heme *b* reduction probably corresponded to the titration of mitochondrial heme *b*, whose redox potential (0 to 50 mV) is significantly more positive than that of phenosafranin. The slight absorbance increase at 444 nm observed at low redox potential was probably due to the contribution of the tail of the

large peak at 426 nm. Introduction of trace amounts of O<sub>2</sub> into the suspension of reduced DC membranes led to the spontaneous reoxidation of heme *b*, as assessed by the decrease of the reduced peak at 426 nm, which allowed us to proceed to the back redox titration of heme *b*. The back titration curve coincided with the direct titration curve, indicating that the system was at equilibrium (Fig. 5C). The mid-point redox potential  $E_{m,7}$  of heme *b* corresponding to the second wave of titration was calculated from the ratio of the reduced to oxidized phenosafranin in the Nernst curve. It was approximated to a value of  $-200$  mV, close to that found for the heme *b* component of the Nox2 subunit of flavocytochrome *b*<sub>558</sub> in neutrophil cells. From the titration data, using a redox absorption coefficient of  $106 \text{ mM}^{-1} \text{ cm}^{-1}$  at 426 nm, it could be calculated that DC membranes contain 90–100 pmol of low potential heme *b* per mg of protein, a value only half of that found in neutrophil membranes (Morel et al., 1985).

#### Assessment of the respective efficiencies of the cytosolic and membrane-bound components of the DC oxidase complex

The previous experiments led us to conclude that whereas DCs are fully equipped in components of the O<sub>2</sub><sup>-</sup>-generating NADPH oxidase, they are barely able to mount a substantial respiratory burst when activated with PMA alone. This paradoxical situation, different from that usually observed in neutrophils, could be explained by an unusual activation mechanism or by the association of an inhibitory factor with one of the components, either membranous or cytosolic, of the oxidase complex. To clarify this point, oxidase activity was assayed in an heterologous cell-free system (Cohen-Tanugi et al., 1991). This system consisted of membrane and cytosolic fractions derived from murine DCs and bovine neutrophils (Table 1). In the cell-free system, only the terminal step of the activation mechanism, namely the binding of the cytosolic factors to the membrane-bound flavocytochrome *b*, is implicated in the elicitation of the oxidase response. As a control, DC membranes preincubated with arachidonic acid were mixed with DC cytosol, GTPγS and ATP, and the elicited oxidase activity was assayed after addition of NADPH and cytochrome *c*. At the optimal concentration of arachidonic acid, the mean rate of O<sub>2</sub><sup>-</sup>

**Table 1. O<sub>2</sub><sup>-</sup> production resulting from NADPH oxidase activation obtained by different combinations of membrane and cytosol fractions from DCs and bovine neutrophils**

Conditions	NADPH oxidase activity (nmol O <sub>2</sub> <sup>-</sup> /minute/ mg membrane protein)
DC membranes+DC cytosol	24±9
DC membranes+neutrophil cytosol	66±13
Neutrophil membranes+DC cytosol	217±52
Neutrophil membranes+neutrophil cytosol	803±175

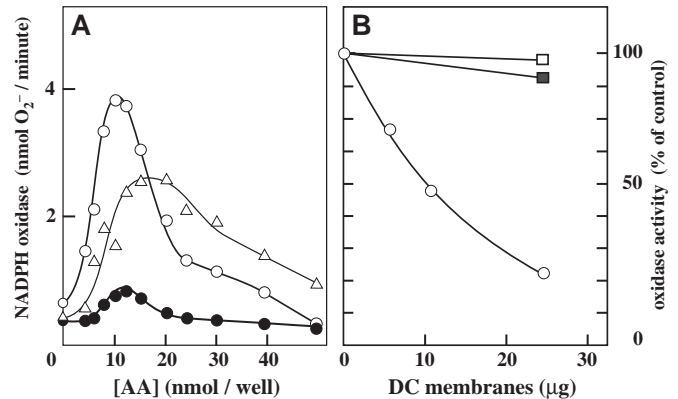
Experimental conditions used for the activation step with membranes, cytosol, MgSO<sub>4</sub>, ATP, GTPγS and arachidonic acid, as well as those for the oxidase assay are described in Materials and Methods. The amounts of membrane and cytosolic proteins used were 4–6 μg and 40–60 μg, respectively, in eight different experiments.

production was 24 nmol O<sub>2</sub><sup>-</sup>/minute/mg membrane protein, which is 3% of that determined in parallel in a cell-free system consisting of membranes and cytosol from bovine neutrophils, namely 803 nmol O<sub>2</sub><sup>-</sup>/minute/mg membrane protein. Addition of KCN to avoid any reoxidation of the reduced cytochrome *c* by contaminant mitochondrial membranes had no effect on the rate of O<sub>2</sub><sup>-</sup> production. The difference in the apparent oxidase activities of DC and neutrophil membranes assayed in a cell-free system confirmed that obtained with PMA activated cells, and it indicates that the defect in the elicitation of oxidase activity in PMA-stimulated DCs is directly related to the final step of the oxidase activation. Through the use of the heterologous cell-free system consisting of murine DC cytosol and bovine neutrophil membranes, the mean rate of O<sub>2</sub><sup>-</sup> production, 217 nmol O<sub>2</sub><sup>-</sup>/minute/mg membrane protein, was nine times higher than that measured with the homologous DC cell-free system. In contrast, the reverse heterologous system obtained by mixing murine DC membranes and bovine neutrophil cytosol was barely more efficient than the homologous DC cell-free system. From these results, we concluded that the limiting factor in O<sub>2</sub><sup>-</sup> production by DCs resides in components of the NADPH oxidase located in the membrane fraction.

As shown in the preceding section, DCs are equipped with a Nox2 protein that is identical to that located in murine macrophages and differs from the human Nox2 by the near absence of glycosylation (Björgvinsdóttir et al., 1996). Nevertheless, murine macrophages, in contrast with murine DCs, are fully competent to generate a respiratory burst upon addition of PMA (Pollock et al., 1995). A substantial amount of Nox2 associated with a low potential heme *b* was present in DC membranes corresponding, on the basis of the heme content, to about half of that reported for neutrophils. We should therefore expect a noticeable production of O<sub>2</sub><sup>-</sup> by activated DC membranes. This was not the case, which led us to hypothesize that a membrane-bound inhibitory factor might interfere with the activation of the membrane-bound Nox2 by cytosolic factors in DCs.

#### Evidence for the presence of an oxidase activation inhibitor of protein nature in DC membranes

If an inhibitory factor of NADPH oxidase activation were present in DC membranes, then mixing DC membranes with



**Fig. 6.** Inhibitory effect of DC membranes on the elicited neutrophil NADPH oxidase activity in a cell-free system. NADPH oxidase activity (nmol O<sub>2</sub><sup>-</sup> produced per minute in each well of the microtiter plate) was determined after preincubation for 10 minutes at room temperature of bovine neutrophil membranes (6 μg protein/assay) or murine DC membranes (10.5 μg protein/assay) or a mixture of neutrophil membranes (6 μg protein/assay) and DC membranes (10.5 μg protein/assay) with neutrophil cytosol (60 μg protein), ATP, GTPγS, MgSO<sub>4</sub>, and increasing amounts of arachidonic acid (AA) (final volume 30 μl). The preincubation step was followed by addition of cytochrome *c*, KCN and NADPH in PBS (see Materials and Methods). The figure shows the rate of production of O<sub>2</sub><sup>-</sup> per minute at different concentrations of arachidonic acid. (A) Neutrophil membranes (○); DC membranes (●); neutrophil membranes mixed with DC membranes (△) (the shift of the optimal concentration of arachidonic acid towards higher values when DC membranes and neutrophil membranes are added together in the medium is due to the higher levels of membrane lipids functioning as an unspecific trap of arachidonic acid). (B) Dose-response of the inhibitory effect of DC membranes on the neutrophil oxidase activity at the optimal concentration of arachidonic acid (○); DC membranes heated for 10 minutes at 100°C and mixed with neutrophil membranes (□); DC membranes treated for 1 hour at 20°C with proteinase K (proteinase K/membrane protein : 1/100) and mixed with neutrophil membranes (■).

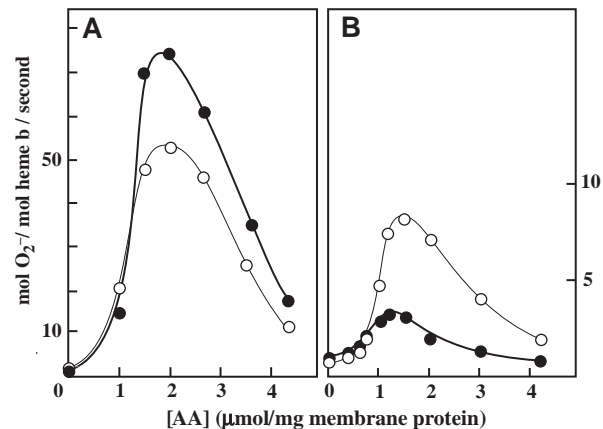
neutrophil membranes in a cell-free system should not result in an additive effect of elicited oxidase activities of the two types of membranes but, on the contrary, in an antagonistic effect. In the experiment illustrated in Fig. 6A, oxidase activation of both murine DC membranes and bovine neutrophil membranes was performed with bovine neutrophil cytosol in the presence of arachidonic acid. Control assays showed that at the optimal concentration of arachidonic acid, DC membranes exhibited a modest oxidase activity. The amount of O<sub>2</sub><sup>-</sup> generated per minute and per well of the microplate amounted to 0.38 nmol (equivalent to 37 nmol O<sub>2</sub><sup>-</sup>/minute/mg membrane protein). This value was much lower than that found with neutrophil membranes under similar conditions, namely 4.0 nmol O<sub>2</sub><sup>-</sup>/minute (equivalent to 660 nmol O<sub>2</sub><sup>-</sup>/minute/mg membrane protein). Upon mixing DC membranes and neutrophil membranes, an intermediate value of O<sub>2</sub><sup>-</sup> production, 2.3 nmol O<sub>2</sub><sup>-</sup>/minute, was obtained. This finding corroborated the hypothesis that an inhibitory factor resides in DC membranes. In the presence of high concentrations of arachidonic acid in the activation step, the inhibitory factor might diffuse from DC membranes to neutrophil membranes. The assay was repeated by varying the



ratio of DC membranes to neutrophil membranes. The dose-effect curve illustrated in Fig. 6B shows that increasing the relative amount of DC membranes resulted in a decrease of the elicited oxidase activity of neutrophil membranes. If the DC inhibitory factor were a protein, its negative effect on the elicitable neutrophil oxidase activity assayed in the cell free system should be relieved either by heating the DC membranes or by subjecting them to a protease treatment. As shown in Fig. 6B, heating DC membranes or incubation of these membranes with proteinase K almost completely abolished their inhibitory effect on the neutrophil oxidase activity.

Based on the hypothesis that a membrane-bound inhibitor interfered with the production of  $O_2^-$  in DCs, it was reasoned that treatment of DC membranes by a detergent should dislodge the inhibitor, the resulting effect being an increased oxidase activity in a cell-free system. DC membranes were lysed by 1%  $\beta$ -octylglucoside ( $\beta$ -OG); addition of cytosol, ATP,  $GTP\gamma S$  and arachidonic acid lowered the final concentration of  $\beta$ -OG to 0.075%, well below its CMC (0.73%) (Hjelmeland and Chrambach, 1984). This condition is expected to reconstitute the oxidase in the phospholipid micelles, without noticeable inhibitory effect of the detergent (Shpungin et al., 1989). A control experiment carried out with neutrophil membranes indicated that the lysed neutrophil membranes supplemented with neutrophil cytosol and the other components of the cell-free system were still able to mount a substantial respiratory burst; their elicited oxidase activity at the optimal concentration of arachidonic acid was decreased by only 30% compared with the control (51.2 versus 74.2 mol  $O_2^-$ /mol heme *b*/second) (Fig. 7A). There was no further decrease in the elicited oxidase activity upon doubling the concentration of  $\beta$ -OG. Addition of FAD, a cofactor of Nox2, did not restore the lost oxidase activity (not shown). With DC membranes treated with  $\beta$ -OG, the opposite effect was observed, consisting in a near tripling of the elicited oxidase activity (8.5 versus 3.0 mol  $O_2^-$ /mol heme *b*/second) (Fig. 7B). Taking into account the decrease in neutrophil oxidase activity due to the presence of residual  $\beta$ -OG, the tripling of oxidase activity in DC membranes incubated with  $\beta$ -OG was clearly an underestimate of the actual NADPH oxidase capacity. Taken together, the above results confirm the location of an inhibitory factor of protein nature that prevents the elicitation of oxidase activity in DC membranes, and its release upon addition of  $\beta$ -OG.

To distinguish between the extrinsic or intrinsic nature of the inhibitory protein, the DC membranes were subjected to two different treatments. One membrane fraction was incubated with 0.5 M KCl and sonicated to remove extrinsic proteins. The other membrane fraction was incubated with 1%  $\beta$ -OG and then diluted 13 fold with PBS supplemented with 0.5 M KCl, to decrease the  $\beta$ -OG concentration below the CMC. The supernatant fractions obtained after high speed centrifugation were dialyzed overnight and added to neutrophil membranes. Oxidase activation was triggered by addition of bovine neutrophil cytosol,  $GTP\gamma S$ , ATP and arachidonic acid. As illustrated in Fig. 8 (inset), the KCl extract displayed a limited inhibitory effect on the elicited oxidase activity, compared with the substantial inhibition observed with the  $\beta$ -OG extract. The inhibition was dose-dependent (Fig. 8, inset), which corroborates the conclusion that the inhibitor is a membrane-bound protein, removable by  $\beta$ -OG. A  $\beta$ -OG extract of



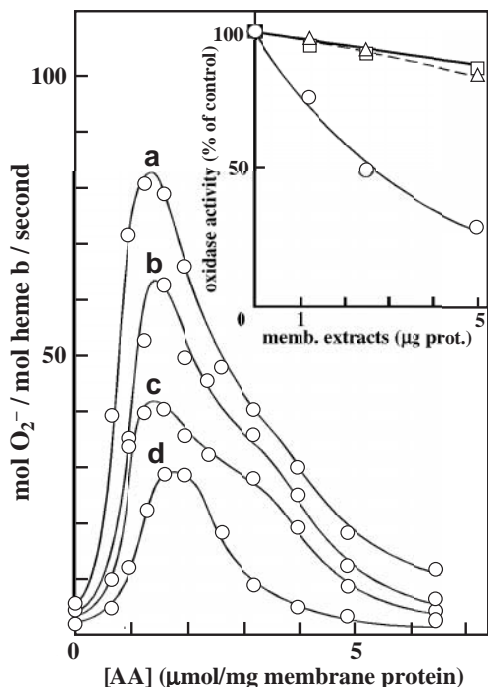
**Fig. 7.** Effects of pretreatment of neutrophil membranes and DC membranes by  $\beta$ -OG on the elicited NADPH oxidase activity in cell-free system. Murine DC membranes (21  $\mu\text{g}$  protein) and bovine neutrophil membranes (6  $\mu\text{g}$  protein) were pretreated by 1%  $\beta$ -OG for 3 minutes at  $0^\circ\text{C}$ . This was followed by the addition of bovine neutrophil cytosol,  $GTP\gamma S$ , ATP, KCN,  $MgSO_4$  and increasing amounts of arachidonic acid (AA) to activate the NADPH oxidase. The elicited oxidase activity was determined by the production of  $O_2^-$  (see Materials and Methods) and expressed in terms of heme *b* turnover. (A) neutrophil membranes, control (●), pretreated by  $\beta$ -OG (○). (B) DC membranes, control (●), pretreated by  $\beta$ -OG (○).

neutrophil membranes added to the neutrophil cell-free system resulted in only a slight inhibition of the oxidase activation, indicating that the inhibitory protein present in DC membranes is not present in neutrophil membranes in detectable amounts (Fig. 8, inset).

In the preceding experiment, the  $\beta$ -OG extract of DC membranes was added to neutrophil membranes prior to the addition of the activating cytosolic factors. When the sequence of addition was reversed, so that the  $\beta$ -OG extract of DC membranes was added to the cell-free system of oxidase activation after arachidonic acid and the cytosolic factors, i.e. after the activation step, the inhibitory effect on the rate of  $O_2^-$  production was much less marked (less than 10%) (not shown). In this case, the effect of the  $\beta$ -OG extract was directed towards the activated NADPH oxidase. This finding led us to eliminate a scavenging effect of the  $\beta$ -OG extract of DC membranes on the superoxide  $O_2^-$  and to conclude that the DC membrane extract inhibits the activation of NADPH oxidase rather than the elicited oxidase activity.

## Discussion

This paper demonstrates that the NADPH oxidase activity of DCs is cryptic because of the presence of a membrane-bound inhibitory factor that precludes the PMA-dependent oxidase activation. The contact of DCs with pro-inflammatory stimuli results in the relief of the inhibition of oxidase activation. These conclusions are supported by the following observations: (1) DCs contain non-limiting amounts of all the components of the NADPH oxidase complex, namely the heterodimeric flavocytochrome *b* with p22phox and the large subunit Nox2 harbouring a low potential heme *b* and all the cytosolic factors of activation. It is noteworthy that Nox2 present in murine macrophages (Björgvinsdóttir et al., 1996)



**Fig. 8.** Differential effects of a KCl extract and a  $\beta$ -OG extract of DC membranes on the NADPH oxidase activity of neutrophil membranes elicited in a cell-free system. Bovine neutrophil membranes (6.2  $\mu$ g protein) were mixed with various amounts of KCl or  $\beta$ -OG extracts from DC membranes, and their NADPH oxidase was activated in the cell-free system consisting of bovine neutrophil cytosol, GTP $\gamma$ S, ATP, MgSO $_4$ , KCN and increasing amounts of arachidonic acid (AA). The elicited oxidase activity was determined by the production of O $_2^-$  (see Materials and Methods) and expressed in terms of heme *b* turnover. Curve a, control (absence of DC membrane extract). Curves b, c and d correspond to 1.15  $\mu$ g, 2.3  $\mu$ g and 4.6  $\mu$ g protein in the  $\beta$ -OG extract of DC membranes, respectively. (Inset) Dose-response of the elicited oxidase activity of neutrophil membranes treated with a  $\beta$ -OG extract of DC membranes ( $\circ$ ), with a  $\beta$ -OG extract of neutrophil membranes ( $\triangle$ ), and with a KCl extract (0.5 M KCl) of DC membranes ( $\square$ ).

and in murine neutrophils (Pollock et al., 1995) has a much smaller size (58 kDa) than that of its human counterpart (90–100 kDa). This holds true for murine DCs. (2) Through the use of heterologous cell-free systems of NADPH oxidase activation, consisting of DC membranes and neutrophil cytosol, or neutrophil membranes and DC cytosol, it was possible to ascribe to the membrane fraction the lack of elicitable oxidase activity in DCs. As substantial amounts of Nox2 and p22phox are present in DC membranes, it was hypothesized that these membranes contain an inhibitory factor. (3) Treatment of DC membranes with the detergent  $\beta$ -OG elicited an increase in the production of O $_2^-$  assayed in the cell-free system. (4) The  $\beta$ -OG extract of DC membranes inhibited the production of O $_2^-$  in a highly efficient cell-free system consisting of membranes and cytosol from bovine neutrophils. Oxidase activation, but not oxidase activity, was inhibited in a dose-dependent manner. From these data, we conclude that a membrane-bound inhibitory factor prevents oxidase activation by DCs. Its protein nature was demonstrated by the abolition of its inhibitory effect upon heat treatment and proteolysis with proteinase K. Thus, among leukocytes, in

addition to neutrophils, macrophages and eosinophils that exhibit, upon stimulation, a strong respiratory burst (Yagisawa et al., 1996), and to B cells in which the oxidative response to stimuli is rate-limited by the low amount of flavocytochrome *b* (Batot et al., 1998), DCs represent a third group of cells that harbour the full panoply of the components of the NADPH oxidase complex, but in which oxidase activation is negatively regulated. As shown here, the efficiency of oxidase activation in DCs is recovered upon contact with pro-inflammatory ligands of microbial origin. There are presumably other favourable conditions, for instance the interaction of DCs with T cells, as suggested by the positive response of DCs to anti-CD40 antibody. The physiological meaning of the cryptic oxidase of DCs is ascertained by the similar responses to pro-inflammatory stimuli of the cultured DCs and DCs directly obtained from mouse spleen.

Confocal analysis of immunocytochemically stained DCs led to the puzzling observation that, in resting cells, a large fraction of p67phox, one of the cytosolic factors of oxidase activation, is predominantly localized to the plasma membrane. It is well recognized that, during the course of oxidase activation in neutrophils, a critical event is the migration of p67phox to the plasma membrane to interact with the membrane-bound flavocytochrome *b* (reviewed by Vignais, 2002). Resting DCs can engulf surrounding antigens by a spontaneous, albeit active, pinocytosis (Steinman and Swanson, 1995). This process might occur concomitantly with the migration of p67phox to the membrane. Under these conditions, to prevent any elicitation of the oxidase activity that would result in an inadvertent and detrimental production of O $_2^-$ , DCs might have developed a mechanism of negative regulation of oxidase activation. Confocal analysis of DCs (Fig. 4) showed the different subcellular localizations of p67phox and p47phox. In contrast to p67phox, p47phox is predominantly present in the cytoplasm. An early report concluded that in activated neutrophils, participation of p47phox precedes that of p67phox in the formation of the active NADPH oxidase by association with Nox2 (Kleinberg et al., 1990). Consistent with this report was the finding that p67phox translocation to the membrane-bound Nox2 does not occur in p47phox-deficient neutrophils (Heyworth et al., 1991). Prevention of migration of p47phox to the cell membrane in DCs, possibly due to the presence of a membrane-bound inhibitor associated with Nox2 or p22phox, might explain the apparent incapability of these cells to mount a respiratory burst.

Myeloid DCs are known to express several members of the Toll-like receptor (TLR) family (reviewed by Akira et al., 2001; Medzhitov, 2001; Janeway and Medzhitov, 2002; Akira and Hemmi, 2003; Imler and Hoffmann, 2003; Kopp and Medzhitov, 2003). TLRs play a critical role in innate immunity as they can recognize and interact with pathogen-associated molecular patterns (PAMPs), and elicit intracellular signals leading to the induction of host defence genes. Importantly, recognition of PAMPs allows the immune system to distinguish infectious non-self from non-infectious self (Janeway and Medzhitov, 2002). In this context, LPS and poly I:C have been found to be ligands for TLR4 and TLR3, respectively (Hoshino et al., 1999; Matsumoto et al., 2002). Recent studies have suggested a role of TLR in the activation of the NADPH oxidase. For instance, the non-phagocytic NADPH oxidase of

gastric pit cells appears to be activated by LPS through a TLR4 mediated signalling pathway (Kawahara et al., 2001). The TLR4 signalling pathway was also reported to mediate upregulation of NADPH oxidase components in gastric mucosal cells (Teshima et al., 1999). Our data indicate that two signalling pathways cooperate to activate the NADPH oxidase in DCs. The first one, a PKC-dependent pathway, has been extensively reported to be involved in the activation of neutrophil and macrophage NADPH oxidase, routinely monitored in the presence of PMA (reviewed by Dinauer, 1993; De Leo and Quinn, 1996; Vignais, 2002). The other pathway that appears to contribute to the oxidase activation in DCs is the TLR-mediated signalling pathway. This pathway might be responsible for the delayed priming of DCs in response to pro-inflammatory stimuli.

The functional significance of the cryptic DC oxidase, activated upon interaction with pro-inflammatory agonists and T cells, remains to be defined. As activated DCs exhibit a high propensity to interact with T cells, and as T cells lack the conventional NADPH oxidase (Hildeman et al., 1999; Hildeman et al., 2003), it is possible that  $O_2^-$  generated by activated DCs and the derived ROS alter the regulation of T-cell redox-sensitive signalling pathways. Along this line, the role of ROS in T-cell apoptosis has been emphasized (Hildeman et al., 2003). It has also been reported that T-cell signalling pathways are differentially affected by oxidative stress, leading for example to the downregulation of PLC- $\gamma$ 1 and to the activation of ERK-2 (extracellular signal-regulated kinase 2) (Cemerski et al., 2002). As an alternative, generation of  $O_2^-$  by activated DCs might lead to an apoptotic process of DCs. In fact, phenotypic maturation induced in vitro by LPS or cytokines such as TNF $\alpha$  was found to end by apoptotic cell death (Winzler et al., 1997). As recently shown (Ingulli et al., 1997), following interaction between antigen-bearing DCs and T cells within the T-cell rich region of lymph nodes, DCs are eliminated by apoptosis, allowing the activated T cells to escape, proliferate and migrate to sites of inflammation. Similarly, it has been reported that, following maturation in response to the bacterial LPS, DCs rapidly die by apoptosis, once they have entered the T-cell areas, unless they receive a survival signal from T cells (De Smedt et al., 1998). It would be interesting to explore whether there is a link between the apoptosis of DCs subjected to pro-inflammatory stimuli and the ability of these stimuli to unmask the cryptic NADPH oxidase of DCs.

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