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Neutrophils and mononuclear cells from patients with chronic granulomatous disease release nitric oxide

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1 Chronic granulomatous disease (CGD) is a group of genetic disorders characterised by recurrent severe suppurative infections due to impaired microbial killing. The principal biochemical defect is an impairment in the production of reactive oxygen intermediates by phagocytes.

2 Nitric oxide (NO) is synthesised from the guanidino nitrogen atom(s) of L-arginine and has recently been proposed to be involved in defence mechanisms. The aim of this study was to investigate the involvement of the oxidative burst in the biosynthesis of NO by neutrophils and mononuclear cells from patients with CGD.

3 NO synthesis was assayed by the ability of neutrophils and mononuclear cells to inhibit thrombin-induced washed platelet aggregation while superoxide anion (O_2^-) production was measured spectrophotometrically by the superoxide dismutase inhibitable reduction of cytochrome c.

4 Neutrophils and mononuclear cells from patients with CGD released NO. This release was inhibited by nitro-L-arginine methyl ester but could be reversed by L-arginine. Zymosan- and PMA-induced O_2^- production was less than 10% as compared with healthy controls.

5 These results indicate that O_2^- production is not essential for NO synthesis in human leucocytes.

Keywords endothelial-derived relaxing factor superoxide anion platelet aggregation respiratory burst human

Introduction

Phagocytes produce two different classes of cytotoxic inorganic oxidants: reactive oxygen intermediates (ROI) (Babior, 1978; Rossi & Zatti, 1964) and reactive nitrogen intermediates (RNI) (Rimele *et al.*, 1988; Stuehr & Marletta, 1985). ROI arise from the one-electron reduction of O_2 to O_2^- according to the reaction NADPH + $2O_2$ --> $NADP^+ + 2O_2^- + H^+$ catalysed by the enzyme NADPH oxidase (Babior, 1988; Babior *et al.*, 1976). RNI originate from the oxidation of a guanidino nitrogen atom of L-arginine by an enzyme that produces a nitrogen-centred radical, nitric oxide (NO) (Hibbs *et al.*, 1987a; Hibbs *et al.*, 1988; Iyengar *et al.*, 1987; Marletta *et al.*, 1988; Palmer *et al.*, 1988; Stuehr *et al.*, 1989). The enzymes responsible for NO synthesis (NO synthases) are NADPH-dependent (McCall *et al.*, 1991) and lead to the formation of stoichiometric amounts of L-citrulline and NO (Hibbs *et al.*, 1987b).

Selvaraj & Sbarra (1966) demonstrated that oxygen was required for the efficient killing of bacteria by neutrophils. Chronic granulomatous disease (CGD) clearly demonstrates the clinical relevance of ROI (Holmes *et al.*, 1967). It is a genetically-determined disease characterised by recurrent severe suppurative infections and impaired bacterial killing, due to defective ROI production by phagocytes (Curnutte & Babior, 1987; Gallin *et al.*, 1983; Tauber *et al.*, 1983). Involvement of RNI in human defence mechanisms is sparse. For instance, a marked increase in urinary NO_3^- excretion was observed in humans with diarrhoea and fever (Hegesh & Shiloah, 1982; Wagner *et al.*, 1984); elevated levels of NO_2^- were observed in both plasma and synovial fluid of patients with rheumathoid arthritis (McCall & Vallance, 1992). However, direct clinical evidence for RNI involvement in defence mechanisms has not yet been demonstrated.

The aim of this work was to evaluate the importance

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of the oxidative burst for RNI generation. Since human neutrophils and mononuclear cells release NO (Salvemini *et al.*, 1989a), we investigated this release in neutrophils and mononuclear cells from patients with CGD.

Methods

Selection of patients

Three patients with CGD (two males, 7 and 1.5 year old and one female, 6 years old) were selected based upon the following criteria: clinical history of recurrent severe suppurative infections, presence of granulomas as determined by histopathological biopsy sections, and impaired phagocyte O_2^- production upon exposure to soluble and particulate stimuli (Curnutte, 1988; Curnutte *et al.*, 1974). The patients' sera immunoglobulin (IgA, IgM, IgG, IgE) and complement (CH_{50}, C_3, C_4) levels, T (CD_4 and CD_8) and B cell counts, lymphocyte proliferative assays, myeloperoxidase, adenosine deaminase and glucose-6-phosphate dehydrogenase assays, chemotactic and phagocytic assays and myelograms were within the normal range. The patients' phagocytes had an impaired *Staphyloccus aureus* killing activity, as assessed by the fluorochrome assay (Bellinati-Pires *et al.*, 1989). The patients were HIV-1 negative, were receiving prophylactic antibiotic chemotherapy and were in a clinically stable condition. This study was approved by the Medical School Ethics Committee.

Preparation of washed platelets

Blood from healthy volunteers was collected by venepuncture into a plastic flask containing 3.8% sodium citrate. Platelet-rich plasma (PRP) was prepared by blood centrifugation at 200 *g* for 12 min at room temperature. The platelets were subsequently collected for washing by a further centrifugation at 900 *g* for 8 min at room temperature in the presence of iloprost (300 ng ml^{-1}). The supernatant was removed and the platelet pellet was resuspended in 15 ml of calcium-free oxygenated (95% O_2/5% CO_2) Krebs' buffer. Iloprost (300 ng ml^{-1}) was again added and the platelets were once more centrifuged at 900 *g* for 8 min at room temperature. The supernatant was aspirated and the pellet was again resuspended in calcium-free Krebs' buffer (Radomski & Moncada, 1983). The platelet count was determined automatically (Coulter Counter model T 890, Hialeah, Fla., U.S.A.) and adjusted to 1×10^8 cells ml^{-1}. Indomethacin (10 μM, to inhibit the formation of cyclooxygenase products) and $CaCl_2$ (1 mM) were added to the final platelet suspension.

Isolation of neutrophils and monuclear cells

Blood from healthy volunteers and from patients with CGD was collected as described above. Neutrophils and mononuclear cells were obtained as relatively pure cell populations using a Sigma Histopaque discontinuous density gradient kit (density 1.077 g ml^{-1} and 1.119 *g* ml^{-1}) according to the manufacturer's instructions (English & Andersen, 1974). Contaminating erythrocytes were hypotonically lysed. The leucocyte count was determined automatically (Coulter Counter model T 890, Hialeah, Fla., U.S.A.) and adjusted to 5×10^7 cells ml^{-1} for platelet agregation assays and to 2×10^6 cells ml^{-1} for the measurement of O_2^- in Hank's balanced salt solution supplemented with 10 mM HEPES (HHBSS) at pH 7.4, and containing 10 μM indomethacin and 1 mM $CaCl_2$.

Nitric oxide release

NO release was assayed by the ability of neutrophils and mononuclear cells to inhibit thrombin-induced human washed platelet aggregation (Salvemini *et al.*, 1989a). Briefly, a suspension of 1×10^8 cells/ml of healthy volunteer washed platelets (500 μl) was incubated at 37° C for 1 min in a Payton dual-channel aggregometer (Born & Cross, 1963) with continuous stirring at 900 rev min^{-1} and then stimulated with submaximal concentrations of thrombin (100–200 mu ml^{-1}). The decrease in optical density was recorded for 5 min after thrombin stimulation.

When required, patients' or healthy control's neutrophils or mononuclear cells (5×10^7 cells ml^{-1}) were added in a volume of 10–50 μl to the platelet suspension and the incubation continued for a further 1 min before stimulation with thrombin. The final leucocyte concentration ranged from $1–5 \times 10^6$ cells ml^{-1}. When necessary, superoxide dismutase (60 u ml^{-1}) was added immediately before addition of the nucleated cells. The NO synthesis inhibitor nitro-L-arginine methyl ester (L-NAME, Moore *et al.*, 1990) alone (300 μM) or in combination with L-arginine (1 mM) was pre-incubated with the neutrophils or mononuclear cells for 60 min before these cells were added to the platelet suspension.

Measurement of superoxide anion

The production of O_2^- by neutrophils and mononuclear cells was measured according to the procedure originally described by McCord & Fridovich (1969). Briefly, a 2×10^6 ml^{-1} neutrophil or mononuclear cell suspension was incubated with phorbol 12-myristate 13-acetate (PMA, 30 nM) or zymosan (100 particles/cell) in the presence of cytochrome c (80 μM) for 60 min at 37° C. The spontaneous superoxide anion release was determined using HHBSS insted of PMA or zymosan. Half of the tubes contained superoxide dismutase (SOD, 90 u ml^{-1}) at the beginning of the experiment as a control for the non-specific reduction of cytochrome c. The reaction was stopped by placing the tubes in an ice-water bath. SOD (90 u ml^{-1}) was then added to the tubes which did not contain it. After centrifugation (2200 *g* for 15 min at 4° C), the absorbance of the supernatant was measured at 550 nm and the O_2^- production was calculated using an extinction coefficient of 21,100 M^{-1} cm^{-1}. All experiments were performed twice in duplicate.

Materials

Superoxide dismutase (from bovine erythrocytes), L-arginine (free base), nitro-L-arginine methyl ester (L-NAME), HEPES, Histopaque 1119, Histopaque 1077,

phorbol 12-myristate 13-acetate (PMA), zymosan, cytochrome c (type III), and indomethacin were obtained from Sigma. Iloprost was a gift from Schering. Thrombin (bovine) was obtained from Roche. The composition of the Krebs' buffer was (mM): 137 NaCl, 2.7 KCl, 11.9 $NaHCO_3$, 0.3 NaH_2PO_4, 0.8 $MgSO_4$, 5.6 glucose, 1 $NaCl_2$ and that of the Hanks' solution was (mM): 137 NaCl, 2.7 KCl, 0.5 Na_2HPO_4, 0.4 KH_2PO_4, 5.5 glucose and supplemented with 10 mM HEPES (HHBSS) at pH 7.4.

Results

Neutrophils and mononuclear cells from patients with CGD inhibit thrombin-induced platelet aggregation due to release of nitric oxide

Thrombin (100–200 mu ml^{-1}) produced submaximal and irreversible platelet aggregation within 5 min. When neutrophils (1–5 × 10^6) or mononuclear cells (1–5 × 10^6) were added to the suspension of washed platelets 1 min before thrombin, platelet aggregation was inhibited according to the number of cells added (Figure 1). On a numerical basis, mononuclear cells were more potent than neutrophils in inhibiting platelet aggregation (Figure 1). Similar results were obtained with phagocytes from healthy controls (Figure 2).

Pre-incubation of neutrophils or mononuclear cells for 60 min with L-NAME (300 μM) reduced their ability to inhibit thrombin (100–200 mu ml^{-1})-stimulated platelet aggregation (Figure 3). Simultaneous pre-incubation of phagocytes with L-arginine (1 mM), reversed the effect of L-NAME (Figure 3). Incubation with L-arginine (1 mM) alone did not affect the ability of phagocytes to inhibit thrombin (100–200 mu ml^{-1})-stimulated platelet aggregation (not shown).

The inhibition of thrombin-induced platelet aggregation by neutrophils or mononuclear cells was enhanced in the presence of superoxide dismutase (60 u ml^{-1}; Figures 1 and 2). The inhibitory activity of neutrophils or mononuclear cells on thrombin-induced platelet aggregation was not seen when a higher concentration of thrombin (400 mu ml^{-1}) was used (data not shown). Superoxide dismutase (60 u ml^{-1}) did not affect thrombin-induced platelet aggregation at the 80–90% maximal level (data not shown).

Superoxide anion

The O_2^- release induced by PMA and Zymosan in CGD patients' cells was below 10% as compared with control cells (Table 1).

Discussion

Our results clearly demonstrate that neutrophils and mononuclear cells from patients with CGD inhibit thrombin-induced healthy volunteer washed platelet aggregation. The inhibition observed is probably due to release of NO, since it was reduced by L-NAME and could be reversed by L-arginine and it was also potentiated by superoxide dismutase. These results indicate that NO synthesis is not dependent on the oxidative burst. Indeed, Iyengar *et al.* (1987) demonstrated release of NO breakdown products (NO_2^- and NO_3^-) by the

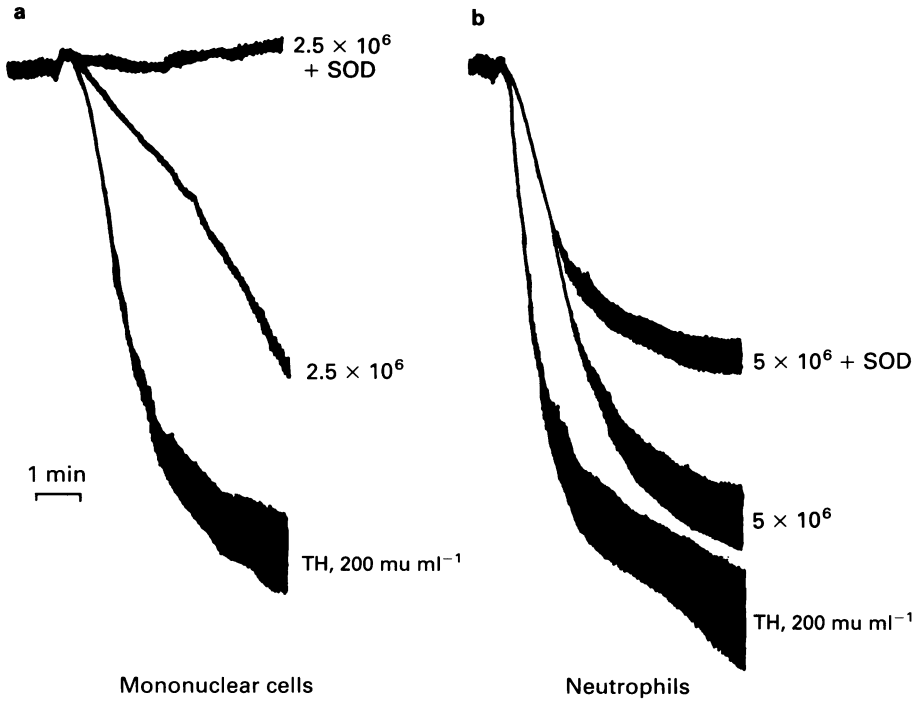

Figure 1 The inhibition of thrombin (200 mu ml^{-1})-induced platelet aggregation by mononuclear cells (2.5 × 10^6 cells ml^{-1}, panel a) or neutrophils (5 × 10^6 cells ml^{-1}, panel b) from patients with CGD is potentiated by superoxide dismutase (SOD, 60 units ml^{-1}). The tracings are representative of three experiments performed with cells from the 7 year old male patient. Similar results were observed in the experiments with cells from the other patients.

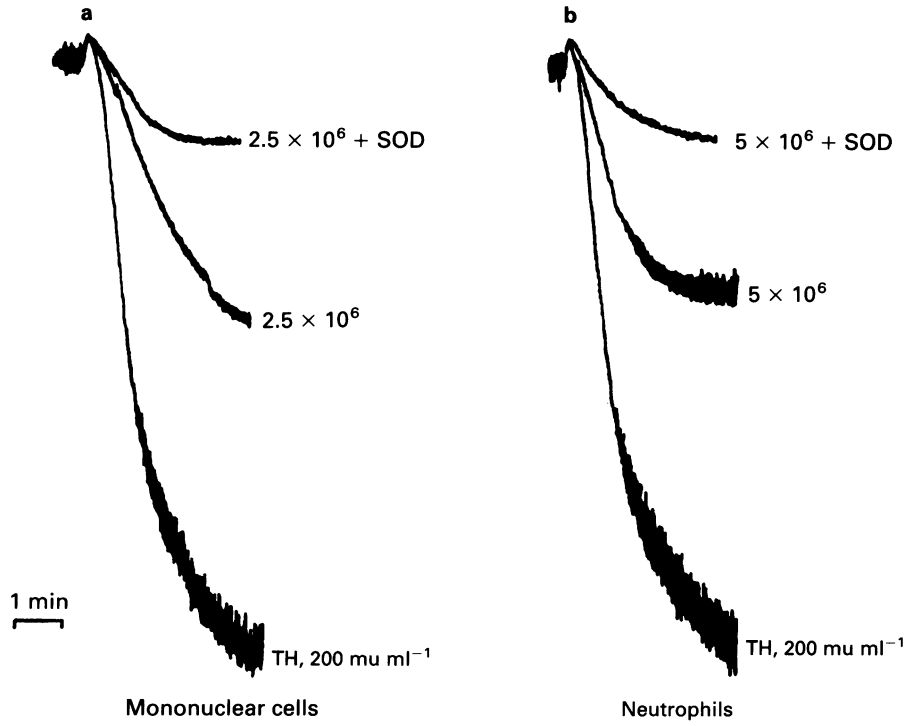

Figure 2 The inhibition of thrombin (200 mu ml^{-1})-induced platelet aggregation by mononuclear cells (2.5 × 10^6 cells ml^{-1}, panel a) or neutrophils (5 × 10^6 cells ml^{-1}, panel b) from healthy subjects is potentiated by superoxide dismutase (SOD, 60 units ml^{-1}). The tracings are representative of over twenty experiments performed with cells from healthy controls.

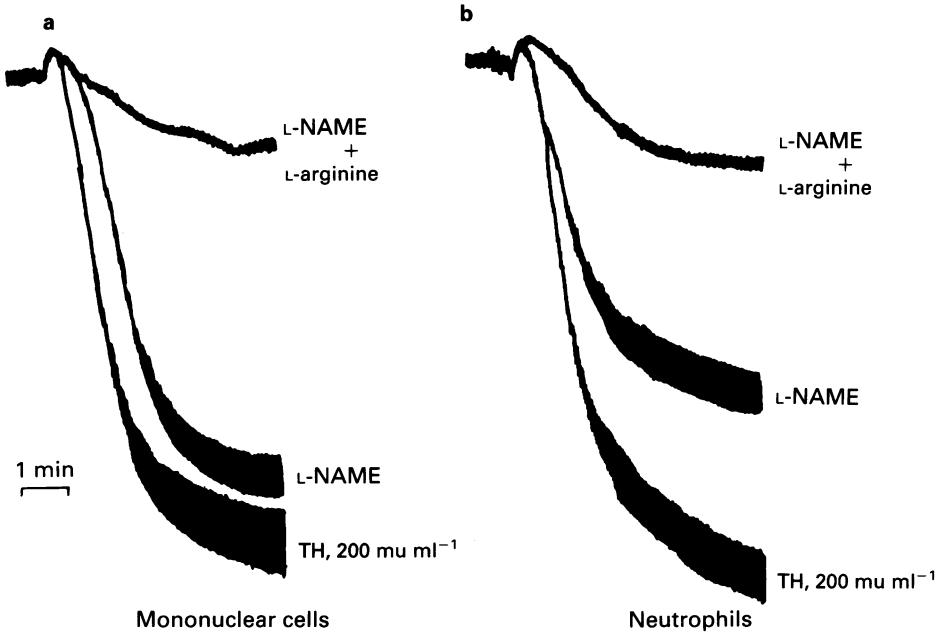

Figure 3 Effect of nitro-L-arginine methyl ester (L-NAME) on the inhibition of thrombin (200 mu ml^{-1})-induced platelet aggregation by mononuclear cells (MN, 2.5 × 10^6 ml^{-1}) and neutrophils (PMN, 5 × 10^6 ml^{-1}). Pre-incubation of mononuclear cells (a) or neutrophils (b) with L-NAME (200 μM), but not with L-arginine (1 mM), reduced their ability to inhibit platelet aggregation. Simultaneous pre-incubation of L-NAME (300 μM) and L-arginine (1 mM) reversed the effect of L-NAME. The tracings are representative of three experiments performed with cells from the 7 year old male patient. Similar results were observed in the experiments with cells from the other patients.

O_2^- deficient J774 C3C cell line and Ding *et al.* (1988) comparing mouse peritoneal macrophage H_2O_2, NO_2^- and NO_3^- production induced by cytokines, also suggested that RNI production is not linked to the respiratory burst.

It is interesting that SOD potentiated CGD patient phagocyte-induced inhibition of platelet aggregation. Since these phagocytes do not produce O_2^-, one has to consider other sources for O_2^- generation, such as the platelets from healthy volunteers (Joseph *et al.*, 1987; Marcus, 1979) and the Krebs' buffer (Warner *et al.*, 1989). Furthermore, SOD itself has also been shown to inhibit human washed platelet aggregation induced by low concentrations of thrombin (Salvemini *et al.*, 1989b).

Table 1 PMA- and zymosan-induced O_2^- production by neutrophils and mononuclear cells from patients with CGD

	PMA (30 nM)		*Zymosan (100 ptc/cell)*	
	Neutrophil	*Mononuclear*	*Neutrophil*	*Mononuclear*
Male 7 years	1–10	1–10	1–10	1–10
Male 1.5 years	< 1	< 1	< 1	1–10
Female 6 years	1–10	1–10	< 1	1–10

Results are expressed as the % of O_2^- production by cells from healthy volunteers.

In conclusion, we present here evidence in a human natural disease model of O_2^- deficiency that the respiratory burst is not essential for NO synthesis. Although there is reasonable *in vitro* evidence that RNI play an important role in modulating the phagocyte cytotoxic activity, we feel that further demonstration of lack of NO production associated with immunodeficiency *in vivo* is fundamental to assess their true relevance in the immune response. Recombinant human interferon γ (rIFN-γ) augments O_2^- production and bacterial killing in neutrophils from CGD patients (Ezekowitz *et al.*, 1987; 1988) and also caused a 70% reduction in the incidence of serious infections in these patients (ICSG, 1991). Interestingly, rIFN-γ has been shown to increase NO_2^- and NO_3^- release via induction of a specific NO synthase (Stuehr & Marletta, 1987). Whether RNI are involved in the beneficial effects of rIFN-γ treatment in these patients remains to be established.

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