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Hydrogen Peroxide and Tumour Necrosis Factor-α Induce NF-κB–DNA Binding in Primary Human T Lymphocytes in Addition to T Cell Lines

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Reactive oxygen intermediates (ROIs), such as hydrogen peroxide (H_2O_2), have been implicated as second messengers in the activation of NF-κB by a variety of stimuli, including tumour necrosis factor-alpha (TNF-α). The aim of the present study was to examine the effects of ROIs on NF-κB activation in primary human CD3+ T lymphocytes and human peripheral blood mononuclear cells (PBMCs). For comparison purposes, Jurkat T cells (subclones JR and JE6.1) were also investigated. Cells were incubated in the presence of either H_2O_2 or TNF-α and nuclear proteins were extracted. NF-κB binding was assessed by electrophoretic mobility shift assays (EMSAs). The concentration of H_2O_2 required to activate NF-κB in human primary CD3+ T lymphocytes was as low as 1 μM. In contrast, much higher concentrations of H_2O_2 were required to activate NF-κB in PBMCs and in the JR subclone of Jurkat T cells. H_2O_2-induced NF-κB activation was not observed in the JE6.1 subclone of Jurkat T cells. NF-κB was activated by TNF-α in all four cell types tested. In PBMCs and Jurkat T cells (subclones JR and JE6.1), this activation could be inhibited by pre-treatment with the antioxidants, pyrrolidine dithiocarbamate (PDTC) and *N*-acetyl-L-cysteine (NAC). Our results support a role for ROIs in NF-κB–DNA binding in human primary T lymphocytes.

Keywords: Reactive oxygen intermediates; Hydrogen peroxide; Tumour necrosis factor-alpha; NF-κB; Primary human T lymphocytes; Antioxidant

Abbreviations: EMSA, electrophoretic mobility shift assay; H_2O_2, hydrogen peroxide; Iκ·Bα, NF-κB inhibitory protein alpha; NAC, *N*-acetyl-L-cysteine; NF-κB, nuclear factor-kappa B; PBMCs, peripheral blood mononuclear cells; PDTC, pyrrolidine dithiocarbamate; ROIs, reactive oxygen intermediates; TNF-α, tumour necrosis factor alpha

INTRODUCTION

NF-κB is a multi-subunit transcription factor which regulates the transcription of many genes involved in inflammation and acute phase

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responses.[1] The predominant inactive form of NF-κB exists as a trimer of p65, p50 and IκB subunits. Stimulation of cells by a variety of pathophysiological agents, including mitogens, cytokines and oxidative stress, leads to the phosphorylation and ubiquitin-regulated proteolysis of IκB.[2] This allows free NF-κB dimers to translocate to the nucleus where they can bind DNA and regulate gene expression.

It has been suggested that ROIs, including H_2O_2, may function as signalling molecules in the activation of NF-κB[3,4] and in the signal transduction of immunoregulatory processes.[5,6] Early investigations demonstrated a role for ROIs in NF-κB activation in T cell lines,[3,7–9] Hela cells,[10] cardiac myocytes,[11] mouse macrophages,[12] rat mesothelial cells[13] and human epithelial cells.[14] However, in certain other cell types including mouse mesangial cell lines,[15] murine thymoma or human epidermal carcinoma cell lines[16] or in some Jurkat T cells (other than subclone JR),[8,17] H_2O_2 does not lead to NF-κB activation. Such observations make it difficult to postulate a role for ROIs, particularly H_2O_2, in NF-κB activation in all T cells. Furthermore, most investigations of this nature have been carried out in transformed T cell lines and there is less evidence to support a role for ROIs in NF-κB activation in primary T lymphocytes.

Several studies in cell lines suggest that stimulation of cells by cytokines may lead to the production of ROIs and consequently NF-κB activation.[18,19] Bonizzi and co-workers have demonstrated a cell type specific role for ROIs in NF-κB activation by IL-1β.[19] They showed that the source of ROIs in lymphoid cells stimulated by IL-lβ is 5-lipoxygenase, whereas the NADPH oxidase complex fulfils the same role in monocytic cells. In contrast, epithelial cells stimulated with IL-lβ activate NF-κB, but do not generate ROIs, suggesting that ROIs are cell type specific second messengers for IL-1 induced NF-κB activation.

Studies which demonstrate inhibition of NF-κB activation by antioxidants lend further support for a role of ROIs in the activation of NF-κB. For example, pyrrolidine dithiocarbamate (PDTC), has been shown to inhibit NF-κB activation by a wide variety of stimuli in the JR subclone of Jurkat T cells.[20] Scavengers of ROIs, such as *N*-acetyl-L-cysteine (NAC), have also been shown to inhibit TNF-α-induced NF-κB activation in the JR subclone of Jurkat T cells,[3] human synovial cells,[21] dendritic cells[22] and the monocytic cell line, U937.[23] However, since NAC and PDTC do not inhibit NF-κB activation in all cell types,[24,25] the extent of NF-κB inhibition by antioxidants appears to vary depending on cell type and signal.

Current evidence, therefore, suggests that the activation of NF-κB by ROIs is cell type-specific and that differences exist in the ability of different cell types to regulate their response to oxidant stress. The purpose of the present study was to establish the role of ROIs in primary human blood cells. This was achieved by incubating both isolated human peripheral blood mononuclear cells (PBMCs) and isolated human CD3+ T lymphocytes with either H_2O_2 or TNF-α and assessing the activation of NF-κB by EMSAs. In addition, the effect of antioxidants on TNF-α-induced NF-κB activation in human primary cells was also investigated.

MATERIALS AND METHODS

Cell Culture and Reagents

Jurkat T cells (subclone JR) (kindly donated by Dr Hieke Pahl, Institute of Biochemistry and Molecular Biology, Albert–Ludwig's University, Frieberg, Germany) and Jurkat T cells (subclone JE6.1) (European Collection of Cell Cultures, Wiltshire, UK) were maintained in tissue culture medium (TCM) comprising RPMI 1640 medium with 25 mM HEPES (Gibco, Paisley, UK), 20% fetal calf serum (FCS) (Globe Pharm, Surrey, UK), 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco, Paisley, UK). Sub-cultures were maintained at between 3 and

6×10^4 cells/ml gassed with 5% carbon dioxide at 37°C. Cells (4×10^6) were treated with either rhTNF-α (Boehringer Mannheim), H_2O_2 (Sigma, Dorset) alone, or after pre-treatment with either NAC or PDTC (Sigma, Dorset). Nuclear extracts were then prepared as described previously.[26]

Isolation of PBMCs

Blood was collected from healthy human volunteers by venipuncture (60 ml) and transferred equally to two 50 ml Falcon tubes (Becton Dickinson, UK) each containing 30 units of sodium heparin (CP Pharm., Wrexham, UK). The blood was diluted 1:2 with Hanks balanced salts solution, layered onto an equal volume of Lymphoprep (Nycomed, Norway) and centrifuged at 600 *g* for 20 min at 20°C. The PBMCs were taken from the interface and washed three times in HBSS; first at 550 *g* for 15 min at 4°C and twice at 400 *g* for 10 min at 4°C. Cells were then re-suspended in TCM for use in experiments.

Immunomagnetic Enrichment of CD3+ T Cells

T lymphocytes were isolated using a negative selection pan T cell isolation kit (Miltenyi Biotec, Surrey, UK) following the supplier's instructions. Briefly, PBMCs were isolated as described in *Isolation of PBMCs* section, and re-suspended in 5 ml of phosphate buffered saline (PBS). Non-CD3+ T cells were labelled using a cocktail of hapten-modified antibodies for 20 min on ice. Cells were washed twice in PBS and centrifuged at 400 *g* for 6 min at 4°C and then magnetically labelled with anti-hapten microbeads for 30 min on ice. Cells were washed twice as described and passed through a depletion column in the magnetic field of a Mini-Mats separator (Miltenyi Biotec, Surrey, UK). Isolated T cells were then washed twice in PBS/2 mM EDTA pH 7.4 as described, and allowed to recover overnight in TCM before experimentation. Purity of T cell preparations (>98%) was checked by immunofluorescence labelling with CD3-FITC (Becton–Dickinson, UK) and various monoclonal antibodies specific for monocytic and B cells. A cell count and Trypan blue test was also performed to assess cell viability and yield.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts (10 μg) were isolated as previously described.[26] Briefly, after stimulation, cells were transferred to 1.5 ml eppendorf tubes, washed twice with 1 ml of ice-cold PBS and centrifuged at 800 *g* for 5 min, 4°C. The pellet was re-suspended in 400 μl of cold RNA lysis buffer [HEPES (pH 7.9) 10 mM, KC1 10 mM, $MgCl_2$ 1.5 mM, DTT 0.5 mM, EDTA 0.1 mM] and incubated on ice for 15 min. Then, 25 μl of 10% NP-40 was added to the cap of each eppendorf tube, inverted five times and centrifuged immediately at 1250 *g* for 5 min at 4°C. The pellet was then re-suspended in 40 μl of nuclear protein extraction buffer [HEPES (pH7.9), NaCl 300 mM, KCl 50 mM, DTT 0.5 mM, EDTA 0.1 mM, glycerol 25%], incubated on ice for 30 min, then centrifuged at 12,000 *g* for 10 min at 4°C. The supernatant (nuclear protein extract) was then transferred to a fresh tube and stored at −70°C until use. The amount of protein was determined using the Bradford Assay Kit (Biorad).

Equal amounts of nuclear protein were then incubated with an NF-κB specific ^{32}P-labelled double-stranded oligonucleotide probe (Promega, Southampton, UK) for 30 min at room temperature. Binding reactions were performed as previously described.[26] In competition experiments, a 100-fold excess of cold NF-κB probe was incubated with the extracts for 10 min prior to the addition of the labelled probe. The samples were then loaded on a 4% non-denaturing polyacrylamide gel and run with 0.5× tris-borate-EDTA (TBE) buffer (pH 8.3) for 1 h at 100 V. Following electrophoresis, the gels were dried under vacuum for 1 h and exposed to

radiographic films (GRI, Essex, UK) in the dark at −70°C overnight.

RESULTS

The activation of NF-κB in primary CD3+ T lymphocytes, PBMCs and Jurkat T cells (subclones JR and JE6.1) in response to H_2O_2 was assessed by EMSA of nuclear protein extracts. Figure 1A–D shows H_2O_2-induced NF-κB–DNA-binding in CD3+ T lymphocytes from four different volunteers after 4 h. The concentration of H_2O_2 which activated NF-κB in the primary CD3+ T lymphocytes was as low as 1 μM in some individuals. However, the concentration of H_2O_2 which activated NF-κB varied between individuals.

We also examined the effect of H_2O_2-induced NF-κB activation in human PBMCs from other volunteers in the study (Fig. 2A and B). Maximum activation was observed after 4 h, as determined by time-course experiments (results not shown). In contrast to primary CD3+ T lymphocytes, concentrations of H_2O_2 <100 μM did not activate NF-κB when compared to control extracts.

Figure 2C shows that micromolar concentrations of H_2O_2 activated NF-κB in the JR subclone of Jurkat T cells, confirming previously reported data.[18] Maximum activation was observed at 150 μM of H_2O_2 after 4 h, as determined by time-course experiments (results not shown). Concentrations of H_2O_2 <150 μM and >200 μM did not activate NF-κB when compared to control extracts. NF-κB activation was not detected at similar concentrations and time-periods in the JE6.1 subclone of Jurkat T cells (results not shown). The absence of NF-κB activation in response to H_2O_2 in these cells has been previously reported.[16] Antibody clearance experiments with antibodies raised against p50 and p65 showed elimination of the band induced by H_2O_2 in these cells, indicating the presence of NF-κB heterodimer (results not shown).

The activation of NF-κB in primary CD3+ T lymphocytes, PBMCs and Jurkat T cells (subclones JR and JE6.1) in response to TNF-α was also studied. Figure 3 shows that NF-κB activation was observed in primary CD3+ T lymphocytes and PBMCs (A and B, respectively) in response to TNF-α after 1 h, in a dose-responsive manner (previous dose-response and time-course experiments revealed that 25 ng/ml TNF-α exposure for 1 h achieved optimum NF-κB–DNA binding).

We also examined the effect of TNF-α-induced NF-κB activation in Jurkat T cells (subclones JR and JE6.1). We found that TNF-α activated NF-κB in both subclones in a dose-responsive manner (Fig. 3C and D). Competition experiments revealed that DNA-binding was specific for NF-κB, since complexes were effectively competed off by excess of unlabelled (cold) NF-κB oligonucleotides (Fig. 3C, lane 5 and Fig. 3D, lane 6).

The antioxidants PDTC and NAC have previously been shown to inhibit TNF-α-induced NF-κB activation in Jurkat T cells (subclone JR), suggesting an involvement of ROIs in this pathway.[3,20] We investigated whether the same effect could be observed in human PBMCs (Fig. 4). NF-κB activation was inhibited in human PBMCs by pre-treatment with 0.1–1 mM PDTC (Fig. 4A). The biphasic dose-response observed with PDTC in these cells has previously been reported for Jurkat T cells,[20] and our data confirms this (Fig. 4B). In addition, TNF-α-induced NF-κB activation was inhibited by PDTC in the JE6.1 subclone of Jurkat T cells (results not shown). NF-κB activation was also inhibited by pre-treatment with 25–50 mM NAC, in both PBMCs and Jurkat T cells (subclone JR) (Fig. 4C and D, respectively).

DISCUSSION

The present study has examined the effects of H_2O_2 and TNF-α on NF-κB activation in human

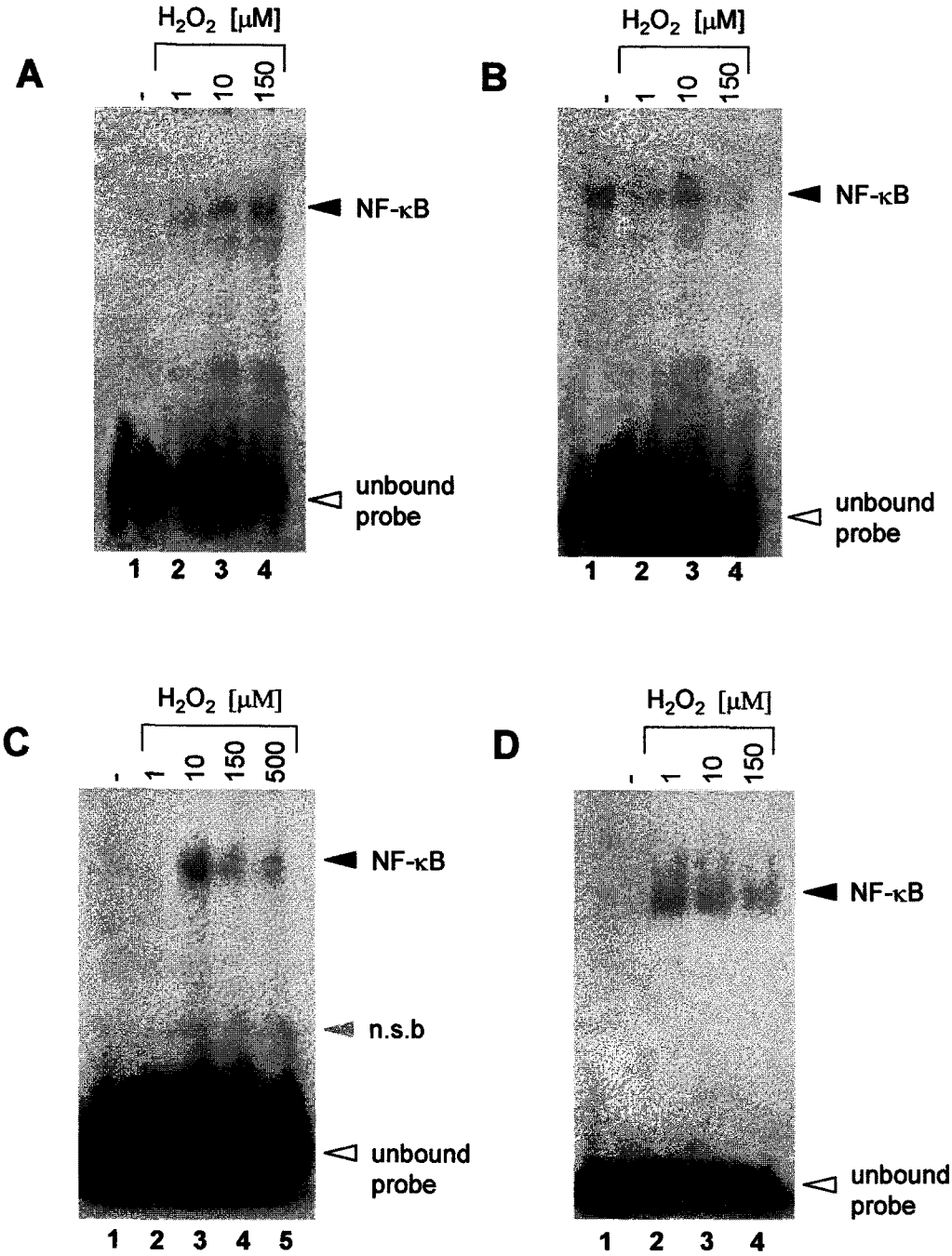

FIGURE 1 The dose-response effect of H_2O_2 on NF-κB activation in human primary CD3+ T lymphocytes taken from 4 different volunteers (A–D) ($n = 4$). Human primary CD3+ T lymphocytes and incubated in the absence (lane 1) or presence of H_2O_2 for 4 h at the concentrations indicated. Nuclear extracts were prepared from these cells and analysed for NF-κB–DNA binding by (EMSAs).

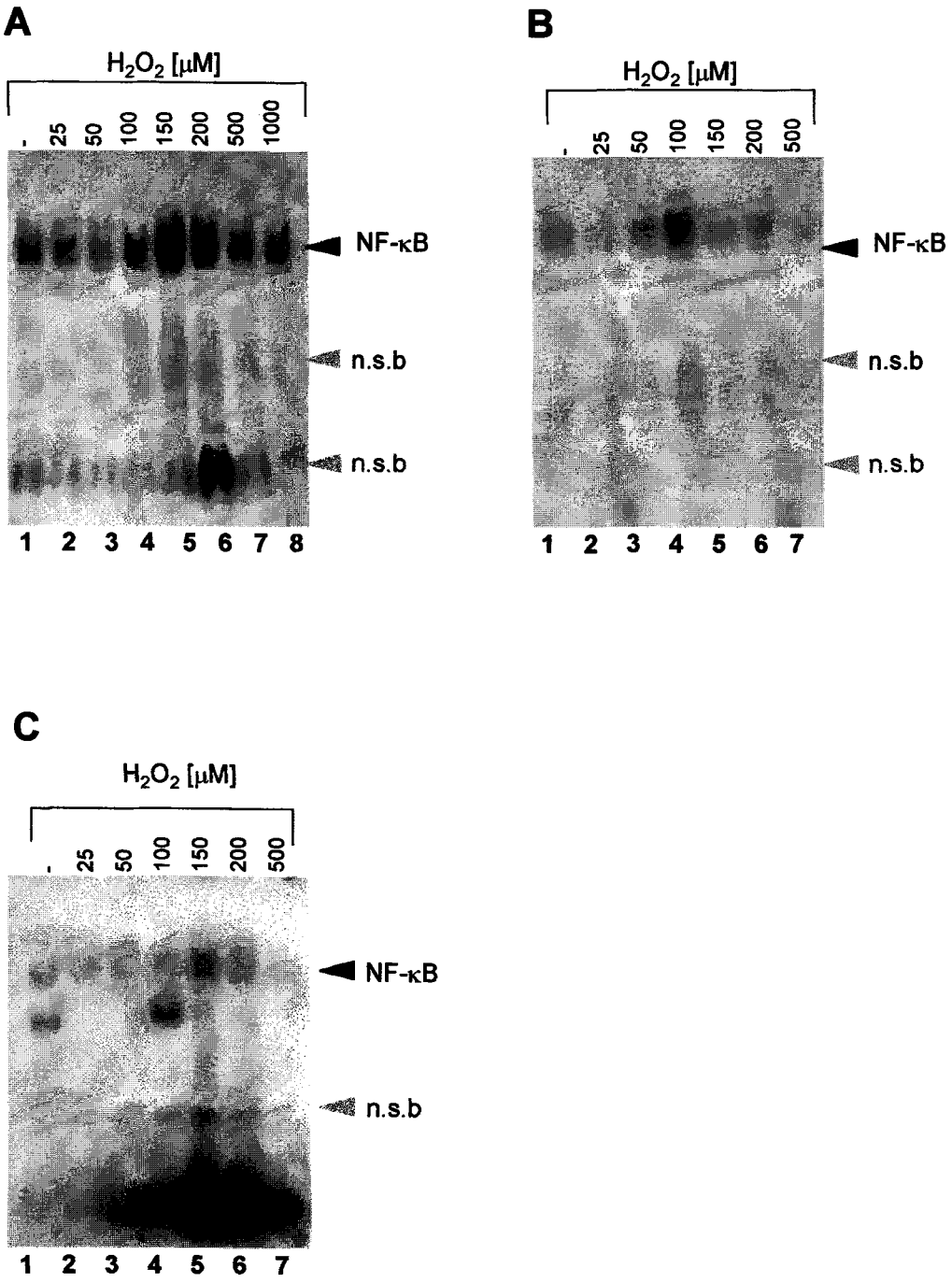

FIGURE 2 The dose-response effect of H_2O_2 on NF-κB activation in human peripheral blood mononuclear cells (PBMCs) from 2 volunteers (A and B) and Jurkat T cells (subclone JR) (C) (for each cell type, $n = 3$). Cells were incubated in the absence (lane 1) or presence of H_2O_2 for 4 h at the concentrations indicated.

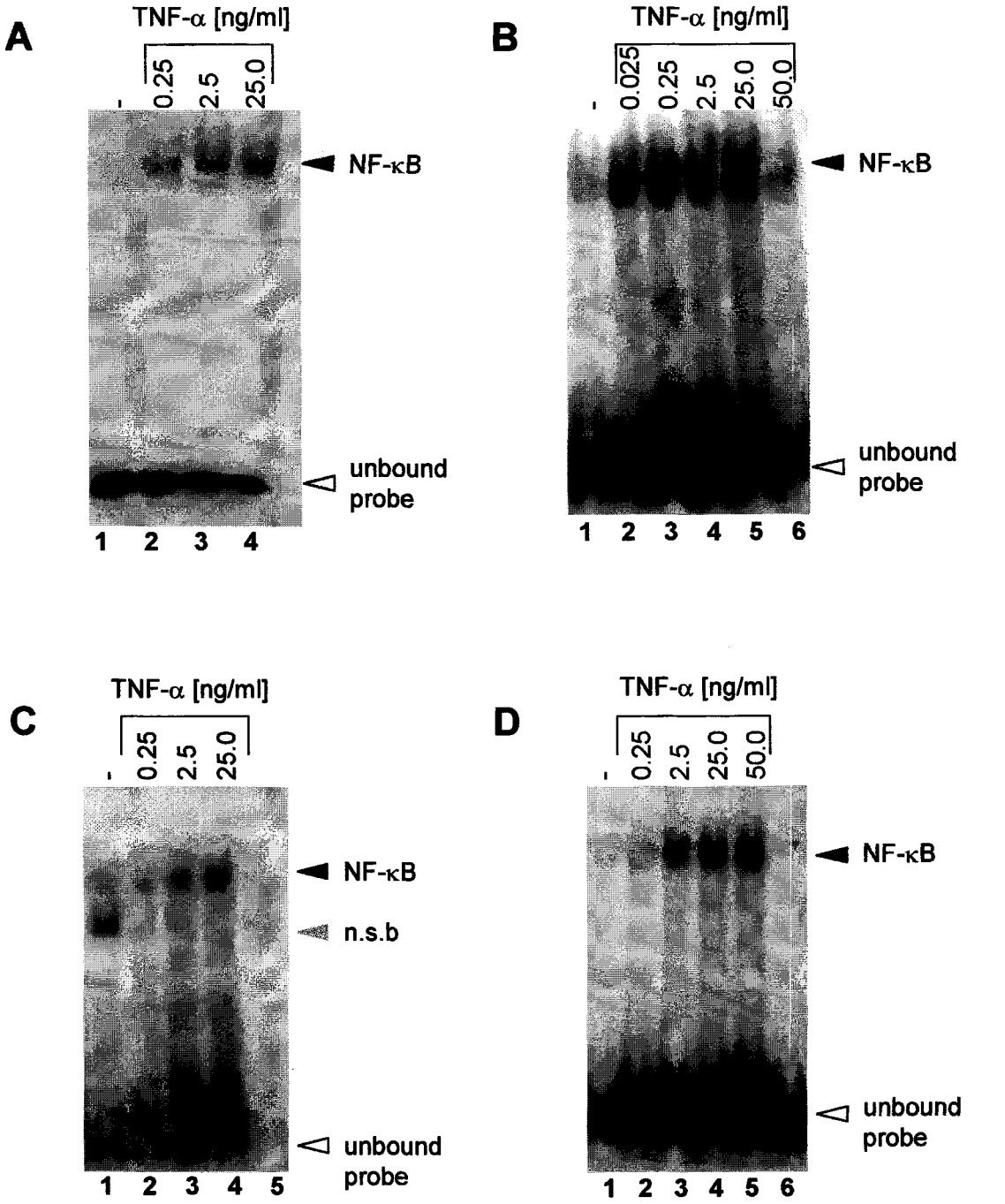

FIGURE 3 The dose-response effect of TNF-α on NF-κB activation. Human primary CD3+ T lymphocytes, PBMCs, Jurkat T cells (subclone JR) and Jurkat T cells (subclone JE6.1) (A–D, respectively) were incubated with TNF-α for 1 h at the concentrations indicated (experiments were repeated at least three times). In (C), lane 5 and (D), lane 6, 100× cold probe was incubated with the 25 ng/ml TNF-α-treated reaction mixture prior to EMSA.

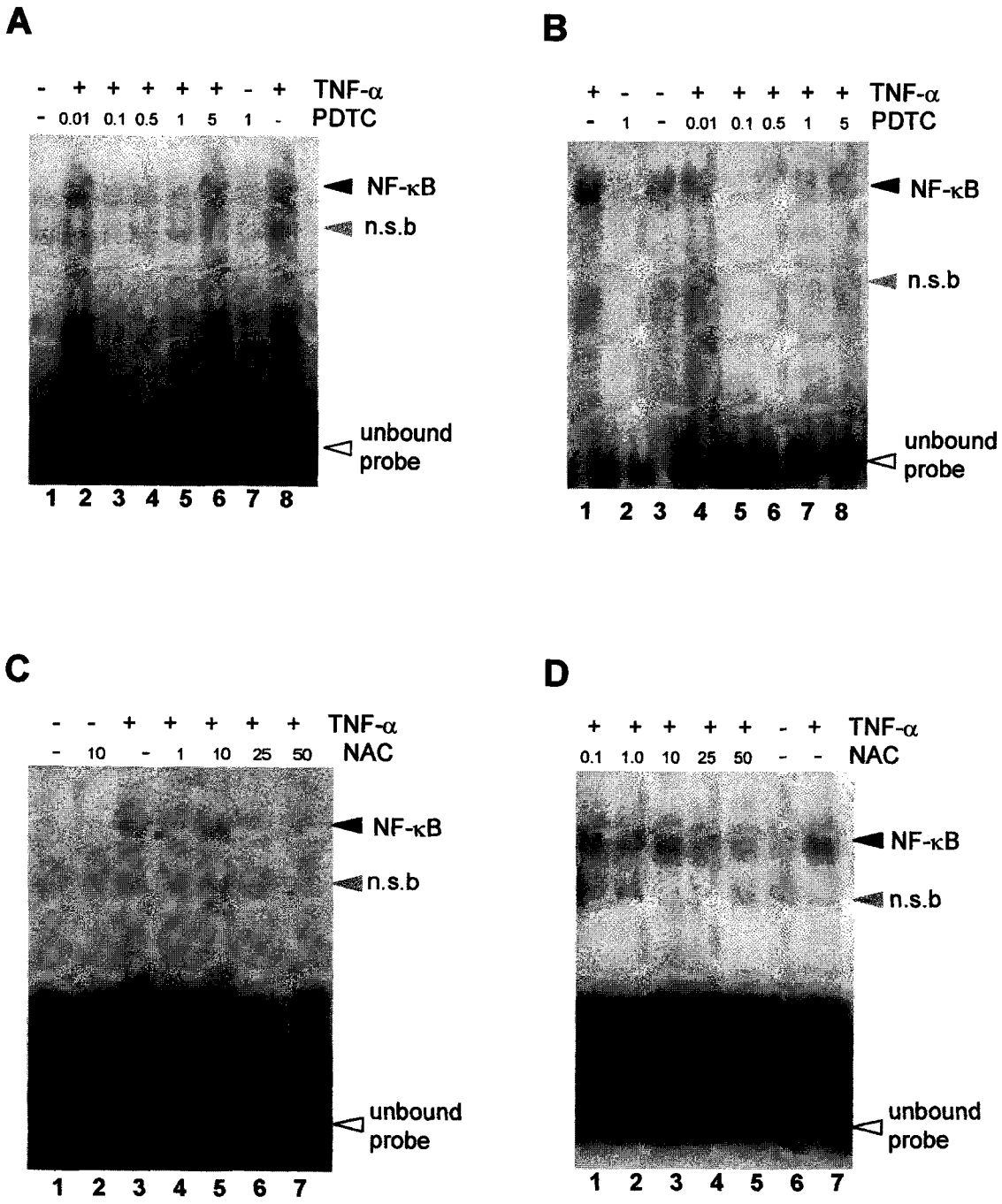

FIGURE 4 The dose-response effect of PDTC and NAC on TNF-α-induced NF-κB activation. PBMCs (A and C) and Jurkat T cells (subclone JR) (B and D), were pre-treated for 1.5 h with either PDTC or NAC, respectively, concentrations (mM) as indicated, followed by a 1 h stimulation with 25 ng/ml TNF-α (for each cell type, $n = 3$).

primary T lymphocytes and Jurkat T cell lines. NF-κB activation was induced by H_2O_2 in normal human primary CD3+ T lymphocytes at lower concentrations of H_2O_2 (1 μM) than those required to achieve the same effect in PBMCs and in the JR subclone of Jurkat T cells (100–150 μM). This lends support to the hypothesis that ROIs are only required at relatively low concentrations for signal transduction in CD3+ T lymphocytes. Furthermore, differences in constitutive and H_2O_2-induced NF-κB activation between primary CD3+ T lymphocytes from different individuals was noted. These results may be due to the cells from some individuals having different levels of antioxidant enzymes, or different capacities to generate ROIs, which render the cells variably responsive to ROIs.

We showed that NF-κB was induced by H_2O_2 in both primary human blood cells (PBMCs) and the JR subclone of Jurkat T cells. However, H_2O_2 did not induce NF-κB activation in the JE6.1 subclone of Jurkat T cells at any of the concentrations or time-periods tested. Others have also failed to reveal H_2O_2-induced NF-κB activation in some subclones of Jurkat T cells (other than subclone JR).[16,27] In contrast, TNF-α did induce NF-κB activation in the JE6.1 subclone of Jurkat T cells, suggesting that the signalling pathway leading to TNF-α-induced NF-κB activation may not require the presence of H_2O_2 in these cells. A possible explanation for the absence of NF-κB activation in Jurkat T cells other than the JR subclone of Jurkat T cells has been put forward by Sen and colleagues.[28] They provided evidence that a sustained elevated concentration of intracellular Ca^{2+} was an important factor in the induction of NF-κB activation by H_2O_2. In the Jurkat cells which were sensitive to oxidant-induced NF-κB activation, the intracellular [Ca^{2+}] elevation was sustained following H_2O_2 challenge. In contrast, in the Jurkat cell subclone, which was insensitive to oxidant-induced NF-κB activation, the Ca^{2+} response to H_2O_2 exposure was rapid and transient. In addition, it was suggested that the NF-κB regulatory effect of NAC and α-lipoate was due to their ability to dampen the elevation of Ca^{2+}which followed oxidant challenge.

The thiol containing compounds PDTC and NAC, effectively inhibited TNF-α-induced NF-κB activation in both PBMCs and Jurkat T cells (subclones JR and JE6.1), consistent with previous reports.[17,27,29] The treatment of these cells with PDTC led to a biphasic dose-response, i.e. concentrations <0.1 mM and >1 mM failed to inhibit NF-κB activation. Interestingly, recent evidence has shown that TNF-α-induced NF-κB activation is inhibited by dimethyldithiocarbamate (DMDTC) in primary human CD4+ T cells, at concentrations two orders of magnitude lower than PDTC's effects in Jurkat T cells, although the incubation time and dosing regime were different from the present study.[30]

However, evidence suggests that PDTC may have oxidative properties at higher concentrations. Brennan and O'Neill[31] showed that while PDTC inhibited the TNF-α-induced activation of NF-κB in DNA binding assays, the PDTC effect was as an oxidiser of the p50 subunit. PDTC inhibition of these nuclear extracts was reversed by treatment with 2-mercaptoethanol by providing reducing conditions for the thiol components. More recently, Moellering *et al.*[32] observed that PDTC alone, at relatively low concentrations (0–25 μM), was a potent inducer of glutathione synthesis in endothelial cells. A pro-oxidative effect of PDTC at higher concentrations may help to explain the biphasic effect of PDTC on TNF-α-induced NF-κB activation seen in the present study.

Interestingly, it has been reported that the effects of NAC and PDTC on NF-κB activation may be stimulus- and/or cell-specific. It was discussed earlier how the effects of oxidants on NF-κB activation were dependent on similar specificities. However, as yet, it remains unclear at which stage in the NF-κB signalling pathway oxidants and antioxidants exert their effect. In the case of NAC and PDTC, it is also unclear

whether their effect can be attributed solely to their antioxidant properties or whether other mechanisms are involved. For example, it has been suggested that these compounds may inhibit NF-κB activation by directly affecting the activity of proteins involved in its signalling pathway.[25]

The present study supports a role for ROIs in NF-κB activation in primary human lymphocytes. Given that ROIs may not play a role in the process in all cell types, it will be important to examine the effects of ROIs and antioxidants on NF-κB activation in other human primary cells, such as macrophages, B cells and endothelial cells. Such experiments may indicate a more specific role for ROIs than previously proposed in relation to NF-κB activation in the immune/inflammatory response.

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