

Induction of cytokines and ICAM-1 by proinflammatory cytokines in primary rheumatoid synovial fibroblasts and inhibition by *N*-acetyl-L-cysteine and aspirin

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Abstract

The role of transcription factor NF- κ B in the induction of cytokines and ICAM-1 upon stimulation with proinflammatory cytokines, IL-1 and tumor necrosis factor (TNF)- α was investigated in primary synovial fibroblasts obtained from patients with rheumatoid arthritis (RA). Nuclear translocation of NF- κ B was demonstrated after 30 min of treatment with IL-1 or TNF- α . Thereafter, the production of several cytokines including granulocyte macrophage colony stimulating factor, IL-6 and IL-8, that are known to be abundantly produced in the synovial cavity of RA patients, was greatly augmented. Similarly, cell surface expression of ICAM-1 was induced by the IL-1 or TNF- α treatment. Since expression of these genes is induced in rheumatoid synovial tissue, this experimental system is considered to represent the *in vivo* situation of RA pathophysiology. Using this cell culture system we attempted to modulate the intracellular signaling cascade for NF- κ B activation and examined the effects of *N*-acetyl-L-cysteine (NAC) and acetylsalicylic acid (aspirin), which were previously reported to inhibit NF- κ B activation. Pretreatment of the primary synovial fibroblasts with NAC inhibited nuclear translocation of NF- κ B. Subsequently, the induction of these cytokines and ICAM-1 was considerably suppressed. On the other hand, pretreatment with aspirin blocked these phenomena only partially. These observations indicate the pivotal role of NF- κ B in RA pathogenesis thus highlighting the possibility of a novel therapeutic strategy.

Introduction

Rheumatoid arthritis (RA) is characterized as a chronic and progressive inflammatory processes with systemic immunological abnormalities leading to synovial hyperplasia and joint destruction. The inflamed synovium is infiltrated by lymphocytes and monocytes, which reinforces the underlying immunological mechanism in this disease process (1–5). Although the primary cause of RA remains unknown, the active involvement of cytokines and cell adhesion molecules (CAM) has been implicated in the rheumatoid inflammation. These cytokines include tumor necrosis factor (TNF)- α , IL-1, IL-6, IL-8, IFN- γ and granulocyte macrophage colony stimulating factor (GM-CSF) (5–7). Additionally, synoviocytes in a hyperplastic rheumatoid synovium are associated with the

augmented expression of ICAM-1 and VCAM-1 (8–11). These cytokines and CAM are considered to play crucial roles in the cognate and non-cognate interaction between synoviocytes and leukocytes that is a prerequisite for chronic inflammation leading to rheumatoid synovitis (10).

Among these cytokines, TNF- α and IL-1 have been studied most extensively because of their actions in inducing the expression of other cytokines and CAM. This notion has been confirmed by clinical trials using anti-TNF- α mAb and IL-1 antagonists in the treatment of RA synovitis (12,13). Furthermore, it is well established that TNF- α and IL-1 stimulate gene expression of these cytokines and CAM through a signal transduction pathway leading to NF- κ B activation (14–21).

NF- κ B is an inducible cellular transcription factor present in the primordial mesenchymal cell lineage including lymphocytes, macrophages and fibroblasts (14,21). NF- κ B regulates a wide variety of cellular genes including those associated with RA, as mentioned above. Although NF- κ B is by no means the sole determinant for inducible expression of these genes, it has been shown to play a significant role in inducing expression of these genes (14–21). Previous studies have demonstrated that NF- κ B binding to the specific DNA sequence (called the ' κ B motif') is essential for transcriptional activation (22,23). Reactive oxygen intermediates (ROI) are known to be involved in the initial stage of NF- κ B activation cascade (for review see 14). The induction of NF- κ B binding is independent of new protein synthesis and involves dissociation of NF- κ B from a cytoplasmic-anchoring protein, I κ B, followed by translocation of NF- κ B to the nucleus, where it activates the target genes (24–26). We have previously demonstrated that the DNA binding activity of NF- κ B is regulated by an oxido-reductive mechanism ('redox regulation') where ROI and the cellular reducing catalyst thioredoxin (TRX) play major roles (27,28), and that a novel serine kinase(s) [named 'NF- κ B kinase(s)'] is involved in NF- κ B activation by directly phosphorylating NF- κ B (29). Although a specific inhibitor of kinases involved in the NF- κ B activation cascade is yet to be identified, antioxidants such as *N*-acetyl-L-cysteine (NAC) (30,31) are known to block NF- κ B cascade. A recent study demonstrated that sodium salicylate and its related compound aspirin could block the proteolytic processing of I κ B and thus inhibit NF- κ B activation (32,33).

These observations led us to investigate the role of NF- κ B in rheumatoid synovitis using primary synovial fibroblasts obtained from patients with RA. We also attempted to block the NF- κ B activation pathway using NAC and examined its effect on the induction of the RA-associated cytokines and CAM. Similarly, the effects of aspirin were investigated.

Methods

Cytokines and reagents

Recombinant human IL-1 β (rhIL-1 β) was kindly provided by Otsuka Pharmaceutical (Tokushima, Japan). Recombinant human TNF- α (rhTNF- α) was purchased from Genzyme (Cambridge, MA). NAC and aspirin were both obtained from Sigma (St Louis, MO). Rabbit polyclonal antibodies to human NF- κ B subunits, p65 and p50, and human I κ B- α were raised from synthetic peptides as previously reported (28,29) or purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibody to human TRX was a generous gift from Dr A. Mitsui (Institute of Basic Science, Ajinomoto, Japan). Mouse mAb to human HLA-DR, von Willebrand factor, desmin, smooth muscle α actin, CD1a, CD68 and the β -subunit of prolyl 4-hydroxylase (5B5) were purchased from Dako (Glostrup, Denmark). Mouse mAb against human ICAM-1 was purchased from Becton Dickinson (San Jose, CA).

Cells

Rheumatoid synovial fibroblasts (RSF) were isolated from the fresh synovial tissue biopsy samples from three RA patients

as previously reported (34,35). We also utilized a single cell clone derived from a primary culture of RA synovial tissue (36). The tissue samples were minced into small pieces and treated with 1 mg/ml collagenase/dispase (Boehringer Mannheim, Mannheim, Germany) for 10–20 min at 37°C. The cells obtained were cultured in F-12 (HAM) (Gibco/BRL, Grand Island, NY) supplemented with 10% FCS (Irvine Scientific, Santa Ana, CA), 100 U/ml of penicillin, 100 mg/ml of streptomycin and 0.5 mM 2-mercaptoethanol. The culture medium was changed every 3–5 days and non-adherent lymphoid cells were removed. Adherent cell subcultures were maintained in the same medium and harvested by trypsinization every 7–10 days before they reached cellular confluency.

All the experiments described here were conducted using the RSF during the third to seventh passage. To characterize the phenotype of adherent cells, the cells were stained with mouse mAb against human HLA-DR, von Willebrand factor, desmin, smooth muscle α actin, CD1a, CD68 and 5B5. Only 5B5 was positive for RSF suggesting its fibroblast-like phenotype.

Immunofluorescence

RSF were cultured in four-well LabTek chamber slides (Nunc, Naperville, IL) and allowed to adhere for 72 h. Cells were then stimulated with 10 ng/ml rhIL-1 β or rhTNF- α for different time periods. Another set of cells was pretreated for 1 h with 10 mM NAC or 2.5 mM aspirin prior to the treatment with these cytokines. Indirect immunofluorescence using specific anti-NF- κ B or anti-TRX antibodies was performed as follows: the cells were fixed in 4.5% paraformaldehyde/PBS for 10 min at room temperature and then permeabilized by 0.5% Triton X-100/PBS for 20 min at room temperature. They were then incubated with rabbit polyclonal antibody against p65 or p50 NF- κ B subunits for 45 min at 37°C. After washing with PBS, the cells were incubated with FITC-conjugated goat anti-rabbit (whole IgG) antibody (Cappel Organon Teknika, Durham, NC) for 20 min at 37°C (37).

Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from RSF by the method described previously (38). Cells ($2\text{--}4 \times 10^6$) were harvested by scraping and washed in cold PBS. Nuclei were isolated by treatment with hypotonic lysis buffer A (10 mM HEPES-KOH, pH 8.0, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 200 mM sucrose, 0.5 mM phenylmethylsulfonyl fluoride, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin and 0.5% NP-40). The nuclei released by lysis were collected by microcentrifugation and the supernatant fraction containing cytoplasmic proteins was frozen on dry ice. After twice washing with buffer A, nuclear proteins were extracted by suspending the nuclei in buffer B (20 mM HEPES-KOH, pH 8.0, 100 mM KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 20% glycerol, and 1 μ g/ml each leupeptin and aprotinin) and sonicated for 20 s by an ultrasonic sonicator (Bioruptor; Cosmo Bio, Tokyo, Japan). After clarification by microcentrifugation the supernatant containing nuclear proteins was frozen on dry ice and stored at -80°C until use. The DNA binding activity was examined by EMSA using the

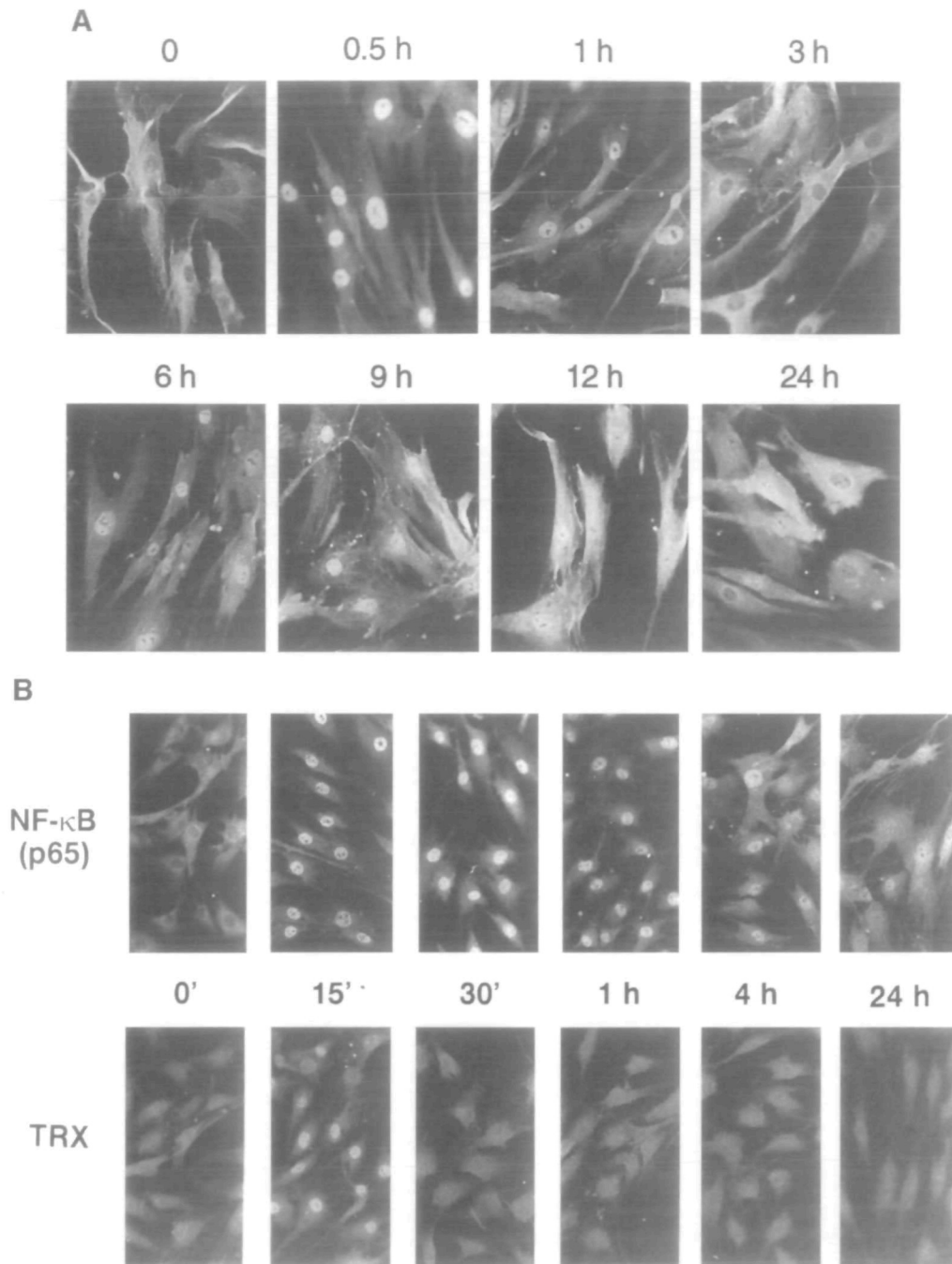


Fig. 1. Nuclear translocation of NF- κ B in RSF. (A) RSF on chamber slides were treated with 10 ng/ml of IL-1 β for various time periods (0, 0.5, 1, 3, 6, 9, 12 and 24 h). The cells were then fixed with 4.5% paraformaldehyde/PBS and permeabilized with 0.5% Triton X-100/PBS. The cells were then reacted with rabbit antibody against p65 of NF- κ B subunit and subsequently stained with FITC-conjugated goat anti-rabbit IgG. (B) RSF were treated with 10 ng/ml of TNF- α for various time periods (0, 0.5, 1, 3, 6, 12 and 24 h). After fixation the cells were stained with antibodies to p65 or TRX.

specific κ B DNA probe as previously described (27,28,39). The sequence taken from the HIV-1 long terminal repeat containing the binding site for NF- κ B is as follows: 5'-TTTCTAGGGACTTTCCGCCTGGGGACTTTCCAG-3'. Competitor oligonucleotides included the wild-type and mutated (GGGACTTTCC to CTCACTTTCC) κ B sequence specified above. For competition experiments, 50-fold molar excess of

unlabeled wild-type κ B or mutated κ B was preincubated with the protein on ice for 5 min before the radioactive probe was added

Immunoblotting analysis

Nuclear and cytoplasmic extracts prepared from the RSF treated with 10 ng/ml of rhIL-1 β were separated on 7.5–15%

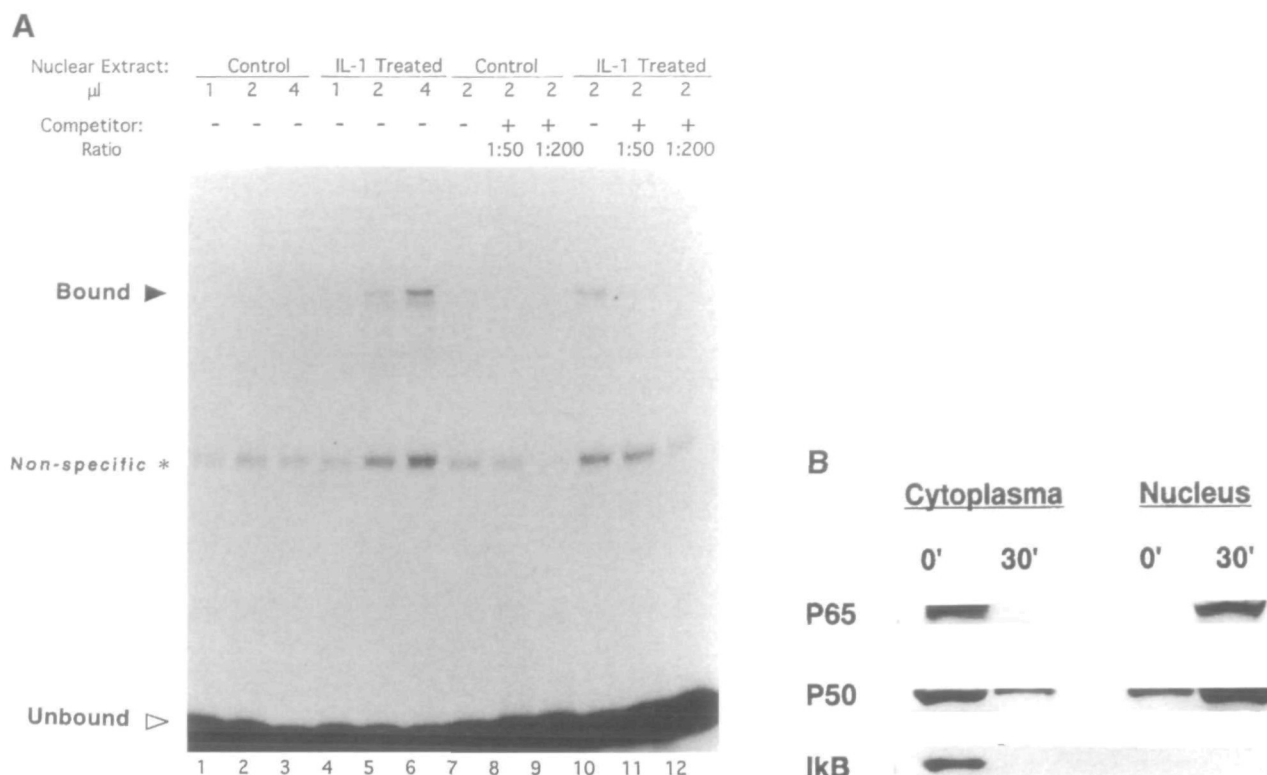


Fig. 2. (A) EMSA using the κ B DNA probe demonstrating the activation of NF- κ B induced by IL-1 β in RSF. Nuclear extracts were prepared from either untreated (lanes 1–3 and 7–9) or IL-1 β -treated (10 ng/ml) RSF (lanes 4–6 and 10–12) and tested for the κ B DNA binding activity by EMSA. Treatment of RSF with IL-1 β was carried out for 30 min. Specificity of the binding was assessed by excess amount of cold competitor oligonucleotides (the ratios to the radiolabeled κ B DNA probe are indicated). Positions of the DNA–protein complex 'Bound' (closed arrow head), 'Unbound' (open arrow head) and 'Non-specific' bind (asterisk) are shown. (B) Immunoblot analysis demonstrating the subcellular distribution of NF- κ B and I κ B in RSF before (0') or after (30') treatment with IL-1 β . Cells were treated with 10 ng/ml of IL-1 β and then harvested for the preparation of nuclear and cytoplasmic extracts at the time indicated. Proteins in each cell extract were separated by 7.5–15% gradient SDS–PAGE, transferred to PVDF membrane, and analyzed for p65, p50 NF- κ B subunits and I κ B- α by immunoblot analysis using specific antibodies.

gradient SDS polyacrylamide gels and transferred for 2 h at 4°C to a PVDF membrane (Immobilon; Millipore, Bedford, MA) in a transfer buffer (20 mM Tris, pH 7.9, 192 mM glycine, 10% methanol, 0.05% SDS) at 10 V/cm². After washing with 0.05% Tween 20/PBS, the PVDF membrane was blocked overnight at 4°C with 5% skim milk/PBS and subsequently incubated with rabbit polyclonal anti-NF- κ B p65 antibody, anti-p50 antibody or anti-I κ B- α antibody at 1:500 to 1:1000 dilution. Immunoreactive proteins were detected according to the enhanced chemiluminescence protocol (ECL System; Amersham, Buckinghamshire, UK) using horseradish peroxidase-linked donkey anti-rabbit antibody. Blots were exposed to film (Hyperfilm-ECL; Amersham) for 0.25–5 min.

Detection of cytokines in RSF culture medium by ELISA

The concentration of cytokines in RSF culture supernatants was determined using cytokine-specific ELISA kits for IL-1 α , IL-1 β and TNF- α (using human IL-1 α , IL-1 β and TNF- α assay kits; Otsuka Pharmaceutical, Tokyo, Japan), IL-6 and IL-8 (human IL-6 and IL-8 ELISA kits; Toray Fuji Bionics, Tokyo, Japan), and GM-CSF (Biotrack Human GM-CSF ELISA System; Amersham) as recommended by the manufacturers. The lower detection limits were 7.8 pg/ml for IL-1 α , 0.125 pg/

ml for IL-1 β , 10 pg/ml for IL-6, 10 pg/ml for IL-8, 7.0 pg/ml for TNF- α and 2.0 pg/ml for GM-CSF. The intra-assay and inter-assay variations of the data were within the ranges of 12 and 45% respectively.

Flow cytometric detection of cell surface ICAM-1 expression

To quantify the cell surface ICAM-1 expression, RSF were inoculated into six-well culture plates and allowed to adhere for 72 h, removed and suspended in cold PBS containing 0.05% NaN₃, and then reacted with mouse mAb to human ICAM-1 (1:200 dilution in PBS containing 0.1% BSA) for 1 h on ice. The cells were then washed, incubated with the FITC-labeled secondary antibody, goat anti-mouse IgG, and analyzed by flow cytometry (FACScan; Becton Dickinson, San Jose, CA). Non-specific fluorescence was assessed by substitution of the non-immunized mouse IgG1 data for that with anti-ICAM-1 mAb. To examine the effects of anti-NF- κ B reagents, cells were pretreated with 10 mM NAC or 2.5 mM aspirin for 1 h before the addition of 10 ng/ml of IL-1 β and the stimulation was terminated by removal of medium followed by twice washing the cells with cold PBS containing 0.05% NaN₃.

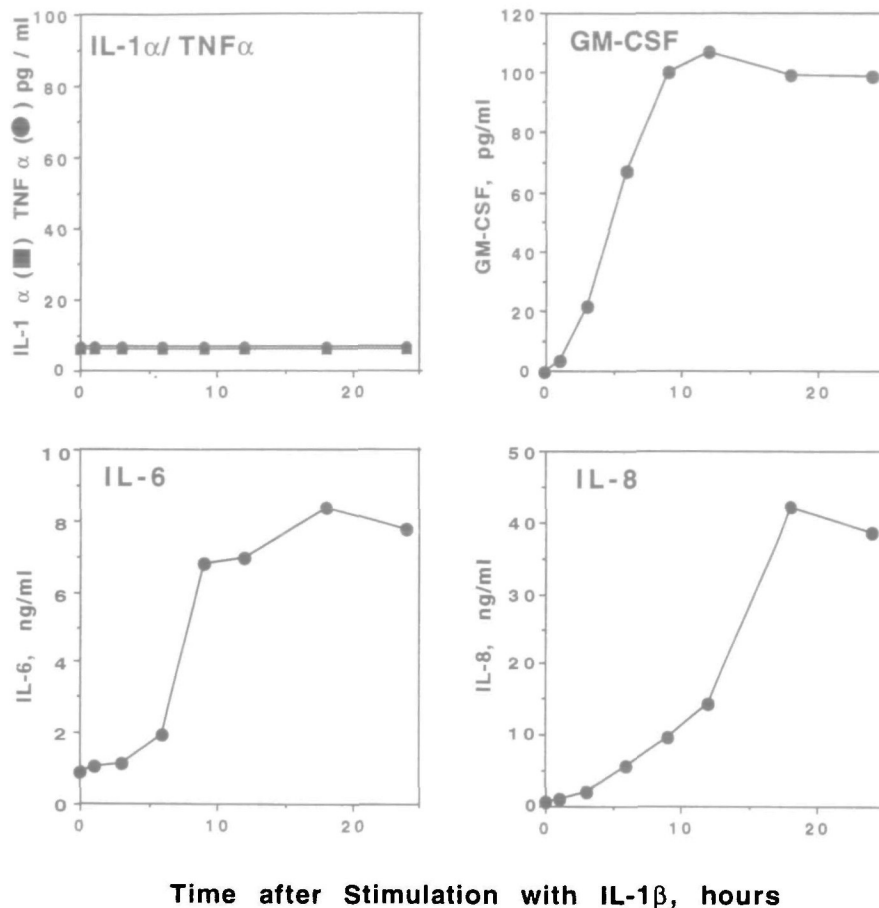


Fig. 3. Induction of cytokines involved in rheumatoid inflammation by stimulation of RSF with IL-1 β . Cells (1.5×10^4) seeded in the wells of culture plates were treated with 10 ng/ml of IL-1 β and then the supernatants were harvested at the end of various incubation periods (0, 1, 3, 6, 9, 12, 18 and 24 h). The concentration of cytokines were determined using cytokine-specific ELISA kits for TNF- α , IL-1 α , IL-6, IL-8 and GM-CSF. Each value represents the mean for duplicate determinations.

Results

Demonstration of nuclear translocation of NF- κ B in RSF by immunofluorescence

To determine the subcellular distribution of NF- κ B, we performed indirect immunofluorescence with rabbit polyclonal antibody against NF- κ B subunit p65 using the primary culture of synovial fibroblasts obtained from patients with RA. As shown in Fig. 1(A), p65 was localized in the cytoplasm of unstimulated RSF. When the cells were stimulated with 10 ng/ml of IL-1 β , p65 was shown to be translocated to the nucleus within 30 min of IL-1 β treatment as reported previously (28). We further examined the time course of the NF- κ B nuclear translocation over an extended time period. The nuclear localization of p65 was observed until 60 min after the IL-1 β treatment. Three hours after the IL-1 β treatment, p65 was found predominantly in the cytoplasm. However, 6–9 h after treatment we found that p65 was again translocated to the nucleus and persisted there until 12 h after treatment although the fluorescence of p65 was less intense than that seen in the earlier phase. Similarly, we examined the nuclear translocation of the other NF- κ B subunit p50. However, since p50 was already present in the nucleus

as well as in the cytoplasm in the untreated cells, the nuclear translocation of p50 staining after IL-1 β treatment (data not shown) was not fully evident using immunofluorescence.

We also examined the effect of TNF- α with a similar result (Fig. 1B). However, when cells were treated with TNF- α , the nuclear translocation of NF- κ B was observed upto 24 h after stimulation. Interestingly, when the cells were stained with antibody to TRX the nuclear translocation of TRX was demonstrated concomitantly with the nuclear translocation of NF- κ B after 15 min of the TNF- α treatment. While nuclear staining of NF- κ B (p65) was observed for 24 h, the nuclear predominance of TRX was no longer detectable after 30 min of TNF- α treatment.

EMSA demonstrate the NF- κ B activation by IL-1 β in RSF

We then applied EMSA to examine whether the κ B-sequence specific DNA binding activity could be induced in the nucleus of RSF after treatment with IL-1 β . The radiolabeled κ B DNA probe was incubated with the nuclear extracts prepared from untreated or IL-1 β -treated RSF. As shown in Fig. 2(A), the nuclear extract from RSF stimulated with 10 ng/ml of IL-1 β demonstrated the κ B DNA binding activity while the nuclear extract from the control RSF did not have such activity even

with the higher amounts of nuclear extract. Competition with excess amounts of the unlabeled κ B oligonucleotide showed the specificity of binding. These data revealed that IL-1 β induced the κ B DNA binding activity in the nucleus.

Subcellular distribution of NF- κ B and I κ B by immunoblotting

To further confirm the subcellular distribution of NF- κ B subunits, p65 and p50, and I κ B- α , we performed immunoblotting with the nuclear and cytoplasmic extracts prepared from RSF with or without treatment with 10 ng/ml of IL-1 β or TNF- α . As shown in Fig. 2(B), p65 was demonstrated in the cytoplasmic extract of the unstimulated RSF, and in the RSF after stimulation with IL-1 β , it was found predominantly in the nucleus. On the other hand, p50 was persistently present both in the cytoplasm and the nucleus before and after the IL-1 β treatment, although the amount of p50 in the nucleus was increased after IL-1 β treatment, which coincided with the decrease of the p50 level in the cytoplasm. I κ B- α was demonstrated only in the cytoplasm of the untreated RSF. After IL-1 β treatment, there was no detectable level of I κ B- α , suggesting its degradation, as reported previously (24,25). Thus, the nuclear translocation of NF- κ B and the induction of the κ B DNA binding activity in the IL-1 β -treated RSF appeared to be well correlated with the I κ B- α degradation, confirming the observations with EMSA (Fig. 2A) and indirect immunofluorescent staining (Fig. 1). Similar results were obtained when RSF was stimulated with 10 ng/ml TNF- α .

Induction of cytokine production in RSF by IL-1 β and TNF- α

IL-1 and TNF have been shown to induce production of various cytokines including IL-6, IL-8 and GM-CSF in lymphocytes, macrophages and fibroblasts (5,6). We, therefore, examined whether RSF could induce these cytokines upon stimulation by IL-1 or TNF, and investigated the role of NF- κ B. In the experiments in Fig. 3, RSF was treated with 10 ng/ml of rhIL-1 β or TNF- α and the levels of IL-6, IL-8 and GM-CSF in the culture supernatant were determined at various times following the treatment. IL-1 β induced the production of each cytokine with a plateau after 12 or 18 h of treatment. The magnitudes of induction were 50-fold for GM-CSF, 4-fold for IL-6 and 24-fold for IL-8. On the other hand, the production of IL-1 α and TNF- α was not detected even after 24 h of treatment. A similar effect was observed with the treatment of TNF- α (data not shown).

NAC and aspirin blocked the NF- κ B activation in RSF

We then examined the effects of the reagents known to inhibit the NF- κ B activation cascade. As shown in Fig. 4, the nuclear translocation of NF- κ B in RSF by IL-1 β was effectively blocked by the pretreatment with 10 mM NAC or 2.5 mM aspirin. The cells were treated with NAC or aspirin 2 h prior to the IL-1 β treatment and the immunostaining was carried out before or after 30 min of stimulation. The RSF pretreated with 10 mM NAC completely inhibited the nuclear translocation of p65 in the primary response of NF- κ B activation induced by IL-1 β (Fig. 4) and also in the later response (data not shown). In contrast, the duration of the effect of aspirin was limited to ~4 h. When the IL-1 β was added after 2 h of pretreatment of the cells with aspirin, it blocked the NF- κ B activation as observed with NAC (Fig. 4). However, when the IL-1 β was added later than 4 h after the aspirin treatment, it was no

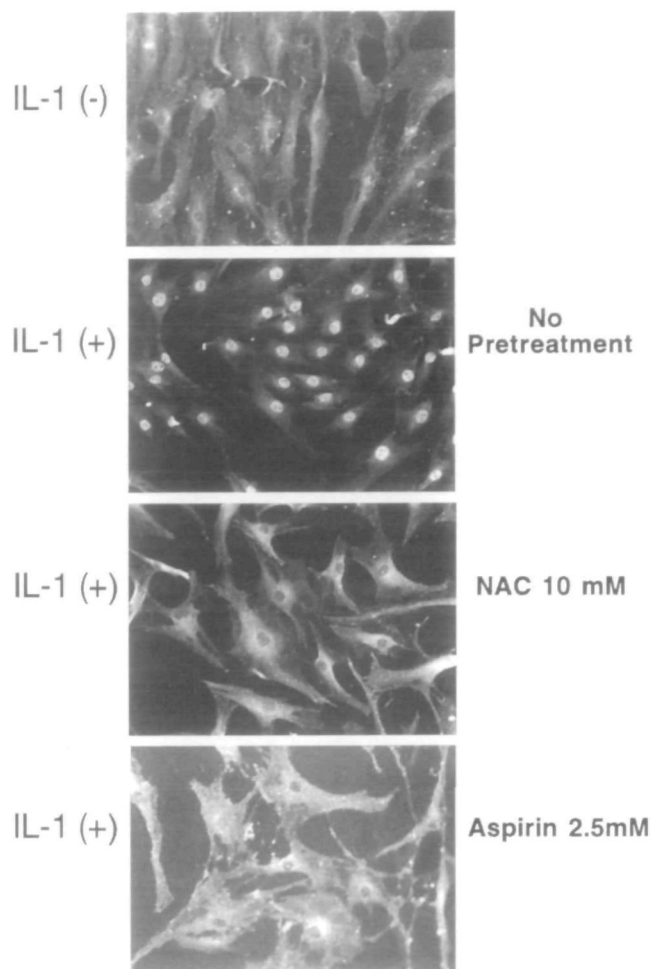


Fig. 4. Effects of NAC and aspirin on nuclear translocation of p65 NF- κ B subunit in RSF. Cells were treated with 10 ng/ml of IL-1 β in the presence or absence of 10 mM NAC or 2.5 mM aspirin. After 30 min of IL-1 β treatment the cells were fixed, permeabilized and incubated with antisera against NF- κ B subunit p65 and visualized by the immunofluorescence technique as described in Fig. 1.

longer able to block the nuclear translocation of NF- κ B (data not shown). We repeated these experiments at least four times and obtained reproducible results for NAC, while with aspirin the results were not quite reproducible because of the significant cytotoxicity with the effective dose of aspirin.

Inhibition of cytokine induction by NAC and aspirin

Having found the inhibitory activity of NAC and aspirin on NF- κ B activation, we then examined their effects on the induction of cytokines elicited by IL-1 β . In Fig. 5, RSF was pretreated with 10 mM NAC or 2.5 mM aspirin for 2 h and the concentrations of IL-6, IL-8 and GM-CSF were measured in the cell culture supernatant before and after 12 h stimulation with IL-1 β . As demonstrated in Fig. 5, NAC strikingly inhibited the induction of these cytokines. These effects of NAC were reproducible for over four times in independent experiments. However, aspirin showed only limited effects: while induction of IL-6 and IL-8 was partially blocked by aspirin, GM-CSF induction was not at all blocked by aspirin.

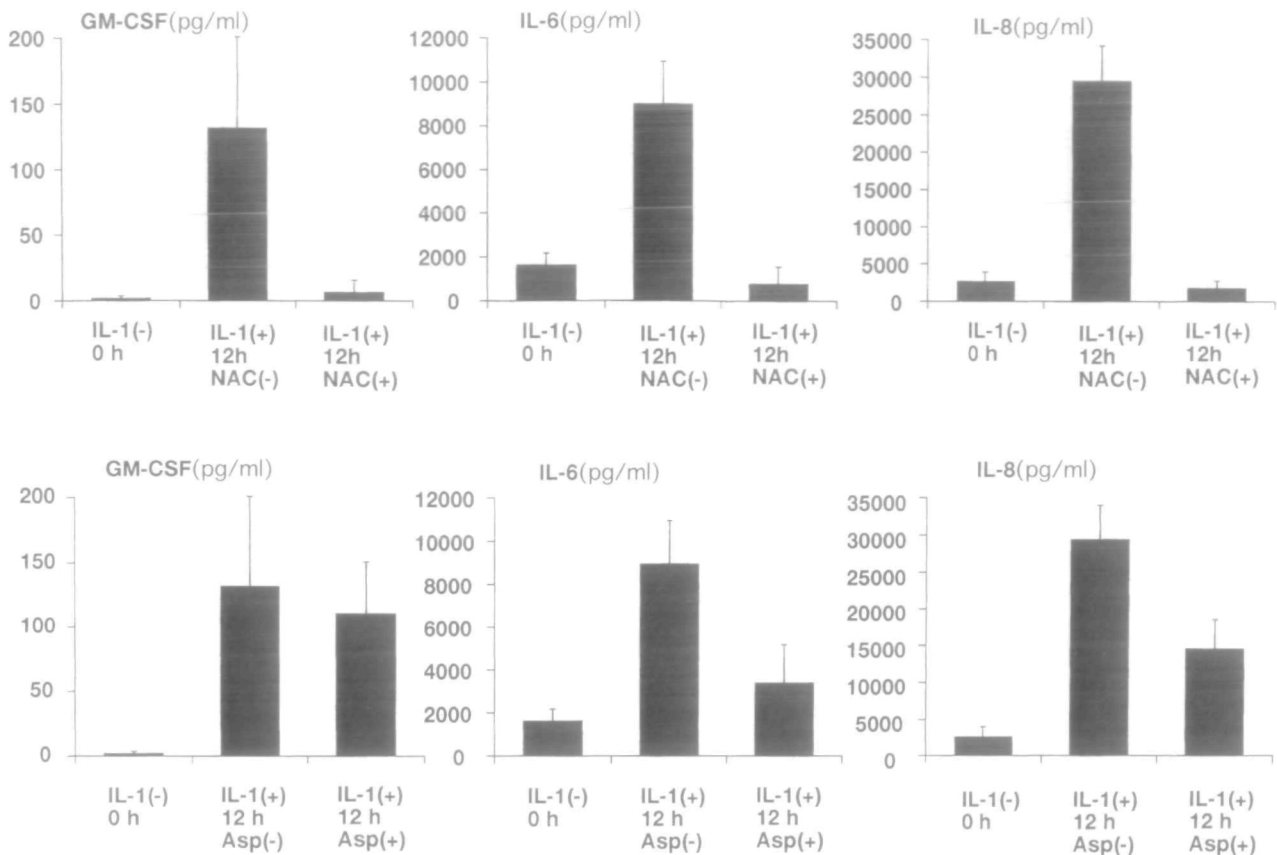


Fig. 5. Effects of NAC and aspirin on the induction of various cytokines by IL-1 β . Cells were treated with 10 ng/ml of IL-1 β in the presence or absence of 10 mM NAC or 2.5 mM aspirin. The supernatants were harvested after 12 h of IL-1 β treatment. The concentration of cytokines was determined using cytokine-specific ELISAs. All samples were assayed in triplicate. Each value indicates the mean. Error bars show SD values.

Induction of ICAM-1 in RSF by IL-1 β and effects of NAC and aspirin

Since ICAM-1 is also known to be under the control of NF- κ B in fibroblasts (21), we first examined the induction of ICAM-1 by IL-1 β . As shown in Fig. 6(A), the ICAM-1 level was greatly augmented by treatment with IL-1 β . We then examined the effects of NAC and aspirin on the induction of ICAM-1 in RSF. The RSF was pretreated with 10 mM NAC or 2.5 mM aspirin for 2 h before the treatment with 10 ng/ml of IL-1 β , fixed and stained with mAb to ICAM-1 after 12 h of IL-1 β treatment. As shown in Fig. 6(A), IL-1 β induced the production of ICAM-1 in the RSF. While NAC completely blocked the ICAM-1 induction, aspirin exhibited only a marginal effect (Fig. 6A). Similarly, flow cytometric analysis was performed to evaluate the effects of NAC and aspirin on the cell surface expression of ICAM-1. Figure 6(B) shows representative data of such experiments. As shown in Fig. 6(B), the level of cell surface ICAM-1 was augmented by IL-1 β and this augmentation was partially blocked by pretreatment with NAC. In contrast, aspirin showed no notable effect. The relative ineffectiveness of these reagents, as compared with the results obtained with the immunostaining using the fixed cells (Fig. 6A), was reproducible and might reflect the relatively longer lifespan of the cell surface ICAM-1 molecule.

Discussion

To investigate the role of NF- κ B in the pathophysiology of RA, we isolated primary RSF from the affected joints of RA patients. Since RSF reacted to mAb 5B5 against the β -subunit of prolyl 4-hydroxylase, which is negative for macrophages, monocytes, dendritic cells and lymphocytes, these cells are considered to belong to a fibroblast lineage. In rheumatoid synovium undergoing chronic and active inflammation, proliferating fibroblasts are considered to be responsible for joint destruction as well as the maintenance of chronic inflammation by supporting sequestration, activation and differentiation of lymphocytes and monocytes through the expression of various cytokines and cell adhesion molecules (3–6,10,11). Therefore, we examined NF- κ B, a common regulator of transcription of these effector genes, GM-CSF, IL-6, IL-8 and ICAM-1 in RSF.

In this report, we demonstrated that the proinflammatory cytokines such as IL-1 β and TNF- α could induce the activation and nuclear translocation of NF- κ B in RSF as revealed by immunostaining, EMSA and immunoblotting analyses. Induction of various cytokines and ICAM-1 was observed following the NF- κ B activation and its nuclear translocation. We then examined the effects of reagents reported to inhibit the NF- κ B activation cascade such as NAC (30,31) and aspirin (32,33) in our RSF system. Antioxidants, particularly NAC,

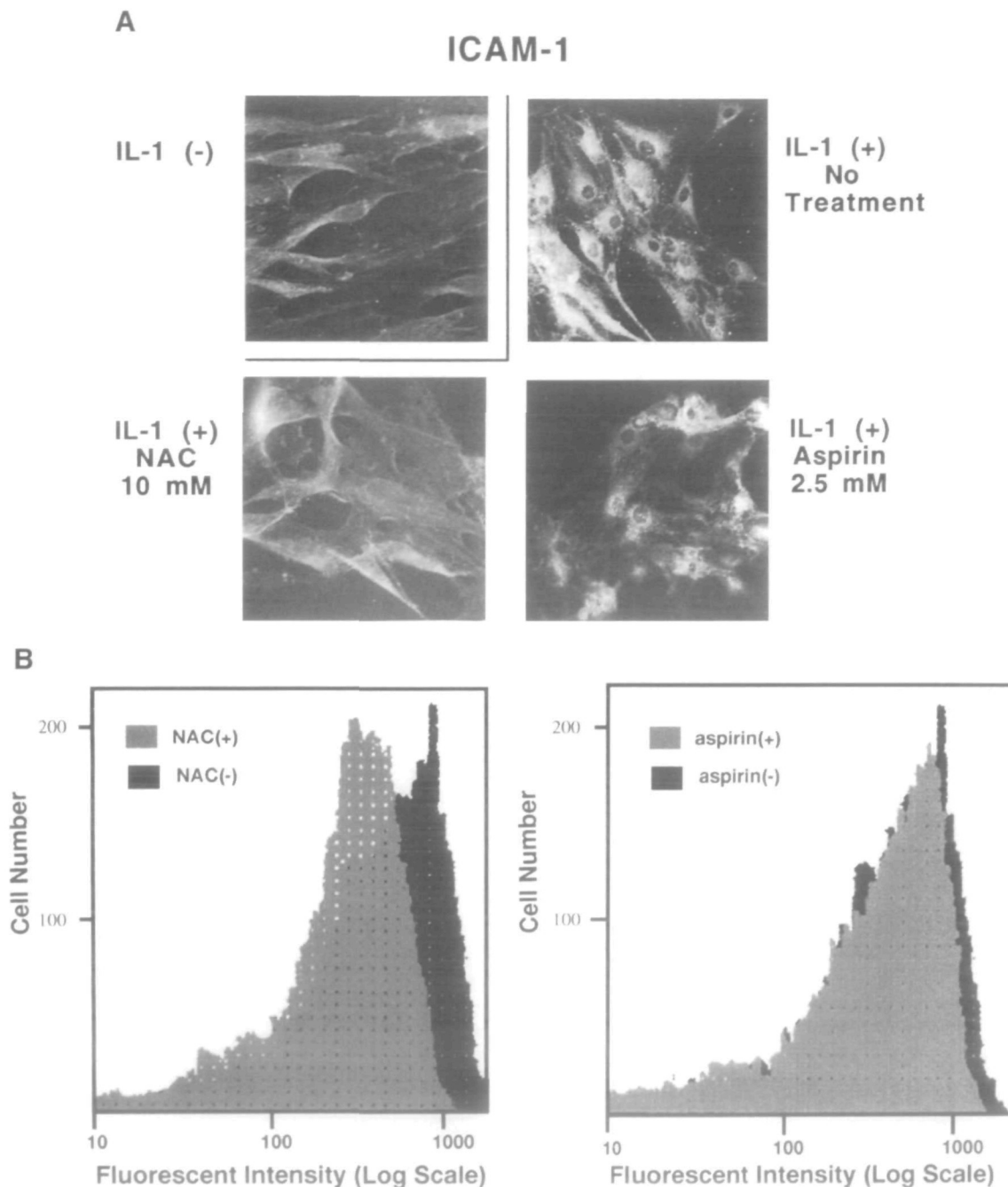


Fig. 6. (A) Effects of NAC and aspirin on the induction of ICAM-1 in RSF. Cells cultured in chamber slides were treated with 10 ng/ml of IL-1 β in the presence or absence of 10 mM NAC or 2.5 mM aspirin. The cells were then fixed and immunostained with mouse mAb against human ICAM-1. Immunofluorescence assay was performed using FITC-conjugated rabbit anti-mouse IgG as the second antibody. (B) Flow cytometric analysis demonstrating the inhibitory effects of NAC and aspirin on the induction of cell surface ICAM-1 induced by IL-1 β . Cells were pretreated with 10 mM NAC or 2.5 mM aspirin for 2 h before the addition of 10 ng/ml IL-1 β and incubated with mouse mAb against human ICAM-1 (primary antibody) and FITC-conjugated rabbit anti-mouse IgG (secondary antibody). Non-specific fluorescence was assessed by substitution of anti-ICAM-1 mAb by mouse IgG1.

were shown to be effective in blocking the NF- κ B translocation and the subsequent induction of target genes. Similar observations have been reported for human monocytes and fibroblasts (30,31,40,41).

Recent studies have revealed that levels of IL-1, IL-6, GM-

CSF and TNF- α are increased in the synovial cavity of patients with RA (3–6,40). Similarly, ICAM-1 expression in the lining layer of the affected synovium is positively correlated with activity of rheumatoid synovitis (41–43). Leukocytes including T lymphocytes and monocytes have been shown to bind to

RSF *in vitro* through interaction with ICAM-1 (9,10). This cell-cell interaction triggers lymphocyte migration and proliferation to generate a germinal center in the synovial lesion. As a consequence, Ig synthesis is enhanced and local immune response is thus triggered in the rheumatoid synovium (44). These observations have highlighted the importance of pro-inflammatory cytokines such as IL-1 and TNF- α in the pathophysiology of RA, since these cytokines are known to induce expression of cell adhesion molecules (19,16,45) and the cytokines involved in rheumatoid inflammatory processes (3–6). IL-1 and TNF- α are also known to be natural inducers of NF- κ B activation in a wide variety of mesenchymal cells including fibroblasts (14,21). Furthermore, NF- κ B is also known to positively regulate the expression of the TNF- α gene (14,25), thus conforming self-perpetuating chronic immune and inflammatory responses. Therefore, NF- κ B is considered to play a key role in RA.

We observed a biphasic pattern of the nuclear translocation of NF- κ B by IL-1 in RSF (Fig. 1A). A similar observation was reported in peripheral blood T lymphocytes (46). It was observed that the activation and nuclear translocation of NF- κ B occurred at ~1 h (immediate), 7–16 h (early) and 40 h (late) after stimulation as demonstrated by EMSA and immunoblotting analysis, and the involvement of *de novo* synthesized TNF- α in the late phase of NF- κ B activation was suggested (46). However, we detected no production of TNF- α or IL-1 α from RSF within 24 h of stimulation by IL-1 β . Currently, we do not have a clear explanation for this. However, it is possible that unknown cytokine(s) that are able to induce NF- κ B activation might be involved. It is also possible that the cyclic nature of the NF- κ B activation might be intrinsic such as through a cell-cycle-associated event since the doubling time of RSF in culture was also ~40 h (data not shown). In contrast to IL-1, TNF- α induced the constitutive nuclear translocation of NF- κ B upto 24 h after the stimulation (Fig. 1B).

It was noted that TRX was translocated to the nucleus concomitantly with the NF- κ B immediately after the stimulation. However, the nuclear localization of TRX was only transient. It has recently been demonstrated in a NMR study that the DNA binding loop of the p50 subunit of NF- κ B makes a complex with TRX *in vitro* (47), thus confirming our earlier observation of TRX involvement in the redox regulation of NF- κ B (27,28). The concomitant nuclear translocation of TRX suggests the binding with NF- κ B through a disulfide bridge between the two molecules during the reduction of NF- κ B by TRX, although the direct TRX binding with NF- κ B within the cell remains to be substantiated.

The effects of NAC in inhibiting the NF- κ B activation cascade as well as the induction of cytokines and ICAM-1 were reproducibly observed in RSF while the effects of aspirin were marginal and often associated with its cytotoxicity. One recent report pointed out that the effects of aspirin in blocking the NF- κ B cascade were not specific and were associated with non-specific inhibition of cellular kinases (48). Similarly, we observed the apparent differences in the effects of NAC and aspirin in blocking the NF- κ B-activation cascade. Additionally, while aspirin could partially block the induction of IL-6 and IL-8, it could not block the GM-CSF induction. It was also reported previously that aspirin could not inhibit the induction of ICAM-1 by NF- κ B activation (33), which was consistent

with our finding in Fig. 6. The partial effect of aspirin could be due to the short duration (4 h) of its action which might be ascribed to the mode of action as suggested by Frantz and O'Neill (48). In addition, we previously demonstrated that gold compounds that are widely used as anti-rheumatic drugs showed direct inhibitory effects on the binding of NF- κ B to DNA *in vitro* (39), again suggesting the role of NF- κ B in RA pathophysiology.

In conclusion, experimental observations demonstrated here support the idea that the apparent complex nature of rheumatoid inflammatory processes involving multiple cytokines and CAM could be explained, at least in part, by the role of a single transcription factor, NF- κ B. Concomitantly, Handel *et al.* have recently demonstrated the presence of NF- κ B subunit proteins, p65 and p50, in the nuclei of synovial lining cells of fresh synovial tissue obtained from patients with RA indicating activation of NF- κ B *in situ* (49). Collectively, NF- κ B and its activation cascade would be a feasible as well as conceivable target for the treatment of RA and related conditions.

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Abbreviations

aspirin	acetylsalicylic acid
CAM	cell adhesion molecule(s)
EMSA	electrophoretic mobility shift assay
GM-CSF	granulocyte macrophage colony stimulating factor
NAC	N-acetyl-L-cysteine
RA	rheumatoid arthritis
ROI	reactive oxygen intermediates
RSF	rheumatoid synovial fibroblasts
TNF	tumor necrosis factor
TRX	thioredoxin.

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