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Inhibitory Effect of E3330, a Novel Quinone Derivative Able to Suppress Tumor Necrosis Factor-α Generation, on Activation of Nuclear Factor-κB

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SUMMARY

(2*E*)-3-[5-(2,3-Dimethoxy-6-methyl-1,4-benzoquinoyl)]-2-nonyl-2-propenoic acid (E3330), is a novel agent with hepatoprotective activity. We report the effect of E3330 on transcriptional activation of tumor necrosis factor (TNF)-α gene and on nuclear factor (NF)-κB activation. Nuclear run-on experiments showed that E3330 decreases transcriptional activation of TNF-α gene induced by lipopolysaccharide (LPS) stimulation in human peripheral monocytes. To investigate the inhibitory mechanisms, we constructed a secreted-type placental alkaline phosphatase (PLAP) reporter gene whose transcription is controlled by a 1.4-kb human TNF-α promoter. A stable transformant of the PLAP reporter gene derived from human monocytic cell line showed very little activity of the promoter before stimulation, whereas LPS stimulation led to a dramatic increase in PLAP activity. E3330 inhibited this induced promoter activity in a dose-dependent manner. There are four putative NF-κB binding sites (κB-1, κB-2, κB-3, κB-4) in human TNF-α promoter. By using mutated promoter-PLAP plasmids, we established that these NF-κB sites were necessary for induction of TNF-α transcription on stimulation with LPS. A gel retardation experiment with synthetic double-stranded oligonucleotides showed that activated NF-κB consisting of p50/p65 heterodimer bound to all four putative NF-κB DNA probes, suggesting that all four putative NF-κB recognition sites play an important role in inducible TNF-α expression. E3330 decreased activated NF-κB in nuclei, suggesting that E3330 inhibits NF-κB activation and/or translocation to the nuclei. Western blotting analysis with anti-IκB-α antibody indicated that E3330 inhibited degradation of IκB-α, which is an inhibitory protein of NF-κB, in LPS-stimulated monocytes. E3330 may suppress the production of active oxygen species serving as common messengers to activate NF-κB.

Monocytes/macrophages play significant roles in inflammatory and immune responses. When monocytes/macrophages are stimulated with various stimuli, such as LPS, PMA, and IL-1, the cells generate cytokines, such as TNF-α, IL-1α, IL-1β, IL-6, and IL-8, which mediate a wide range of biological activities (1). Among these cytokines, TNF-α shows various activities, including induction of pyrogen, activation of macrophages and neutrophiles, induction of expression of adhesion molecules on endothelial cell membrane, enhancement of fibroblast growth, and activation of HIV-1 expression (2, 3). Recently, it was proposed that TNF-α is involved in the pathogenesis of human liver injury, such as fulminant hepatic failure, alcoholic hepatitis, and chronic liver disease (4–6). Therefore, an inhibitor of TNF-α generation could be a useful therapeutic drug in the treatment of these diseases.

E3330 was reported to inhibit LPS-induced TNF-α generation in human monocytes, rat resident peritoneal macrophages, and *Propionibacterium*-elicited peritoneal macrophages, rat Kupffer cells and rat spleen macrophages (7). A therapeutic effect of E3330 and an inhibitory effect of E3330 on *in vivo* TNF-α generation in plasma in mice with endotoxin-mediated hepatitis and in rats with galactosamine-induced hepatitis were also reported (8, 9). Northern blot analysis indicated that the inhibitory effect of E3330 on TNF-α generation is due to inhibition of mRNA biosynthesis and/or destabilization of mRNA.

Biosynthesis of TNF-α mRNA is regulated at transcriptional and post-transcriptional levels. Binding of NF-κB to NF-κB recognition sites in the TNF-α gene 5′ upstream region is important for transcription of human and murine TNF-α gene (10–12). NF-κB is a critical regulator of several genes involved in immune and inflammatory responses (13,

ABBREVIATIONS: LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α; NF-κB, nuclear factor-κB; IL, interleukin; IκB, inhibitor of NF-κB; HIV-1, human immunodeficiency virus type-1; LTR, long terminal repeat; PLAP, human placental alkaline phosphatase; AIDS, acquired immune deficiency syndrome; SDS, sodium dodecyl sulfate; PMA, phorbol-12,13-myristate acetate; fMLP, *N*-formyl-MET-LEU-PHE; E3330, (2*E*)-3-[5-(2,3-dimethoxy-6-methyl-1,4-benzoquinoyl)]-2-nonyl-2-propenoic acid; HBSS, Hanks' balanced salt solution; NP-40, Nonidet-40; bp, base pair(s); SSC, standard saline citrate; BGH, bovine growth hormone; TK, thymidine kinase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium.

14) and was first identified as a nuclear factor that activates the expression of the κ light chain gene. Soon, it became evident that this factor binds NF-κB recognition sequences (e.g., 5′-GGGACTTTCC-3′) that exist in the 5′ upstream region of cytokine, cytokine receptor, and virus genes. NF-κB transcription complexes are dimeric proteins consisting of one or two members of a family related to the c-Rel proto-oncogene, such as NF-κB1 (p105, p50), NF-κB2 (p100, p52), NF-κB (p50/p65), Rel, RelA (p65), RelB, dorsal, Dif, and Cif. NF-κB p50/p65 heterodimer is present in the cytosol of resting cells as the bound form with an inhibitory protein, IκB-α. When the cells are stimulated with various agents such as LPS, PMA, and TNF-α, the cytosolic NF-κB/IκB-α complex is dissociated and free NF-κB translocates to the nuclei.

To clarify the mechanism of the inhibitory effect of E3330 on TNF-α gene expression, we examined the effects of E3330 on the transcriptional activation of TNF-α gene and on the activation of NF-κB.

Materials and Methods

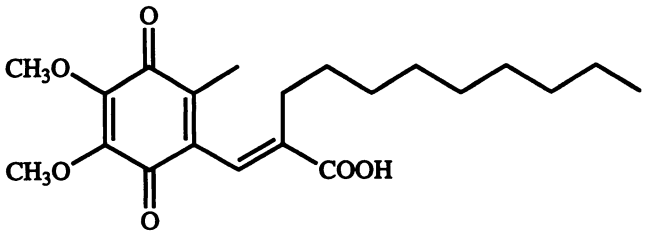
Cells

THP-1 (acute monocytic leukemia human) and Raw264.7 (macrophage, Abelson leukemia virus-transformed, BALB/c) were obtained from the American Type Culture Collection (Rockville, MD).

Drugs and Chemicals

LPS (*Escherichia coli* 0127:B08), TRI zol, polydeoxyinosinic-deoxycytidylic acid, cytochrome *c*, superoxide dismutase, catalase, PMA, fMLP, xanthine, xanthine oxidase, Triton X-100, NP-40, deoxycholate, chymostatin, pepstatin A, NaF, Na_3VO_4, MTT, dithiothreitol, geneticin (G418), and PMSF were purchased from Sigma Chemical Co. (St. Louis, MO); RPMI 1640, HBSS without Ca^{2+} and Mg^{2+}, penicillin/streptomycin, and HEPES, GIBCO-BRL (Grand Island, NY); Ficoll-Paque, NAP-5 columns, ultrapure NTP set, and CellPhect Transfection Kit (DEAE-dextran), Pharmacia (Uppsala, Sweden); aprotinin and leupeptin, Boehringer Mannheim Yamanouchi (Tokyo, Japan); NF-κB (p50, human), Promega Co. (Madison, WI); endotoxin-free fetal calf serum, Nippon B.M.A. Co. (Osaka, Japan); [α-^{32}P]dCTP (220 TBq/mmol), [α-^{32}P]UTP (110 TBq/mmol), anti-rabbit Ig horseradish peroxidase-linked whole antibody (from donkey), Hybond-ECL (nitrocellulose), and Hyperfilm-ECL and ECL Western blotting detection reagents, Amersham (Arlington Heights, IL); human placenta cDNA library (HL1075b), genomic library (HL1067J), and the vector pTKβ, Clontech Laboratories (Palo Alto, CA); vent DNA polymerase, New England Biolabs, Inc. (Beverly, MA); pRC/CMV, Invitrogen Corp. (San Diego, CA); anti-p50 antibody, anti-p52 antibody, anti-p65 antibody, anti-Rel antibody, anti-RelB antibody, and anti-IκB-α antibody, Santa Cruz Biotechnology (Santa Cruz, CA); Klenow fragment, Takara Shuzo Co. (Kyoto, Japan); restriction enzymes and DNA modifying enzymes, New England Biolabs, Toyobo KK (Osaka, Japan), or Takara Shuzo Co.; EDTA and EGTA, Nacalai Tesque (Kyoto, Japan); Tris, 40% acrylamide/bis solution (19:1), 10× Tris/boric acid/EDTA buffer (1× = 89 mM Tris, 89 mM boric acid, and 2 mM EDTA), ammonium persulfate, glycine, and boric acid, Bio-Rad Laboratories (Richmond, CA); 20× SSC (1× = 150 mM NaCl and 15 mM sodium citrate), reaction buffer building set, and glycerol, Wako Pure Chemical Industries, Ltd. (Osaka, Japan); T7-GEN *in vitro* mutagenesis kit, United States Biochemical Corp. (Cleveland, OH); Multigel 4/20, Daiichi Pure Chemicals Co. (Tokyo, Japan); and Smilight (Lumistain; 4-methoxy-4-(3-phosphatephenyl)spiro[1,2-dioxetane-3,2′-adamantane]), Sumitomo Kinzoku (Tokyo, Japan).

E3330 (Fig. 1) was synthesized in our laboratories. DNA probes of human TNF-α NF-κB-like sites and HIV-1 NF-κB site were synthesized according to a solid-phase phosphate triester method with a model 394 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA).



E3330

(2E)-3-[5-(2,3-dimethoxy-6-methyl-1,4-benzoquinoyl)]-2-nonyl-2-propenoic acid

Fig. 1. Chemical structure of E3330.

Nuclear Run-on Assay

Human monocytes from peripheral blood of healthy male volunteers were obtained through the method previously reported (15). Briefly, human mononuclear cells were isolated through Ficoll-Paque density gradient sedimentation. The cells were suspended at a concentration of 2×10^6 cells/ml in RPMI 1640 containing 10% heat-inactivated autologous serum, seeded at 20 ml/dish onto 150-mm dishes (Falcon 3025, Becton Dickinson, NJ), and cultured for 1.5 hr. Nonadherent cells were removed through rinsing, and the remaining cells were used as the monocyte preparation. The monocytes were cultured in the presence or absence of E3330 for 30 min, followed by stimulation with 100 ng/ml LPS for 30 min. Then, the cells were rinsed free of HBSS and lysed in NP-40 lysis buffer (10 mM Tris·HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40), and the nuclei were isolated by spinning for 5 min in a microfuge. The nuclei were resuspended in 100 μl of glycerol buffer (50 mM Tris·HCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA), frozen in liquid N_2, and stored at −80°. For the nuclear run-on assay, isolated nuclei were mixed with an equal volume of reaction buffer (10 mM Tris·HCl, pH 8.0, 5 mM $MgCl_2$, 300 mM KCl, 1 mM concentrations of ATP, GTP, and CTP, and 37 MBq of [α-^{32}P]UTP) and incubated at 30° for 30 min. RNA was extracted with TRI zol and purified through ethanol precipitation. For the preparation of filters, both purified human TNF-α cDNA and β-actin cDNA were used. These fragments were denatured with 0.1 M NaOH at room temperature for 30 min. After the addition of an equal volume of 6× SSC, the mixture was heated at 95° for 10 min and quickly chilled on ice. The solution containing DNA fragments was filtered through nitrocellulose with use of a slot-blot manifold. Each slot contained 5 μg of DNA. The filter was baked at 80° for 2 hr, prehybridized in 6× SSC at 42°, and hybridized to 0.75–1 × 10^6 cpm [^{32}P]RNA preparation in 6× SSC at 42° for 2 days. After the hybridization, the filter was washed three times in 2× SSC/0.1% SDS at room temperature and twice in 0.2× SSC/0.1% SDS and then exposed to an X-ray film for 3 days.

Construction of DNA Coding Secreted Form of PLAP

Human PLAP cDNA has been sequenced (16), and its modification to secreted-type PLAP (truncation of the carboxyl-terminal 24 amino acids) was also reported (17). We isolated the 1276-bp 5′ part of this cDNA through polymerase chain reaction from a human placenta cDNA library (HL1075b). Two sets of primers (A, 5′-CCAGAATTC-CTGCCTCGCCACTGTCC-3′ and 5′-TTAGGATCCTGGCAGCTGT-CAC-3′; B, 5′-GTGACAGCTGCCAGGATCCTAA-3′ and 5′-AGGAC-CGTGTAGGCCTCCCTGT-3′) gave two overlapping DNA fragments

(273 and 1028 bp, respectively). Digestion of these DNA fragments with restriction enzyme *Bam*HI and ligation with *Bam*HI cohesive ends yielded the 1276-bp 5′ part of the PLAP-coding DNA. The isolated 1276-bp fragment was inserted into pBlueScript KS plasmid with the use of the *Eco*RI and *Sma*I restriction sites that are located at the 5′ end and 22 bp upstream of the 3′ end of this fragment. The nucleotide sequence of this 1276-bp insert was identical to the reported cDNA sequence (16).

The 3′ part of PLAP-coding DNA was not amplified by polymerase chain reaction, due to its GC-rich sequence. We designed an artificial DNA that codes 108 amino acid residues [amino acid residues 382–489 in Millan (16)], has a translational stop codon TAA instead of the Arg^{490} codon, and has a restriction enzyme recognition site for *Bgl*II in its 3′ end. We synthesized two pairs of 98–100 base oligonucleotides (Fig. 2; model 394 DNA/RNA synthesizer, Applied Biosystems). Oligonucleotides of each pair were mixed, annealed via the 17-nucleotide complementary sequence of the 3′ ends, and subjected to polymerase chain reaction (vent DNA polymerase) to yield double-stranded DNA segments (179 and 181 bp, respectively). These two DNA fragments were subcloned in the *Hin*cII site of pUG131 [multilinker of pUC18 was replaced with that of M13tg131 vector (18)], and their nucleotide sequences were confirmed with the dideoxy termination method. Plasmids with the correct sequence were digested with *Xma*I and *Aat*II or *Aat*II and *Bgl*II to yield *Xma*I/*Aat*II 165-bp fragment and *Aat*II/*Bgl*II 164-bp fragment, respectively. These two fragments were purified on agarose and inserted between the *Xma*I and *Bgl*II sites of pUG131. The 329-bp insert of this plasmid was cut out with *Xma*I and *Bgl*II and ligated with 1276-bp *Hin*dIII/*Xma*I fragment (the *Hin*dIII site is located in the multilinker site of KS plasmid). The resulting 1593-bp PLAP-coding DNA was inserted between the *Hin*dIII and *Bgl*II sites of pUG131. The 5′ noncoding region and 15 bp of coding DNA were cut out with *Eco*RI and *Sph*I, and then *Eco*RI/*Hin*dIII/*Sph*I[d] adapter (5′-pAATTCAAGCTTACCATG-3′ and 5′-pGTAAGCTTG-3′) was inserted into the deletion site. This plasmid contains DNA coding the secreted form of PLAP (1548 bp, *Hin*dIII to *Bgl*II) and is named pUG-PLAP (Fig. 3A).

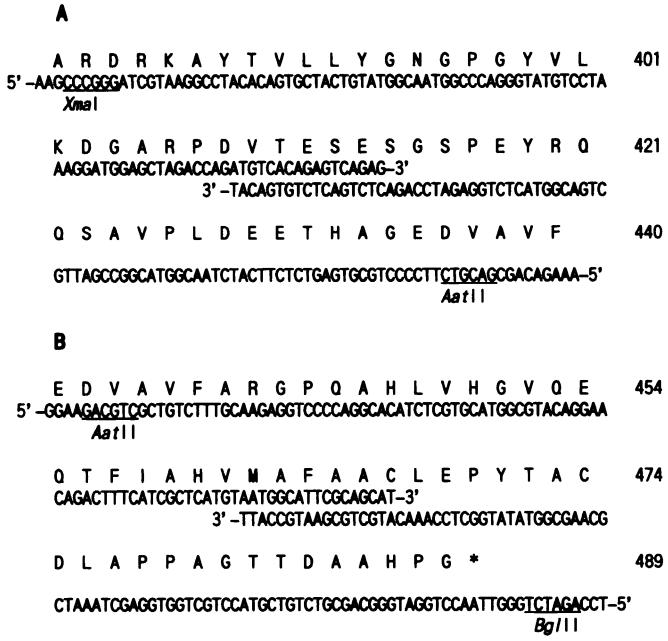


Fig. 2. Nucleotide sequences of the synthetic oligonucleotides for the 3′ end of PLAP-coding DNA. Two pairs of artificial 98–100-mer oligonucleotides were synthesized. The nucleotide sequences shown in A and B code 59 and 55 amino acid residues, respectively [corresponding to amino acid residues 382–489 in Millan (16); *top row*]. *, Stop codon.

Construction of PLAP Reporter Plasmids

pSV2-PLAP. pSV2-PLAP was derived from pSV2-dhfr plasmid (19). PLAP-coding DNA was cut out from pUG-PLAP through digestion with *Hin*dIII and *Bgl*II, and the 1.5-kb DNA fragment was ligated to the large fragment of pSV2-dhfr plasmid cut with the same enzymes to produce pSV2-PLAP (Fig. 3B). PLAP gene expression is controlled by the SV40 early promoter in this vector.

pUG-BGH-PLAP. To minimize read-through transcription, BGH terminator was introduced in the upstream region of the reporter expression cassette. Digestion of pRC/CMV with *Sac*I and *Sph*I gave the 217-bp BGH terminator. This fragment was ligated with pUG-131 that had been cut with *Sac*I and *Sph*I to generate pUG-BGH. A DNA fragment that has the PLAP coding region, SV40 splice sites, and a poly(A)$^+$ signal (2.5 kb) was isolated from pSV2-PLAP by cutting completely with *Hin*dIII and then partially with *Bam*HI. This 2.5-kb fragment was ligated with pUG-BGH that had been cut with *Hin*dIII and *Bam*HI to give pUG-BGH-PLAP (Fig. 3C).

TNF-α-PLAP. The complete DNA sequence of human lymphotoxin and TNF-α gene locus (7 kb) has been reported (20). A 1.4-kb TNF-α promoter region DNA (−1211 to +167 relative to TNF-α cap site) was isolated as three overlapping DNA fragments by polymerase chain reaction from a genomic library (HL1067J, Clontech Laboratories). Three pairs of primers (A, 5′CGATCTAGAACTTCCCAGTCTATCTAAG-3′ and 5′-TTTTCATGAAGCTCTCACTTCTC-3′; B, 5′-GAGAAGTGAGAGCTTCATGAAAA-3′ and 5′-CTTTTGGGGACCAGGTCTGTG-3′; C, 5′-CACAGACCTGGTCCCCAAAAG-3′ and 5′-TAGAAGCTTTCCAGGGGAGAGAGGGTG-3′) gave a 470-bp *Xba*I/*Bsp*HI fragment, a 382-bp *Bsp*HI/*Bst*XI fragment, and a 524-bp *Bst*XI/*Hin*dIII fragment, respectively. These three fragments were inserted into pUG-BGH-PLAP that had been cut with *Xba*I and *Hin*dIII. The resulting plasmid, named TNF-α-PLAP (Fig. 3D), was sequenced, and seven nucleotides were found to be different than the reported sequence (+146/C, −315/A, −448/delete, −511/insertion A, −575/C, −655/A, and −892/G).

Mutated TNF-α-PLAP. Putative NF-κB recognition sequences in the TNF-α 5′ upstream enhancer region (κB-1, −634 to −625; κB-2, −605 to −596; κB-3, −220 to −211; κB-4, −105 to −96) were mutated with the use of a T7-GEN *in vitro* mutagenesis kit. A 1.4-kb TNF-α enhancer/promoter region was subcloned in M13 mp18 and mp19 plasmids to produce a single-stranded template for *in vitro* annealing of mutagenic primers (for κB-1, 5′-CACCCCCGGGAATTTATAGACCCCACTGGGG-3′; for κB-2, 5′-CAAGCCTGGGACAGTTTCGGGGAGTGAAATC-3′; for κB-3, 5′-AGCATCAAGGATAGTTTTCACACTCCCCATC-3′; for κB-4, 5′-CCAGATGAGCTCATAAATTTCTCCACCAAGG-3′) and synthesis of the complementary strand. Correct mutations were selected through sequencing and then introduced into pUG-BGH-PLAP reporter plasmid as *Xba*I/*Hin*dIII fragments.

HIV-1 κB-PLAP. For convenience in inserting multicopy enhancer elements, a *Spe*I site was introduced into the pUG-BGH-PLAP. pUG-BGH-PLAP was digested with *Sph*I and *Xba*I and then ligated with *Sph*I/*Spe*I/*Xba*I adapter (5′-pCACTAGTAAAT-3′ and 5′-pCTAGATTTACTAGTGCATG-3′) to yield pUG-BGH-PLAP (*Spe*I). Truncated herpes simplex virus TK promoter (−51 to +105 from the cap site of TK) was isolated through polymerase chain reaction (21, 22). Primers (5′-GGATCTAGACCCCGCCCAGCGTCTTGTCA-3′ and 5′-GGAAAGCTTGCGGCACGCTGTTGACGCTG-3′) that have *Xba*I or *Hin*dIII sites in their 5′ ends were used to amplify the 157-bp TK promoter fragment from the vector pTKβ. After digestion with *Xba*I and *Hin*dIII, 157-bp *Xba*I/*Hin*dIII fragment was ligated to pUG-BGH-PLAP (*Spe*I) that had been cut with the same enzymes to yield TK-PLAP. The HIV-1 κB-PLAP (Fig. 3E) construct contains four copies of the synthetic oligonucleotides 5′-pCTAG*TGGGACTTTCCT*-3′ and 3′-*ACCCTGAAAGG*AGATCp-5′, corresponding to the 10-bp NF-κB-responsive element (−103 to −94 in HIV-1 LTR. Italics show NF-κB sequences.) inserted upstream of a truncated

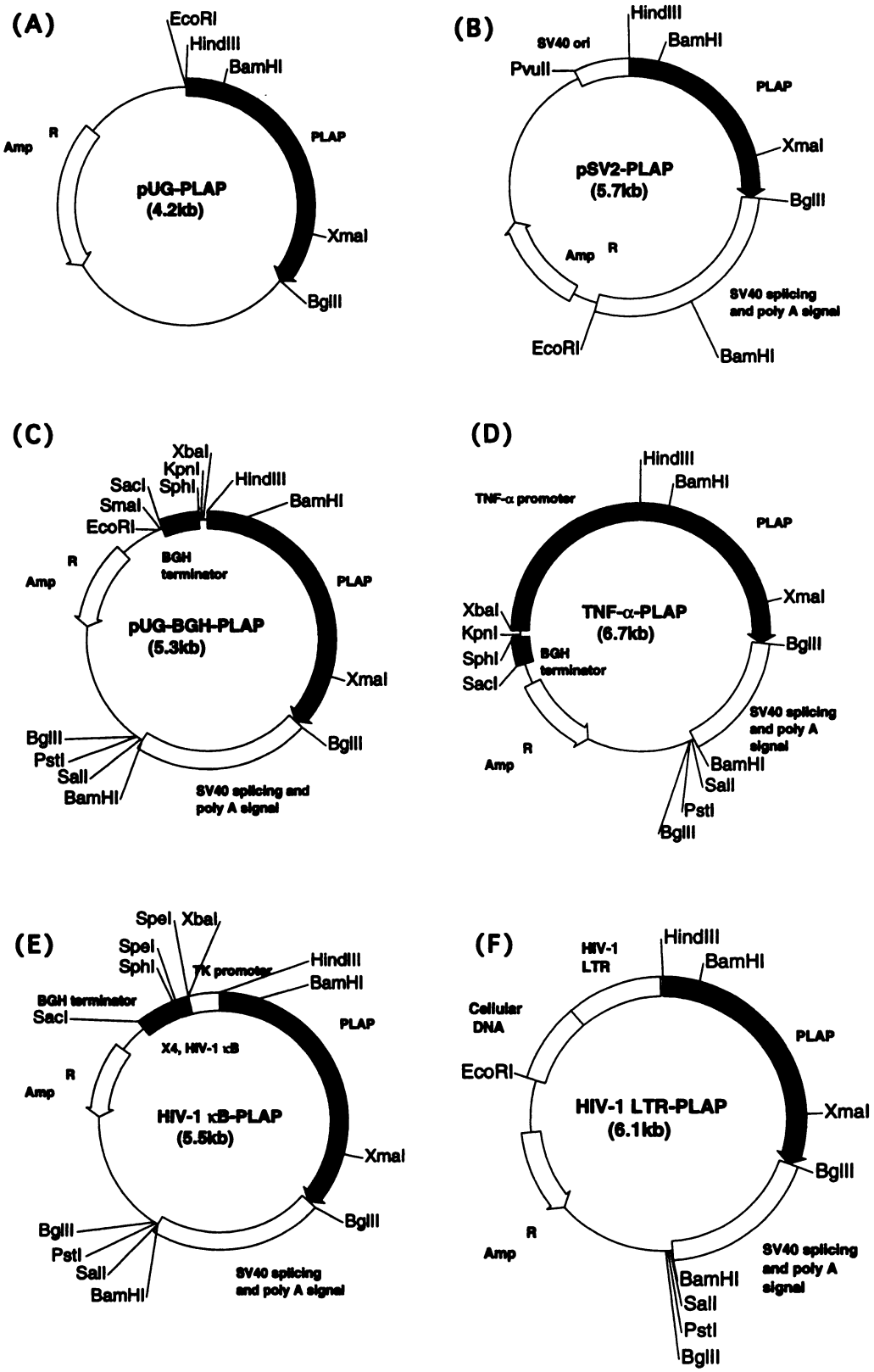


Fig. 3. Structure of plasmid vectors containing secreted human PLAP. *Solid arrow of each plasmid*, a 1.5-kb fragment of secreted form of human PLAP that extends from *Hin*dIII to *Bgl*II. The *open area* from *Pvu*II to *Hin*dIII in pSV2-PLAP (B) is the SV40 sequence that contains the SV40 origin of replication and the early promoter. Other *open areas* from *Bgl*II to *Bam*HI (or *Eco*RI) in pSV2-PLAP (B), pUG-BGH-PLAP (C), TNF-α-PLAP (D), HIV-1 κB-PLAP (E), and HIV-1 LTR-PLAP (F) are SV40 sequences encoding the intron for small t antigen and the polyadenylation signal for early mRNA. The *lightly stippled areas* in pUG-BGH-PLAP (C), TNF-α-PLAP (D), and HIV-1 κB-PLAP (E) are a 217-bp fragment of BGH terminator that extends from *Sac*I to *Sph*I. The *heavily stippled area* in TNF-α-PLAP (D) is a 1.4-kb TNF-α promoter fragment. The *open area* in HIV-1 κB-PLAP (E) is 157 bp of truncated TK promoter segment that extends from *Spe*I/*Xba*I to *Hin*dIII. The *solid segment* from *Spe*I to *Spe*I/*Xba*I in HIV-1 κB-PLAP (E) contains four copies of HIV-1 NF-κB element. The *open area* in HIV-1 LTR-PLAP (F) is the HIV-1 proviral 5′ LTR sequence (531 bp).

TK promoter in TK-PLAP through the use of restriction sites *Spe*I and *Xba*I. The orientation of NF-κB-responsive elements was found to be forward, forward, backward, and backward relative to that in HIV-1 LTR by sequencing.

HIV-1 LTR-PLAP. HIV-1 LTR-containing DNA fragment was isolated from pUC-BENN-chloramphenicol acetyltransferase plasmid (Dr. M. A. Martin, provided through the National Institute of Allergy and Infectious Diseases, AIDS Research and Reference Reagent Program) (23). pUC-BENN-chloramphenicol acetyltransferase plasmid was cut with *Eco*RI and *Hin*dIII, and then a 981-bp fragment containing cellular DNA and HIV-1 LTR was purified and ligated to pUG-BGH-PLAP to yield HIV-1 LTR-PLAP (Fig. 3F).

Preparation of TR-1 Cells through Stable Transfection of TNF-α Reporter Plasmid into Genome of THP-1 Cells

To produce stable reporter cells responsive to LPS stimulation, a 1.2-kb *SalI/XhoI* fragment of the PGK-neo expression cassette was introduced into the *SalI* site of TNF-α-PLAP plasmid (24). The resulting plasmid TNF-α-PLAP-PGK-neo was transfected into THP-1 cells through electroporation (Bio-Rad Gene Pulser), and the cells were subjected to G418 (1 mg/ml) selection in 96-well plates. The G418-resistant clones were isolated to check their LPS responsiveness. One clone (TR-1) produced PLAP enzyme and secreted it into the culture medium on stimulation with LPS.

Stimulation of TR-1 with LPS

TR-1 cells were maintained in RPMI 1640 containing 10% heat-inactivated endotoxin-free fetal calf serum and G-418 (1 mg/ml). The cells, in RPMI 1640 medium without G418, were seeded at a density of 1.0×10^5 cells/well onto 48-well plates (Costar 3548, Costar Corp., Cambridge, MA). The TR-1 cells were cultured in the presence or absence of E3330 for 30 min, followed by stimulation with 100 ng/ml of LPS for 18 hr. After the stimulation, culture supernatant was drawn from each well and assayed for alkaline phosphatase activity (described below).

Transfections and PLAP Assay

Cells of Raw264.7, a mouse macrophage cell line, in RPMI 1640 containing 10% heat-inactivated, endotoxin-free fetal calf serum were seeded before transfection at a density of 1.5×10^6 cells/well onto six-well plates (Falcon 3046, Becton Dickinson, NJ). These cells were transfected with PLAP reporter plasmid (15 μg) through the DEAE-dextran method (25). At 16 hr after transfection, cells (5×10^6 cells/well, six-well plate) were incubated in the presence or absence of E3330 for 30 min, followed by stimulation with 1 μg/ml of LPS for 48 hr. After the stimulation, culture supernatant was drawn from each well and assayed for alkaline phosphatase (described below). Alkaline phosphatase activity secreted into the culture medium was quantified with the use of a chemiluminescent substrate, 4-methoxy-4-(3-phosphatephenyl)spiro[1,2-dioxetane-3,2′-adamantane] (Lumistain reagent) (26). To inactivate tissue-nonspecific alkaline phosphatase that was contained in the culture medium, samples were heated at 65° for 30 min before measurements were made. Aliquots of 10 μl were mixed with 90 μl of assay buffer (0.28 M Na_2CO_3-$NaHCO_3$, pH 10.0, containing 8.0 mM $MgSO_4$) in a microplate (Dynatech, Chantilly, VA), and then 100 μl of Lumistain reagent was added and mixed. After 60 min at room temperature, steady state chemiluminescence was measured with a microplate luminometer (LB96P, EG&G Bertold, Bad Wildbad, Germany).

Preparation of Nuclear Extracts and Gel Shift Assay

Human monocytes were separated from peripheral blood according to the method described above. The cells were suspended at a concentration of 1×10^6 cells/ml in RPMI 1640 containing 10% heat-inactivated autologous serum, seeded at 10 ml/dish onto 100-mm dishes (Costar 3100), and cultured for 1.5 hr. Nonadherent cells were removed by rinsing, and the remaining cells were used as the monocyte preparation. The monocytes were cultured in the presence or absence of E3330 for 30 min, followed by stimulation with 10 ng/ml of LPS for 2 hr. Then, the nuclear extracts were isolated according to the known rapid preparation method with a slight modification (27). Briefly, cells were collected with a cell scraper, washed with 1 ml of HBSS, and pelleted by spinning for 10 min in a microfuge. The cell pellet was resuspended in 100 μl of cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM PMSF). The cells were allowed to swell on ice for 15 min, mixed with 10 μl of 10% solution of NP-40, and vigorously vortexed for 10 sec. The cell homogenate was centrifuged for 10 min in a microfuge. After removal of the supernatant, the nuclear pellets were resuspended in 25 μl of buffer C (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM PMSF), and the tube was vigorously shaken at 4° for 15 min on a shaking platform. The nuclear extracts were centrifuged for 10 min in a microfuge, and the supernatant was frozen in aliquots at −80°.

The NF-κB probes of TNF-α used for gel shift assay consisted of the NF-κB-like sites located at −634 bp (κB-1, 5′-GG-GTCT *GTGAATTCCC*GGGGGTGA-3′), −605 bp (κB-2, 5′-GG-CTCCCC *GGGGCTGTCCC*AGCT-3′), −220 bp (κB-3, 5′-GG-TGTGA *GGGGTATCCT*TGATGCT-3′), and −105 bp (κB-4, 5′-GG-CTCAT *GGGTTTCTCC*ACCAAGG-3′) from the transcription start point of TNF-α gene (20) (italics show NF-κB-like sequences). The NF-κB probe of HIV-1 used for gel shift assay consisted of the NF-κB site located between −103 and −90 (5′-GG-CTACAA *GGGACTTTCC*GCT *GGGGACTTTCC*AGG-3′) nucleotides from the transcription start point of HIV-1 (28) (italics show NF-κB sequences). Double-stranded oligonucleotides were labeled with [α-^{32}P]dCTP with use of the Klenow fragment. The labeled DNA probes were purified with use of NAP-5 columns. Gel shift assay was performed according to the previously described method with a slight modification (29). The nuclear extracts (2 μg/ml) were incubated with ^{32}P-labeled NF-κB probe of TNF-α or HIV-1 (10,000–20,000 cpm) in the binding buffer (10 mM Tris·HCl, 40 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 1% NP-40, 1% deoxycholate, 3 μg/ml polydeoxyinosinic-deoxycytidylic acid) at room temperature for 30 min. DNA/protein complexes were separated from free DNA probes on native 5% polyacrylamide gel. The gels were vacuum-dried and exposed to an X-ray film or an imaging plate of the BAS 2000 system (Fuji Photo Film Co., Tokyo, Japan).

To characterize the bands, we carried out supershift assay. The nuclear extract and oligonucleotide were mixed, specific antibody (3 μg) against nuclear factor was added, and the mixture was incubated at 4° for 1 hr; then, gel shift assay was conducted according to the procedure described above.

To examine the effect of E3330 on direct binding of NF-κB protein to NF-κB probes, 2 μg of nuclear extract was isolated from human monocytes that had been stimulated with 10 ng/ml of LPS for 2 hr, or 2 μg of the human recombinant p50 protein was incubated with TNF-α or HIV-1 κB probe in the presence or absence of E3330 for 30 min at room temperature.

Western Blotting Analysis of IκB-α Degradation

The monocytes attached to 150-mm dishes were cultured in the presence or absence of E3330 for 30 min, followed by stimulation with 100 ng/ml of LPS for 30 min. After the stimulation, the cells were rinsed with HBSS and then lysed with 200 μl of ice-cold lysis buffer (0.2 M HEPES, pH 7.2, 1% Triton X-100, 10% glycerol, 0.1 mM leupeptin, 1 mM PMSF, 1 mM Na_3VO_4, 50 mM NaF). The lysed samples were centrifuged for 10 min in a microfuge at 4° to remove insoluble debris. Then, 50 μl of 5-fold-concentrated Laemmli buffer and 10 μl of 2-ME were added, and the entire mixture was boiled for 3 min. The resulting samples (20 μl) were analyzed with electrophoresis on 10–20% Tris-glycine-buffered polyacrylamide gradient gels in SDS. The separated proteins were transferred to 0.22-μm nitrocellulose membrane with an electroblotter. The membrane was analyzed for the degradation of IκB-α proteins with rabbit anti-IκB-α antibody and horseradish peroxidase-linked donkey anti-rabbit IgG antiserum. The membranes were dried, incubated in ECL reagents, covered with Saran wrap, and exposed to ECL hyperfilms.

Superoxide Anion Production

Human monocytes were separated from peripheral blood through the method described above. The cells were suspended at a concentration of 1×10^7 cells/ml in RPMI 1640 containing 10% heat-inactivated autologous serum, seeded at 100 μl/well onto 96-well plates (Falcon 3092), and cultured for 1.5 hr. After removal of nonadherent cells through rinsing, the reaction buffer [HBSS (−) containing 0.1% glucose and 25 μg/ml catalase, in the

presence or absence of 25 μg/ml of superoxide dismutase] was added to the monocytes. The monocytes were incubated in the presence or absence of E3330 for 30 min, followed by the addition of 80 μM cytochrome *c* and stimulation with PMA (10 nM), LPS (1 μg/ml), or fMLP (10 μM) for 60 min in the final volume of 200 μl.

Superoxide anion-scavenging activity of E3330 was determined in a cell-free system. Xanthine oxidase (10 munits/ml) and xanthine (100 μM) were incubated in the presence or absence of a test compound for 30 min.

The amount of superoxide anion production was measured in terms of superoxide dismutase-inhibitable cytochrome *c* reduction with an automatic microplate reader (EL 340I, Bio-Tek Instruments, Winooski, VT).

Cell Viability

Cell viability was tested with the MTT colorimetric method (30) or lactate dehydrogenase assay.

Results

Inhibitory effect of E3330 on transcriptional rate of TNF-α gene. Northern blot analysis for TNF-α mRNA indicated that the inhibitory effect of E3330 on TNF-α generation is due to inhibition of TNF-α mRNA biosynthesis (7). To confirm this, nuclear run-on experiments were performed. The transcription rate of TNF-α gene in human monocytes immediately rose after LPS (100 ng/ml) stimulation and reached a maximum at 30 min. Based on densitometry with a BAS 2000 image analyzer, the increase of TNF-α gene transcription over the basal level reached 12.0-fold. Pretreatment with E3330 decreased the transcription rate of TNF-α gene in dose-dependent manner but did not significantly affect transcription of actin mRNA (Fig. 4A). Image analysis indicated that the inhibition by E3330 at 30 and 100 μM amounted to 65% and 85%, respectively (Fig. 4B). At 100 μM, E3330 did not show measurable cytotoxicity in the MTT assay. These results indicate that LPS stimulation increases the TNF-α gene transcription rate and E3330 inhibits this transcriptional activation.

Inhibitory effect of E3330 on transcriptional activation of TNF-α gene. To assess the inhibitory effect of E3330 on the transcriptional activation of TNF-α gene, we performed the assay with TNF-α-PLAP reporter gene. We used the expression system of secreted-type PLAP gene as a reporter because the product of PLAP gene was secreted into the culture medium and could be detected sensitively with a chemiluminescent substrate, 4-methoxy-4-(3-phosphatephenyl)spiro[1,2-dioxetane-3,2′-adamantane] (26).

As shown in Fig. 5A, Raw264.7 cells transiently transfected with PLAP reporter plasmids containing 1.4-kb TNF-α promoter showed very little PLAP activity before stimulation. However, stimulation of the cells with LPS led to a ~10-fold increase in PLAP activity. In the presence of E3330, this inducible expression was inhibited. Inhibition by 30 μM E3330 amounted to 54%, and this concentration of E3330 did not show any cytotoxicity as measured with MTT assay.

Next, we prepared a stable transformant of THP-1 cell (TR-1) in which the TNF-α-PLAP reporter gene was integrated in the genome. TR-1 cells also showed very little

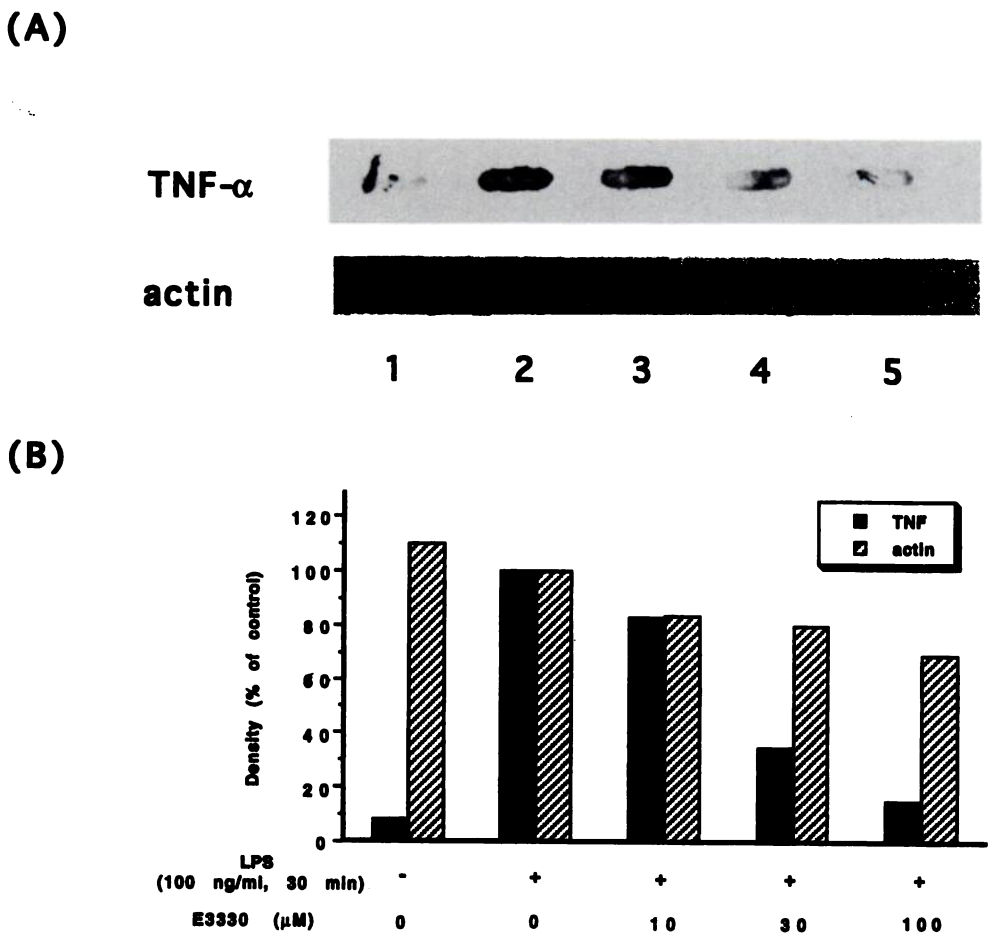


Fig. 4. Inhibitory effect of E3330 on steady state level of TNF-α mRNA. A, Nuclear run-on assay. Human monocytes were incubated in the presence or absence of E3330 for 30 min, followed by stimulation with LPS for 30 min. Isolation of nuclei and elongation, purification, and hybridization of RNA are described in Materials and Methods. *Lane 1*, without LPS; *lane 2*, LPS (100 ng/ml); *lane 3*, LPS (100 ng/ml) + E3330 (10 μM); *lane 4*, LPS (100 ng/ml) + E3330 (30 μM); *lane 5*, LPS (100 ng/ml) + E3330 (100 μM). B, Densitometry of the spots. The value of *lane 2* was defined as 100%. The 100% values of TNF-α and actin were 9,345 and 11,160, respectively. *Solid bars*, TNF-α mRNA; *hatched bars*, actin mRNA.

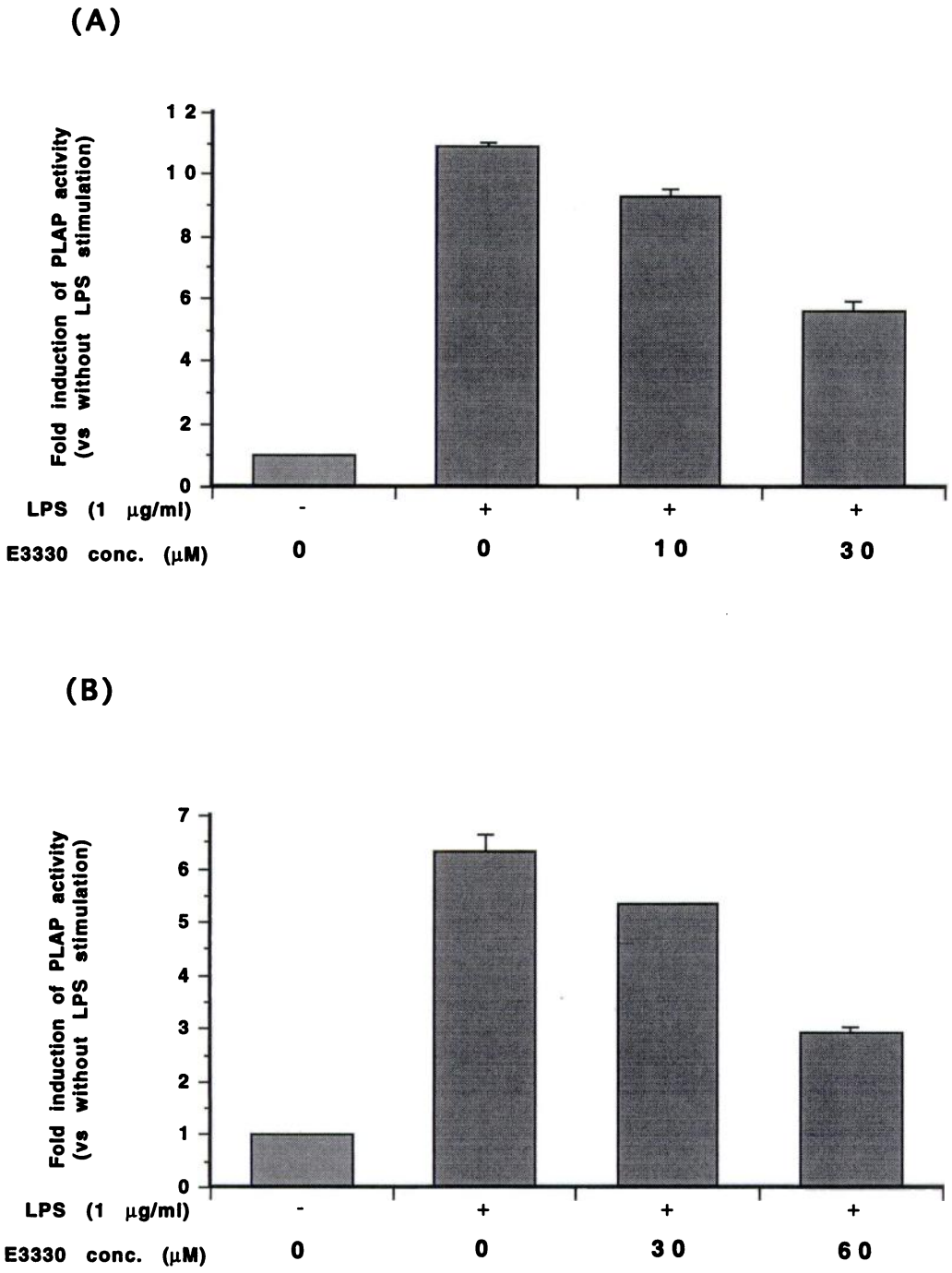


Fig. 5. Inhibitory effect of E3330 on transcription of TNF-α gene induced by LPS. A, Effect of E3330 on PLAP transcription of LPS-stimulated Raw264.7 cells. TNF-α PLAP plasmid was transiently transfected into the Raw264.7 cells with the DEAE-dextran method (see Materials and Methods). After the transfection, the cells were incubated in the presence or absence of E3330 for 30 min, followed by stimulation with LPS (1 μg/ml) for 48 hr. Culture supernatant was collected from each well and assayed for alkaline phosphatase activity. The amounts of alkaline phosphatase activity were estimated as arbitrary units by chemiluminescence counting with a microLumat LB 96. The linearity of the standard placental alkaline phosphatase activity was maintained from 0.25 ng/ml (256 ± 13) to 25 mg/ml (3,480,546 ± 40,641, mean ± standard error; five experiments). The mean value of 1.0-fold induction was 23,943 ± 467 (three experiments). Each value is the mean ± standard error of three experiments. B, Effect of E3330 on PLAP transcription of LPS-stimulated TR-1 cells. TR-1 cells were incubated in the presence or absence of E3330 for 30 min, followed by stimulation with LPS (100 ng/ml) for 18 hr. The mean value of 1.0-fold induction was 415 ± 6. Each value is the mean ± standard error of three experiments.

PLAP activity before stimulation, whereas stimulation of the cells with LPS led to a ~6.8-fold increase in PLAP activity. As shown in Fig. 5B, E3330 inhibited the production of PLAP in a dose-dependent manner. The inhibitory effect of E3330 at 60 μM was 64% and LDH release from the E3330-treated cells was <10% compared with that of lysed cells, indicating that the inhibitory effect of E3330 is not due to cytotoxicity. These results indicate that E3330 inhibits the induced expression of TNF-α-PLAP reporter gene.

NF-κB is related to transcription of TNF-α gene. NF-κB is a critical transcriptional factor of several genes involved in immune and inflammatory responses (13, 14). It has been reported that transcription of murine TNF-α gene was regulated by NF-κB activation (11, 12). We identified four NF-κB recognition site-like sequences located at −634 (κB-1), −605 (κB-2), −220 (κB-3), and −105 (κB-4) bp from the transcription start site in the 5′ upstream region of human TNF-α gene. The locations of these NF-κB recognition site-like sequences are indicated in Fig. 6A. To characterize the functional role of the putative NF-κB binding sites, we introduced mutations by using synthetic oligonucleotides (Fig. 6B), and we ligated them with PLAP reporter plasmid to generate TNF-α-PLAP containing mutant NF-κB recognition site-like sequences. These constructs were transformed into Raw264.7 cells, and their

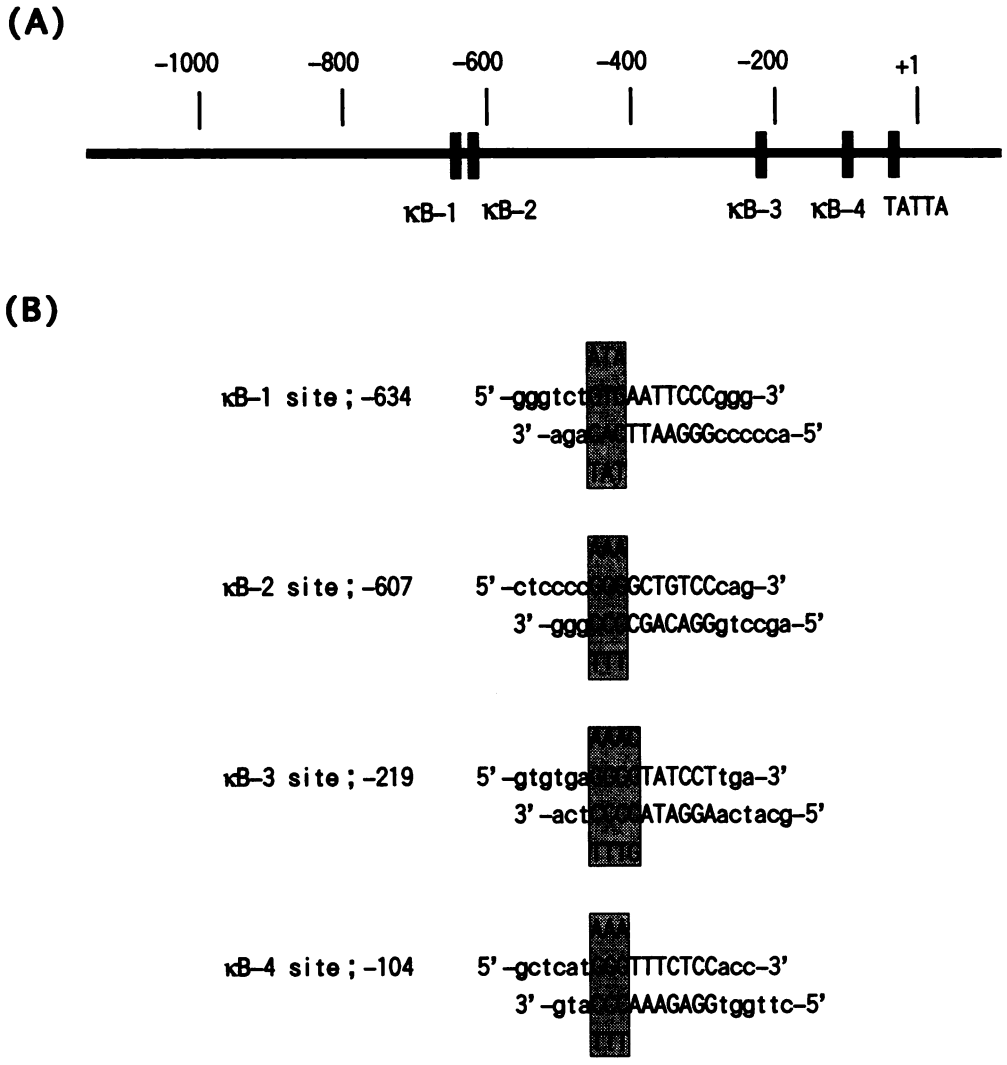


Fig. 6. Effects of NF-κB binding site mutations in the TNF-α 5′ upstream region on transcription. A, Structure of the human TNF-α promoter. The sequence of the NF-κB-like sites and their locations relative to the mRNA start site are indicated. B, Mutant sequences of NF-κB sites in the TNF-α 5′ upstream region. *Shaded regions*, nucleotide changes in the mutants. C, Effects of NF-κB binding site mutations in the TNF-α 5′ upstream region on transcription. Plasmids containing NF-κB binding site-mutated forms of the TNF-α 5′ upstream region were transfected into Raw264.7 cells with the DEAE-dextran method. The cells were stimulated with LPS (1 μg/ml) for 18 hr, and the amounts of alkaline phosphatase activity in the culture supernatant were estimated. *Solid bars*, LPS-stimulated cells; *hatched bars*, unstimulated cells.

inducibility in response to LPS stimulation was examined. We chose Raw264.7 cells because this cell line showed excellent transfection competency, was easy to transfect, and was suitable for comparing the transcription levels among plural plasmids. As shown in Fig. 6C, the expression of wild-type TNF-α-PLAP reporter gene was low before induction, whereas this gene was highly inducible in Raw264.7 cells on stimulation with LPS. On the other hand, induced expression of the mutant genes was dramatically reduced. These data indicated that each of the NF-κB recognition site-like sequences plays a critical role in LPS stimulation of human TNF-α gene transcription.

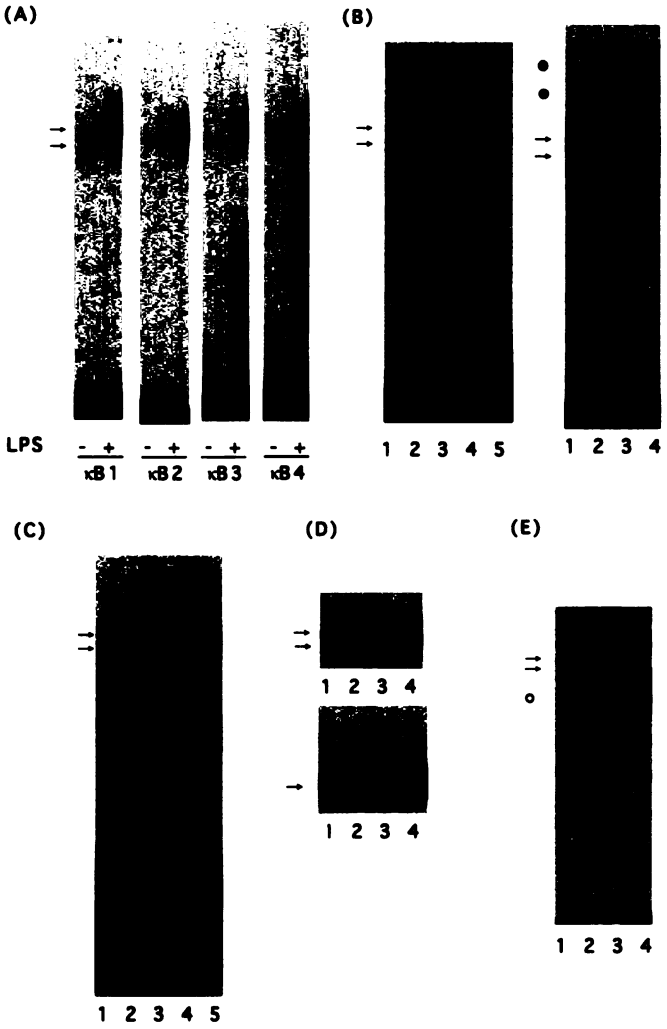


Fig. 7. Effect of E3330 on NF-κB activity. A, Detection of NF-κB binding by TNF-α NF-κB oligonucleotides. Nuclear extracts from LPS (10 ng/ml, 60 min)-stimulated human monocytes were incubated with ^{32}P-labeled TNF-α κB-1, κB-2, κB-3, and κB-4 double-stranded oligonucleotide. *Arrows*, specific binding. B, Specificity of bindings detected by TNF-α NF-κB-1 oligonucleotides. *Left*, competition for binding of LPS-induced nuclear NF-κB with NF-κB-related or unrelated oligonucleotides. Nuclear extracts from human monocytes stimulated with LPS (10 ng/ml) for 60 min were incubated with ^{32}P-labeled double-stranded TNF-α κB-1 oligonucleotide in the presence or absence of NF-κB-related or unrelated oligonucleotides. *Lane 1*, without competitor; *lane 2*, 10-fold excess of unlabeled double-stranded TNF-α κB-1 oligonucleotide; *lane 3*, 100-fold excess of unlabeled double-stranded TNF-α κB-1 oligonucleotide; *lane 4*, 10-fold excess of unlabeled double-stranded cAMP-responsive element oligonucleotide; *lane 5*, 100-fold excess of unlabeled double-stranded cAMP-responsive element oligonucleotide. *Arrows*, specific binding. *Right*, identification of NF-κB in the gel shift assay. Nuclear extracts from human monocytes stimulated with LPS (10 ng/ml) for 60 min were incubated with ^{32}P-labeled double-stranded TNF-α κB-1 oligonucleotide, followed by the addition of antibodies at 4° for 60 min. *Lane 1*, without antibody; *lane 2*, anti-p50 antibody; *lane 3*, anti-p65 antibody; *lane 4*, anti-p52 antibody. *Arrows*, specific binding. ●, Supershifted binding. C, Inhibitory effect of E3330 on the induction of NF-κB activity in human monocyte nuclei detected by binding to double-stranded TNF-α κB oligonucleotides. Human monocytes were incubated in the presence or absence of E3330 for 30 min, followed by stimulation with LPS for 120 min. The nuclear extracts prepared from these cells were incubated with ^{32}P-labeled double-stranded oligonucleotide of TNF-α κB-1. *Lane 1*, without LPS; *lane 2*, LPS (10 ng/ml); *lane 3*, LPS (10 ng/ml) + E3330 (10 μM); *lane 4*, LPS (10 ng/ml) + E3330 (30 μM); *lane 5*, LPS (10 ng/ml) + E3330 (100 μM). *Arrows*, specific binding. D, No inhibitory effect of E3330 on the direct binding of NF-κB to TNF-α κB-1 oligonucleotide. *Top*, effect of E3330 on the direct binding of nuclear extracts from LPS-stimulated human monocytes to TNF-α κB-1 oligonucleotide. Nuclear extracts from human monocytes stimulated with LPS (10 ng/ml) for 60 min were incubated with ^{32}P-labeled double-stranded TNF-α κB-1 oligonucleotide in the presence or absence of E3330. *Lane 1*, without drug; *lane 2*, E3330 (10 μM); *lane 3*, E3330 (30 μM); *lane 4*, E3330 (100 μM). *Arrows*, specific binding. *Bottom*, effect of E3330 on the direct binding of human recombinant p50 protein to TNF-α κB-1 oligonucleotide. Human recombinant p50 protein was incubated with ^{32}P-labeled double-stranded TNF-α κB-1 oligonucleotide in the presence or absence of E3330. The order of lanes was the same as on the *top*. *Arrow*, p50 homodimer binding. E, Effect of E3330 on the induction of NF-κB binding activity to HIV-1 κB oligonucleotide in nuclei of human monocytes. The experimental procedure was the same as in C except for the use of HIV-1 κB oligonucleotide instead of TNF-α κB-1 oligonucleotide. *Lane 1*, without LPS; *lane 2*, LPS (10 ng/ml); *lane 3*, LPS (10 ng/ml) + E3330 (10 μM); *lane 4*, LPS (10 ng/ml) + E3330 (30 μM). *Arrows*, specific binding. ○, Nonspecific binding.

Induction and characterization of NF-κB in nuclei of LPS-stimulated human monocytes and inhibitory effect of E3330 on induction of NF-κB activity detected by binding to oligonucleotides corresponding to TNF-α NF-κB-recognition site-like sequences. NF-κB p50/p65 heterodimer is present in the cytosol of resting cells (13, 14). After stimulation of the cells with various agents, the cytosolic NF-κB/IκB complex is dissociated, and free NF-κB translocates to the nuclei. We performed gel retardation assay with four oligonucleotides having NF-κB recognition site-like sequences in the TNF-α 5′ upstream region and the same nuclear extract prepared from LPS-stimulated human monocytes (Fig. 7A). Although a small amount of a faster-migrating band was detected in the nuclei of resting monocytes, the slower-migrating band in the nuclei increased after stimulation of the cells with LPS (10 ng/ml) for 60 min. The result with a ^{32}P-labeled κB-1 oligonucleotide showed that the amount of faster-migrating band in the nuclei was unchanged, whereas the increase in the slower-migrating band after stimulation for 60 min amounted to 2.4-fold from the resting level on the basis of densitometry with a BAS 2000 image analyzer. The intensity of binding was in the order of κB-1 > κB-2 > κB-4 > κB-3. There was no difference in migration among the κB oligonucleotides. These results indicate that activated-NF-κB binds to all four κB sites of TNF-α gene.

To confirm the specificity of these bands that were detected with gel shift assay, we carried out competition assay with cold oligonucleotides. These bands between ^{32}P-labeled κB-1 oligonucleotide and nuclear extracts from LPS-stimulated human monocytes were effectively eliminated by competition by excess unlabeled κB-1 oligonucleotide but not by an unrelated oligonucleotide of cAMP-responsive element (-TGACGTTCA-) (Fig. 7B, *left*). Furthermore, the results of supershift assay with specific antibodies reactive with NF-κB protein suggested that these bands were NF-κB proteins. As shown in Fig. 7B (*right*), the faster- and slower-migrating bands were both shifted by antibody reactive with p50, whereas only the latter band was shifted by antibody reactive with p65. Anti-p52, anti-Rel, and anti-relB antibodies did not affect the migration of these bands (data not shown). On the other hand, the band between ^{32}P-labeled κB-1 oligonucleotide and nuclear extracts from unstimulated human monocytes was shifted only by anti-p50 antibody (data not shown).

Similar results were obtained by using κB-2, κB-3, and κB-4 oligonucleotides instead of κB-1 oligonucleotide with nuclear extract of LPS-stimulated human monocytes (data not shown). These data indicated that the faster-migrating band was composed of p50 homodimer and the slower-migrating band was composed of p50/p65 heterodimer, implying that stimulation with LPS induces p50/p65 heterodimer in nuclei of human monocytes. Next, we examined the effect of E3330 on the appearance of NF-κB binding activity in the nuclei. The results of gel shift assay with a ^{32}P-labeled κB-1 oligonucleotide and nuclear extracts of LPS-stimulated human monocytes are shown in Fig. 7C. E3330 inhibited the induction of slower-migrating NF-κB binding to κB-1 oligonucleotide in a dose-dependent manner. The inhibitory effect of E3330 at 30 μM was 92%. Similar results were obtained by using κB-2, κB-3, and κB-4 oligonucleotides instead of κB-1 oligonucleotide (data not shown).

The effect of E3330 on direct binding of NF-κB to κB-1 oligonucleotide is shown in Fig. 7D. This compound did not inhibit the direct binding of nuclear extracts from LPS-stimulated monocytes (*top*) or human recombinant p50 homodimer (*bottom*) to κB-1 oligonucleotide, even at 100 μM. These results suggest that E3330 inhibits NF-κB activation and/or translocation to the nuclei from cytosol.

Although NF-κB-recognized sequences have been found the in 5′ upstream regions of many genes, HIV-1 is of particular interest because HIV-1 is the causal retrovirus of AIDS (31). It has been reported that tandem NF-κB sequences exist in the LTR of HIV-1, and NF-κB positively regulates transcription of this gene (3, 13, 14, 28). Next, we examined the inhibitory effects of E3330 on the induction of NF-κB binding activity through the use of gel shift assay with HIV-1 κB oligonucleotide (Fig. 7E). E3330 inhibited the induction of the NF-κB binding activity of the slower-migrating band. The inhibitory effect at 30 μM amounted to 86%.

Inhibitory effect of E3330 on expression of NF-κB reporter gene. TNF-α gene activation in response to LPS stimulation requires four putative NF-κB binding sites in the 5′ upstream region of the TNF-α gene. To determine the effect of E3330 on NF-κB activation, Raw264.7 cells were transfected with other NF-κB activatable plasmids, and the effect of E3330 on the gene transcription was measured (Table 1). When Raw264.7 cells that had been transiently transfected with HIV-1 κB-PLAP, containing four copies of the HIV-1 κB motif, were stimulated with LPS (1 μg/ml) for 48 hr, PLAP activity in the culture supernatant increased to 3.2 times the basal level. E3330 at 30 μM reduced the induction of PLAP expression to 81% of the control. Transfection with HIV-1 LTR-PLAP and stimulation with LPS under the same conditions gave a 2.6-fold induction of PLAP enzyme. E3330 at 30 μM reduced the induction of PLAP activity to 41% of the control. In addition, when Raw264.7 cells were transfected with pSV2 PLAP and stimulated with LPS under the same conditions, PLAP activity increased to 12.0 times the basal activity, and E3330 at 30 μM decreased the induction of PLAP activity to 71% of the control. During these experiments, we did not detect significant cytotoxicity with the MTT assay, so E3330 clearly affected the NF-κB-containing promoters, but the degree of the inhibition differed, depending on the reporter constructs. Possibly, the active NF-κB concentration required in the nuclei for gene activation might be different for each NF-κB binding element or promoter. In addition, other inducible transcription factors might be involved in the activation of pSV2 and HIV-1 LTR and act synergistically in the promoter activation. These results suggest that E3330 can inhibit NF-κB activation or a signaling pathway that leads to NF-κB activation.

TABLE 1

Inhibitory effect of E3330 on transcriptional activity of HIV-1 κB- and HIV-1 LTR-reporter gene

The plasmids were transfected into Raw 264.7 cells according to the DEAE-dextran method. The cells were incubated in the presence or absence of E3330 for 30 min, followed by stimulation with LPS (1 μg/ml) for 48 hr. Culture supernatant was collected from each well and assayed for alkaline phosphatase activity.

Plasmid	LPS	E3330	PLAP activity[a]	Percent of induced PLAP activity
	μg/ml	μM		%
HIV-1 κB-PLAP	0	0	0.1 ± 0.1	0
	1	0	2.6 ± 0.1	100
	1	30	1.3 ± 0.1	17
HIV-1 LTR-PLAP	0	0	1.0 ± 0.1	0
	1	0	3.2 ± 0.0	100
	1	30	1.9 ± 0.1	40
pSV2-PLAP	0	0	1.0 ± 0.0	0
	1	0	12.1 ± 0.1	100
	1	30	8.9 ± 0.2	71

[a] The amounts of alkaline phosphatase activity were estimated as arbitrary units by chemiluminescence counting with an LB96P. The mean values of 1.0-fold induction were 40,996 ± 4,251 (HIV-1 κB-PLAP), 54,096 ± 2,917 (HIV-1 LTR-PLAP), and 13,321 ± 210 (pSV2-PLAP) for three experiments.

Inhibitory effect of E3330 on IκB-α degradation. NF-κB is present in the cytosol of resting cells as a complex with an inhibitory protein, IκB-α (13, 14). A variety of cellular stimuli cause the dissociation of NF-κB from IκB-α, allowing free NF-κB to translocate to the nuclei and activate gene expression. The translocation of NF-κB is preceded by the phosphorylation and degradation of IκB-α (33–35). We therefore examined the effect of E3330 on IκB-α phosphorylation and degradation. The results of Western blotting with anti-IκB-α antibody showed that IκB-α was separated with SDS-polyacrylamide gel electrophoresis as a 37-kDa band from resting human monocytes (Fig. 8, *lane 1*). After the stimulation of human monocytes with LPS (100 ng/ml) for 30 min, the band of IκB-α decreased, and the phosphorylated form of IκB-α appeared as a slower-moving band (Fig. 8, *lane 2*). E3330 had no distinct effect on the appearance of slower-moving band, but it inhibited the decay of native IκB-α in a dose-dependent manner (Fig. 8, *lanes 3–5*). These results indicated that E3330 might inhibit some step in the mechanisms, leading to the degradation of IκB-α protein.

Effect of E3330 on superoxide anion production. Because active oxygen species serve as common messengers that activate NF-κB (13, 14, 32, 36, 37), we examined the effect of E3330 on superoxide anion production of human monocytes (Table 2). When human monocytes attached to a plastic plate were stimulated with LPS (1 μg/ml), PMA (10 nM), or fMLP (10 μM), superoxide production was 11.1 ± 0.6, 12.3 ± 1.3, or 22.1 ± 1.7 nmol/ml/60 min, respectively. The inhibitory effect of E3330 at 100 μM amounted to 74% for LPS, 81% for PMA, and 84% for fMLP. In the superoxide production system with xanthine

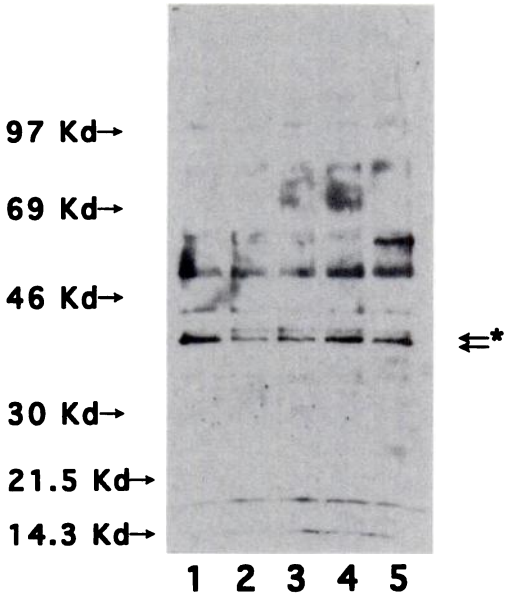


Fig. 8. Inhibitory effect of E3330 on phosphorylation of IκB-α. Human monocytes were incubated in the presence or absence of E3330 for 30 min, followed by stimulation with LPS for 30 min. Preparation of the whole-cell lysate and electrophoresis and blotting were as described in Materials and Methods. The membrane was analyzed for the degradation of IκB-α proteins with rabbit anti-IκB-α antibody and horseradish peroxidase-linked donkey anti-rabbit IgG antiserum. The membranes were dried, incubated in ECL reagents, covered with Saran wrap, and exposed to ECL hyperfilms. *Lane 1*, without LPS; *lane 2*, LPS (10 ng/ml); *lane 3*, LPS (10 ng/ml) + E3330 (10 μM); *lane 4*, LPS (10 ng/ml) + E3330 (30 μM); *lane 5*, LPS (10 ng/ml) + E3330 (100 μM). *Arrows*, IκB-α proteins. *, Phosphorylated form of IκB-α.

oxidase and xanthine, the corresponding value was only 22%. These results suggest that one mechanism of E3330 action on NF-κB activation might be inhibition of superoxide anion production.

Discussion

E3330 is a novel synthetic quinone derivative that was discovered in our laboratories and has protective effects against endotoxin-mediated hepatitis in mice and galactosamine-induced hepatitis in rats (8, 9). The main mechanism of the protective effect of E3330 in the animal models is considered to be inhibition of TNF-α generation. E3330 inhibits TNF-α generation from several types of macrophages/monocytes, and the effect is considered to be based on the suppression of TNF-α mRNA expression (7). The results of the nuclear run-on assay indicated that E3330 decreases the transcription rate of TNF-α mRNA without altering that of actin mRNA (Fig. 4). Furthermore, E3330 decreased LPS-induced PLAP gene expression in Raw264.7 cells transiently transfected with TNF-α reporter gene and in TR-1 cells in which TNF-α reporter gene was stably integrated in the genome (Fig. 5, A and B). These data strongly suggest that the inhibitory effect of E3330 on TNF-α biosynthesis is caused at least in part by the suppression of transcriptional activation.

There have been many studies on critical regulatory regions for human TNF-α gene transcription. Deletion and/or mutation experiments showed that significant regions include the region −95 to −35 upstream from the transcription start site for PMA-stimulated U937 cells (10), the region −125 to −82 upstream for TNF-stimulated K652 cells (38), the region −100 to −74 upstream for PMA-treated Jurkat cells expressing C/EBP-β (39), and the region −115 to −98 upstream for PMA-induced Hut78 cells (40). On the other hand, it has been reported that NF-κB binding sites (especially that −510 to −501 upstream from the transcription start site) are important for LPS-stimulated TNF-α transcription in murine peritoneal macrophages (11, 12). NF-κB is a critical regulator of several genes involved in immune and inflammatory responses (13, 14). We therefore examined the role of NF-κB recognition sites in the 5′ upstream region of the human TNF-α gene.

Because the NF-κB binding sequence at −510 to −501 upstream exists in murine TNF-α gene but not in human TNF-α gene, transcription of the gene is presumably regulated by other NF-κB binding sequences. We searched for NF-κB recognition site-like sequences in the 5′ upstream region of human TNF-α gene and found sites located at −634 (κB-1), −605 (κB-2), −220 (κB-3), and −105 (κB-4) bp from the transcription start site (Fig. 6A). We prepared human TNF-α PLAP reporter plasmids, each containing one NF-κB site mutation in the 1.4-kb 5′ upstream region, and transiently transfected Raw264.7 cells with these plasmids. We chose Raw264.7 cells because this cell line showed excellent transfection competency and was suitable for comparison of the transcription level among plural plasmids. Although Raw264.7 cells are murine cell lines, the cells that were transfected with wild-type human TNF-α-reporter plasmids showed high inducibility (Fig. 6C). Furthermore, we confirmed the presence of NF-κB in nuclear extracts from LPS-stimulated Raw264.7 through binding studies to oligonucleotides corresponding to the human TNF-α NF-κB-recognition site-like sequences described above (data not shown). The suppression of transcriptional activity in Raw264.7 cells that were transfected with various human TNF-α-reporter plasmids containing a single NF-κB site mutation suggested that each of the NF-κB sites plays a critical role in LPS-stimulated TNF-α transcription.

Through the use of gel shift assay with oligonucleotides corresponding to human TNF-α NF-κB-recognition site-like sequences, two specific bands of NF-κB was clearly detected in the nuclear extracts prepared from LPS-stimulated human monocytes (Fig. 7A). The results of supershift assay indicated that the faster-migrating band was composed of p50 homodimer and the slower-migrating band was composed p50/p65 heterodimer (Fig. 7B). The amount of p50/65 heterodimer increased after stimulation with LPS from 30 to 120 min, but that of p50 homodimer did not. These data suggest that the increase of p50/65 heterodimer in the nuclei may lead to up-regulation of TNF-α transcription. This result should be confirmed by transcription experiments involving cotransfection with p50 and p65 protein expression vectors in the TR-1 and Raw264.7 cell systems. The order of binding activity between NF-κB (p50/65 heterodimer) and κB oligonucleotides was κB-1 > κB-2 > κB-4 > κB-3. Recently, it has been reported that the 5′ upstream region of the human TNF-α gene contains four potential NF-κB recognition sites at positions −104, −220, −605, and −633, and the order of binding strength is −605 > −104 > −220 > −633 (41). The

TABLE 2
Inhibitory effects of E3330 on the production of superoxide anion by human monocytes

	Stimuli	E3330 (μM)	O_2^- production	Percent of control
			nmol/ml/60 min	%
Human monocytes[a]	LPS (1 μg/ml)	0	11.0 ± 0.6	100
		10	8.8 ± 1.1	79
		30	7.0 ± 0.5	64
		100	2.9 ± 1.0	26
	PMA (10 nM)	0	22.1 ± 1.7	100
		10	14.5 ± 1.7	66
		30	14.0 ± 0.6	63
		100	4.2 ± 0.1	19
	fMLP (10 μM)	0	12.3 ± 1.3	100
		10	9.8 ± 0.2	80
		30	6.9 ± 0.2	56
		100	2.0 ± 0.8	16
Xanthine oxidase/xanthine[b]	Xanthine oxidase (10 m units/ml) Xanthine (0.1 mM)	0	14.9 ± 0.1	100
		10	14.8 ± 0.3	99
		30	13.5 ± 0.1	91
		100	11.6 ± 0.1	78

[a] Human monocytes were incubated in the presence or absence of E3330 for 30 min, followed by stimulation with LPS (1 μg/ml), PMA (10 nM), or fMLP (10 μM) for 60 min. Each value is the mean ± standard error of three experiments.
[b] Superoxide anion was generated with a xanthine oxidase/xanthine system in the presence or absence of E3330 for 30 min. Each value is the mean ± standard error of three experiments.

difference of that order from ours may be due to differences of experimental conditions, including the cells (e.g., freshly isolated human monocytes versus Mono Mac 6 cells), the culture conditions (e.g., autologous heat-inactivated serum versus fetal calf serum), and so on.

Next, we investigated the effect of E3330 on the appearance of NF-κB in the nuclei of LPS-stimulated human monocytes. Recently, it was reported that cyclosporin A inhibits TNF-α generation by anti-CD3-, anti-T cell receptor-α/β-, or ionomycin-stimulated murine Ar 5 T cells due to inhibition of the induction of NF-κB binding to the site located at −104 bp from the transcription start site (42). Our results suggest that the inhibitory effect of E3330 on TNF-α mRNA biosynthesis is caused by inhibition of the induction of NF-κB binding to the four κB sites of the TNF-α 5′ upstream region (Fig. 7C). Because E3330 did not inhibit the direct binding of nuclear extracts from LPS-stimulated monocytes and of human recombinant p50 homodimers to κB-1 oligonucleotide (Fig. 7D), the compound might affect signal transduction, leading to NF-κB activation after LPS stimulation.

NF-κB is known to activate not only TNF-α gene but also many other genes, including HIV-1 (13, 14). Tandem NF-κB-recognized sequences exist in the LTR of HIV-1 (28). It has been reported that monocytes/macrophages infected with HIV-1 play an important role as a reservoir of HIV-1, and LPS is a potent stimulator of HIV-1 expression in the cells (43, 44). We examined whether the inhibitory effect of E3330 on the induction of NF-κB binding activity is recognized by an oligonucleotide corresponding to HIV-1 κB. E3330 inhibited the induction of NF-κB recognized by HIV-1 κB oligonucleotide in the same way as seen with TNF-α κB oligonucleotides (Fig. 7E). The results of the HIV-1 κB plasmid transfection experiment suggest that the inhibitory effect of E3330 on the transcriptional activity may be caused by inhibition of NF-κB activation (Table 1). Similar results were obtained in Raw264.7 cells transfected with HIV-1 LTR-PLAP and TNF-α PLAP that contain NF-κB binding sites (Table 1 and Fig. 5A). These results indicate that the inhibitory effect of E3330 on the transcription might be specific to NF-κB-inducible genes.

NF-κB activation is regulated by its cytoplasmic inhibitor IκB-α (13, 14). The activation of NF-κB is associated with phosphorylation and degradation of IκB-α (32, 34, 35, 45, 46). The inducible phosphorylation of IκB-α is not sufficient for its dissociation from NF-κB (45–47) but is a signal for its proteolytic degradation by ubiquitin-dependent proteasome (34, 45, 48). This degradation process is absolutely required for NF-κB activation. To clarify the effect of E3330 on the IκB-α degradation, we performed Western blotting analysis with anti-IκB-α (Fig. 8). IκB-α was detected at 37 kDa through SDS-polyacrylamide gel electrophoresis in resting human monocytes. After LPS stimulation, the phosphorylated form of IκB-α was detected at a position corresponding to larger molecular size. Native IκB-α decreased as a result of the phosphorylation and the subsequent degradation (Fig. 8, *lane 2*). E3330 inhibited the decay of native IκB-α protein (Fig. 8, *lanes 4 and 5*). These data suggest that the inhibitory effect of E3330 on the induction of NF-κB is due to repression of IκB-α degradation.

Recently, it was reported that oxygen radicals are involved in the activation of NF-κB (13, 14, 36, 37). Therefore, we examined the effect of E3330 on superoxide anion production by human monocytes. The amounts of superoxide anion production by plastic plate-attached monocytes stimulated with LPS were comparable with those after PMA or fMLP stimulation. E3330 suppressed the production of superoxide anion in monocytes stimulated with not only LPS but also PMA and fMLP, although this compound showed no scavenging activity (Table 2).

The mechanism by which the inhibitory effect of E3330 on oxygen radical production would lead to suppression of IκB-α degradation is unclear. One possibility is that oxygen radicals might regulate the phosphorylation level of IκB-α. This is supported by a recent study demonstrating that the phosphorylation of IκB-α is blocked by the antioxidant pyrrolidine dithiocarbamate (32). The degradation of IκB-α is preceded by phosphorylation on Ser^{32} and Ser^{36}

(33–35). If phosphorylation of IκB-α is essential for IκB-α degradation, the inhibitory effect of E3330 on the decay of native IκB-α protein might reflect modulation by E3330 of the phosphorylation or dephosphorylation of IκB-α, although the compound did not distinctly suppress the formation of phosphorylated IκB-α (Fig. 8, *lanes 4 and 5*). It has been reported that protein kinase C and some tyrosine kinases can phosphorylate IκB-α in cell-free systems (13, 14), but E3330 did not inhibit either protein kinase C or the tyrosine phosphorylation of $p56^{lyn}$, which is associated with CD14 in LPS-stimulated human monocytes (data not shown). These data suggest that E3330 might affect phosphorylation at some other step of signal transduction that is regulated by oxygen radicals after LPS stimulation. A second possibility is that IκB-α protein could be damaged directly by oxygen radicals. Oxidative damage is involved in the irreversible inactivation of α1-proteinase inhibitor by radicals and in the reversible regulation of glyceraldehyde-3-phosphate dehydrogenase by oxidant stress (36).

In summary, we clarified that one of the inhibitory mechanisms of E3330 on TNF-α generation is the inhibition of NF-κB activation through suppression of IκB-α degradation. The inhibitory effect of E3330 on IκB-α protein degradation might be a consequence of a decrease of superoxide anion production.

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