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Suppression by Azelastine Hydrochloride of NF-κB Activation Involved in Generation of Cytokines and Nitric Oxide

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ABSTRACT—The influence of the anti-allergy agent azelastine hydrochloride (Azeptin®) on NF-κB activation associated with the generation of cytokines and nitric oxide (NO) was investigated in various kinds of human and mouse cells. Azeptin dose-dependently suppressed both DNA and protein synthesis in human gingival fibroblasts (HF) and also suppressed blastogenesis of human peripheral blood lymphocytes (PBL). Generation of tumor necrosis factor-alpha, interleukin 1-beta, granulocyte-macrophage colony-stimulating factor and interleukin-6 from 10^{-5} M Azeptin-treated PBL and human monocytes (HM) was decreased to approximately 1/3 to 2/3 of the control levels. In parallel with the decreased cytokine generation, each cytokine mRNA was less expressed in the presence of 10^{-5} M Azeptin. In addition, both inducible nitric oxide synthase-mRNA level and NO generation in mouse peritoneal macrophages were suppressed by 10^{-5} M Azeptin. Being compatible with those results, Azeptin (10^{-5} M) suppressed activation of NF-κB in PBL, HM and HF. These results appear to indicate that suppression of cytokine and NO generation by Azeptin results at least partially from the inhibition of NF-κB activation.

Keywords: Azelastine hydrochloride, Cytokine, Nitric oxide synthase, NF-κB

Azelastine hydrochloride (Azeptin®, Eisai Co., Tokyo), an anti-allergy agent, has been used for the treatment of many disorders, especially allergic rhinitis, bronchial asthma and Behçet's disease (1－3). Azeptin suppresses leukocyte function and stabilizes cell membranes (4), and the anti-allergic therapeutic effect of Azeptin seems to depend on these biological actions. Recently, suppressive actions of Azeptin in reactive oxygen intermediates (ROI) generation and cytokine release from many kinds of cells have been clarified (5, 6). ROI and cytokines are mediators of inflammation and regulaters of cell growth and differentiation. Therefore, Azeptin may be used as a therapeutic agent for many disorders in a variety of fields.

Nitric oxide (NO), which is produced from macrophages, endothelial cells and nerve cells, is known to be a mediator of inflammation and immunological responses (7, 8). The reaction between NO and O_2^- results in the formation of peroxynitrite anion $ONOO^-$, which has powerful cytotoxic properties and induces tissue damage (9). In addition, NO upregulates to release ROI and inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-1α (IL-1α) from macrophages (10). These coactions appear to play critical roles in both protection and impairment of the body.

When cells are stimulated by cytokines, receptor type or non-receptor type tyrosine kinases are activated and the signaling downstreams finally into the nucleus, resulting in activation of transcription factors that leads to protein synthesis, cell proliferation and differentiation. NF-κB is one of the mammalian transcriptional activators involved in the transmission of various signals from the cytoplasm to the nucleus. Recent findings indicate that ROI and cytokines can activate NF-κB, which induces cytokine-mRNA and inducible isoform of nitric oxide synthase (iNOS) (11－14).

The mechanism by which Azeptin suppresses cytokine and ROI generation has not yet been clarified. The mechanism of Azeptin's action in leukocytes, fibroblasts and many other cells should be clarified because we observed suppression of peplomycin (PLM)-induced pulmonary fibrosis by Azeptin in mice. It has been demonstrated that PLM, a bleomycin (BLM) derivative, upregulates signal transduction and increases cytokine and ROI generation from leukocytes (15). PLM also enhances proliferation and collagen synthesis of fibroblasts. The action of Azeptin and PLM is contradictive (6). Considering these facts, we examined the influence of Azeptin

on a nuclear transcription factor, NF-κB in human peripheral blood lymphocytes (PBL), human monocytes (HM), human gingival fibroblasts (HF) and mouse peritoneal macrophages (MPM) and attempted to clarify the mechanism of the suppression of cytokine and NO generation.

MATERIALS AND METHODS

Cell preparation

Peripheral blood mononuclear cells were isolated from heparinized venous blood by Ficoll-Paque (Pharmacia Fine Chemical, Piscataway, NJ, USA) density separation. PBL were separated from HM by culturing in plastic dishes for 1 hr and passing through nylon-wool columns. HM were removed by gentle scraping with a cell scraper after the addition of cold phosphate-buffered saline (PBS). HM contamination in PBL and PBL contamination in HM were ascertained to be less than 0.5% and 2%, respectively, by non-specific esterase staining. Isolated PBL and HM were suspended in RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo) supplemented with 10% fetal bovine serum (FBS).

HF were separated from healthy human gingivae. The resected tissues were minced to pieces measuring approximately 2 mm, and these were cultured in Dulbecco's modified Eagle's medium (DMEM, Nissui) containing 10% FBS. After two to three passages, the proliferated fibroblasts were subjected to the following experiments. MPM were collected by washing the peritoneal cavity of the ICR male mouse with 5 ml PBS and suspended in RPMI 1640 medium containing 10% FBS.

DNA and protein syntheses in fibroblasts, and blastogenesis in PBL

HF (1×10^4 cells) were cultured with or without Azeptin (10^{-7} to 10^{-4} M) in 96-well, flat-bottomed microplates for 72 hr. Five μCi/ml [^{3}H]thymidine (New England Nuclear, Boston, MA) or [^{3}H]proline (Amersham International Plc, Amersham, England) was added during the last 24 hr of culture. The cells were then removed with 0.1% trypsin and harvested on filter papers. The radioactivity of [^{3}H]thymidine or [^{3}H]proline incorporated in the cells was measured with a beta counter.

PBL (1×10^5 cells) were incubated with or without Azeptin (10^{-7} to 10^{-4} M) in the presence of interleukin-2 (IL-2), (Shionogi Pharmaceutical Co., Osaka) or phytohemagglutinin-P (PHA) (Difco, Detroit, MI, USA) in 96-well, round-bottomed microplates. Blastogenesis was quantified by adding [^{3}H]thymidine during the final 6 hr of the 72-hr incubation and measuring the amount incorporated.

Titration of cytokines

PBL, HM and HF were cultured with or without 10^{-5} and 10^{-6} M of Azeptin for 48 hr. Cytokines in the culture supernatants were measured with enzyme-amplified sensitivity immunoassay kits for IL-1β, TNF-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Medgenix Diagnostics, Brussels, Belgium) and an ELISA kit for IL-6 (Toray-Fuji Bionics, Tokyo).

NO determination

NO, quantified by the accumulation of nitrite as a stable end product, was determined by a microplate assay (16). Briefly, 100 μl samples (culture supernatants) were removed from the supernatants and incubated with an equal volume of Griess reagent (1% sulfanilamide, 0.1% *N*-1-naphthylethylenediamine dihydrochloride, 2.5% H_3PO_4) at room temperature for 10 min. The absorbance at 540 nm was determined with a microplate reader. The nitrite concentration was calculated from a sodium nitrite standard curve.

Western blot analysis

After cultivation of MPM with or without Azeptin in the presence of lipopolysaccharide (LPS) (Sigma Chemical Co., St. Louis, MO, USA), the cells were harvested and lysed with sodium dodecyl sulfate (SDS) sample buffer (125 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.5 mM sodium orthovanadate). Immediately after cell lysis, the SDS buffer solution was boiled for 5 min. The samples were then separated by SDS-PAGE under reducing conditions and electrophoretically transferred to an Immobilon-P filter (Nippon Millipore Ltd., Tokyo). After incubation for 60 min in 3% powdered-skim milk at room temperature, the filter was incubated with anti-macNOS mAb (Transduction Laboratories, Lexington, KY, USA). The antibody was detected with peroxidase-conjugated rabbit anti-mouse IgG (Cappel, Inc., West Chester, PA, USA). Peroxidase-positive bands were detected by an enhanced chemiluminescence Western blotting detection system (Amersham).

Nuclear extracts and electrophoretic mobility shift assay (EMSA) for NF-κB

Cells (1×10^6) were washed in PBS and then lysed in buffer solution containing 10 mM Hepes (pH 7.8), 10 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml antipain (Sigma), 5 μg/ml leupeptin (Sigma) and 0.5% Nonidet P-40 for 15 min. By centrifugation at $12{,}000 \times g$ for 5 min, nuclei were pelleted and washed again in lysis buffer. The nuclear proteins were then extracted in buffer containing 50 mM Hepes (pH 7.8), 50

mM KCl, 300 mM NaCl, 0.1 mM EDTA,1 mM DTT, 0.1 mM PMSF, and 10% glycerol for 20 min and centrifuged at 12,000×*g* for 5 min. The supernatant containing the nuclear proteins was collected, and protein concentration was determined according to the method of Bradford (17). EMSA was performed as described elsewhere (18). Briefly, the nuclear extract protein (2 μg), 1 μg poly (dI-dC) and ^{32}P-labeled NF-κB probe (Stratagene, La Jolla, CA, USA) were mixed in binding buffer [10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM DTT and 5% glycerol], and the binding reaction mixtures (20 μl) were electrophoresed on native 4% polyacrylamide gels; the protein-bound isotope was developed by autoradiography. NF-κB probes were labeled with [γ-^{32}P] (Amersham) by using T4 polynucleotide kinase and purified on a G-50 spin column.

RNA extraction and reverse transcription

Total cellular RNA was extracted from PBL, HM and HF in 4 M guanidinium thiocyanate and subsequent centrifugation through a 5.7 M CsCl cushion, according to standard protocols. The RNA was quantitated by measuring the optical density at 260 nm. The extracted RNA (1 μg) was added to 20μl of reverse transcription buffer (50 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA, 10 μg/ml bovine serum albumin, and 1 mM DTT) containing 10 mM deoxyribonucleoside triphosphate (dNTP), 50 U RNase inhibitor, 1 μg random hexadeoxynucleotide primer, and 50 U avian myeloblastosis virus reverse transcriptase (all from Boehringer Mannheim, Germany). This mixture was incubated at 42℃ for 40 min and then heated at 95℃ for 5 min. The polymerase chain reaction (PCR) was performed with 4 μl of cDNA preparation.

PCR

The prepared cDNA was added to the reaction mixture containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 0.2 mM dNTP, 0.4 μM of both 5′ and 3′ primers, and 2 U taq polymerase (Perkin-Elmer Co., Hayward, CA, USA) in a total volume of 50 μl. All reaction mixtures were overlaid with 50 μl mineral oil and incubated in a thermal cycler (Perkin-Elmer) for 45 sec at 94℃, 45 sec at 60℃, and 2 min at 72℃ per cycle. Aliquots (5 μl) of the reaction mixture were removed at suitable intervals during PCR. PCR fragments were visualized by agarose gel electrophoresis and ethidium bromide staining and were positively identified by size. Primers for human TNF-α, IL-1β, GM-CSF, IL-6 and mouse iNOS were purchased from Clontech Laboratories, Inc. (Palo Alto, CA, USA). β-Actin served as the internal control.

RESULTS

DNA and protein syntheses

Azeptin suppressed both DNA and protein syntheses in a dose-dependent manner (Table 1). Incorporated [^{3}H]thymidine levels in HF that had been pretreated with 10^{-6}, 10^{-5} and 10^{-4} M Azeptin were 3739±86, 2543±293 and 35±9 cpm/10^4 cells, respectively. These were less than the control value of 4359±275 cpm/10^4 cells. In addition, IL-2 and PHA-induced blastogenesis in PBL were significantly suppressed by the addition of 10^{-5} or 10^{-4} M Azeptin into the culture medium. When PBL were stimulated with 100 U/ml IL-2 for 72 hr, they incorporated labeled thymidine of 348±28×10^2 cpm /10^5 cells during the last 6 hr in the absence of Azeptin, while incorporated thymidine levels were 85±2 and 4±2×10^2 cpm/10^5 cells in the presence of 10^{-5} and 10^{-4} M Azeptin, respectively. Similar suppression of thymidine incorporation by Azeptin was induced in PHA-stimulated PBL.

Compared to DNA synthesis, proline incorporation

Table 1. Inhibition by Azeptin of DNA and protein syntheses in PBL and fibroblasts

Cells	DNA synthesis					Protein synthesis				
	Azeptin (M)					Azeptin (M)				
	0	10^{-7}	10^{-6}	10^{-5}	10^{-4}	0	10^{-7}	10^{-6}	10^{-5}	10^{-4}
HF	4359±275※	4257±182	3739±86	2543±293	35±9	3441±52※	3222±97	3158±88	3069±95	38±9
PBL										
IL-2-activated	348±28†	324±27	312±14	85±2	4±2	ND	ND	ND	ND	ND
PHA-activated	1776±386†	1805±391	1871±438	429±120	5±1	ND	ND	ND	ND	ND

※mean±1 S.D. (cpm/10^4 cells) of quadruplicate cultures. †mean±1 S.D. (×10^2 cpm/10^5 cells) of 3 healthy donors. ND: not determined. Fibroblasts (1×10^4 cells/well) were cultured in the presence or absence of the indicated concentrations of Azeptin for 72 hr. [^{3}H]Thymidine or [^{3}H]Proline was added during the last 24 hr, and isotope incorporated in the cells was measured. PBL (1×10^5 cells/well) were treated with 100 U/ml IL-2 or 1 μg/ml PHA in the presence or absence of the indicated concentrations of Azeptin for 72 hr. [^{3}H]Thymidine incorporated into the cells during the last 6 hr was determined.

was slightly suppressed by Azeptin. In HF, 10^{-5} M Azeptin only slightly decreased the incorporation of labeled proline. However, 10^{-4} M Azeptin almost completely inhibited proline incorporation in fibroblasts. These results suggest that Azeptin at concentrations of more than 10^{-4} M is cytotoxic to PBL and HF. Therefore, we performed the following experiments with 10^{-5} or 10^{-6} M Azeptin.

Cytokine generation

Generation of TNF-α, IL-1β, GM-CSF, and IL-6 from PBL, HM and HF were suppressed by 10^{-6} and 10^{-5} M Azeptin (Fig. 1). TNF-α and IL-1β from 10^{-5} M Azeptin-treated PBL and HM were decreased to about two thirds of the control level. Suppression of these cytokines by Azeptin was supported by each mRNA expression level (Fig. 2). In both PBL and HM, TNF-α-mRNA expression was decreased to less than a half of the control level by a 6 hr-treatment with 10^{-5} M Azeptin, and IL-1β-mRNA expression was almost completely inhibited by the same concentration. Azeptin also blocked the expression of GM-CSF- and IL-6-mRNA in both kinds of cells.

NO generation and NOS

MPM generated nitrite even in the absence of LPS (Fig. 3), and they generated approximately 8.3 μM of nitrite per 10^5 cells in the presence of 10 ng/ml LPS. When Azeptin and LPS were concomitantly added to the culture medium, nitrite generation was decreased to less than 1 $\mu M/10^5$ cells. Being compatible with this inhibition, Azeptin suppressed both iNOS, a 130-kDa protein, and its mRNA (Fig. 4: A and B).

Activation of NF-κB

DNA-bound NF-κB was increased by treatment of PBL, HM, and HF with 20 U/ml interferon-γ (IFN-γ), 5 μg/ml PLM and 100 U/ml IL-1β, respectively (Fig. 5). These NF-κB activations were inhibited by 10^{-5} M Azeptin. When these cells were pretreated with 10^{-6} M Azeptin for a long time ($\geqq$4 hr), a decrease of intranuclear NF-κB was also observed.

DISCUSSION

Cytokines as well as NO and ROI regulate cell differentiation, proliferation and activation. These agents are associated with many pathophysiologies, and an unbalanced network of these agents may cause impaired homeostasis of the body. In some diseases, production of cytokines, NO or ROI is upregulated and suppression of

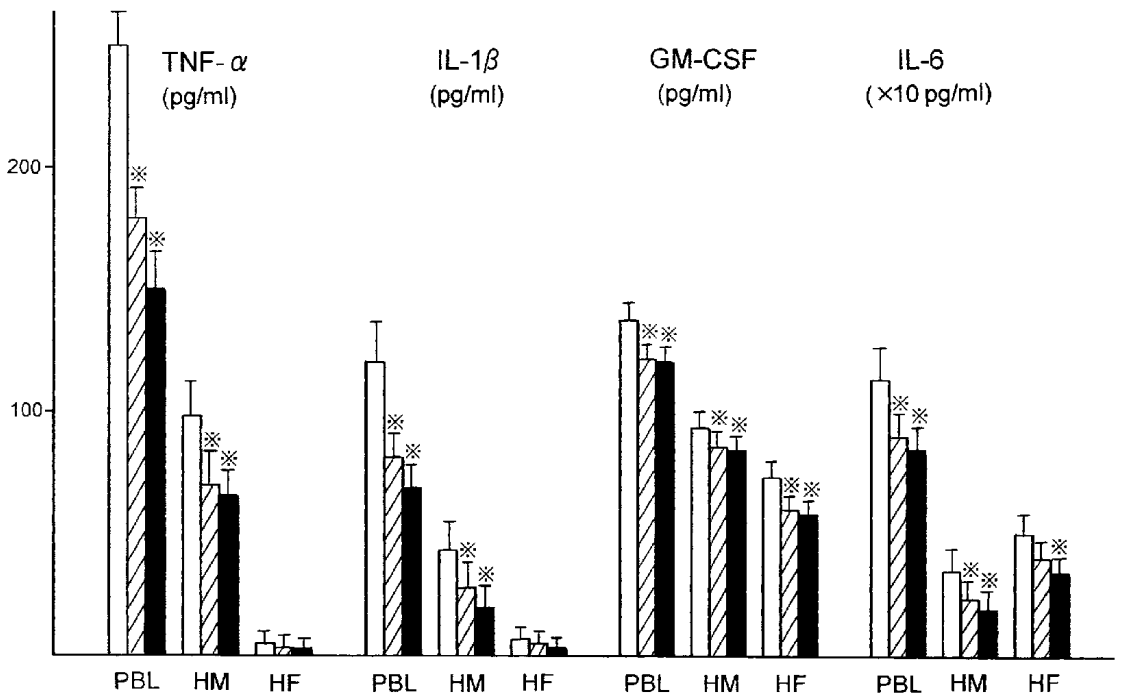


Fig. 1. Inhibitory effect of Azeptin on cytokine production from cultured human cells. PBL (10^6 cells/ml), HM (10^5 cells/ml) and HF (10^5 cells/ml) were incubated with each culture medium in the presence or absence of Azeptin (10^{-6} or 10^{-5} M) for 48 hr. Cytokines in the culture supernatants were measured as described in Materials and Methods. Each bar indicates the mean $\pm$ S.D. of triplicate determinations. $^{※}P<0.05$ vs control (without Azeptin), *U*-test. □: medium alone, ▨: Azeptin (10^{-6} M), ■: Azeptin (10^{-5} M).

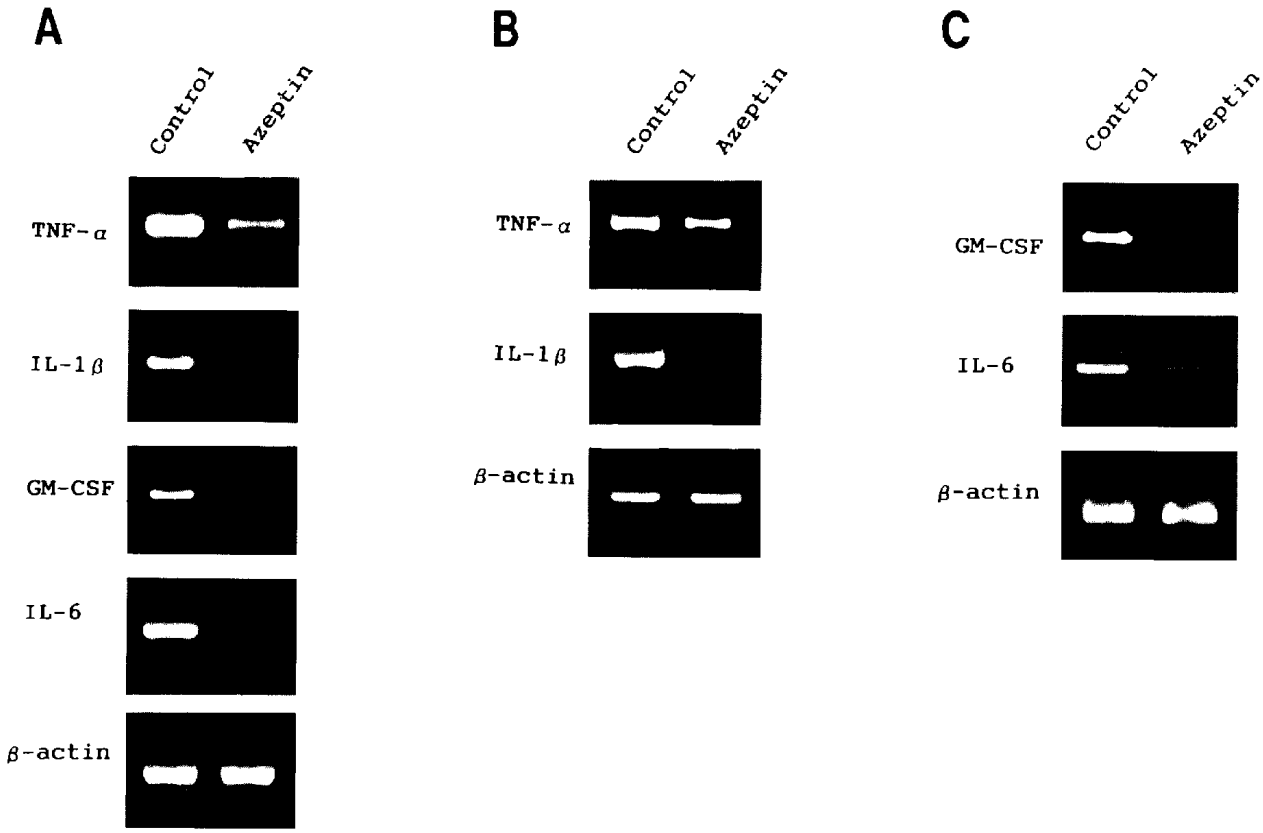


Fig. 2. Suppression by Azeptin of cytokine mRNA expression in PBL (A), HM (B) and HF (C). Cells were cultured in the presence or absence of 10^{-5} M Azeptin for 6 hr. Total cellular RNA was then extracted, reverse-transcribed and subjected to PCR. The results are representative of three separate experiments.

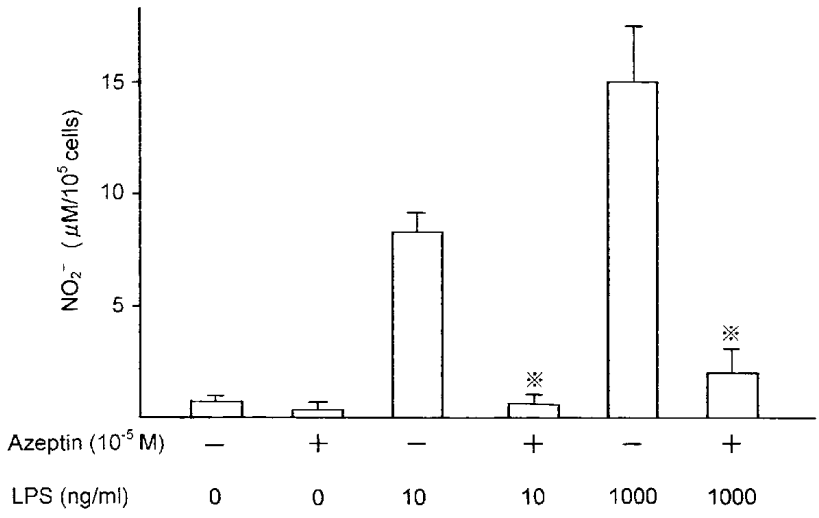


Fig. 3. Influence of Azeptin on NO production by MPM. MPM from 3 mice were cultured for 48 hr in the presence or absence of the indicated concentrations of LPS and Azeptin. NO in the culture supernatants was measured as described in Materials and Methods. Each bar indicates the mean±S.D. of triplicate determinations. $^{※}P<0.05$ vs control (without Azeptin), *U*-test.

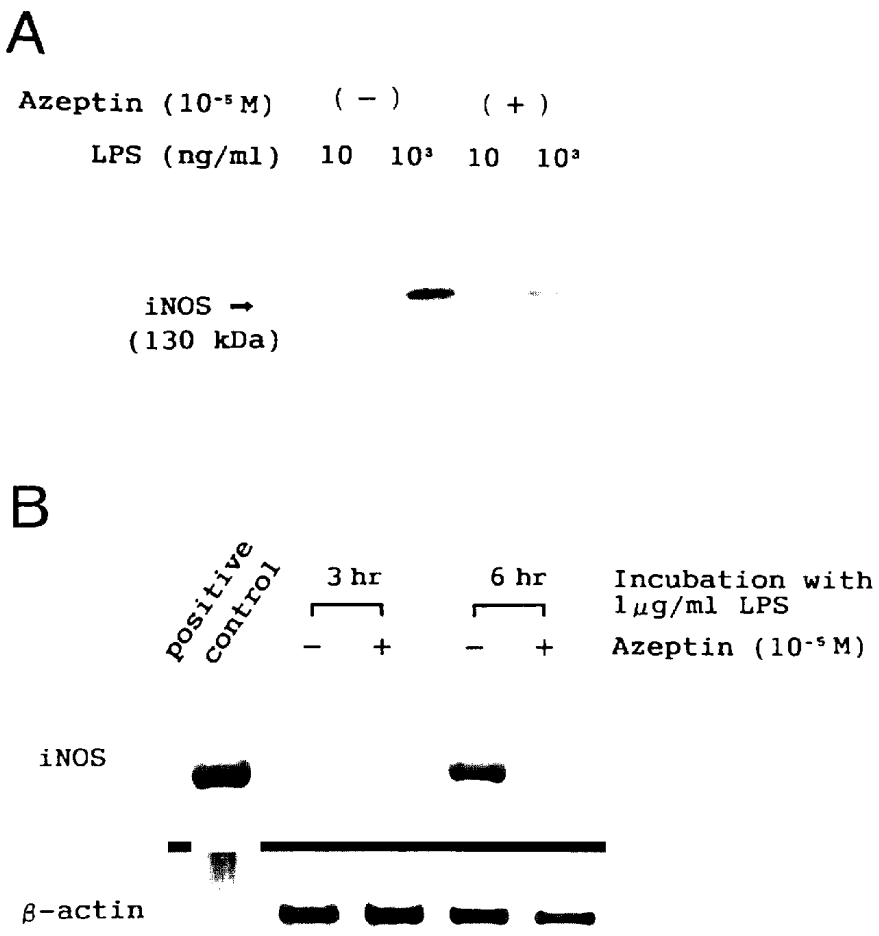


Fig. 4. Inhibition by Azeptin of iNOS (A) and its mRNA (B) in MPM. MPM were incubated for 24 hr in the presence or absence of the indicated doses of LPS and 10^{-5} M Azeptin (A). Lysates from the cells were electrophoresed and blotted as described in Materials and Methods. MPM were stimulated with LPS in the presence or absence of Azeptin for 3 and 6 hr (B). Total cellular RNA was then extracted, reverse-transcribed and subjected to PCR. The results are representative of three separate experiments.

the production of these mediators may induce improvement of the diseased condition (19–21). Allergy is one example of the pathophysiology of such diseases.

Azeptin has been used in allergic diseases with the expectation of its leukocyte function-suppressing activity. However, other than our studies, few biochemical or physiological analyses concerning Azeptin's action have been reported. In the present study, we examined a wide range of biophysiological activities induced by Azeptin using several kinds of human and mouse cells. As expected, Azeptin suppressed DNA and protein syntheses in HF and suppressed DNA synthesis of human PBL. In parallel with this suppression, generation of cytokines such as TNF-α, IL-1β, GM-CSF, and IL-6 by PBL, HM, and HF was suppressed in cells treated with 10^{-6} M Azeptin. Both TNF-α (22, 23) and IL-1β (24) are key elements in the cytokine network involved in bone resorption and collagen synthesis. With these cytokines, IL-6 plays a critical role in inflammation and IL-6 enhances cytokine generation (25, 26). In addition, GM-CSF activates the functions of polymorphonuclear leukocytes (PMN), PBL and epithelial cells (27, 28). Therefore, these suppressing activities of Azeptin appear to contribute to down-regulation of inflammation and cell proliferation.

ROI upregulate the bacteriocidal activity of PMN (29, 30) and signal transduction of lymphocytes and other cells (31, 32). ROI also impair DNA and cause cellular and tissue impairment for example, pulmonary impairment may be induced by ROI. We previously described that Azeptin suppressed chemiluminescence in rabbit peripheral PMN and RAM (5, 6). This suppression by Azeptin appears to indicate that Azeptin suppresses signal

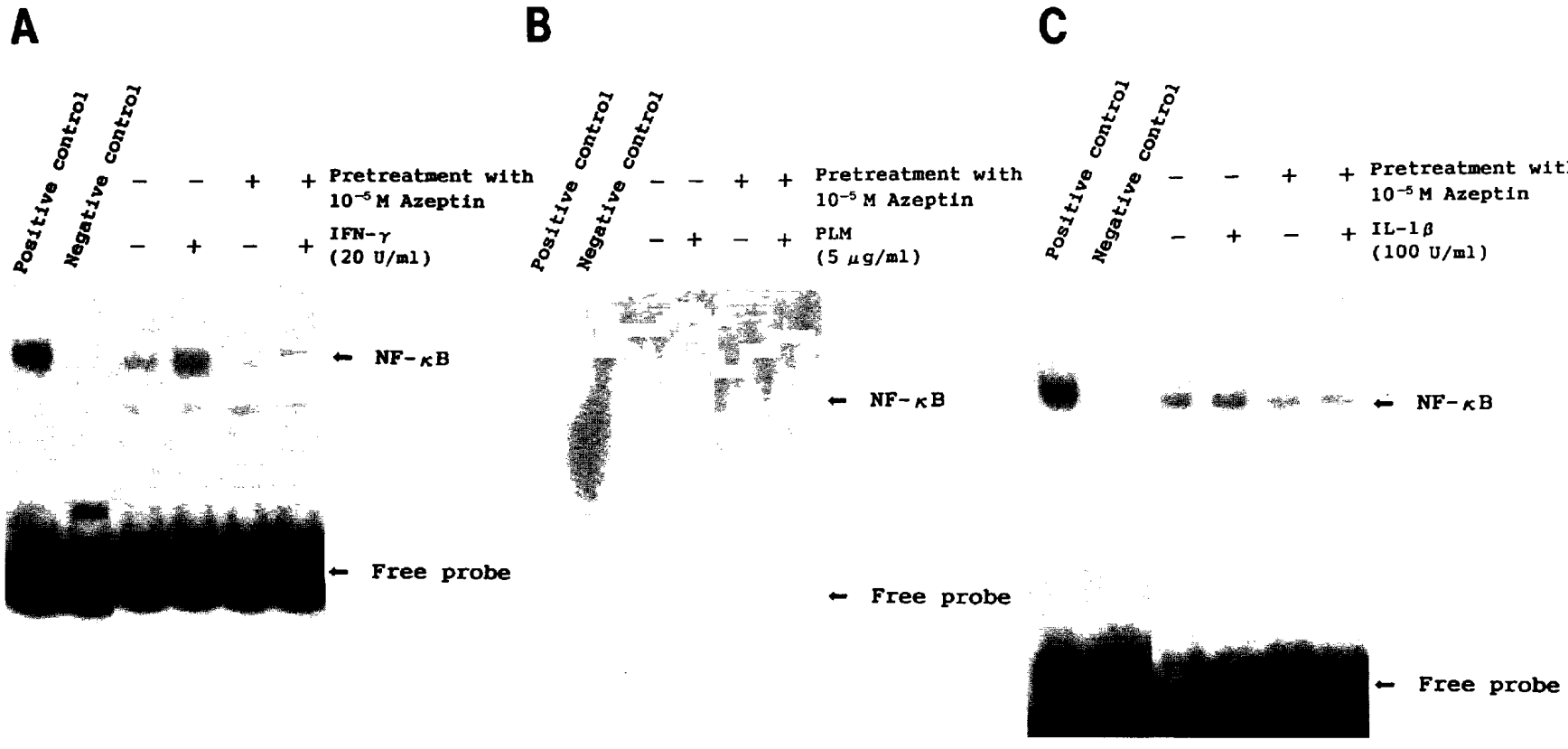


Fig. 5. Inhibitory effect of Azeptin on NF-κB activation by IFN-γ, PLM and IL-1β in PBL (A), HM (B) and HF (C), respectively. Cells (1×10^6/ml) were pretreated with or without 10^{-5} M Azeptin for 4 hr followed by incubation for 1 hr in the presence or absence of the indicated reagents. Nuclear extracts were then obtained, and EMSAs were performed as described in Materials and Methods with the nuclear extract from HeLa cells for the positive contorl. Negative control tests were performed in the absence of any nuclear extracts. The results are representative of three separate experiments.

transduction and protects the tissues from impairment induced by ROI. Azeptin also inhibited NO generation and iNOS message expression. Together with the suppression of DNA and protein syntheses, Azeptin appears to suppress various aspects of cell metabolism.

Signal transduction is necessary for generation of ROI and cytokines. As one of the signal pathways in ROI generation, activation of phospholipase Cγ followed by phosphorylation of phosphatidyl inositol and increase of intracellular ionized free calcium ($[Ca^{2+}]_i$) and protein kinase C (PKC) is needed for the final activation of nicotinamide diphosphate hydrase (33–35). By Azeptin, the levels of the second messengers (inositol 1,4,5-trisphosphate and diacyl glycerol), $[Ca^{2+}]_i$ and PKC activity in formyl-methionyl-leucyl-phenylalanine-stimulated PMN were lowered (5). This down-regulation of signal transduction was compatible with decreased protein tyrosine phosphorylation (data not shown). We and others have reported that protein tyrosine phosphorylation was necessary for ROI generation from TNF-α stimulated PMN (6, 36). Therefore, suppression of ROI generation by Azeptin appears to depend on the down-regulated signal transduction.

NF-κB is one of the transcription factors associated with a variety of genes related to cytokines, NOS, cell surface adhesion molecules and receptors. Among cytokine genes, those of IL-2, IL-1β, IL-6, TNF-α, IFN-γ and GM-CSF are the targets of NF-κB (37–40). Therefore, the suppression of NF-κB activation by Azeptin appears to be compatible with suppressed generation of these cytokines. However, the basal level of NF-κB seems too low to regulate each cytokine expression. Recently, regulation of cytokine expression by other transcription factors, nuclear factor of activated T cells, AP-1, and Oct-1,2 were reported (41). Together, our results and the observations from these reports indicate that these transcription factors might take part in the regulation of cytokine expression.

Our results indicate that Azeptin suppresses generation of ROI, NO, and cytokines by suppression of signal transduction including intranuclear transcription factors. When these activities are considered, Azeptin may be utilized as an immunosuppressant. This drug may be useful in pathophysiologies associated with cytokines and ROI, for instance, pulmonary fibrosis.

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