Antiinflammatory Effects of Glucocorticoids in Brain Cells, Independent of NF-κB[1]

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Glucocorticoids are potent antiinflammatory drugs. They inhibit the expression of proinflammatory cytokines and adhesion molecules. It has recently been proposed that the underlying basis to such inhibition is the induction of the protein IκB, which inhibits the transcription factor NF-κB. The latter is a key activator of the genes encoding cytokines and adhesion molecules. The present study shows that the synthetic glucocorticoid, dexamethasone, inhibits the induction of the proinflammatory cytokine IL-8 and the adhesion molecules VCAM-1 and ICAM-1 in human 1321N1 astrocytoma and SK.N.SH neuroblastoma cells. However, dexamethasone failed to induce IκB or inhibit activation of NF-κB by IL-1 in the two cell types. EMSA confirmed the identity of the activated NF-κB by demonstrating that an oligonucleotide, containing the wild-type NF-κB-binding motif, inhibited formation of the NF-κB-DNA complexes whereas a mutated form of the NF-κB-binding motif was ineffective. In addition, supershift analysis showed that the protein subunits p50 and p65 were prevalent components in the activated NF-κB complexes. The lack of effect of dexamethasone on the capacity of IL-1 to activate NF-κB correlated with its inability to induce IκB and the ability of IL-1 to cause degradation of IκB, even in the presence of dexamethasone. The results presented in this paper strongly suggest that glucocorticoids may exert antiinflammatory effects in cells of neural origin by a mechanism(s) independent of NF-κB. *The Journal of Immunology,* 1999, 163: 2113–2119.

G lucocorticoids are widely used immunosuppressive and antiinflammatory agents (1). They exert such effects by inhibiting the expression of genes encoding various adhesion molecules and cytokines (2–6). Much work has recently probed the molecular mechanism by which glucocorticoids may modulate the expression of these genes and has concentrated on the NF-κB-Rel transcription factor family as a prime target (7). The most common form of active NF-κB is a heterodimer consisting of two DNA binding subunits of 50 kDa (p50) and 65 kDa (p65) that together recognize and contact a specific decameric DNA sequence (8). Most cells contain latent NF-κB in the cytoplasm, where it is rendered inactive via its association with an inhibitory protein IκB (9). The latter not only prevents nuclear uptake of NF-κB by masking nuclear location signals (10) but also inhibits the binding of NF-κB to its DNA recognition sequence (9). Several different types of IκB[3] have been described including IκBα, IκBβ, and IκBε (11, 12). Activation of cells by proinflammatory cytokines such as IL-1 effects the dissociation of IκB from NF-κB via serine phosphorylation and subsequent proteasomal proteolysis of the IκB inhibitory subunit, thus releasing an active NF-κB heterodimer that is translocated into the nucleus (13, 14). This facilitates induction of NF-κB-regulated genes such as those encoding adhesion molecules and cytokines (15). Recent reports have suggested that glucocorticoids exert their immunosuppressive effects by inhibiting the action of NF-κB (16, 17). The studies

report that glucocorticoids induce the transcription of the IκBα gene, causing increased IκBα protein synthesis, which serves to sequester active NF-κB in inactive cytoplasmic complexes. The reduction in levels of active NF-κB would presumably culminate in decreased expression of adhesion molecules and cytokines.

One of the authors has previously reported that IL-1 can cause proinflammatory effects in glial cells such as the induction of the NF-κB-regulated adhesion molecules VCAM-1 and ICAM-1 (18). We predicted that glucocorticoids would act to modulate the induction of these molecules by virtue of their reported inhibitory effects on NF-κB. Indeed, the present study demonstrates that the synthetic glucocorticoid, dexamethasone, inhibits the induction of these adhesion molecules and IL-8, another protein whose expression is regulated by NF-κB. However, we also found that glucocorticoids can reduce the expression of these proteins in circumstances where they fail to modulate the levels of IκB or active NF-κB.

Materials and Methods

Materials

The human 1321N1 astrocytoma and SK.N.SH neuroblastoma were obtained from European Collection of Animal Cell Cultures (Salisbury, U.K.). RPMI, penicillin, streptomycin, and FCS were obtained from Life Technologies (Grand Island, NY). IL-1β was obtained from the National Cancer Institute (Frederick, MD). Dexamethasone was obtained from Sigma (Poole, U.K.). The matched Ab pairs against IL-8 were obtained from R&D Systems (Abingdon, U.K.). The 21-bp oligonucleotides containing the wild-type NF-κB binding motif from the human IL-8 promoter (underlined) (5′-ATCG<u>TGGAATTTCC</u>TCTGACA-3′) or the mutated NF-κB motif (mutated bases in bold) (5′-ATCG**TTAACTTTCC**TCTGACA-3′) were obtained from MWG Biotech (Milton Keynes, U.K.). T4 polynucleotide kinase was obtained from Promega (Madison, WI). [γ-³²P]ATP (10 mCi/mmol) and poly(dI-dC) were obtained from Amersham (Bucks, U.K.). The anti-p50, p65, and c-*rel* antisera were gifts from Dr. Luke O'Neill (Trinity College, Dublin, Ireland). Anti-IκBα (Mad 3) (C-15) Abs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). HRP-conjugated sheep anti-rabbit IgG and the Boehringer Mannheim (Mannheim, Germany) chemiluminescence Western blotting kit were obtained from Boehringer Mannheim. The luciferase assay system with reporter lysis buffer was obtained from Promega.

Cell culture

1321N1 and SK.N.SH cells were cultured in DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% (v/v) FCS. The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 and passaged using 0.25% (w/v) trypsin in DMEM. IL-1β stimulation was performed on cells in serum-containing medium at 37°C for all experiments.

Assay of cell adhesion molecules and IL-8

1321N1 and SK.N.SH cells (5×10^4 cells/0.25 ml) were plated into 96-well microtiter plates and allowed to adhere for 72 h. Cells were then stimulated with various concentrations of IL-1β for 24 h. In some experiments, cells were pretreated with various concentrations of dexamethasone for 15 h before IL-1β (10 ng/ml) treatment for an additional 24 h. Stimulation was terminated by removal of medium, which was subsequently assayed for IL-8 using matched Ab pairs (R&D Systems) in a sandwich ELISA system according to the supplier's instruction. The adherent cells were measured for expression of VCAM-1 and ICAM-1 as described previously (18).

Preparation of subcellular fractions

1321N1 and SK.N.SH (2×10^5 cells/ml; 5 ml) were plated into 25-cm^2 flasks and allowed to adhere for 72 h. Cells were pretreated for 15 h in the absence or presence of various concentrations of dexamethasone and then stimulated with and without IL-1β (10 ng/ml) for 30 min. Stimulation was terminated by removal of medium and cells were washed with 5 ml ice-cold PBS. Cells were then scraped into 1 ml ice-cold hypotonic buffer (10 mM HEPES-NaOH buffer, pH 7.9, containing 1.5 mM MgCl$_2$, 10 mM KCl, 0.5 mM DTT, and 0.5 mM PMSF). Cells were pelleted by centrifugation at $21,000 \times g$ for 10 min and then lysed for 10 min on ice in hypotonic buffer (20 μl) containing 0.1% (v/v) Nonidet P-40. Lysates were centrifuged at $21,000 \times g$ for 10 min. The resulting supernatants constituted cytosolic fractions and were measured for levels of IκB by Western immunoblotting as described below. The pellets were resuspended in 20 mM HEPES-NaOH buffer, pH 7.9 (15 μl), containing 420 mM NaCl, 1.5 mM MgCl$_2$, 0.2 mM EDTA, 25% (w/v) glycerol, and 0.5 mM PMSF and incubated for 15 min on ice. Incubations were then centrifuged at $21,000 \times g$ for 10 min and the supernatants removed into 10 mM HEPES/NaOH buffer, pH 7.9 (75 μl), containing 50 mM KCl, 0.2 mM EDTA, 20% (w/v) glycerol, 0.5 mM PMSF, and 0.5 mM DTT. Such samples constituted nuclear extracts and were assayed for NF-κB by EMSA as described previously (18).

EMSA

Nuclear extracts (4–10 μg protein) were incubated with 20,000 cpm of a 21-bp oligonucleotide, containing the wild-type NF-κB binding motif from the human IL-8 promoter, which had been previously labeled with [γ-^{32}P]ATP (10 mCi/mmol) by T4 polynucleotide kinase (19). Incubations were performed for 30 min at room temperature in the presence of 2 μg poly(dI-dC) and 10 mM Tris-HCl buffer, pH 7.5, containing 100 mM NaCl, 1 mM EDTA, 5 mM DTT, 4% (w/v) glycerol, and 0.1 mg/ml nuclease-free BSA. In some experiments, unlabeled oligonucleotides, containing the wild-type or mutated NF-κB motif from the IL-8 promoter (20 pmol each), or polyclonal antisera (1 μl) against the NF-κB subunits, p50, p65, and c-rel, were added to the extracts 30 min before incubating with labeled oligonucleotide. All incubations were subjected to electrophoresis on native 4% (w/v) polyacrylamide gels, which were subsequently dried and autoradiographed.

Western immunoblotting

Cytosolic fractions (20 μg protein) were subjected to SDS-PAGE on 12% polyacrylamide slab gels. The separated proteins were electrophoretically transferred (80 mA, 1 h) from the gels to nitrocellulose in 0.192 M glycine/25 mM Tris, pH 8.3, containing 1.3 mM SDS and 15% (v/v) methanol, using a Hoefer TE70 Semiphor semidry transfer unit. The nitrocellulose was blocked overnight at room temperature in 20 mM Tris buffer, pH 7.5, containing 0.5 M NaCl and 5% (w/v) powdered milk (Marvel) (Premier Beverages, Stafford, U.K.), and was washed three times (10 min each) with 20 mM Tris buffer, pH 7.5, containing 0.5 M NaCl and 0.05% (v/v) Tween 20. The washed nitrocellulose was incubated with a 1 μg/ml of anti-IκBα (Mad 3) (C-15) Abs in 20 mM Tris buffer, pH 7.5 containing 0.5 M NaCl, 0.05% (v/v) Tween 20 and 1% (v/v) Marvel for 2 h at room temperature. The nitrocellulose was then washed six times (5 min each) as above before incubation for 1 h at room temperature with a 1:1000 dilution of HRP-conjugated sheep anti-rabbit IgG in 20 mM Tris buffer, pH 7.5, containing 0.5 M NaCl, 0.05% (v/v) Tween 20, and 1% (v/v) Marvel. After a further

six washes (5 min each) as above, immunoreactive bands were visualized using the Boehringer Mannheim chemiluminescence Western blotting kit (Boehringer Mannheim) as recommended by the manufacturers.

Electroporation and assay of luciferase

Trypsinized 1321N1 astrocytoma (1×10^7 cells) were resuspended in PBS (250 μl) containing a NF-κB reporter plasmid (5 μg of DNA). The latter consisted of five copies of the NF-κB consensus site cloned into the luciferase reporter construct pGL3-Basic (Promega). The suspended cells were electroporated (250 V, 25 mA, maximum capacitance) by means of an Electroporator II (Invitrogen, San Diego, CA). Aliquots (3 ml) of electroporated cells were seeded into 6-well plates (4×10^5 cells/well) and incubated for 24 h in DMEM supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10% (v/v) of FCS in a humidified atmosphere of 5% CO_2 at 37°C. Cells were subsequently treated in the absence or presence of various concentrations of dexamethasone for 15 h before being stimulated in the absence or presence of IL-1β (10 ng/ml) for a further 24 h. Cells extracts were generated and measured for luciferase activity using the luciferase assay system with reporter lysis buffer from Promega.

Results

Dexamethasone inhibits the IL-1β-induction of VCAM-1, ICAM-1, and IL-8 in 1321N1 astrocytoma

Human 1321N1 astrocytoma were stimulated with various concentrations of IL-1β for a 24-h period and measured for VCAM-1, ICAM-1, and IL-8 by use of ELISA-based detection systems. As shown previously by one of the authors (18), neither VCAM-1 or ICAM-1 were expressed at detectable levels in untreated cells, but IL-1 caused a dose-dependent induction of both adhesion molecules (Fig. 1, *A* and *B*). We also demonstrate in the present study that IL-1 induces expression of the chemokine IL-8 in these cells (Fig. 1*C*). Because the induction of these genes in glial cells may be crucial in mediating leukocytic infiltration of brain leading to neuropathological states, the search for pharmacological agents that could modulate the induction was considered valuable. The presence of NF-κB-binding sites in the promoter regions of the genes coupled with the previous reports describing the inhibitory effects of glucocorticoids on NF-κB promoted these agents as lead candidates for regulating the expression of adhesion molecules and chemokines in glial cells.

Human 1321N1 astrocytoma were thus incubated with various concentrations of the synthetic glucocorticoid, dexamethasone, for 15 h before stimulation with IL-1β (10 ng/ml) for a further 24 h. Cell-surface expression of VCAM-1 and ICAM-1 was then quantitated, and supernatants removed from these cells were analyzed for IL-8 content (Fig. 2). Dexamethasone caused concentration-dependent inhibition of IL-1-induced VCAM-1 and ICAM-1 expression, inhibiting VCAM-1 expression more strongly than ICAM-1. Similarly, the IL-1-induced expression of IL-8 was inhibited by dexamethasone in a concentration-dependent manner. The effects of dexamethasone on IL-1 activation of NF-κB was next examined to correlate the inhibitory effects on induction of adhesion molecules and IL-8 with an action on NF-κB.

Dexamethasone fails to influence the IL-1β activation of NF-κB in 1321N1 astrocytoma

1321N1 astrocytoma were incubated in the absence or presence of various concentrations of dexamethasone for 15 h before stimulation with IL-1β for a further 1-h period. Nuclear extracts were prepared and assayed for NF-κB activity by EMSA (Fig. 3*A*). Because the objective was to correlate effects on NF-κB with effects on NF-κB regulated genes, it was decided to use the specific NF-κB motif from the IL-8 promoter. The motif from the IL-8 promoter was chosen in preference to that of either VCAM-1 or ICAM-1 because the inhibitory effects on gene induction were
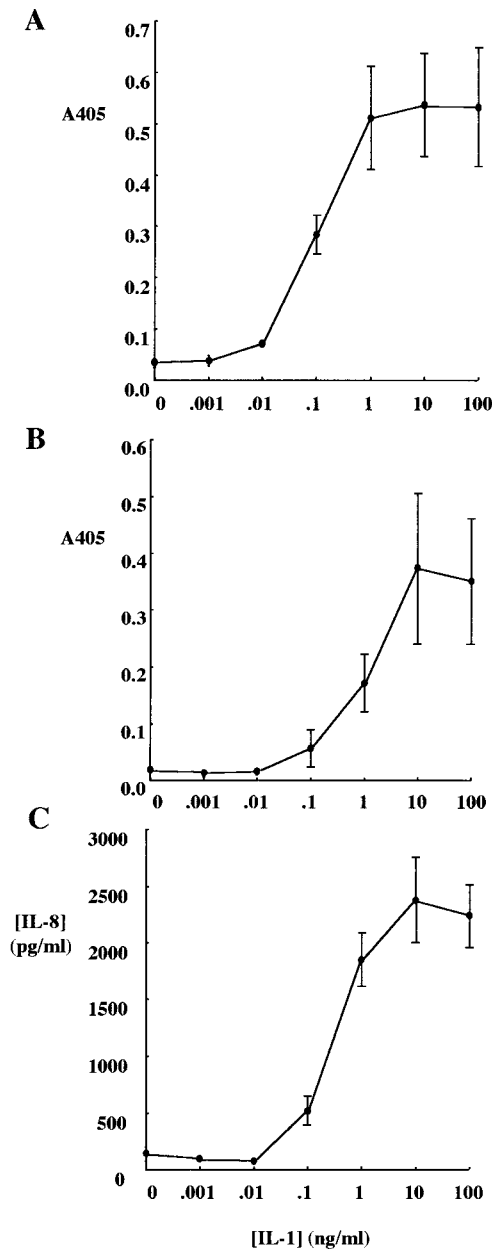
FIGURE 1. Induction of adhesion molecules and chemokines by IL-1β in 1321N1 astrocytoma. 1321N1 astrocytoma were treated for 24 h with various concentrations of IL-1β. The cells were then measured for (*A*) VCAM-1 and (*B*) ICAM-1 expression by direct ELISA, and supernatants were examined for levels of (*C*) IL-8 by sandwich ELISA as described in *Materials and Methods*. The levels of adhesion molecules are expressed in terms of absorbance at 405 nm. The data represent mean ± SEM of three independent experiments.
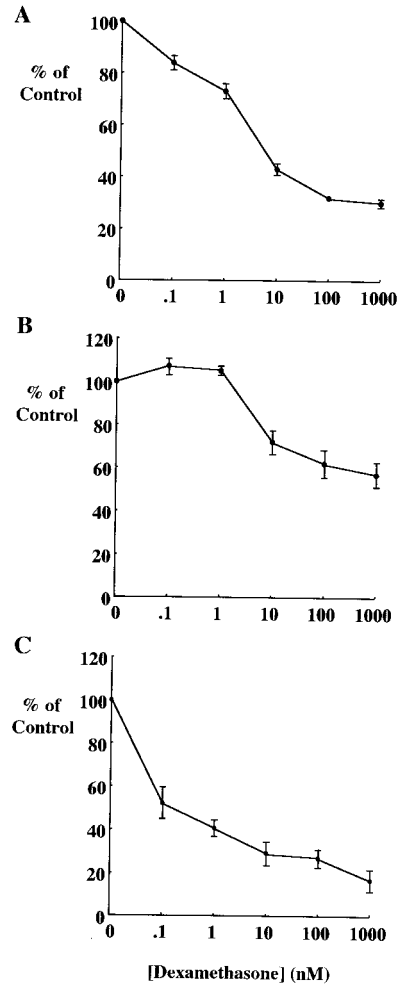

FIGURE 2. Inhibition of IL-1β-induction of adhesion molecules and IL-8 by dexamethasone in 1321N1 astrocytoma. 1321N1 astrocytoma were pretreated for 15 h with various concentrations of dexamethasone and stimulated for a further 24-h period with IL-1β (10 ng/ml). The cells were then measured for (*A*) VCAM-1 and (*B*) ICAM-1 expression by direct ELISA, and supernatants were examined for levels of (*C*) IL-8 by sandwich ELISA as described in *Materials and Methods*. The results are expressed as percentages of values obtained in IL-1β-treated cells in the absence of dexamethasone. The data represent mean ± SEM of three independent experiments.

greatest with respect to IL-8. NF-κB-DNA complexes were undetectable in extracts from unstimulated cells (Fig. 3*A*, *lane 1*). A prominent protein-DNA complex was apparent in these extracts but it represented a nonspecific protein-DNA interaction because the formation of the complex was inhibited by the addition of an unlabeled oligonucleotide containing either the wild-type or mutated form of the NF-κB binding motif (Fig. 3*B*). The addition of IL-1β to 1321N1 cells resulted in the formation of two additional DNA-protein complexes of lower electrophoretic mobility (Fig. 3*A*, *lane 7*). These complexes represented the specific recognition of the NF-κB-binding motif by proteins in the nuclear extracts

because their formation was inhibited by unlabeled oligonucleotide, containing the wild-type NF-κB-binding motif but not by one containing a mutated NF-κB site (Fig. 3*B*). The identity of the NF-κB subunits in the complexes induced by IL-1 was investigated by treating nuclear extracts from IL-1-stimulated cells with antisera against p50, p65, or c-*rel* and observing any effects on DNA-binding activity (Fig. 3*C*). Interestingly, the band of lowest mobility appears as a doublet in these experiments, rather than a singlet as in Fig. 3, *A* and *B*. This discrepancy may be due to the extended running time applied for the subunit analysis, which may facilitate superior resolution of complexes of similar sizes. The IL-1-induced complexes were abolished by pretreating the extracts with Abs against the p65 and p50 subunits of NF-κB but not by anti-c-*rel* or an isotype control anti-human IgG. Although the composition of each complex cannot be definitively stated from these studies, the analysis indicates that IL-1 activates forms of NF-κB in 1321N1 cells that bind to the NF-κB-binding motif from the IL-8 promoter as complexes containing p50 and p65.

A

B

C

FIGURE 3. Effect of dexamethasone on activation of NF-κB in 1321N1 astrocytoma. *A*, 1321N1 astrocytoma were pretreated for 15 h with various concentrations of dexamethasone and stimulated for a further 30 min in the absence (C) or presence (+IL-1) of IL-1β (10 ng/ml). Nuclear extracts were prepared and assessed for NF-κB activity as described in *Materials and Methods*. *B*, Cultures of 1321N1 cells were incubated in the absence (C) and presence (IL-1) of IL-1β (10 ng/ml) for 1 h, after which nuclear extracts were prepared and assessed for NF-κB activity in the absence or presence of unlabeled oligonucleotides (20 pmol) containing the wild-type (WT) or mutated (MUT) NF-κB motif from the IL-8 promoter. *C*, Extracts were also examined for NF-κB binding activity in the absence or presence of polyclonal antisera against the NF-κB subunits p50, p65, and c-*rel*. Nonimmune IgG was used a control. The arrows indicate the mobility of protein-DNA complexes and unbound oligonucleotide (Free Probe). The data are representative of three independent experiments.
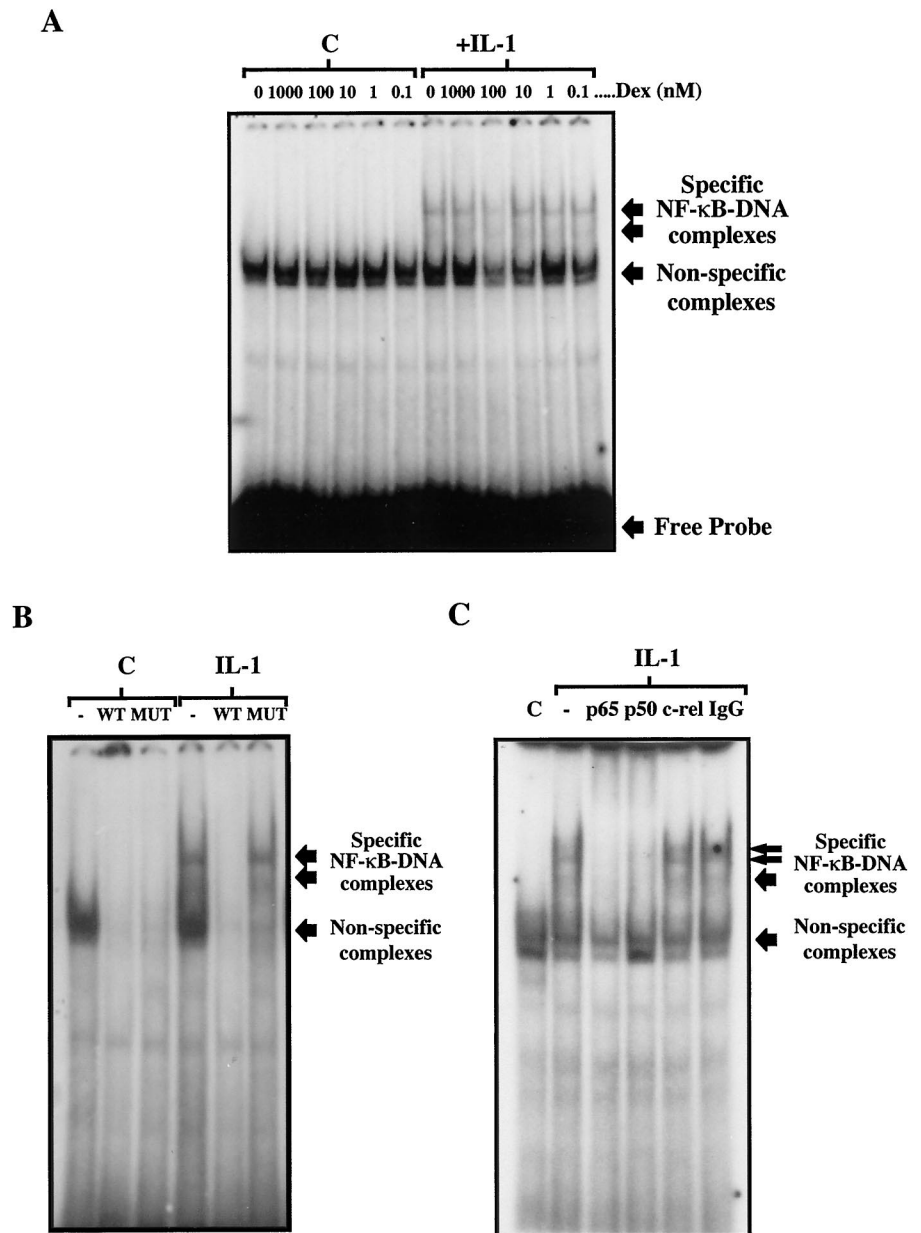
Dexamethasone was assessed for its ability to inhibit such IL-1 activation of these NF-κB complexes. Three independent experiments revealed that none of the wide-ranging concentrations of dexamethasone displayed any reproducible effect on basal levels of NF-κB or on the efficacy of IL-1 in activating NF-κB in 1321N1 cells (Fig. 3*A*). The inability of dexamethasone to block NF-κB activation prompted a characterization of its influence on levels of IκB in these cells. Indeed previous reports had suggested that activation of NF-κB is sensitive to dexamethasone by virtue of the latter's ability to induce IκBα (16, 17). Western immunoblotting analysis of cytosolic extracts from untreated and IL-1-stimulated cells demonstrated that IL-1 effects proteolysis of IκBα in 1321N1 astrocytoma (Fig. 4), which is coincident with increased levels of NF-κB in the nuclear extracts. However, dexamethasone failed to induce IκBα in cells treated in the absence or presence of IL-1. This lack of induction correlates with the failure of dexamethasone to inhibit IL-1 activation of NF-κB. Thus, dexamethasone fails to affect the nuclear translocation or DNA-binding activity of NF-κB in 1321N1 astrocytoma.

Dexamethasone fails to affect the IL-1 induction of a NF-κB-regulated reporter gene in 1321N1 astrocytoma

Previous studies had suggested that dexamethasone may exert inhibitory effects on NF-κB by modulating its transactivating ability (20–23). To determine whether this was a potential mechanism by which dexamethasone mediates its inhibitory effects on adhesion molecule and IL-8 expression in 1321N1 cells, the influence of dexamethasone on the ability of NF-κB to induce the expression of a reporter construct was next examined. Human 1321N1 astrocytoma were transfected with a NF-κB-luciferase reporter construct and incubated for 15 h with dexamethasone before stimulation in the absence or presence of IL-1β for a further 24 h. Cell lysates were prepared and assayed for luciferase activity (Fig. 5). Untreated cells demonstrated some basal expression of luciferase, which was induced 17-fold with the addition of IL-1β. Pretreatment of cells with various concentrations of dexamethasone failed to affect basal or IL-1-induced levels of luciferase. Therefore, it appears that dexamethasone has no effect on the transactivating
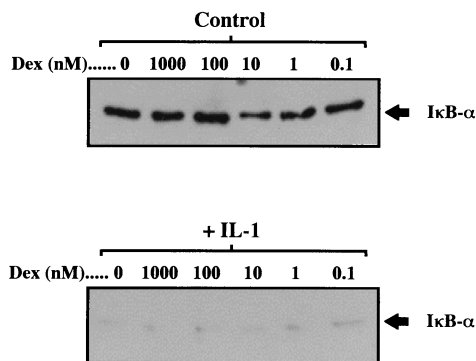
FIGURE 4. Effect of dexamethasone on levels of IκBα protein in 1321N1 astrocytoma. 1321N1 astrocytoma were pretreated for 15 h with various concentrations of dexamethasone and stimulated for a further 30 min in the absence (Control) or presence (+IL-1) of IL-1β (10 ng/ml). Cytosolic extracts were prepared and analyzed by Western immunoblotting for levels of IκBα using anti-IκBα (Mad 3) (C-15) from Santa Cruz Biotechnology. The data are representative of three independent experiments.

ability of NF-κB in glial cells. These results propose that glucocorticoids can produce well-defined antiinflammatory responses in 1321N1 astrocytoma by inhibiting the induction of adhesion molecules and IL-8 yet by a mechanism independent of NF-κB.

Dexamethasone fails to affect IL-1 activation of NF-κB but inhibits IL-1 induction of VCAM-1, ICAM-1, and IL-8 in SK.N.SH neuroblastoma

To ensure that the above effects are not simply artifacts of the 1321N1 astrocytoma cell line, the effects of dexamethasone on SK.N.SH neuroblastoma, another cell of neural origin, was next examined. The ability of dexamethasone to affect IL-1 activation of NF-κB in these cells was first examined. SK.N.SH cells were pretreated with various concentrations of dexamethasone for 15 h before stimulation for an additional 1 h with or without IL-1. Nuclear extracts from these cells were analyzed for NF-κB by EMSA (Fig. 6). SK.N.SH cells contained basal levels of a NF-κB-DNA complex, and the IL-1 caused an increase in the quantity of this complex. Dexamethasone failed to affect the basal or IL-1-stimulated levels of the complex, indicating that it has no influence on
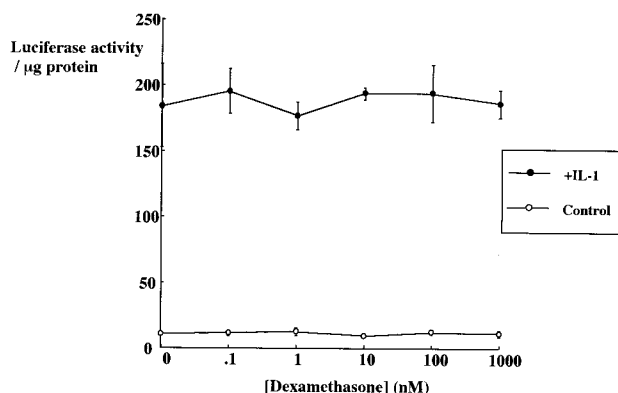

FIGURE 5. Failure of dexamethasone to repress induction of a NF-κB-regulated reporter gene in 1321N1 astrocytoma. 1321N1 astrocytoma, previously transfected with a plasmid containing a NF-κB-regulated luciferase gene, were pretreated for 15 h with various concentrations of dexamethasone and stimulated for a further 24 h in the absence (Control) or presence (+IL-1) of IL-1β (10 ng/ml). Cell extracts were measured for luciferase activity, and the results are expressed as light units normalized for protein. The data represent mean ± SEM of three independent experiments.
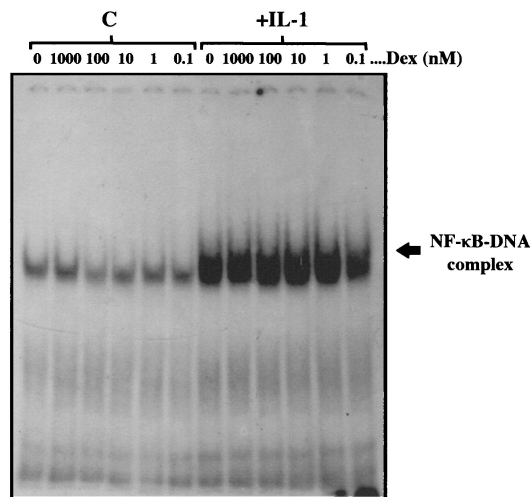

FIGURE 6. Effect of dexamethasone on activation of NF-κB in SK.N.SH neuroblastoma. SK.N.SH neuroblastoma were pretreated for 15 h with various concentrations of dexamethasone and stimulated for a further 30 min in the absence (C) or presence (+IL-1) of IL-1β (10 ng/ml). Nuclear extracts were prepared and assessed for NF-κB activity as described in *Materials and Methods*. The arrow indicates the mobility of the NF-κB-DNA complex. The data are representative of three independent experiments.

the ability of IL-1 to induce nuclear translocation and DNA binding activity of NF-κB. Experiments were then performed to assess if dexamethasone could produce antiinflammatory effects in SK.N.SH cells despite its inability to affect NF-κB. IL-1 was initially shown to strongly induce the expression of VCAM-1, ICAM-1, and IL-8 in SK.N.SH cells (data not shown). The inhibitory effects of dexamethasone on the IL-1 induction of the adhesion molecules and IL-8 was then assessed (Fig. 7). Dexamethasone caused a dose-dependent inhibition of the IL-1-induced expression of VCAM-1 and ICAM-1 (Fig. 7, *A* and *B*) with the expression of both adhesion molecules being reduced by comparable degrees. Similarly the IL-1-induced expression of IL-8 was also inhibited in a dose-dependent fashion by dexamethasone in these cells (Fig. 7C). These studies, in conjunction with the equivalent ones in 1321N1 astrocytoma, emphasize that the antiinflammatory effects of dexamethasone are not always dependent on inhibition of NF-κB.

Discussion

Many recent reports have indicated NF-κB as being an important target for mediating the antiinflammatory effects of glucocorticoids (16, 17). This promoted glucocorticoids as being efficacious drugs in controlling the expression of NF-κB-responsive genes. The latter include the genes encoding leukocytic adhesion molecules such as VCAM-1 (24) and ICAM-1 (25) and chemokines such as IL-8 (26). Indeed, it has been shown that the NF-κB-binding site in the promoter regions of these genes is the crucial *cis*-element in conferring IL-1 responsiveness on adhesion molecule (27) and IL-8 (28) expression. The present study was thus initiated to characterize the potential inhibitory effects of glucocorticoids on the IL-1-induction of these proinflammatory molecules in cells of neural origin. This is of major clinical relevance because the inappropriate expression of adhesion molecules and chemokines in brain cells can lead to the generation of neuropathological conditions. The initial experiments demonstrated that the synthetic glucocorticoid dexamethasone could produce the predicted inhibitory effects on the IL-1 induction of VCAM-1, ICAM-1, and IL-8 in
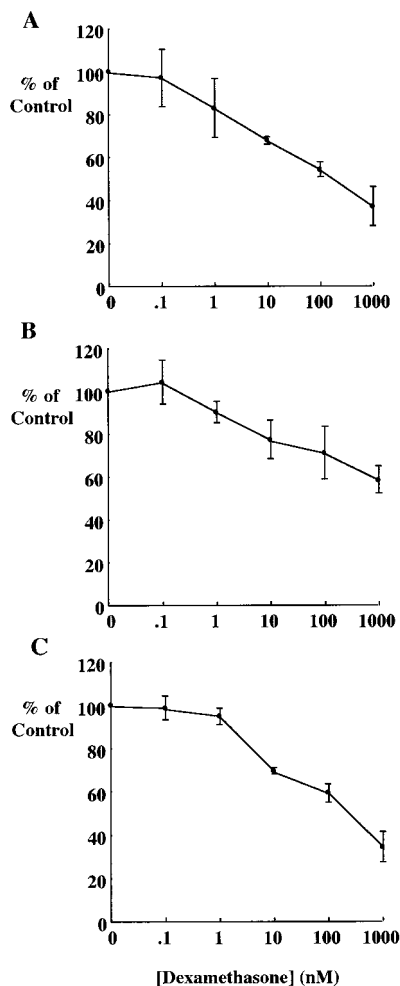
FIGURE 7. Inhibition of IL-1β-induction of adhesion molecules and IL-8 by dexamethasone in SK.N.SH neuroblastoma. SK.N.SH neuroblastoma were pretreated for 15 h with various concentrations of dexamethasone and stimulated for a further 24-h period with IL-1β (10 ng/ml). The cells were then measured for (*A*) VCAM-1 and (*B*) ICAM-1 expression by direct ELISA, and supernatants were examined for levels of (*C*) IL-8 by sandwich ELISA as described in *Materials and Methods*. The results are expressed as percentages of values obtained in IL-1β-treated cells in the absence of dexamethasone. The data represent mean ± SEM of three independent experiments.

1321N1 astrocytoma. The expression of IL-8 was inhibited to the greatest extent, suggesting that the glucocorticoid-sensitive pathway plays a major role in the induction of IL-8. However, the range of concentrations of dexamethasone that inhibited the expression of the proinflammatory molecules were completely ineffectual in affecting the activation of NF-κB. Dexamethasone failed to block IL-1 from stimulating the nuclear translocation and DNA-binding activity of NF-κB as determined by EMSA analysis. The NF-κB-DNA complexes that were induced by IL-1 contained both p50 and p65 based on the ability of Abs against these two subunits to block formation of the complexes. The inability of dexamethasone to block translocation of these subunits into the nucleus is in apparent contrast to previous studies (16, 17) that have shown that dexamethasone may reduce the amount of translocated nuclear p65 in response to proinflammatory cytokines such as TNF. This discrepancy may be explained by the proposal that up-regulation of IκBα in response to steroids, which was the suggested model by which translocation of p65 was decreased, may not be a universal

mechanism. Indeed, a recent report (29) has shown that the levels of IκB in the neuronal cortex and hippocampus are lower in dexamethasone-treated rats than in untreated animals. In contrast, peripheral cells from the same animals show elevated IκB levels following treatment with dexamethasone. The present study, where dexamethasone fails to affect levels of IκB, confirms further that induction of IκBα protein by glucocorticoids, and the consequential reduction in nuclear levels of active NF-κB is not a universal phenomenon and is cell-type specific.

The inability of dexamethasone to affect the IL-1-induced nuclear translocation or DNA-binding activity of NF-κB did not preclude the possibility that it may produce its antiinflammatory effects in 1321N1 cells by reducing the transactivation potential of NF-κB. Indeed, previous reports had proposed that glucocorticoids elicit their immunosuppressive effects by mediating direct protein-protein interactions between members of the steroid-receptor family and the DNA-binding subunits of NF-κB. Several studies have reported such interactions in vitro, which were found to suppress NF-κB transcriptional activity (20–23). De Bosscher et al. (23) showed that these direct protein interactions may interfere with the transactivation potential of NF-κB. They observed that gene expression driven by a chimeric protein containing the transactivating p65 subunit of NF-κB linked to the DNA-binding domain of the yeast GAL4 protein was strongly suppressed by glucocorticoids. Because the DNA-binding activity of the GAL4 fusion protein was not affected, it was concluded that the glucocorticoids must therefore interfere with the transactivating potential of the NF-κB p65 subunit. Further support for this model emerged when deletion analysis revealed an absolute requirement for the C-terminal transactivation domain of p65 in repression of transcriptional activity by glucocorticoids (30). Because the present studies had shown that IL-1 could induce the formation of NF-κB-DNA complexes containing p65 (Fig. 3*C*), it was decided to assess the ability of dexamethasone to affect the ability of IL-1 to induce the expression of a NF-κB-regulated reporter gene, luciferase, which had previously been transfected into 1321N1 cells. While IL-1 effected an impressive 17-fold induction of the reporter gene, dexamethasone failed to affect this induction. This again suggests that the antiinflammatory effects of dexamethasone in 1321N1 cells are mediated by a mechanism(s) independent of NF-κB.

To ensure that the observed effects are not simply artifacts of the 1321N1 cell system similar studies to the above were performed on SK.N.SH neuroblastoma, another widely used model cell system of neural origin. The effects that were observed in 1321N1 cells were mirrored in the SK.N.SH cells. Dexamethasone caused a dose-dependent inhibition of the IL-1 induction of the adhesion molecules VCAM-1 and ICAM-1 and the chemokine IL-8, but the same range of concentrations failed to affect activation of NF-κB. This suggests that the scenario whereby glucocorticoids may produce immunosuppressive and antiinflammatory effects in cells and NF-κB need not be involved in mediating the effects may be widespread and is not limited to a single cell type. Thus the mechanism(s) by which glucocorticoids are immunosuppressive and antiinflammatory are likely to be multifactorial. Some reports have shown glucocorticoids to down-regulate NF-κB activity by inducing IκBα protein (16, 17), whereas other studies have also described an inhibition of NF-κB but by a process independent of IκB (20, 31). In contrast, another recent report has demonstrated that glucocorticoids can also potentiate activation of NF-κB in some cell types (32).The present study adds further to the complexity and debate relating to the mechanism of glucocorticoid action in that glucocorticoids fail to affect in any way the NF-κB system in astrocytoma or neuroblastoma cells, yet retain their ability to produce effects that are antiinflammatory in nature.

References

1. Cupps, T. R., and A. S. Fauci. 1982. Corticosteroid-mediated immunoregulation in man. *Immunol. Rev. 65:133*.
2. Lee, S. W., A. P. Tsou, H. Chan, J. Thomas, K. Petrie, E. M. Eugui, and A. C. Allison. 1988. Glucocorticoids selectivley inhibit the transcription of the interleukin 1β gene and decrease the stability of interleukin 1β mRNA. *Proc. Natl. Acad. Sci. USA 85:1204*.
3. Arya, S. K., F. Wong-Stahl, and R. C. Gallo. 1984. Dexamethasone-mediated inhibition of human T cell growth factor and γ-interferon messenger RNA. *J. Immunol. 133:273*.
4. Beutler, B., N. Krochin, I. W. Milsark, C. Leudke, and A. Cerami. 1986. Control of cachectin (tumor necrosis factor) synthesis: mechanism of endotoxin resistance. *Science 232:977*.
5. Peppel, K., J. M. Vinci, and C. Baglioni. 1991. The AU-rich sequences in the 3′ untranslated region mediate the increased turnover of interferon mRNA induced by glucocorticoids. *J. Exp. Med. 173:349*.
6. Mukaida, N., C. C. O. Zachariae, G. L. Gusella, and K. Matsushima. 1991. Dexamethasone inhibits the induction of monocyte chemotactic-activating factor production by IL-1 or tumor necrosis. *J. Immunol. 146:1212*.
7. Dumont, A., S. P. Hehner, M. L. Schmitz, J. A. Gustafsson, J. Liden, S. Okret, P. T. Van Der Saag, S. Wissink, B. Van Der Burg, P. Herrlich, G. Haegeman, K. De Bosscher, and W. Fiers. 1998. Cross-talk between steroids and NF-κB: what language? *Trends Biochem. Sci. 23:233*.
8. Narayanan, R., K. A. Higgins, J. R. Perez, T. A. Coleman, and C. A. Rosen. 1993. Evidence for differential functions of the p50 and p65 subunits of NF-κB with a cell adhesion model. *Mol. Cell Biol. 13:3802*.
9. Baeuerle, P. A., and D. Baltimore. 1988. IκB: a specific inhibitor of the NF-κB transcription factor. *Science 242:540*.
10. Ganchi, P. A., S. C. Sun, W. C. Greene, and D. W. Ballard. 1992. IκB/MAD-3 masks the nuclear localization signal of NF-κB p65 and requires the transactivation domain to inhibit NF-κB p65 DNA binding. *Mol. Cell Biol. 3:1339*.
11. Thanos, D., and T. Maniatis. 1995. NF-κB: a lesson in family values. *Cell 80:529*.
12. Whiteside, S. T., J. Epinat, N. R. Rice, and A. Israel. 1997. A new member of the IκB protein family, IκBε, inhibits RelA (p65)-mediated NF-κB transcription. *EMBO J. 16:1413*.
13. Beg, A. A., T. S. Finco, P. V. Nantermet, and A. S. Baldwin. 1993. Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of IκB: a mechanism for NF-κB activation. *Mol. Cell Biol. 13:3301*.
14. Henkel, T., T. Machleidt, I. Alkalay, M. Kronke, Y. Ben-Yeriah, and P. A. Baeuerle. 1993. Rapid proteolytic degradation of IκB-α is necessary for activation of transcription factor NF-κB. *Nature 365:182*.
15. Baeuerle, P. A. 1991. The inducible transcription factor NF-κB: regulation by distinct protein subunits. *Biochim. Biophys. Acta 1072:63*.
16. Scheinman, R. I., P. C. Cogwell, A. K. Lofquist, and A. S. Baldwin, Jr. 1995. Role of transcriptional activation of IκBα in mediation of immunosuppression by glucocorticoids. *Science 270:283*.
17. Auphan, N., J. A. DiDonato, C. Rosette, A. Helmberg, and M. Karin. 1995. Immunosuppression by glucocorticoids: inhibition of NF-κB activity through induction of IκB synthesis. *Science 270:286*.
18. Moynagh, P. N., D. C. Williams, and L. A. J. O'Neill. 1994. Activation of NF-κB and induction of VCAM-1 and ICAM-1 expression in human glial cells by IL-1. *J. Immunol. 153:2681*.
19. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. In *Molecular Cloning: A Laboratory Manual*. N. Ford, C. Nolan, and M. Ferguson, eds. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, p.5.68.
20. Scheinman, R. I., A. Gualberto, C. M. Jewell, J. A. Cidlowski, and A. S. Baldwin, Jr. 1995. Characterisation of mechanisms involved in transrepression of NF-κB by activated glucocorticoid receptors. *Mol. Cell Biol. 15:943*.
21. Liden, J., F. Delaunay, I. Rafter, J. Gustafsson, and S. Okret. 1997. A new function for the C-terminal zinc finger of the glucocorticoid receptor: repression of RelA transactivation. *J. Biol. Chem. 272:21467*.
22. Ray, K. P., S. Farrow, M. Daky, F. Talabot, and N. Searle. 1997. Induction of the E-selectin promoter by interleukin 1 and tumour necrosis factor α, and inhibition by glucocorticoids. *Biochem. J. 328:707*.
23. De Bosscher, K., M. Lienhard Schmitz, W. Vanden Berghe, S. Plaisance, W. Fiers, and G. Haegeman. 1997. Glucocorticoid-mediated repression of nuclear factor-κB dependent transcription involves direct interference with transactivation. *Proc. Natl. Acad. Sci. USA 94:13504*.
24. Cybulsky, M. I., J. W. U. Fries, A. J. Williams, P. Sultan, R. Eddy, M. Byers, T. Shows, M. A. Gimbrone, and T. Collins. 1991. Gene structure, chromosomal location, and basis for alternative mRNA splicing of the human VCAM1 gene. *Proc. Natl. Acad. Sci. USA 88:7859*.
25. Voraberger, G., R. Schafer, and C. Stratowa.1991. Cloning of the human gene for intercellular adhesion molecule 1 and analysis of its 5′-regulatoryregion. *J. Immunol. 147:2777*.
26. Mukaida, N., M. Shiroo, and K. Matsushima. 1989. Genomic structure of the human monocyte-derived neutrophil chemotactic factor IL-8. *J. Immunol. 143: 1366*.
27. Ledebur, H.C., and T. P. Parks. 1995. Transcriptional regulation of the intercellular adhesion moelcule-1 gene by inflammatory cytokines in human endothelial cells: essential roles of a variant NF-κB site and p65 homodimers. *J. Biol. Chem. 270:933*.
28. Harant, H., R. de Martin, P. J. Andrew, E. Foglar, C. Dittrich, and I. J. D. Lindley. 1996. Synergisitc activation of interleukin-8 gene transcription by all-trans-retinoic acid and tumour necrosis factor-α involves the transcription factor NF-κB. *J. Biol. Chem. 271:26954*.
29. Unlap, M. T., and R. S. Jope. 1997. Dexamethasone attenuates NF-κB DNA binding activity without inducing IκB levels in rat brain in vivo. *Mol. Brain Res. 45:83*.
30. Wissink, S., E. C. van Heerde, M. L. Schmitz, E. Kalkhoven, B. van der Burg, P. A. Baeuerle, and P. T. van der Saag. 1997. Distinct domains of the RelA NF-κB subunit are required for negative cross-talk and direct interaction with the glucocorticoid receptor. *J. Biol. Chem. 272:22278*.
31. Heck, S., K. Bender, M. Kullmann, M. Gottlicher, P. Herrlich, and A. C. B. Cato. 1997. IκBα-independent downregulation of NF-κB activity by glucocorticoid receptor. *EMBO J. 16:4698*.
32. Wang, Y., J. J. Zhang, W. Dai, K. Y. Lei, and J. W. Pike. 1997. Dexamethasone potently enhances phorbol ester-induced IL-1β gene expression and nuclear factor NF-κB activation. *J. Immunol. 159:534*.