

The Immunosuppressive Fungal Metabolite Gliotoxin Specifically Inhibits Transcription Factor NF- κ B

By Heike L. Pahl,* Beate Krauß,* Klaus Schulze-Osthoff,* Thomas Decker,‡ E. Britta-Mareen Traenckner,* Markus Vogt,* Christina Myers,§ Tom Parks,§ Paul Warring,|| Arno Mühlbacher,|| Armin-Peter Czernilofsky,¶ and Patrick A. Baeuerle*

From the *Institute of Biochemistry and Molecular Biology, Albert-Ludwigs-University, D-79104 Freiburg, Germany; ‡Vienna Biocenter, Institute for Microbiology and Genetics, A-1030 Vienna, Austria; the §Department of Inflammatory Diseases, Boehringer Ingelheim, Ridgefield, Connecticut 06877; the ||Division of Cell Biology, John Curtin School of Medical Research, Australian National University, Canberra, Australian Capital Territory 2601, Australia; and ¶Boehringer Ingelheim Research Vienna, A-1120 Vienna, Austria

Summary

Opportunistic infections, such as aspergillosis, are among the most serious complications suffered by immunocompromised patients. *Aspergillus fumigatus* and other pathogenic fungi synthesize a toxic epipolythiodioxopiperazine metabolite called gliotoxin. Gliotoxin exhibits profound immunosuppressive activity in vivo. It induces apoptosis in thymocytes, splenocytes, and mesenteric lymph node cells and can selectively deplete bone marrow of mature lymphocytes. The molecular mechanism by which gliotoxin exerts these effects remains unknown. Here, we report that nanomolar concentrations of gliotoxin inhibited the activation of transcription factor NF- κ B in response to a variety of stimuli in T and B cells. The effect of gliotoxin was specific because, at the same concentrations, the toxin did not affect activation of the transcription factor NF-AT or of interferon-responsive signal transducers and activators of transcription. Likewise, the activity of the constitutively DNA-binding transcription factors Oct-1 and cyclic AMP response element binding protein (CREB), as well as the activation of protein tyrosine kinases p56^{lck} and p59^{lyn}, was not altered by gliotoxin. Very high concentrations of gliotoxin prevented NF- κ B DNA binding in vitro. However, in intact cells, inhibition of NF- κ B did not occur at the level of DNA binding; rather, the toxin appeared to prevent degradation of I κ B- α , NF- κ B's inhibitory subunit. Our data raise the possibility that the immunosuppression observed during aspergillosis results in part from gliotoxin-mediated NF- κ B inhibition.

Because of the development of intensive combination chemotherapy and bone marrow transplant preparatory regimens, both of which cause prolonged myelosuppression, the incidence of fungal infections in cancer patients has risen sharply in the past decades (1). Fungal infections are now the cause of death in 40% of patients with acute leukemia at some hospitals. In a recent international survey (2), 30% of mycotic infections represented cases of aspergillosis, which is caused by the fungi *Aspergillus fumigatus*, *A. flavus*, *A. niger*, and *A. terreus*. Aspergillosis carries a very high mortality rate, survival ranging only between 5 and 20%.

The precise molecular mechanism of *Aspergillus* pathogenesis remains unclear. One possible etiological agent is the secondary fungal metabolite gliotoxin. This metabolite is produced in large quantities by fungal cultures and can be isolated from mice undergoing systemic experimental as-

pergillosis (3). Gliotoxin belongs to the epipolythiodioxopiperazine class of compounds and is lethal at relatively low concentrations (LD₅₀ in mice and rats: 25–50 mg/kg) (4). Gliotoxin exhibits profound immunosuppressive effects in vivo. Sublethally irradiated mice injected intraperitoneally with gliotoxin showed a significant delay in the recovery of immune cells compared with untreated irradiated controls (5). Whereas LPS-responsive B cells were detected by proliferation assays in control animals 14 d after irradiation, animals treated with a single dose of 100 μ g gliotoxin showed no measurable B cell response until 30 d after irradiation. By contrast, the recovery of the T cell response, as measured by Con A-stimulated cell proliferation, was equal in both mock- and gliotoxin-treated animals. The in vivo data differ somewhat from observations in vitro, where gliotoxin inhibits mitogen-induced proliferation of both B and T cells. Furthermore, in MLCs, pretreatment of stimu-

lator cells with gliotoxin completely abrogates the induction of alloreactive cytotoxic T cells (6). When low concentrations (100 ng/ml) of gliotoxin are used, this inhibition can be overcome by the addition of IL-2. However, at higher concentrations of gliotoxin, T cell activation is suppressed even in the presence of cytokines. In addition to the inhibition of lymphocyte stimulation and proliferation, gliotoxin also induces apoptosis in these cells as well as in macrophages, L929 fibroblasts, and squamous carcinoma cells *in vitro* (7–9). *In vivo*, gliotoxin induces apoptosis in thymocytes, spleen cells, and cells of the mesenteric lymph nodes (5).

Gliotoxin exerts its immunosuppressive effects in part by inhibiting activation and proliferation of B and T cells. We therefore investigated whether the toxin interferes with the activation of transcription factors that are crucially involved in B and T cell activation and proliferation: nuclear factor (NF- κ B¹/Rel), nuclear factor of activated T cells (NF-AT), and signal transducer and activator of transcription (STAT) factors. NF- κ B is a central mediator of the immune response (10). It regulates the transcription of various inflammatory cytokines, hematopoietic growth factors, growth factor receptors, and cell adhesion molecules (listed in detail in reference 10). In most cell types, NF- κ B is sequestered in an inactive, cytoplasmic complex by binding to I κ B, its inhibitory subunit (11). A large variety of T or B cell-activating stimuli rapidly induce NF- κ B, stimulating κ B-dependent transcription. Many NF- κ B target genes encode proteins controlling cell proliferation. NF- κ B activation requires phosphorylation and proteolytic degradation of I κ B subunits (12–16). The active NF- κ B complex, which translocates to the nucleus, is frequently composed of a heterodimer of p50 and RelA (p65) subunits (17). Whereas mouse null mutants of p50 are viable and show only a very weak immunodeficient phenotype (18), null mutants of RelA die *in utero* and show massive apoptosis in the liver (19). Mice lacking *c-rel* are defective in lymphocyte proliferation, humoral immunity, and IL-2 expression (20). Pharmacological inhibition of NF- κ B *in vivo* may thus affect immune regulation as well as cell survival.

The NF-AT family of transcription factors, whose members share weak sequence similarity with NF- κ B/Rel proteins, also plays an important role in T cell activation. These proteins regulate the expression of the genes for IL-2, -3, -4, GM-CSF, and TNF- α (21). Activation of cytoplasmic NF-AT factors requires the calcium- and calmodulin-dependent phosphatase calcineurin, nuclear translocation of NF-AT, and the association of NF-AT with an AP-1 factor on the DNA (for reviews see references 22, 23). The importance of NF-AT in T cell activation is illus-

trated by the effect of the fungal metabolites cyclosporin A and FK506. These agents indirectly inhibit calcineurin thereby preventing NF-AT activation (24, 25). Treatment of patients with cyclosporin A or FK506 causes a potent immunosuppression that is exploited clinically for organ transplantation (26–28). Natural immunosuppressive compounds with specificity for NF- κ B have not yet been reported.

Stimulation of cells in response to IFNs is mediated by the STAT family of transcription factors. Like NF-AT and NF- κ B/Rel proteins, STATs are maintained in a cytoplasmic form in unstimulated cells (29). STAT activation occurs via direct phosphorylation by receptor-associated tyrosine kinases, subsequent dimerization via SH2 domains, and nuclear translocation (30). Stimulation of cells with type I IFN (IFN- α/β) causes activation of STAT1 and STAT2, which subsequently form a transcription factor, ISGF3, which consists of a STAT1-STAT2 heterodimer in association with a 48-kD DNA-binding protein. This 48-kD subunit belongs to the IRF family of proteins (30). ISGF3 activates transcription of target genes like MHC class I by associating with a promoter DNA sequence designated the interferon-stimulated response element (ISRE). In contrast, type II IFN (IFN- γ) exclusively activates STAT1 so that a homodimer is formed that binds to a distinct response element, the gamma interferon activation site (GAS; 30). Other cytokines and growth factors, including prolactin, epidermal growth factor, IL-2, -3, -4, -6, -12, LIF, erythropoietin, and growth hormone also use members of the STAT factor family as signal transducers (31–36).

We show here that nanomolar concentrations of gliotoxin specifically inhibited the activation of NF- κ B in various cell types, whereas the activation of NF-AT and STAT factors was not affected at such low concentrations. The toxin prevented stimulation of κ B-controlled reporter gene activity by TNF, IL-1, and PMA whereas it did not affect induction in response to IFN- γ . NF- κ B activation in response to five different stimuli (LPS, IL-1 β , TNF- α , PMA/PHA, and okadaic acid) was blocked, suggesting that gliotoxin interfered with a late step in a common signaling cascade leading to NF- κ B activation. Very high concentrations of gliotoxin inhibited NF- κ B DNA binding *in vitro*, most likely because of oxidation of a cysteine residue by the toxin's sulfhydryl group. However, this modification did not occur *in vivo*: overexpressed p50 and cytoplasmic NF- κ B complexed with I κ B were not inactivated by the toxin. Inhibition of NF- κ B activation most likely results from inhibition of I κ B- α proteolysis. Our data suggest that the immunosuppressive activity of the fungal metabolite gliotoxin may rely on inhibition of the transcription factor NF- κ B in T and B cells.

Materials and Methods

Cell Culture

Jurkat, 70Z/3, D10.G4.1, and stably transfected A549 cells were maintained in RPMI 1640 medium; HeLa S3 and 293 cells

¹Abbreviations used in this paper: CREB, cyclic AMP response element binding protein; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; ICAM-1, intercellular adhesion molecule 1; IRF, interferon regulatory factor; ISGF3, IFN-stimulated gene factor 3; ISRE, IFN-stimulated response element; LIF, leukemia inhibitory factor; NF-AT, nuclear factor of activated T cells; NF- κ B, nuclear factor κ B; STAT, signal transducer and activator of transcription.

were maintained in DMEM. Both were supplemented with 10% FCS and 50 µg/ml penicillin-streptomycin (all from GIBCO BRL, Eggenstein, Germany). Medium for D10.G4.1 cells contained 50 µg/ml β-ME and 50 U/ml IL-2 in addition. The 70Z/3 cells transfected with the p55 TNF receptor were generously given by Dr. M. Krönke (Kiel, Germany); gliotoxin, methylthiogliotoxin (bis-dethio-bis(methylthio)-gliotoxin), PMA, and LPS were purchased from Sigma Chemical Co. (Münich, Germany). Other chemicals were obtained from the following suppliers: okadaic acid (Paesel and Lorei GmbH & Co., Hanau, Germany), IL-1β (Genzyme Corp., Cambridge, MA) and TNF (Boehringer-Mannheim, Mannheim, Germany).

Transfections

293 cells were plated 12–16 h before transfection at a density of 10⁶ cells per 60-mm dish. Transfections were performed using calcium phosphate precipitation as previously described (16).

Transactivation Assays

A549 cells, stably transfected with the plasmid pGLH3 carrying a luciferase reporter gene driven by a fragment of the intercellular adhesion molecule (ICAM) 1 promoter (bp -277 to -9 relative to the start of transcription) carrying response elements for both TNF-α and IFN-γ (37–39), were seeded in microtiter plates. Cells were pretreated with either 1 µg/ml gliotoxin or 3 µg/ml bis-dithio-bis(methylthio)-gliotoxin for 30 min before stimulation with either 300 U/ml TNF-α, 1 ng/ml IL-1β, 20 ng/ml PMA, or 300 U/ml IFN-γ for 6 h. Cells were harvested in situ using a commercial lysis buffer and luciferase assay system (Promega Corp., Heidelberg, Germany) and luciferase activity was determined on a microplate scintillation counter (TopCount; Packard Instruments Co., Inc., Meriden, CT).

Plasmids

The expression vector containing amino acids 1–399 of the NF-κB p50 subunit has been previously described in detail (40).

Electrophoretic Mobility Shift Assays

NF-κB, Oct-1, and CREB. Total cell extracts were prepared using a high salt detergent buffer (Totex buffer) (20 mM Hepes, pH 7.9, 350 mM NaCl, 20% (vol/vol) glycerol, 1% (wt/vol) NP-40, 1 mM MgCl₂, 0.5 mM EDTA, 0.1 mM EGTA, 0.5 mM dithiothreitol [DTT], 0.1% PMSF, and 1% aprotinin). Cells were harvested by centrifugation, washed once in ice-cold PBS (Sigma) and resuspended in four cell volumes of Totex buffer. The cell lysate was incubated on ice for 30 min, then centrifuged for 5 min at 13,000 g at 4°C. The protein content of the supernatant was determined and equal amounts of protein (10–20 µg) were added to a reaction mixture containing 20 µg BSA (Sigma), 2 µg poly (di-dC) (Boehringer Mannheim), 2 µl buffer D⁺ (20 mM Hepes, pH 7.9, 20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25% NP-40, 2 mM DTT, and 0.1% PMSF), 4 µl buffer F (20% Ficoll-400, 100 mM Hepes, 300 mM KCl, 10 mM DTT, 0.1% PMSF), and 100,000 cpm (Cerenkov Counting) of a ³²P-labeled oligonucleotide in a final volume of 20 µl. Samples were incubated at room temperature for 25 min. NF-κB, CREB, and Oct-1 oligonucleotides (Promega) were labeled using γ-[³²P]ATP (3,000 Ci/mmol; Amersham International, Braunschweig, Germany) and T4 polynucleotide kinase (Promega).

DOC Activation. DOC activation was performed precisely as

previously described (11); the detergent (purchased from Sigma) was added to a final concentration of 0.4%.

NF-AT. Nuclear extracts were prepared as previously described (41). Electrophoretic mobility shift assay (EMSA)-binding reactions were identical to those used for NF-κB, Oct-1, and CREB except that only 0.2 µg/ml poly (di-dC) was used.

STAT Factors

ISGF3 activation was determined in an EMSA using the ISRE from the promoter of the ISG15 gene (42) as a probe. HeLa S3 cells were treated for 12 h with 1 ng/ml IFN-γ followed by a 30-min stimulation with 1,000 U/ml of IFN-α before harvest. Where indicated, gliotoxin was added 30 min before addition of IFN-α and remained in the culture medium during IFN stimulation. The EMSA conditions have been described previously (29). For in vitro treatment of ISGF3 with gliotoxin, the extracts were treated with the indicated concentrations of the drug for 30 min at room temperature before addition to the EMSA-binding reaction.

Immunoprecipitation and Kinase Assays

Jurkat cells (2 × 10⁷) were pretreated with different concentrations of gliotoxin for 1 h and subsequently stimulated with 50 ng/ml PMA for 30 min. Cells were washed with ice-cold PBS containing 400 µM EDTA and 400 µM sodium vanadate and lysed in 250 µl of RIPA buffer (5 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 100 µM Na₃VO₄, 1 mM DTT, 1 mM PMSF, 10 µg/ml leupeptin, and 10 mM sodium phosphate, pH 7.4). Nuclei and cell debris were removed by centrifugation at 16,000 g for 15 min at 4°C. The supernatant was precleared with protein A-Sepharose (Pharmacia LKB, Freiburg, Germany) and immunoprecipitated with 1 µg of rabbit anti-p56^{lck} or anti-p59⁶ⁿ antibody (Upstate Biotechnology, Inc., Lake Placid, NY). Immune complexes were collected with protein A-Sepharose and washed twice with lysis buffer, once with 10 mM Hepes, pH 8.0, containing 50 mM NaCl, and once with Hepes buffer without NaCl. Immune complex kinase assays were performed by incubation at 25°C in 50 µl kinase buffer (30 mM Hepes, pH 7.2, 5 mM MgCl₂, 5 mM MnCl₂, 1 µM ATP, and 10 µCi γ-[³²P]ATP) containing 5 µg of acid-treated rabbit muscle enolase (Sigma). After 15 min, the reaction was ended by the addition of Laemmli buffer. The samples were analyzed by 10% SDS-PAGE and subjected to autoradiography.

Western Blotting

Total cell extracts (100 µg) were boiled in Laemmli sample buffer and subjected to SDS-PAGE. Before transfer, gels were equilibrated in ice-cold blotting buffer (48 mM Tris, 39 mM glycine, 15% methanol, and 0.037% SDS). Proteins were transferred at 2.5 mA/cm² for 1 h onto Immobilon P membranes (Millipore Corp., Eschborn, Germany) using a semi-dry blotting apparatus (Bio-Rad Laboratories, Munich, Germany). Transfer efficiency was monitored by Ponceau S staining of membranes. Nonspecific binding sites were blocked by immersing the membrane in TBST blocking solution (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20 [vol/vol] containing 5% low-fat dried milk powder) overnight at 4°C. After a short wash in TBST, the membranes were incubated in a 1:700 dilution of anti-IκB-α antibody (12) in blocking solution for 1 h at room temperature, followed by extensive washing with TBST. Bound antibody was decorated with goat anti-rabbit/horseradish peroxidase conjugate (Bio-

Rad; diluted 1:3,000 in blocking solution) for 30 min at room temperature. After washing for 45 min, the immunocomplexes were detected using enhanced chemiluminescence Western blotting reagents (ECL; Amersham). Exposure to Eastman Kodak XAR-5 film (Rochester, NY) was performed for 30 s.

Results

Gliotoxin Is a Potent Inhibitor of NF- κ B Activation. To investigate whether gliotoxin interferes with the activation of transcription factors involved in T cell activation, Jurkat T cells were incubated with various concentrations of gliotoxin

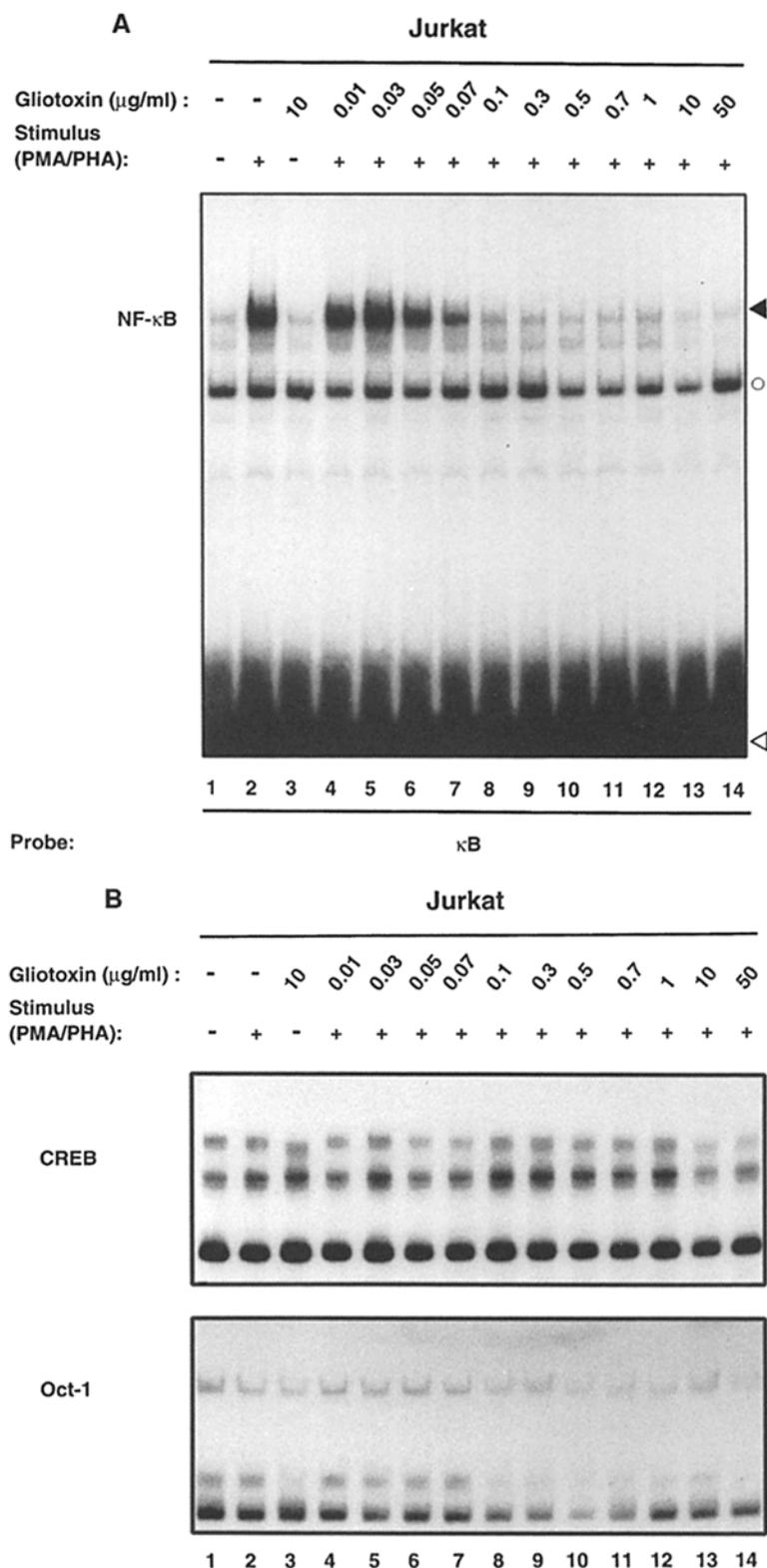


Figure 1. (A) The effect of gliotoxin on NF- κ B DNA binding. Jurkat cells were treated for 1 h with gliotoxin at the concentrations indicated and were subsequently stimulated with 50 ng/ml PMA and 5 μ g/ml PHA for 1 h. (Lane 1) Unstimulated control cells; (lane 3) cells were treated with gliotoxin alone. Equal amounts of protein from cell extracts were analyzed for NF- κ B activity by EMSA. A section of a fluorogram is shown. (\blacktriangle) Position of NF- κ B DNA complexes. (\circ) A nonspecific activity binding to the probe; (\triangleleft) unbound oligonucleotide. (B) The effect of gliotoxin on DNA binding of transcription factors Oct-1 and CREB. The Jurkat cells extracts used in A were analyzed for CREB and Oct-1 DNA binding by EMSA. A section of a fluorogram is shown.

for 1 h. Subsequently, cells were stimulated for 1 h with PMA/PHA, after which total cell extracts were prepared. The extracts were analyzed for NF- κ B DNA-binding activity in an EMSA. Stimulation with PMA/PHA induced one novel DNA-binding activity in Jurkat T cells (Fig. 1 A, lane 2). Antibody reactivity and competition assays identified this complex as a NF- κ B p50/p65 heterodimer (data not shown; 15, 43). NF- κ B activation was significantly reduced by treatment of Jurkat cells with 70 ng/ml of gliotoxin, whereas 100 ng/ml (300 nM) gliotoxin and higher concentrations completely prevented NF- κ B induction (Fig. 1 A, lanes 7 and 8). The weak basal activity of NF- κ B (lane 1) was not reduced even at very high gliotoxin concentrations (lanes 10–14), indicating that the toxin did not directly inhibit NF- κ B DNA binding. A very similar dose–response was observed in the HeLa tumor cell line as well as in various other cell lines (see below; data not shown). A faster migrating, nonspecific DNA-binding activity was not altered by gliotoxin treatment, even when doses \leq 50 μ g/ml were used (Fig. 1 A, lane 14).

We analyzed the Jurkat T cell extracts shown in Fig. 1 A in EMSAs using DNA probes containing consensus motifs for the constitutively bound transcription factors Oct and CREB (Fig. 1 B). Treatment with gliotoxin concentrations \leq 50 μ g/ml did not detectably affect DNA binding of Oct and CREB proteins to their respective DNA probes. Taken together, these data show that treatment of cells

with gliotoxin at nanomolar concentrations specifically inhibits the activation of transcription factor NF- κ B, whereas the DNA-binding activity of other transcription factors remained unaffected at up to 500-fold higher concentrations.

Gliotoxin Is an Inhibitor of NF- κ B-dependent Gene Expression. The inducible expression of the ICAM-1 gene in response to TNF- α , IL-1 β , and PMA is controlled by a NF- κ B-binding site in its upstream promoter, whereas the inducibility in response to IFN- γ is controlled by a binding motif for STAT1 (37–39). Here, we tested the effect of gliotoxin on the expression of a stably integrated firefly luciferase reporter gene under control of a fragment of the human ICAM-1 promoter in A549 cells (37). As shown in Fig. 2, TNF- α , IL-1 β , PMA, and IFN- γ caused a substantial induction of the reporter gene. 1 μ g/ml gliotoxin, which did not affect the basal ICAM-1 promoter activity, suppressed reporter gene induction by TNF- α , IL-1 β , and PMA, which act via the κ B motif. By contrast, gliotoxin could not suppress induction of the ICAM-1 promoter by IFN- γ , which is mediated by STAT transcription factors. These data provide compelling evidence for the specificity of the toxin. The inhibitory activity of gliotoxin on κ B-dependent gene expression relied on intact sulfhydryl groups; 3 μ g/ml of the immunologically inactive derivative methylthiogliotoxin (44, 45) did not display any inhibitory potential (Fig. 2). At higher concentrations of gliotoxin, inhibitory as well as stimulatory effects on the activity of var-

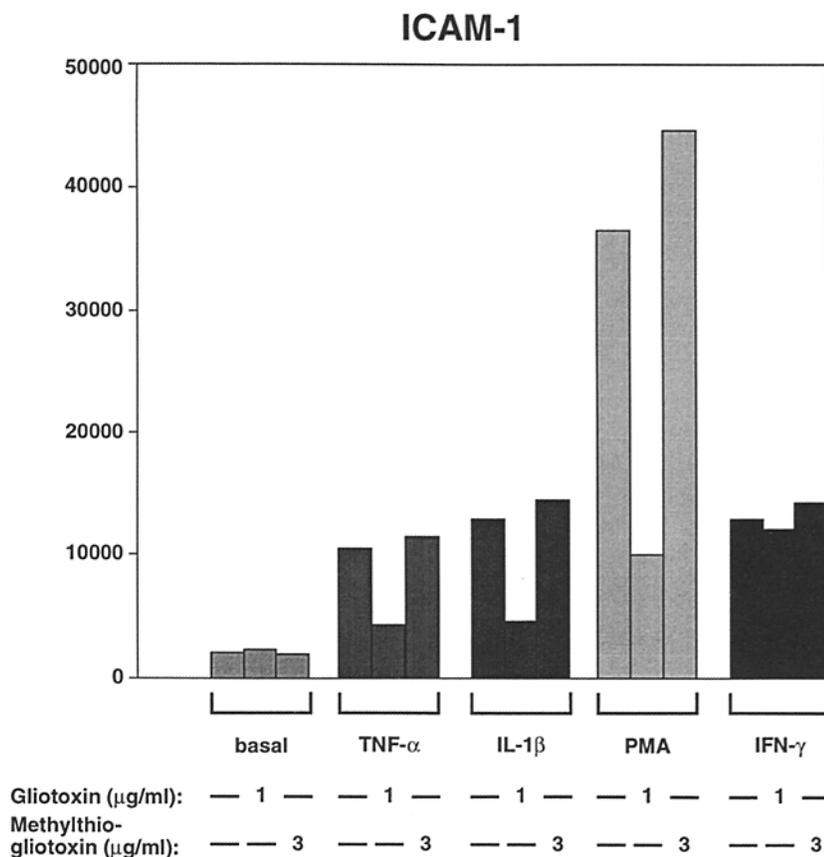


Figure 2. The effect of gliotoxin on ICAM-1 promoter-driven reporter gene activity. A549 cells stably transfected with a luciferase reporter gene driven by a fragment of the ICAM-1 promoter were treated for 30 min with either 1 μ g/ml gliotoxin or 3 μ g/ml methyl gliotoxin as indicated. Subsequently, cells were induced with either 300 U/ml TNF- α , 1 ng/ml IL-1 β , 20 ng/ml PMA, or 300 U/ml IFN- γ for 6 h and luciferase activity was determined. The results are given in arbitrary relative light units and are representative of three experiments.

ious unrelated promoters and enhancers were observed in reporter gene assays (data not shown), suggesting that only low concentrations of gliotoxin specifically affected NF- κ B. Functional analysis of the ICAM-1 promoter thus corroborates data obtained from EMSAs showing that nanomolar concentrations of gliotoxin potently and specifically inhibit NF- κ B activation.

Gliotoxin Does Not Impair Activation of STAT and NF-AT Factors. To further investigate the specificity of gliotoxin, we studied the activation of STAT and NF-AT factors by IFNs and PMA/ionomycin, respectively. The effect of gliotoxin on the activation of STAT factors was tested in HeLa S3 cells. In these cells, NF- κ B activation is also inhibited by pretreatment with 100 ng/ml of gliotoxin (data not shown). Extracts from control or IFN- α/γ -stimulated cells were assayed in an EMSA using an ISRE as the DNA probe (Fig. 3 A). Pretreatment of HeLa S3 cells with IFN- γ for 12 h increases the amount of the 48-kD DNA-binding subunit of ISGF3 (p48) and therefore the amount of ISGF3 generated after a subsequent pulse with IFN- α (46). Moreover, under these conditions, elevated amounts of ISGF2/IRF1, another ISRE-binding factor, are observed. Since induction of the ISGF2/IRF1 gene by IFN depends on STAT1

binding to a GAS sequence in the IRF1 promoter (47), these conditions also determine whether STAT1 homodimer activation is intact. Doses of ≤ 10 μ g/ml gliotoxin did not significantly perturb the activation of STAT1, STAT2, IRF-2, and p48 in response to IFN- α/γ stimulation of HeLa S3 cells (Fig. 3 A, lanes 4–7). Similarly, treatment of extracts from IFN- α/γ -stimulated HeLa S3 cells with gliotoxin under cell-free conditions had no effect on the DNA-binding of these factors (Fig. 3 B).

D10.G4.1 T cells behaved similarly to Jurkat T cells with respect to the inhibition of NF- κ B activation by gliotoxin (data not shown). In nuclear extracts of these cells, treatment with PMA/ionomycin strongly induced an activity that binds to the distal NF-AT site in the murine IL-2 enhancer (Fig. 4, compare lanes 1 and 2). This protein–DNA complex is specifically competed by an excess of unlabeled oligonucleotide (data not shown). T cells were treated for 1 h with increasing concentrations of gliotoxin followed by a 1-h stimulation with PMA/ionomycin. Activation of NF-AT from its cytoplasmic form was not affected by a treatment of D10.G4.1 T cells with gliotoxin, even at a concentration of 10 μ g/ml, 100-fold the concentration that abrogated activation of NF- κ B (see Fig. 1). As seen

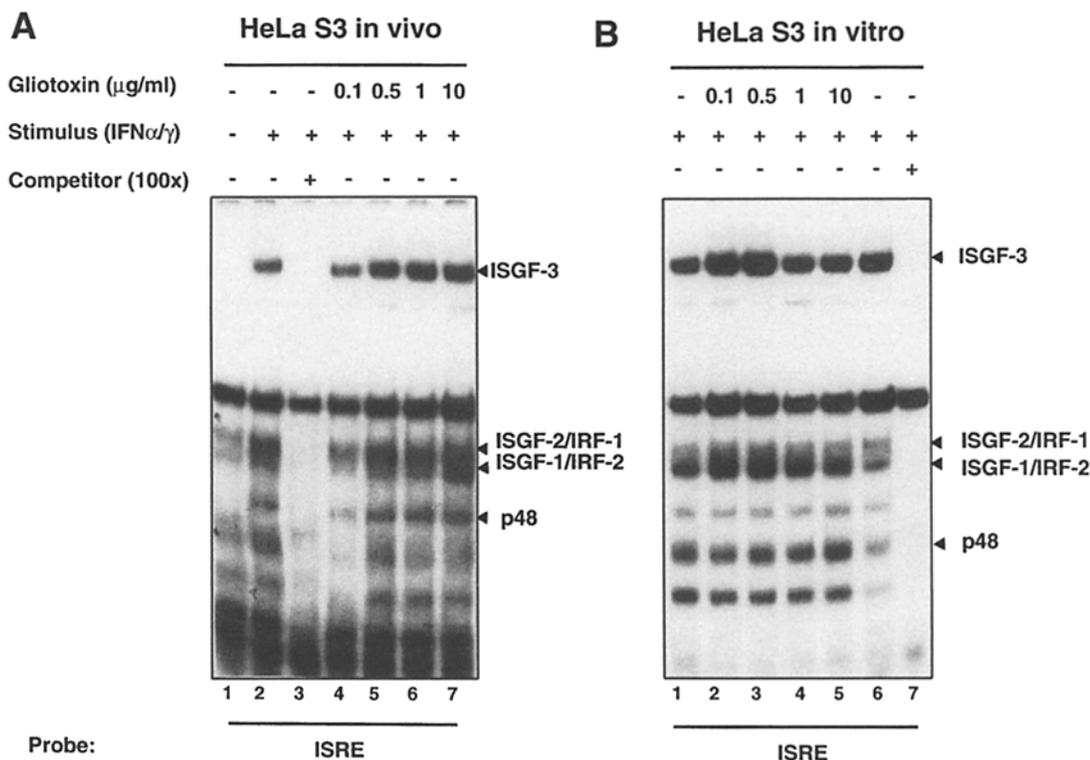


Figure 3. The effect of gliotoxin on DNA binding of STAT factors in vitro and in vivo. (A) HeLa S3 cells were treated in vivo for 12 h with IFN- γ and subsequently with IFN- α for 30 min. The indicated concentrations of gliotoxin were added 30 min before addition of IFN- α and remained in the culture medium during IFN stimulation. Equal amounts of protein from whole cell extracts were analyzed for STAT factor binding by EMSA. (Lane 7) A 100-fold excess of unlabeled oligonucleotide was added as a competitor. A section of a fluorogram is shown. (◀) Positions of various STAT factor DNA complexes. (B) Unstimulated HeLa S3 cells (lane 1) or cells stimulated with IFN- γ and IFN- α as described for A (lanes 2–7) were used to prepare whole cell extracts. The extracts were treated with various concentrations of gliotoxin in vitro as indicated. Equal amounts of protein from cell extracts were analyzed for STAT factor binding by EMSA. (Lane 3) A 100-fold excess of unlabeled oligonucleotide was added as a competitor. A section of a fluorogram is shown. (◀) Positions of various STAT factor DNA complexes.

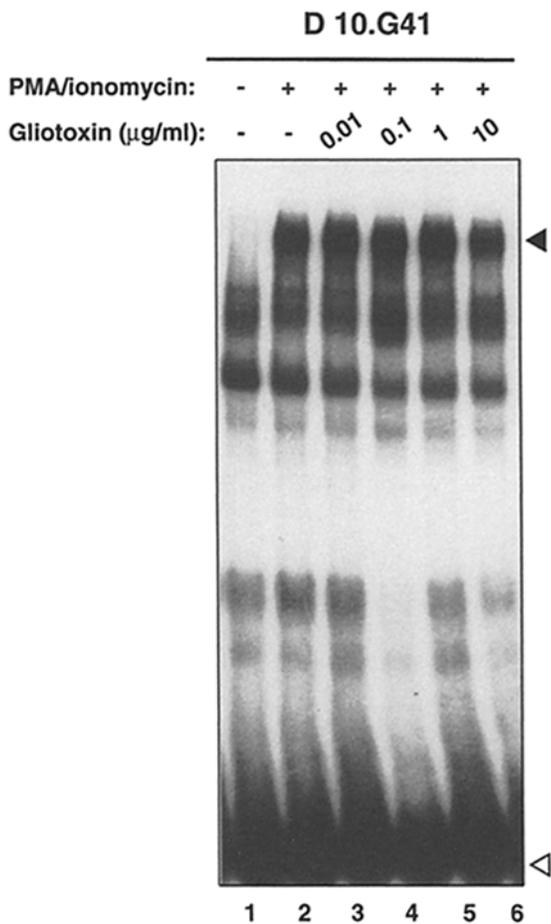


Figure 4. The effect of gliotoxin on NF-AT DNA binding. D10.G4.1 cells were treated for 1 h with gliotoxin at the concentrations indicated and were subsequently stimulated with 50 ng/ml PMA and 1 μM ionomycin for 1 h. (Lane 1) Unstimulated control cells were used. Equal amounts of protein from nuclear extracts were analyzed for NF-AT activity by EMSA. (◄) Position of NF-AT DNA complexes. (◄) Unbound oligonucleotide.

in previous experiments (Figs. 1–3), none of the unrelated nonspecific DNA-binding activities was sensitive towards gliotoxin. Taken together, these data show that treatment of cells with gliotoxin specifically inhibits the activation of transcription factor NF-κB whereas other constitutive and inducible factors remain unaffected at up to 500-fold higher concentrations. In the following, we examined several steps in the signal transduction pathway, which lead to NF-κB activation in B and T cells, for their susceptibility to gliotoxin.

Gliotoxin Acts on a Common Mechanism of NF-κB Activation. NF-κB can be activated by numerous stimuli (10). Reporter gene assays using the ICAM-1 promoter (Fig. 2) showed that gliotoxin inhibits NF-κB activation in response to several different stimuli, suggesting that the toxin targets a signal transduction step shared by many inducers. To test this hypothesis, we used the pre-B cell line 70Z/3, in which NF-κB can be activated by such diverse stimuli as PMA, LPS, IL-1β, or the phosphatase 2A inhibitor okadaic

acid. In a stably transfected 70Z/3 clone carrying the p55 TNF receptor (48), NF-κB can also be induced with TNF-α. 70Z/3 or 70Z/3-p55 cells were preincubated with 1 μg/ml of gliotoxin for 1 h before stimulation with the various NF-κB inducers. The stimuli induced NF-κB DNA-binding activity to different levels (Fig. 5). Pretreatment with gliotoxin completely prevented the activation of NF-κB by all five stimuli, indicating that the toxin interfered with a common event shared by all inducers.

Only Very High Concentrations of Gliotoxin Inactivate NF-κB DNA Binding. A simple explanation for the inhibitory effect of gliotoxin would be a direct interference with the DNA-binding assay detecting NF-κB. Gliotoxin is a sulfhydryl compound with the potential to covalently modify proteins by formation of mixed disulfides with exposed cysteine residues (3). NF-κB subunits harbor a redox-sensitive cysteine residue in their DNA-binding domains (49–51). In addition, gliotoxin has been shown to cause DNA breaks at 90 μg/ml *in vitro* (52), such that high gliotoxin concentrations may impair DNA-binding reactions by damaging the oligonucleotide probe. To test these possibilities, we incubated cell extracts from PMA/PHA-activated Jurkat T cells containing activated NF-κB with gliotoxin. Addition of 25 μg/ml gliotoxin to the DNA-binding reaction did not detectably inhibit NF-κB DNA binding *in vitro* (Fig. 6, lane 3). Only concentrations ≥250 μg/ml impaired DNA binding of NF-κB (Fig. 6, lanes 4 and 5).

It is possible that in intact cells gliotoxin is converted into a metabolite that directly modifies NF-κB. We investigated this hypothesis by transfecting 293 cells with an expression vector for the p50 DNA-binding subunit of NF-κB. p50 was previously shown to be highly susceptible to modification of Cys₆₂ by oxidants (49, 51). Overexpressed p50 is not subject to significant IκB inactivation and will therefore constitutively bind DNA as a homodimer (53). Transfected 293 cells expressing p50 NF-κB were treated with increasing concentrations of gliotoxin for 2 h after which cell extracts were analyzed by EMSA. Concentrations of ≤50 μg/ml gliotoxin, 500-fold the inhibitory dose for NF-κB activation, did not impair the p50 DNA-binding activity *in vivo* (Fig. 7). In addition, we found that the NF-κB/IκB complex in nonstimulated 70Z/3 pre-B cells, was not inactivated by gliotoxin treatment; deoxycholate treatment (11) recovered similar amounts of NF-κB activity in cell extracts from control and gliotoxin-treated cells (Fig. 8, compare lanes 2 and 4). Taken together, these data suggest that gliotoxin does not directly modify NF-κB DNA-binding subunits *in vivo*.

Gliotoxin Does Not Prevent Activation of Tyrosine Kinases p56^{lck} or p59^{lyn} upon T Cell Activation. An early event after stimulation of the TCR is the activation of the Src-like tyrosine kinases p56^{lck} and p59^{lyn} (54, 55). Extracts from control and gliotoxin-treated Jurkat T cells were used for immunoprecipitation with antibodies against p56^{lck} or p59^{lyn}. Kinase activities were determined by incubation of immunoprecipitates with the substrate rabbit muscle enolase and γ-[³²P]ATP. Tyrosine-phosphorylated enolase was then separated by SDS-PAGE and visualized by autoradiography

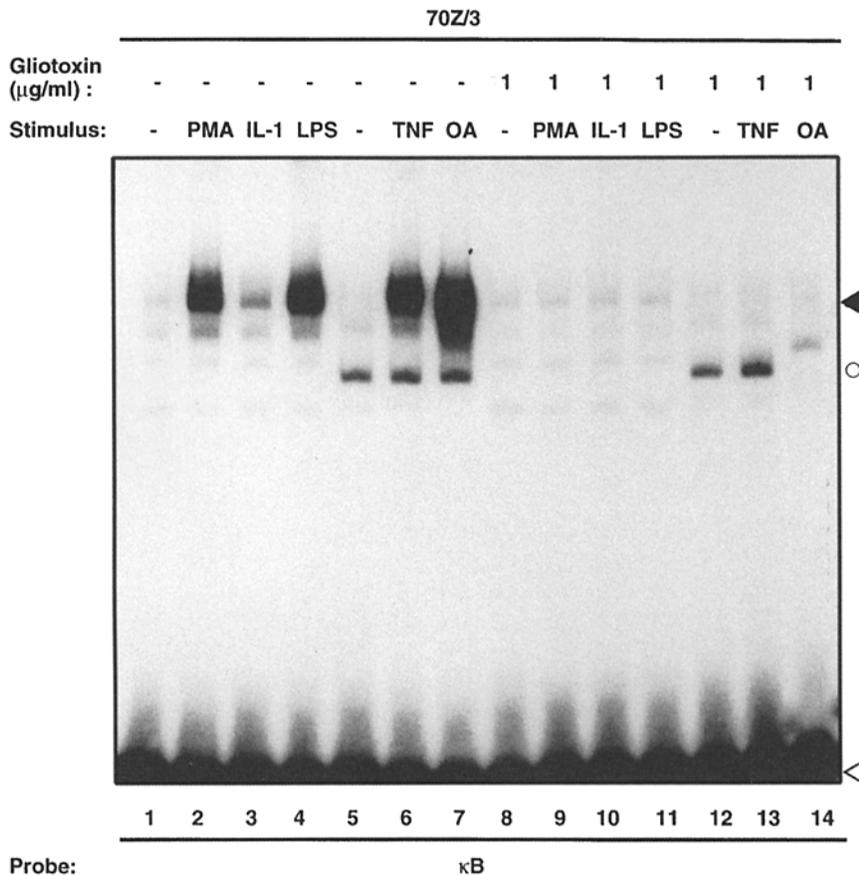


Figure 5. The effect of gliotoxin on NF-κB activation in response to five distinct stimuli. 70Z/3 pre-B cells were stimulated with 50 ng/ml PMA (lanes 2 and 9), 50 U/ml of IL-1-β (lanes 3 and 10), or 10 μg/ml of LPS (lanes 4 and 11). A stably transfected 70Z/3 clone, expressing the p55 TNF receptor, was stimulated with 200 U/ml of TNF (lanes 6 and 13) or 0.5 μM okadaic acid (lanes 8 and 14). In lanes 1, 5, 8, and 12 the appropriate unstimulated control cells were used. The cells analyzed in lanes 8–14 were pretreated with 1 μg/ml gliotoxin for 1 h before stimulation. Equal amounts of protein from cell extracts were analyzed for NF-κB activity by EMSA. A section of a fluorogram is shown. (◄) Position of NF-κB DNA complexes. (○) A nonspecific activity binding to the probe; (◄) unbound oligonucleotide.

(Fig. 9). Stimulation of Jurkat cells with PMA resulted in 8.8- and 6.7-fold induction of enolase phosphorylation by p56^{lck} and p59^{lyn} immunoprecipitates, respectively (data not shown). Treatment of Jurkat T cells with gliotoxin concentrations ≤10 μg/ml did not detectably affect the ability of either p56^{lck} or p59^{lyn} to phosphorylate rabbit muscle enolase (Fig. 9), suggesting that gliotoxin did not prevent NF-κB activation by inhibiting TCR-associated tyrosine kinases.

Gliotoxin Allows IκB-α Phosphorylation but Prevents Its Degradation. Release of IκB-α from NF-κB requires phosphorylation of IκB-α on serines 32 and 36 and subsequent proteolytic degradation of IκB-α by the ubiquitin/proteasome pathway (12–16). The inducible phosphorylation of

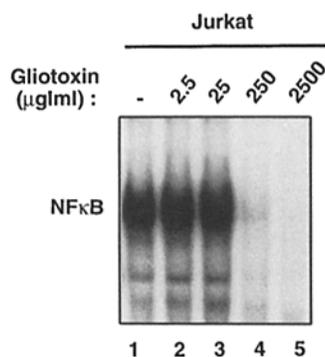


Figure 6. The effect of in vitro addition of gliotoxin on NF-κB DNA binding. Extracts from PMA/PHA-stimulated Jurkat cells were analyzed for NF-κB DNA binding by EMSA. The indicated amounts of gliotoxin were added to the EMSA-binding reactions in vitro.

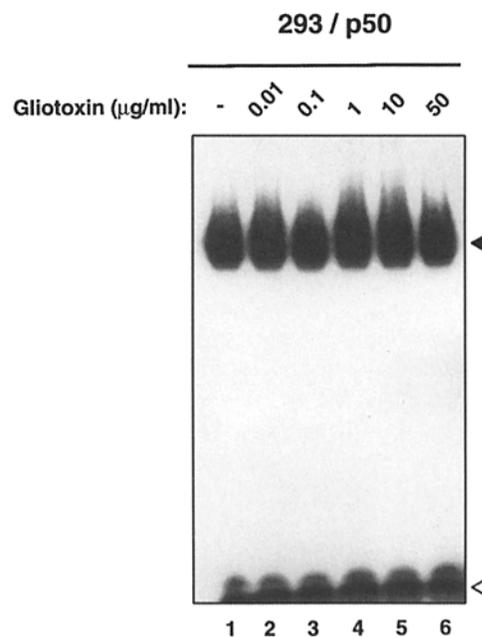


Figure 7. The effect of gliotoxin on p50 DNA binding. 293 cells were transiently transfected with 6 μg of a p50 expression vector. 24 h after transfection, cells were treated with various concentrations of gliotoxin, as indicated, for 2 h. Total cell extracts were prepared and analyzed by EMSA using a ³²P-labeled oligonucleotide probe containing a NF-κB-binding site. A section of a fluorogram is shown. (◄) The NF-κB p50 homodimer DNA complex. (◄) Unbound oligonucleotide.

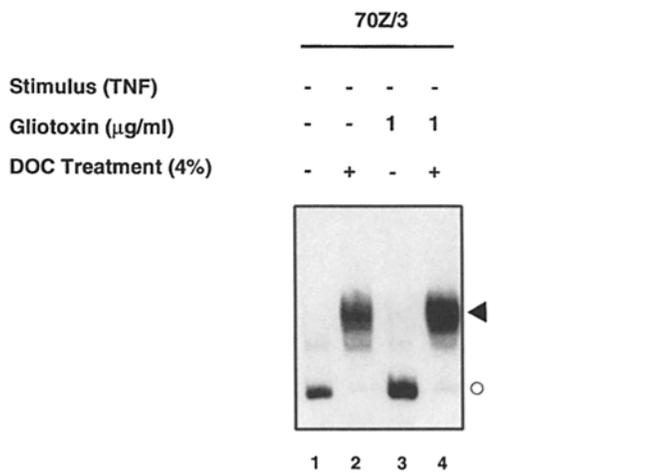


Figure 8. Activation of NF- κ B DNA binding by DOC sodium deoxycholate treatment. 70Z/3 pre-B cells were treated with 1 $\mu\text{g/ml}$ gliotoxin (lanes 3 and 4) or left untreated (lanes 1 and 2). In lanes 2 and 4 the detergent DOC was added to cell extracts for 15 min to a final concentration of 0.4%. Subsequently, the cell extracts were analyzed for NF- κ B activity by EMSA. A section of a fluorogram is shown. (\blacktriangleleft) Position of NF- κ B DNA complexes. (\circ) A nonspecific activity binding to the probe.

I κ B is readily detected in Western blots as a slight decrease in protein mobility. Jurkat T cells were treated with gliotoxin for 1 h and stimulated with 0.5 μM okadaic acid for 60 min. I κ B- α was detected on Western blots using a polyclonal antibody (12). In control cells, okadaic acid stimulation led to both a decreased mobility of I κ B- α and a reduction in the total amount of I κ B (Fig. 10, top, lane 2), which coincided with NF- κ B activation in EMSAs (bottom, lane 2). In gliotoxin-treated cells, the inducible mobility decrease of I κ B- α in response to okadaic acid is still observed (Fig. 10, top, lane 4), indicating that the toxin did not inhibit the I κ B- α kinase. However, more phosphorylated I κ B- α was present and, consequently, less NF- κ B activated (Fig. 10, bottom, lane 4) than under control conditions (compare lanes 2 and 4), suggesting that gliotoxin interfered with I κ B- α degradation.

Discussion

Invasive aspergillosis represents one of the most difficult therapeutic problems in immunocompromised patients. De-

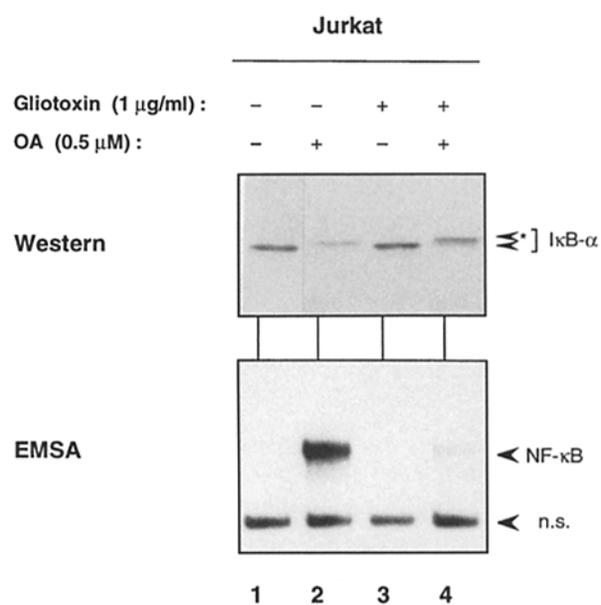


Figure 10. The effect of gliotoxin on I κ B- α phosphorylation and proteolytic degradation. Jurkat T cells were treated with 1 $\mu\text{g/ml}$ gliotoxin for 1 h before stimulation with 0.5 μM okadaic acid for 60 min. (Top) Cell extracts were analyzed by SDS-PAGE and Western blotting with an anti-I κ B- α polyclonal antibody. (Bottom) Extracts were analyzed for NF- κ B activity by EMSA. A section of a fluorogram is shown. (\blacktriangleleft) Position of NF- κ B DNA complexes and nonspecific binding to the DNA probe (n.s.)

spite therapy with the antifungal agent Amphotericin B, mortality rates range between 75 and 100% (56). This challenge is compounded by the lack of knowledge about the molecular mechanism of *Aspergillus* pathology. The secondary fungal metabolite gliotoxin, synthesized by *A. fumigatus* and other pathogenic fungi, can be isolated from peritoneal lavages of mice undergoing systemic experimental aspergillosis (3). To date, however, gliotoxin has not been implicated directly in the pathogenesis of aspergillosis and its mechanism of action remains a matter of debate (57, 58).

We show here that gliotoxin inhibits the activation of transcription factor NF- κ B, a central mediator of the human immune response. NF- κ B activates the transcription of various cytokine genes including those for IL-1, IL-2, GM-CSF, G-CSF, M-CSF, and IL-6 (10). Gliotoxin inhibits

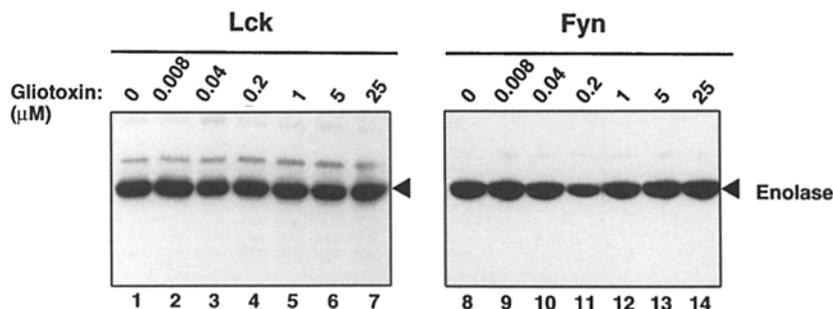


Figure 9. The effect of gliotoxin on the activity of p56^{lck} and p59^{fyn}. Jurkat cells were treated with various concentrations of gliotoxin as indicated for 1 h before stimulation with 50 ng/ml PMA for 10 min. Cell extracts were immunoprecipitated with antibodies against the kinases p56^{lck} and p59^{fyn}. Precipitated proteins were incubated with rabbit muscle enolase and γ -[³²P]ATP for 15 min after which the reaction products were analyzed by SDS-PAGE.

IL-1 synthesis in macrophages and the release of IL-2 from activated T cells (6, 59). These effects can now be explained by the inhibition of NF- κ B activation. Similarly, gliotoxin treatment of stimulator cells prevents the induction of alloreactive cytotoxic T cells in MLCs. This most likely results from the loss of NF- κ B-mediated IL-2 expression (60), since the inhibition can be overcome by the exogenous addition of IL-2. Gliotoxin's inhibition of mitogen-activated T and B cell proliferation (45) may also be a consequence of NF- κ B inhibition: NF- κ B is induced by many B and T cell mitogens such as LPS, Con A, anti-CD28, anti-CD3, anti-IgM, and lymphotropic viruses (10). By inhibiting NF- κ B activation, gliotoxin may interrupt one signal transduction pathway leading to T and B cell proliferation. The selective delay in the recovery of LPS-responsive B cells in gliotoxin-treated mice (5) can be explained in the same manner.

The mechanism of action by which gliotoxin exerts its effect remains a matter of debate. It has been shown that gliotoxin causes DNA fragmentation in vitro and in vivo. In vivo, treatment of L929 fibroblasts, a sheep squamous cell carcinoma line, macrophages, or spleen cells with 30 ng-1 μ g/ml of gliotoxin for 1 h causes DNA fragmentation (9). Other cell types, for example the monkey kidney cell line Vero, the rat mammary carcinoma MAT, or the mouse mastocytoma cell line P815 are resistant to gliotoxin-induced DNA damage. In vitro, gliotoxin shows no effect on DNA on its own; high concentrations of the toxin (0.3 mM or 90 μ g/ml) need to be incubated with reductants such as DTT or glutathione in order to produce DNA damage (52).

Eichner et al. (52) proposed that the DNA damage is caused by the production of H₂O₂ through redox-cycling of gliotoxin. In this model, gliotoxin undergoes intracellular processing to the dithiol derivative, generating a redox cycle that produces superoxide anions and ultimately, H₂O₂. This hypothesis has been contradicted by Schweizer and

Richter (57), who find no evidence for either redox-cycling or H₂O₂ production by gliotoxin in isolated mitochondria. Rather, they propose that gliotoxin reacts with thiol groups to cause a transient release of Ca²⁺ from mitochondria. Contrary to this report, Beaver and Waring (58) found no increase in intracellular Ca²⁺ levels when thymocytes were treated with concentrations of gliotoxin between 50 nM and 10 μ M. Only doses >500 nM produced large increases in intracellular Ca²⁺ levels.

Based on the data presented in this paper, we propose a new model for the mechanism of action of gliotoxin. We suggest that gliotoxin's effects on immune cells are mainly due to the inhibition of NF- κ B activation. An inability to activate NF- κ B is also achieved in transgenic mice, which carry a deletion in the gene for the p65 subunit of NF- κ B. These animals die in utero and show severe apoptosis in the liver (19). It is interesting to note that mice treated with gliotoxin show extensive apoptosis in spleen cells by light and electron microscopy and by DNA fragmentation analysis (5). Both the fetal liver and the adult spleen are hematopoietic organs. Perhaps certain hematopoietic cells require the ability to activate NF- κ B in order to avoid programmed cell death. Spleens from gliotoxin-treated mice are selectively depleted of B cells (5). FACS[®] analysis performed 16 d after gliotoxin treatment identified 37% of the splenocytes as B cells whereas in control animals, 70% of the population were stained with a B cell marker. It appears that the inability to activate NF- κ B leads to apoptosis in B cells.

We propose the following model for the mechanism of action of the fungal metabolite gliotoxin. By inhibiting activation of the transcription factor NF- κ B, gliotoxin abrogates B and T cell stimulation, causing a severe immunosuppression. Furthermore, the inability to activate NF- κ B may cause apoptosis of B cells, compounding the immunosuppressive effect. Our data suggest a direct role for gliotoxin in the pathology of *Aspergillosis*.

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Address correspondence to Dr. Heike L. Pahl, Institute of Biochemistry, Albert-Ludwigs University, Hermann-Herder-Str. 7, D-79104 Freiburg, Germany. Dr. Baeuerle's present address is Tularik Inc., South San Francisco, CA 94080.

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