Brief Definitive Report

The Effect of Immunosuppressive Agents on the Induction of Nuclear Factors that Bind to Sites on the Interleukin 2 Promoter

By Angela Granelli-Piperno, Patricia Nolan, Kayo Inaba, and Ralph M. Steinman

From the Laboratory of Cellular Physiology and Immunology, and Irvington Institute, The Rockefeller University, New York, New York 10021

Summary

Cyclosporin A (CSA), FK506, and glucocorticosteroids all inhibit the production of lymphokines by decreasing lymphokine gene expression. Previous experiments have defined six different sites that may contribute to the transcriptional control of the interleukin 2 (IL-2) promoter, and for each, active nuclear binding factors are induced upon mitogenic stimulation. While dexamethasone markedly blocks the increase in IL-2 mRNA in stimulated human blood T cells, we found that the drug does not block the appearance of factors that bind to the transcriptional control sites termed AP-1, AP-3, NF-kB, OCT-1, B site, and NF-AT. In contrast, both CSA and FK506 have similar effects: the drugs cause modest decreases in AP-3 and NF-kB, and marked decreases in the activity of AP-1 and NF-AT. Therefore, CSA and FK506, while chemically different, seem to act upon a similar pathway that leads to IL-2 gene expression, whereas glucocorticoids do not affect this pathway.

A variety of immunosuppressants block T cell prolifera tion and lymphokine production. A major site of action of these immunosuppressive drugs is at the level of lymphokine gene expression. This was noted first for cyclosporin A (CSA) (1–4), which acts primarily at the level of lymphokines rather than other components of T cell activation, such as the p55 IL-2R and c-*fos* (2). Two other drugs, dexamethasone and FK506, also act primarily at the level of IL-2 gene expression (5–7). It is likely that glucocorticoids have a different mechanism of action than CSA and FK506, since steroids affect many cell types, whereas CSA and FK506 are more T cell restricted.

To gain more insight into the mechanism of action of immunosuppressive drugs, we have taken advantage of recent progress in defining nuclear factors that bind to the IL-2 promoter. An activation-dependent enhancer within sequences −326 to −52 of the 5′ flanking region of the IL-2 gene has been identified (for review see reference 8). In this enhancer reside several elements common to other genes, like the NFkB, AP-1, AP-3, and OCT-1 sites, as well as a site that seems restricted to activated lymphoid cells and is called the nuclear factor for activated T cells (NF-AT) (9). Here, we report the induction of these nuclear factors in primary populations of human blood T cells. We show that FK-506 and CSA markedly inhibit the activation of factors that bind to the AP-1 and NF-AT sites, whereas dexamethasone has no effect on all six nuclear binding factors tested.

Materials and Methods

Cell Cultures. Briefly, human mononuclear cells were isolated from buffy coats on Ficoll-Hypaque density gradients, washed in PBS, and rosetted with neuraminidase-treated sheep erythrocytes. The rosette-positive fraction was further purified by passage over a nylon wool column and used as a source of T cells. T cells were cultured at 5 × 10^6/ml in RPMI 1640 supplemented with 10% heat-inactivated FCS, 20 μg/ml gentamicin sulfate, and 5 × 10^{-5} M 2-ME. Cells were stimulated with PHA (Gibco Laboratories, Grand Island, NY) at 1 μg/ml and PMA (Sigma Chemical Co., St. Louis, MO) at 5 ng/ml in the presence or absence of CSA or CSH (Sandoz, Basel, Switzerland) at 1 μg/ml; FK506 (Fujisawa Pharmaceutical Co. Ltd., Osaka, Japan) at 100 ng/ml; or dexamethasone (Sigma Chemical Co.) at 10^{-7} M.

Nuclear Extracts. These were prepared from 2–4 × 10^8 T cells by homogenization in two-cell pellet volumes of 10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, and 10% glycerol (10). Nuclei were centrifuged at 1,000 *g* for 5 min, washed, and resuspended in two volumes of the above solution. 3 M KCl was added drop by drop to reach 0.39 M KCl. Nuclei were extracted at 4°C for 1 h and centrifuged at 100,000 *g* for 30 min. The supernatants were dialyzed in 20 mM Hepes, pH 7.9, 50 mM KCl, 20% glycerol, 0.5 mM PMSF, and 1 mM EDTA, and then clarified by centrifugation and stored at −80°C. Protein concentration was determined using the Bradford method.

DNA-Protein Binding Assay. 0.2 ng (~10^4 cpm) of end-labeled DNA fragments were incubated at room temperature for 20 min with 5–10 μg of nuclear protein in the presence of 2 μg Poly(dI-

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/90/12/1869/04 $2.00
Volume 172 December 1990 1869–1872

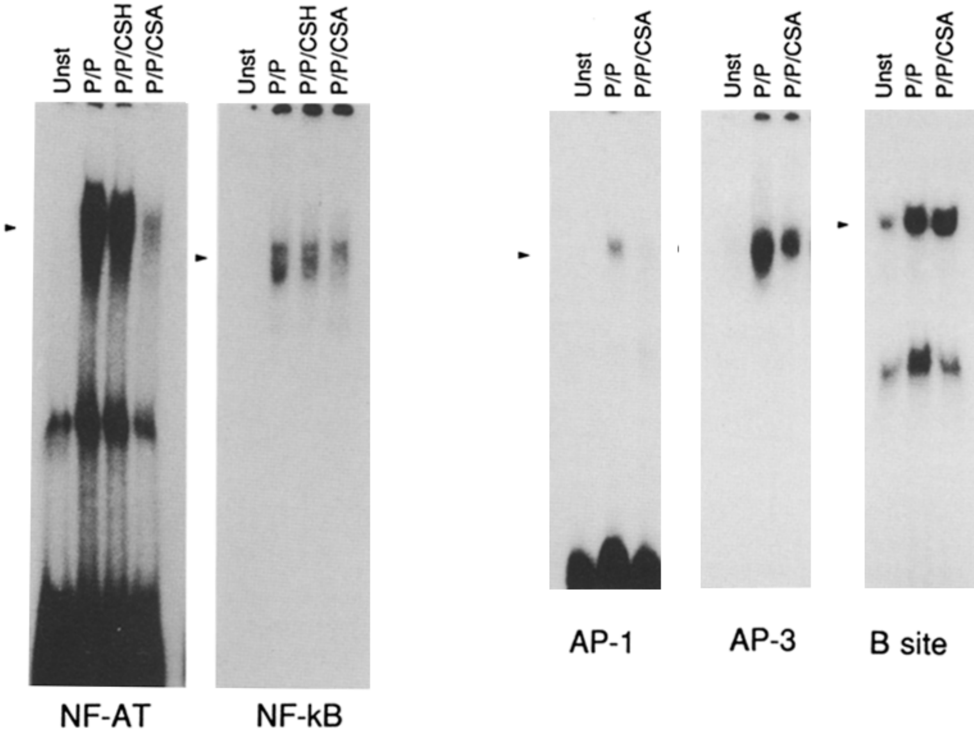

Figure 1. Effects of CSA on the induction of nuclear factors that bind to elements of the IL-2 enhancer. Human blood T cells were left unstimulated (*Unst*) or were stimulated with PHA (1 μg/ml) and PMA (5 ng/ml) (*P/P*) in the presence or absence of CSA or the nonimmunosuppressive analogue CSH (1 μg/ml). After 5 h, the cells were collected and nuclear extracts prepared. 10 μg of nuclear proteins were incubated with 0.2 ng of the indicated ^{32}P end-labeled oligonucleotides. Protein-DNA complexes (*arrows*) were separated from free oligonucleotide (lower part of each lane) in low ionic strength 4% polyacrylamide gels.

dC) in 20 μl of 10 mM Tris HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, and 5% glycerol (this buffer was used for NF-kB, NF-AT, and B sites). For AP-1, AP-3, and OCT-1 sites, the buffer was 20 mM Hepes, pH 7.9, 4% Ficoll, 2.5 mM $MgCl_2$, 1 mM DTT, and 40 mM KCl. Protein-DNA complexes were separated from free probe on a 4% polyacrylamide gel in 0.25× TBE at 150 V for 1.5 h at room temperature. The gels were dried and exposed to X-ray film. For each site, we verified that a 20-fold molar excess of specific cold oligonucleotide would compete the binding of proteins to a radiolabeled probe, whereas a similar excess from another site would not.

DNA Probes. Oligonucleotides were synthesized by The Rockefeller University Facility with a DNA synthesizer (Applied Biosystems, Inc., Foster City, CA). The following probes were used: the NF-AT binding site, −254 to −285 of the IL-2 promoter, 5′-GGAGGAAAAACTGTTTCATACAGAAGGCGT-3′ (9); the NF-kB binding site, −206 to −195 region of the IL-2 promoter, 5′-GGGATTTCACCT-3′ (11); the OCT-1 binding site, 5′-ACTCTTCACCTTATTTGCATAAGCGATTCTA-3′, used for the purification of the OTF-1 transcription factor and kindly provided by Dr. N. Heintz (The Rockefeller University); the AP-1 binding site, −157 to −140 of the IL-2 promoter, 5′-TTCCAAAGAGTCAT-

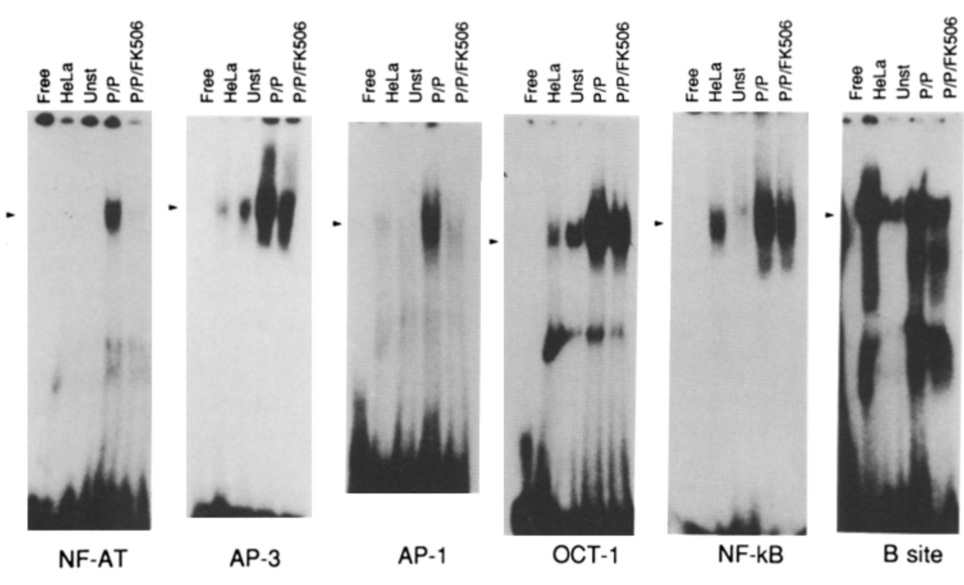

Figure 2. Effects of FK506 on the induction of nuclear factors that bind to elements of the IL-2 enhancer. The experiments were the same as those in Fig. 1, except that FK506 (100 ng/ml) was used as the immunosuppressant, and nuclear extracts from HeLa cells were also tested. Arrows indicate the specific DNA-protein complexes.

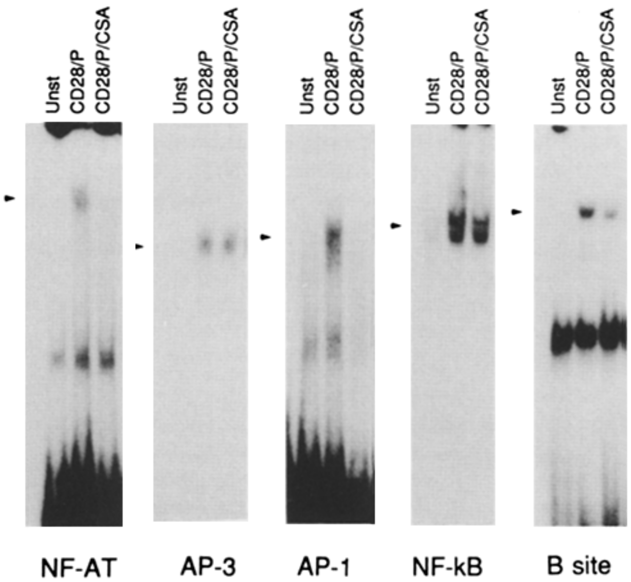

Figure 3. Effects of CSA on the induction of nuclear factors in response to mitogenic anti-T cell mAb. The experiments were the same as those in Fig. 1, except that an anti-CD28 mAb, 9.3 (ascites, kindly provided by Dr. P. Martin, Seattle, WA, and used at 1:1,000), was used instead of PHA as the mitogen.

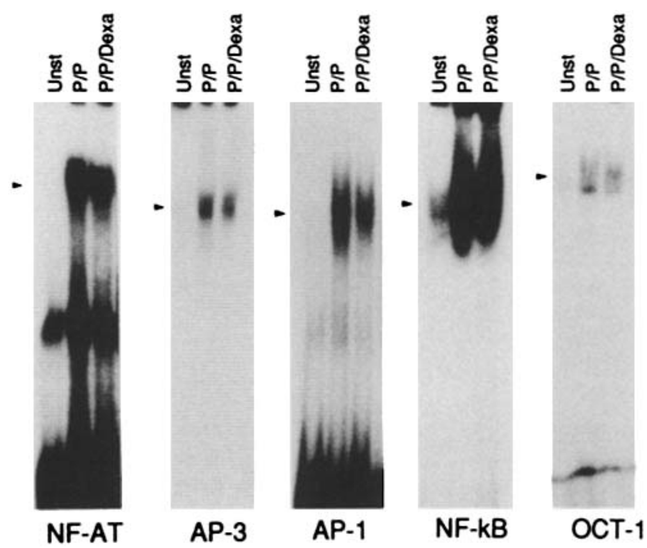

Figure 4. Effects of dexamethasone on the induction of nuclear factors that bind to elements of the IL-2 enhancer. The experiments were the same as those in Fig. 1, except that dexamethasone (10^{-7} M) was used as the immunosuppressive. Arrows indicate the specific DNA-protein complexes.

CAG-3′ (11); the AP-3 binding site used for the purification of AP-3, 5′-TGTGGAAAGTCCCA-3′ (12); and the B site, −82 to −67 of the IL-2 promoter, 5′-TAATATGTAAAACATT-3′ (13). Probes were labeled with [^{32}P]ATP.

Results and Discussion

DNA-nuclear protein interactions were monitored with standard electrophoretic mobility shift assays (EMSA). Extracts of nuclei from mitogen stimulated T cells were prepared 5 h after application of the mitogen in the presence or absence of an immunosuppressive drug. Resting T cells did not contain active factors that bind to the NF-AT, NF-kB, AP-1, AP-3, and B sites, but these activities were induced by stimulation with PHA and PMA (Fig. 1). The immunosuppressive CSA, but not the nonimmunosuppressive analogue CSH, markedly inhibited the induction of NF-AT and AP-1, but only partially blocked induction of NF-kB and AP-3 (Fig. 1). Inhibition by CSA of NF-AT was also observed in stimulated Jurkat cells (14). The fact that CSA primarily acts on NF-AT and AP-1 is of interest. Both are distinct from other transcription factors in requiring new protein synthesis as well as two signals, in this case lectin plus PMA (Granelli-Piperno, A., manuscript submitted for publication).

We next examined the effect of another immunosuppressive drug, FK506. FK506, like CSA, inhibits T cell proliferation and IL-2 gene expression (5), which we confirmed. FK506 proved to be similar to CSA at the level of nuclear transcription factors. The inductions of NF-AT and AP-1 were markedly reduced, whereas AP-3, NF-kB, OCT-1, and the factor that binds to the B site were not (Fig. 2). We simultaneously evaluated extracts of HeLa cell nuclei. These contained all the nuclear factors that were inducible in T cells, except for NF-AT, thus confirming that NF-AT is at T cell–restricted activity.

In additional experiments, CSA and FK506 blocked the induction of nuclear factors in response to the other mitogens, anti-CD3 (not shown) or anti-CD28 mAb (Fig. 3). Some authors find that stimulation with anti-CD28 is CSA resistant (15), but we noted that CSA reduced the induction of IL-2 mRNA as well as nuclear factors that bind the IL-2 promoter (16) (Fig. 3).

We last tested dexamethasone, a glucocorticosteroid that also inhibits the increase in IL-2 mRNA that occurs during mitogenesis (6, 7). While the drug clearly blocks the induction of IL-2 mRNA (not shown), dexamethasone did not alter the induction of any of the factors that bind to the elements we have examined (Fig. 4). These data indicate that the IL-2 transcriptional control pathway that is suppressed by glucocorticoids is different from that of CsA and FK506.

This work was supported by grant GM-37643 from the National Institutes of Health to A. Granelli-Piperno.

Address correspondence to Angela Granelli-Piperno, Laboratory of Cellular Physiology and Immunology, Box 280, The Rockefeller University, 1230 York Avenue, New York, NY 10021.

Received for publication 7 August 1990.

References

1. Granelli-Piperno, A., K. Inaba, and R.M. Steinman. 1984. Stimulation of lymphokine release from T lymphoblasts: requirement for mRNA synthesis and inhibition by Cyclosporin A. *J. Exp. Med.* 160:1792.
2. Granelli-Pipeno, A., L. Andrus, and R.M. Steinman. 1986. Lymphokine and nonlymphokine mRNA levels in stimulated human T cells: kinetics, mitogen requirements, and effects of cyclosporin A. *J. Exp. Med.* 163:922.
3. Kronke, M., W.J. Leonard, J.M. Depper, S.K. Arya, F. Wong-Staal, R.C. Gallo, T.A. Waldman, and W.C. Greene. 1984. Cyclosporin A inhibits T-cell growth factor gene expression at the level of mRNA transcription. *Proc. Natl. Acad. Sci. USA.* 81:5214.
4. Elliot, J.F., Y. Lin, S.B. Mizel, R.C. Bleakley, D.G. Harnish, and V. Paetkau. 1984. Induction of interleukin 2 messenger RNA inhibited by Cyclosporin A. *Science (Wash. DC).* 226:1439.
5. Tocci, M.J., D.A. Matkovick, K.A. Collier, P. Kwok, F. Dumont, S. Lin, S. Degudicibus, J.J. Siekierka, J. Chin, and N.I. Hutchinson. 1989. The immunosuppressant FK506 selectively inhibits expression of early T cell activation genes. *J. Immunol.* 143:718.
6. Arya, S.K., F. Wong-Staal, and R.C. Gallo. 1984. Dexamethasone-mediated inhibition of human T cell growth factor and γ-interferon messenger RNA. *J. Immunol.* 133:273.
7. Vacca, A., S. Martinotti, I. Screpanti, M. Maroder, M.P. Felli, A.R. Farina, A. Gismondi, S. Santoni, L. Frati, and A. Gulino. 1990. Transcriptional regulation of the interleukin 2 gene by glucocorticoid hormones. *J. Biol. Chem.* 265:8075.
8. Crabtree, G.R. 1989. Contingent genetic regulatory events in T lymphocyte activation. *Science (Wash. DC).* 243:355.
9. Shaw, J.P., P.J. Utz, D.B. Durand, J.J. Toole, E.A. Emmel, and G.R. Crabtree. 1988. Identification of a putative regulator of early T cell activation genes. *Science (Wash. DC).* 241:202.
10. Dignam, J.D., R.M. Lebovitz, and R.G. Roeder. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* 11:1475.
11. Holbrook, N.J., M. Lieber, and G.R. Crabtree. 1984. DNA Sequence of the 5′ flanking region of the human interleukin 2 gene: homologies with adult T-cell leukemia virus. *Nucleic Acids Res.* 12:5005.
12. Chiu, R., M. Imagawa, R.J. Imbra, J.R. Bockoven, and M. Karin. 1987. Multiple cis- and trans-acting elements mediate the transcriptional response to phorbol esters. *Nature (Lond.).* 329:648.
13. Nabel, G.J., C. Gorka, and D. Baltimore. 1988. T-cell-specific expression of interleukin 2: evidence for a negative regulatory site. *Proc. Natl. Acad. Sci. USA.* 85:2934.
14. Emmel, E.A., C.L. Verweij, D.B. Durand, K.M. Higgins, E. Lacy, and G.R. Crabtree. 1989. Cyclosporin A specifically inhibits function of nuclear proteins involved in T cell activation. *Science (Wash. DC).* 246:1617.
15. June, C.H., J.A. Ledbetter, M.M. Gillespie, T. Lindsten, and C.B. Thompson. 1987. T-cell proliferation involving the CD28 pathway is associated with cyclosporine-resistant interleukin 2 gene expression. *Mol. Cell. Biol.* 7:4472.
16. Granelli-Piperno, A. 1988. In situ hybridization for interleukin 2 and interleukin 2 receptor mRNA in T cells activated in the presence or absence of cyclosporin A. *J. Exp. Med.* 168:1649.