JOURNAL OF VIROLOGY, Nov. 1992, p. 6288–6293
0022-538X/92/116288-06$02.00/0

Vol. 66, No. 11

Antioxidants Selectively Suppress Activation of NF-κB by Human T-Cell Leukemia Virus Type I Tax Protein

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Received 19 May 1992/Accepted 24 July 1992

Oxygen radical scavengers, such as dithiocarbamates and cysteine derivatives, inhibit activation of the ubiquitous transcription factor nuclear factor κB (NF-κB) after treatment of cells with tumor necrosis factor, phorbol ester, and interleukin-1. An involvement of oxygen radicals was more directly evident from the induction of NF-κB by low concentrations of H_2O_2 and the demonstration that cells stimulated with various NF-κB inducers release H_2O_2 and superoxide. In this study, we used the antioxidant pyrrolidine dithiocarbamate (PDTC) to investigate whether the activation of NF-κB by the viral transactivator Tax from human T-cell leukemia virus type I also depends on the production of reactive oxygen intermediates. The Tax-induced activation of the DNA-binding activity of NF-κB in Jurkat T cells was potently suppressed by micromolar concentrations of PDTC. Within the same concentration range, PDTC and two other dithiocarbamates also strongly interfered with transactivation of the long terminal repeat (LTR) of human immunodeficiency virus type 1 by Tax but had no effect on transactivation of the same LTR by Tat. Transactivation of the human T-cell leukemia virus type I LTR by Tax was also barely influenced. Tax seems to activate NF-κB by a mechanism shared with all other inducers of NF-κB tested so far. It appears that one of the pleiotropic activities of Tax leads to an enhanced production of oxygen radicals that are required for activation of NF-κB.

Nuclear factor κB (NF-κB) is a transcriptional activator important for the expression of human immunodeficiency virus type 1 (HIV-1) upon T-cell-activating stimuli (14, 28). Most of the target genes of NF-κB in T cells and other cell types encode proteins involved in immune, inflammatory, and acute-phase responses (for reviews, see references 3 and 6). A cytoplasmic form of NF-κB is stabilized by an inhibitory subunit called IκB (4, 5). Stimulation of cells with a wide variety of pathogens and inflammatory cytokines releases IκB and allows mobilization of the factor via nuclear uptake and DNA binding of a heterodimer of p50 and p65 subunits (49). NF-κB shares DNA binding and inhibitory subunits with the proto-oncogene c-Rel, suggesting that NF-κB and c-Rel constitute one transcription factor system (8, 16, 20).

Among the various conditions that induce NF-κB in T cells is oxidative stress as generated by the presence of micromolar concentrations of H_2O_2 in cell cultures (37). Agents known to scavenge oxygen radicals, such as dithiocarbamates, cysteine and its derivatives, and agents interfering with the synthesis of hydroxyl radicals (e.g., iron chelators), can potently suppress the induction of NF-κB by H_2O_2 (36). *N*-Acetyl-L-cysteine, cysteine, and pyrrolidine dithiocarbamate (PDTC) were also shown to suppress activation of NF-κB by tumor necrosis factor (TNF), interleukin-1, phorbol 12-myristate 13-acetate (PMA), double-stranded RNA, cycloheximide, and lectin (26, 36, 37, 41). This suggested that oxygen radicals are used as intracellular messengers for the activation of NF-κB by all inducers investigated so far (for a review, see reference 35).

The antioxidant diethyl dithiocarbamate (DDTC) is currently being tested in clinics to determine whether it can slow the progression of AIDS (17, 31). In cell culture systems, a 100 μM concentration of the pyrrolidine derivative (PDTC) was sufficient to almost completely suppress the activation of NF-κB and the expression of HIV-1 long terminal repeat (LTR)-controlled reporter genes following TNF or PMA-lectin treatment of cells (36). The activity of other factors and the activation of reporter genes not controlled by NF-κB were not affected by the agent. Because PDTC did not interfere with the DNA binding and nuclear translocation of activated NF-κB, it most likely prevented a reaction required to release IκB from the cytoplasmic complex of NF-κB. It is not known where PDTC acts in the cell.

The transactivator Tax from human T-cell leukemia virus type I (HTLV-I) (7, 22, 32), the X protein from hepatitis B virus (38, 47, 48), and the IE1 protein from cytomegalovirus (CMV) (34) have been described as activators of NF-κB, but the mechanism by which these proteins mobilize the cytoplasmic form of NF-κB is not yet understood. In this report, we show that the mobilization of NF-κB in response to the expression of the Tax protein is blocked by micromolar concentrations of PDTC. The same doses of PDTC also suppressed transactivation of the HIV-1 LTR by Tax. However, the drug still allowed a strong induction of the HTLV-I LTR by Tax and of the HIV-1 LTR by Tat and had no influence on basal activities of reporter constructs controlled by factors other than NF-κB. The antioxidant PDTC seemed to selectively block only one of the pleiotropic activities of Tax. We discuss the possibility that Tax can induce a prooxidant state in cells.

MATERIALS AND METHODS

Cell culture and transfection. The J6 subclone of Jurkat T cells was obtained from the European Collection of Animal Cell Cultures (ECACC 88052401). Cells were grown in RPMI

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1640 medium supplemented with 10% fetal calf serum and 1% (wt/vol) penicillin-streptomycin (all purchased from GIBCO Laboratories). Transfections were performed by the DEAE-dextran method, and chloramphenicol acetyltransferase (CAT) activity was determined as described earlier (15, 30).

Oligonucleotides and plasmids. Oligonucleotides were synthesized as described recently (37). The sequences of the κB and octamer oligonucleotide probes as well as the ^{32}P-labeling procedures are detailed elsewhere (37). The CAT reporter constructs HIV-1-LTR-CAT (28) and HIV-1-LTRmu-CAT (29) contained sequences of the HIV-1 LTR from −453 to +80. The construction of the HTLV-I-LTR CAT reporter construct, pU3R-I CAT (40), and of the Tax expression plasmid pHISLdsph were described in detail previously (15a). The Tat expression plasmid pCT21 (1) was constructed by insertion of a *SalI-BamHI* fragment from pCV-1 (1a) into a vector containing CMV immediate-early promoter-enhancer sequences and a simian virus 40 polyadenylation signal. The IE1 expression plasmid SVCC2 contains promoter regulatory and coding sequences from the IE1 gene of CMV (43) and was kindly provided by M. Stinski.

Electrophoretic mobility shift assays (EMSAs). Cells were fractionated, and nuclear extracts were prepared as described previously (4). Binding conditions for mobility shift assays, native-gel electrophoresis, and purification of NF-κB were previously described in great detail (52). Binding reactions with nuclear extracts contained 2 μg of poly(dI-dC). Further details are given in the figure legends. The antiserum against the p50 subunit of NF-κB was a kind gift from A. Israel.

RESULTS

PDTC blocks the activation of NF-κB by Tax. Jurkat T cells were transfected with an expression plasmid encoding the Tax protein from HTLV-I. Consistent with earlier reports (7, 22, 32), expression of Tax strongly induced a κB-specific DNA-binding activity which was detectable in nuclear extracts by EMSAs (Fig. 1A; compare lanes 1 and 2). The induced complex comigrated with that of purified NF-κB containing p50 and p65 subunits (Fig. 1B; compare lanes 1 and 2). It was partially abrogated in the presence of an antiserum specific for the p50 subunit of NF-κB, and a novel, more slowly migrating complex appeared (Fig. 1B, lane 4). The remaining complex could contain c-Rel and p65, which do not cross-react with the p50 antiserum (2). Treatment of Tax-expressing Jurkat T cells with 40 or 90 μM PDTC led to a dose-dependent reduction of the amount of NF-κB-specific complex(es) (Fig. 1A, lanes 3 and 4). A 90 μM concentration of PDTC suppressed the activity of NF-κB to a level seen in mock-transfected cells (Fig. 1A; compare lanes 1 and 4), and 40 μM suppressed the activity to approximately 50%, as determined by Cerenkov counting of the radioactivity in protein-DNA complexes. This effect was not due to inhibition of the DNA binding activity of NF-κB because the NF-κB in nuclear extracts from Tax-induced cells was insensitive to PDTC (data not shown; see also reference 36). Treatment with pyroglutamic acid, a cyclic molecule structurally related to PDTC, had no effect (Fig. 1A, lane 5). PDTC had no significant influence on other activities nonspecifically binding to the κB probe (Fig. 1A) or on complexes formed with a ^{32}P-labeled DNA probe containing an octamer protein binding motif (Fig. 1C). The latter experiment showed that nuclear extracts contained the same amounts of protein and had identical integrities. In conclusion, the data demonstrate that the antioxidant PDTC can block the mobilization of NF-κB by Tax.

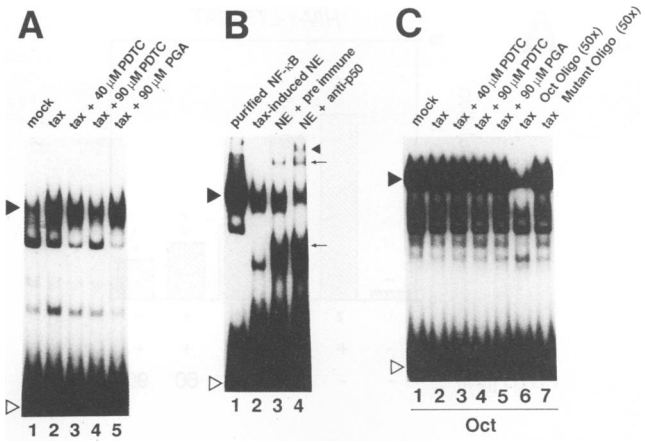

FIG. 1. Effect of PDTC on the activation of NF-κB by Tax. (A) Dose-dependent suppression of NF-κB activation by PDTC. Human Jurkat T cells were mock transfected (lane 1) or transfected with a Tax expression plasmid (7.5 μg; lanes 2 to 5). The indicated concentrations of PDTC or pyroglutamic acid (PGA) were added 20 to 24 h after transfection to cultures for 10 h. Thereafter, nuclear extracts were prepared and analyzed by EMSA by using a ^{32}P-labeled κB oligonucleotide as described previously (52). Fluorograms of native gels are shown. (B) Characterization of the Tax-induced complex. The nuclear extracts (NE) from Tax-expressing Jurkat cells (lane 2) were comigrated with purified human NF-κB (approximately 50 pg; lane 1). A preimmune serum (lane 3) or an antiserum specific for the p50 subunit of NF-κB (1.5 μl each; lane 4) was incubated with the nuclear extract. Small, filled arrowhead on the right, position of a novel, highly retarded complex. Arrows, positions of two novel complexes also seen with the preimmune serum. (C) Analysis of nuclear extracts with a DNA probe detecting octamer-binding proteins. The same extracts tested in panel A by EMSA with a κB probe were analyzed here with an octamer probe (Oct). Octamer-specific complexes were evident from competition with a 50-fold molar excess of unlabeled octamer probe (lane 6) and of a nonspecific κB mutant oligonucleotide (lane 7). Filled arrowhead, κB-specific complex (A and B) and octamer-specific complex (C); open arrowhead, unbound DNA probe.

PDTC blocks induction of the HIV-1 LTR by Tax. One target element for Tax is the LTR of HIV-1 (12, 39, 53). The transactivation of the LTR by Tax was shown to rely on intact NF-κB binding sites. In our experiments, cotransfection of a construct of an HIV-1 LTR-controlled CAT reporter gene with the Tax expression plasmid caused a 21-fold induction of CAT activity in Jurkat T cells (Fig. 2A). Treatment of cells with increasing amounts of PDTC led to a dose-dependent decrease of this CAT enzyme induction. The dose response paralleled that seen with the suppression of the DNA binding activity of NF-κB by PDTC (Fig. 1A). In the presence of 90 μM PDTC, the CAT induction was blocked by 80% (Fig. 2A). In order to test whether this effect of PDTC was dependent on κB sites, we tested the effect of PDTC on the activity of the HIV-1 LTR reporter construct in uninduced Jurkat cells. In contrast to the situation in Tax-induced cells, the basal activity of the construct was reproducibly enhanced when cells were treated with 30 μM PDTC and barely influenced by a treatment with 90 μM PDTC (Fig. 2B). PDTC also did not suppress the activity of an HIV-1 LTR reporter construct in which both NF-κB binding sites are inactivated by mutation (Fig. 2B). This mutant construct

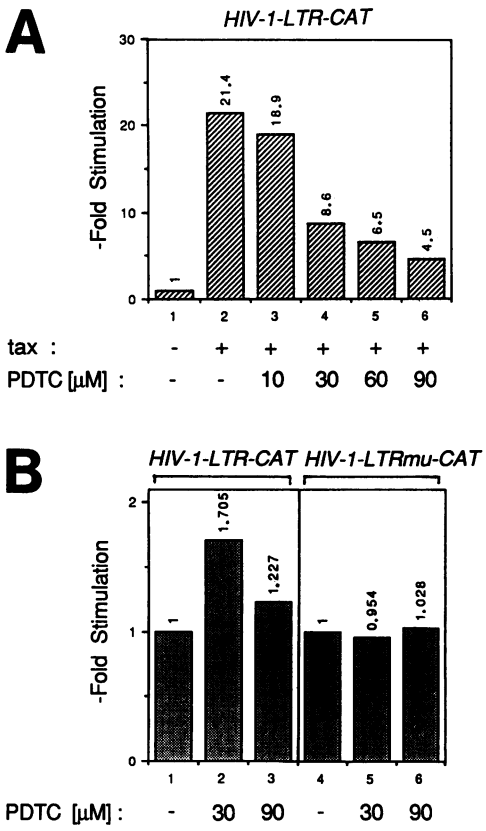

FIG. 2. Effect of PDTC on the activity of HIV-1-LTR-controlled reporter genes. (A) Effect of PDTC on gene induction by Tax. Jurkat T cells were transfected with 7.5 μg of HIV-1-LTR-CAT reporter plasmid alone (column 1) or together with 5 μg of Tax expression plasmid (columns 2 to 6). At 21 to 24 h after transfection, cells were treated for 10 to 14 h with the indicated amounts of PDTC. The CAT activity in column 1 corresponds to a rate of conversion of [^{14}C]chloramphenicol into acetylated forms of 0.2%. (B) The effect of PDTC on basal activities of two HIV-1 LTR reporter plasmids. The plasmid HIV-1-LTRmu-CAT has two mutations abolishing the NF-κB binding sites (1, 27). The two constructs gave rates of chloramphenicol conversion in the absence of PDTC of 4.4% (column 1) and 0.5% (column 4). Mock-transfected cells gave a conversion rate of <0.1% (data not shown). Mean values from two independent transfection experiments are shown. There was less than 10% deviation from the mean.

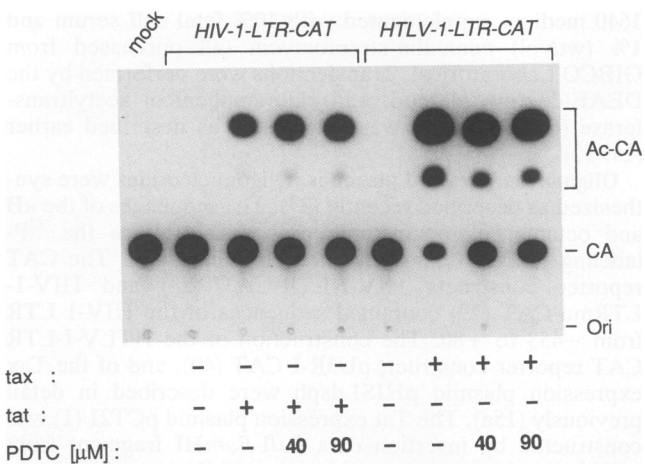

FIG. 3. Effect of PDTC on homologous transactivations by Tat and Tax. Jurkat T cells were mock transfected (first lane) or transfected with 7.5 μg of the indicated CAT reporter plasmids under the control of the HIV-1 LTR (second to fifth lanes) or the HTLV-I LTR (sixth to ninth lanes). Where indicated, 7.5 μg of expression plasmids encoding the HIV-1 transactivator Tat or Tax was cotransfected. For incubation periods, see the legend to Fig. 2. An autoradiogram of a thin-layer chromatography plate is shown. The positions of [^{14}C]labeled chloramphenicol (CA), acetylated forms (Ac-CA; bracket), and the origin (Ori) are indicated at the right. The rate of conversion of chloramphenicol to acetylated forms in mock-transfected cells was 0.14%. Very similar effects were obtained in both laboratories. A representative CAT assay is shown.

is mainly dependent in its activity on three Sp1 binding sites. In conclusion, PDTC selectively suppressed the κB-dependent induction of the HIV-1 LTR by Tax and did not interfere with gene activation governed by other factors.

We also tested the effect of two other dithiocarbamates which have aliphatic side chains different from that of PDTC. One is DDTC, a drug used for decades for the treatment of heavy-metal poisoning and, more recently, tested for suppression of the onset of AIDS symptoms (for a review, see reference 44). The other is disulfiram, a disulfide-linked form of DDTC, which is used to prevent alcoholics from drinking (for a review, see reference 13). At a concentration in cell cultures of 90 μM, both agents could suppress the induction of the HIV-1 LTR by Tax (see Fig. 4). While disulfiram was almost as potent as PDTC, DDTC gave only 50% inhibition. It is possible that these differences came from different half-lives or rates of cellular uptake of the drugs in cultured cells.

Homologous LTR activation by Tax and Tat is not suppressed by PDTC. Another protein strongly enhancing the expression of HIV-1 LTR-controlled genes is the HIV-1 transactivator Tat. This viral protein requires a sequence called TAR, which is downstream from the transcription start site (for a review, see reference 50) and binding sites for the factor Sp1 in the LTR (10, 53). Cotransfection of a Tat expression plasmid with the homologous HIV-1 LTR-controlled CAT reporter gene led to strong induction of CAT activity which was not seen in the absence of Tat (Fig. 3). This transactivation of the LTR by Tat was not suppressed in the presence of 40 or 90 μM PDTC; rather, a slight stimulation was noted. Also, no effect of PDTC on transactivation of a c-*fos* promoter-enhancer by two transactivators from hepatitis B virus, HBx and MHBs^t, was observed (25a).

In order to test whether PDTC interfered simply with expression of the transfected *tax* gene or generally with the biological activity of the Tax protein, we investigated the influence of the antioxidant on the homologous transactivation of the HTLV-I LTR by Tax. Among other elements, three 21-bp repeats in the LTR were found to be required for this activity of Tax. These elements can bind various proteins belonging to the ATF/CREB family of transcription factors (19, 46, 51) and proteins called HEB1 and HEB2 (27) but apparently not NF-κB. While a CAT reporter gene under control of the HTLV-I LTR was fairly inactive in Jurkat T cells, a very strong induction of CAT activity was seen upon cotransfection of the Tax expression plasmid (Fig. 4). In the presence of 40 μM PDTC, the CAT induction was reduced by approximately 30%. Unlike with the HIV-1 LTR, 90 μM PDTC could not further suppress the transactivation of the HTLV-I LTR by Tax. This experiment shows that, firstly, cells treated with PDTC contain biologically active Tax

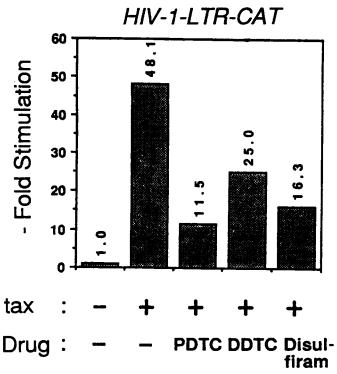

FIG. 4. Effect of various dithiocarbamates on Tax-induced activation of the HIV-1 LTR. For details, see the legend to Fig. 2. DDTC, disulfiram, and PDTC were tested at a concentration of 90 μM PDTC. Assays were performed in duplicate.

protein and that, secondly, PDTC seems to selectively suppress the κB-dependent transactivation by Tax. The findings above are summarized in a model (Fig. 5).

IE1 is an immediate-early transactivator of CMV (for a review, see reference 42). One group reported that IE1 transactivates the CMV enhancer via multiple NF-κB-binding sites (34). Other groups could not confirm this idea when constructs under control of the HIV-1 LTR were used to test the effect of IE1 (9, 11). Unlike expression of Tax (Fig. 1A), transient expression of the IE1 protein under the same conditions barely induced a κB-specific DNA binding activity in Jurkat T cells (data not shown). This explained why there was only a twofold increase in CAT activity from the HIV-1-LTR-CAT reporter after cotransfection with IE1 expression vector. This weak induction was suppressed by 40 or 90 μM PDTC but not by 40 or 90 μM pyroglutamic acid. It is therefore possible that IE1 also (weakly) activates NF-κB by a mechanism shared with Tax and all other inducers of NF-κB tested so far. However, compared with the effects seen with Tax, no safe conclusions can be drawn from the data obtained with IE1.

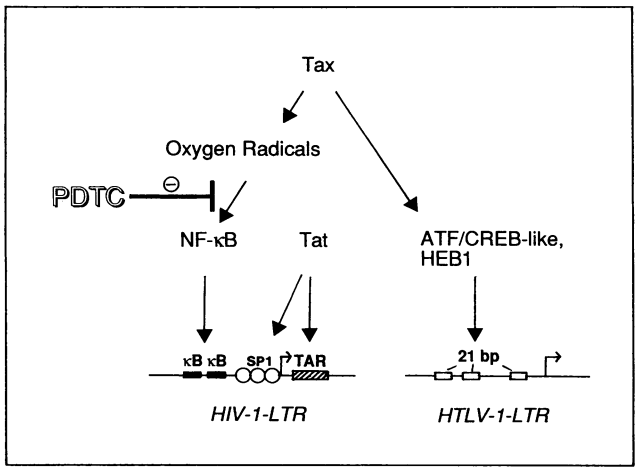

FIG. 5. Model illustrating the selective inhibiting effect of PDTC on the activation of NF-κB by Tax.

DISCUSSION

Recent studies have shown that T cells latently infected with HIV-1 start producing virus when exposed to mild oxidative stress by hydrogen peroxide (21, 37). The treatment leads to the appearance of active NF-κB in nuclei and activates the LTR, depending on its two NF-κB-binding sites. These effects of H_2O_2 are efficiently suppressed by antioxidants (37), suggesting a causal relationship between the activation of NF-κB by oxidative stress and the onset of virus production. The present study demonstrates that PDTC, a potent and well-characterized antioxidant, is a fairly selective inhibitor of the effects of the Tax transactivator from HTLV-I on the activation of NF-κB and subsequent transactivation of the HIV-1 LTR. Thus, it appears that Tax activates NF-κB and the HIV-1 LTR by a mechanism which also depends on reactive oxygen intermediates. Tax could increase the production of hydrogen peroxide, as was observed with the NF-κB inducers TNF, PMA, and interleukin-1 in many different cell types (24, 25, and references therein). By the use of antioxidants, only indirect evidence for this idea can be provided at present. In future studies, more direct support may be obtained by the use of temperature-sensitive Tax mutants or by experiments in which recombinant Tax is added to cell cultures. Production of reactive oxygen species in culture supernatants in response to temperature shift or addition of Tax could then be directly measured.

Tax could induce oxidant stress either by direct activation of radical-producing systems or by downregulation of radical-scavenging enzymes. Alternatively, Tax could activate the expression of a gene encoding, for instance, an NF-κB-activating cytokine. Only the latter mechanism would necessarily depend on new protein synthesis. Recent experiments showed that NF-κB can be activated by addition of recombinant Tax protein to cell cultures (23). This can occur in the presence of protein kinase C inhibitors. Only 15 min after addition of Tax, active NF-κB appeared in nuclei, a period during which cellular uptake of the Tax protein also had to occur. This fast kinetic of activation would be consistent with a direct effect of Tax on the NF-κB–IκB complex which does not require de novo protein synthesis.

If Tax is an inducer of oxidant stress, several observations recently made for HTLV-I and the Tax protein would gain novel aspects. For instance, it was shown that mice transgenic for HTLV-I develop inflammatory arthropathy at an age of 2 to 3 months, correlating with expression of Tax in joints (18). The symptoms were very similar to those observed for rheumatoid arthritis. Rheumatoid arthritis is associated with high levels of tissue-damaging reactive oxygen intermediates, which is why antioxidants are important antirheumatoid drugs. Tax as an inducer of oxidant stress could directly contribute to the production of radicals in the transgenic mouse system.

Ruben and Rosen (33) established Jurkat T-cell lines constitutively expressing Tax. Whereas cells transiently transfected with the *tax* gene showed a strong induction of NF-κB, stably transfected cells displayed only very low levels of NF-κB activity in nuclei. These cells were also desensitized for the induction of NF-κB by PMA and TNF. The latter finding is consistent with the idea that Tax, PMA, and TNF use a common pathway to mobilize NF-κB. In our model, Tax would lead to an elevated production of oxygen radicals. The T cell could counteract the oxidative stress by enhanced production of enzymes eliminating radicals and repairing radical damage, such as glutathion peroxidases and

transferases, thioredoxins, superoxide dismutases, catalases, and peroxidases. The enhanced level of such enzymes in Tax-overexpressing cells would then prevent a permissive production of NF-κB-activating radicals in response to a treatment of cells with PMA and TNF. Support for this idea comes from the observation that Tax was found to induce a protein called adult T-cell leukemia-derived factor, which is equivalent to human thioredoxin (45). Although it is not known whether Tax can induce this protein in a PDTC-sensitive manner, this observation demonstrates that Tax can upregulate an enzyme involved in protecting cells from oxidative damage.

ACKNOWLEDGMENTS

We are grateful to Inge Radant for excellent technical assistance and E.-L. Winnacker for his continuing support.

This work was supported by grants from the Bundesministerium für Forschung und Technologie (P.A.B.) and Deutsche Forschungsgemeinschaft awarded to P.A.B. (SFB 217, Ba-957/1-2) and B.F. (DFG Forschergruppe "DNA Viren des hämatopoetischen Systems").

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