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Transcriptional Activation of the Vascular Cell Adhesion Molecule-1 Gene in T Lymphocytes Expressing Human T-Cell Leukemia Virus Type 1 Tax Protein

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Recruitment and extravasation of T cells through the blood-brain barrier are favored by adhesion molecule-mediated interactions of circulating T cells with endothelial cells. Since a common pathological finding in human T-cell leukemia virus type 1 (HTLV-1)-associated diseases is the infiltration of HTLV-1-infected T lymphocytes into various organs, we have looked for the profile of adhesion molecules expressed by HTLV-1-transformed T cells. Flow cytometry analysis indicated that these cells were expressing high levels of vascular cell adhesion molecule 1 (VCAM-1 [CD106]), a 110-kDa member of the immunoglobulin gene superfamily, first identified on endothelial cells stimulated with inflammatory cytokines. This adhesion molecule was also expressed by T cells obtained from one patient with HTLV-1-associated myelopathy/tropical spastic paraparesis but not by activated T cells isolated from one normal blood donor. The role of the viral *trans*-activator Tax protein in the induction of VCAM-1 was first indicated by the detection of this adhesion molecule on Jurkat T-cell clones stably expressing the *tax* gene. The effect of Tax on VCAM-1 gene transcription was next confirmed in JPX-9 cells, a subclone of Jurkat cells, carrying the *tax* sequences under the control of an inducible promoter. Furthermore, deletion and mutation analyses of the VCAM-1 promoter performed with chloramphenicol acetyltransferase constructs revealed that Tax was *trans* activating the VCAM-1 promoter via two NF-κB sites present at bp −72 and −57 in the VCAM-1 gene promoter, with both of them being required for the Tax-induced expression of this adhesion molecule. Finally, gel mobility shift assays demonstrated the nuclear translocation of proteins specifically bound to these two NF-κB motifs, confirming that VCAM-1 was induced on Tax-expressing cells in a κB-dependent manner. Collectively, these results therefore suggest that the exclusive Tax-induced expression of VCAM-1 on T cells may represent a pivotal event in the progression of HTLV-1-associated diseases.

Most of individuals infected by human T-cell leukemia virus type 1 (HTLV-1) remain asymptomatic, indicating that exposure to the virus leads to persistent infection that rarely results in disease progression. Indeed, only 1 to 5% of infected persons develop either adult T-cell leukemia (ATL) or a nonmalignant neurologic disorder termed HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (13, 24, 39, 41, 50, 54, 56). The mechanisms involved in progression to either disease remain to be clarified, but might include genetic predisposition, age at infection, routes of transmission, and differences of the host immune responses.

The interplay of two viral regulatory proteins, Tax and Rex, strictly regulates HTLV-1 gene expression. Tax, a 40-kDa nuclear protein, increases transcription by acting via 21-bp repeats found in the proviral 5′ long terminal repeat (LTR). Rex, a 27-kDa nuclear/nucleolar protein, acts at the posttranscriptional level via an mRNA Rex-responsive element located in the 3′ LTR to facilitate the export of mRNAs required for the synthesis of viral enzymes and structural proteins (7, 10, 47). In addition, numerous investigations have been devoted to unraveling the effects of Tax in the onset and development of diseases linked to HTLV-1 T-cell infection. Indeed, the *trans*-activating capacity of Tax enables this protein to intervene in the induction and/or the increased expression of a large collection of cellular genes, such as those coding for cytokines, cytokine receptors, proto-oncogenes, and cytoskeletal proteins such as vimentin (10, 12, 55). Tax does not bind DNA directly (2, 38). Indeed, the activation of these cellular genes is correlated with the ability of Tax to interact with the activating transcription factor/cyclic AMP-responsive element-binding (ATF-1/CREB) family and the serum response factor (4, 11). Likewise, by interacting with IκBα or NF-κB precursors, Tax is able to increase the nuclear localization of the NF-κB-related family of transcription factors (3, 17, 34, 49). Thus, by deregulating the transcription of cellular genes involved in T-cell activation and proliferation, Tax is playing a crucial role in viral pathogenesis.

A pathological finding common to ATL and HAM/TSP is the infiltration of lymphocytes into various organs. Indeed, ATL cells are able to invade organs such as the liver, spleen, lungs, and skin (50, 54). Furthermore, infiltration of HTLV-1-infected T cells into the central nervous system may be a hallmark of the evolution of the pathogenic process and may act by triggering the chronic inflammatory lesions observed in HAM/TSP patients (18, 20–22, 25). The emigration of lymphocytes from the periphery has been shown to be dependent on three distinct interactions of adhesion molecules with endothelial cells, selectin-carbohydrate, chemoattractant-receptor, and

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integrin-immunoglobulin (Ig) (for reviews, see references 5 and 48). Recent observations have provided evidence for the Tax-induced expression of adhesion molecules, which might be involved in HTLV-1-infected T-cell infiltration. Thus, it has been observed that Tax is responsible for the upregulation of both intercellular cell adhesion molecule 1 (ICAM-1 [CD54]), a member of the Ig gene superfamily, and lymphocyte function antigen 3 (LFA-3 [CD58]) in HTLV-1-infected T cells (32, 51, 52). The L-selectin gene, which is constitutively overexpressed in fresh ATL cells, can be transactivated by Tax (53). Besides these conventional adhesion models, the OX40/gp34 interactions define another adhesion pathway. Indeed, OX40, a type I transmembrane protein of the tumor necrosis factor (TNF) receptor family expressed by activated T cells, mediates the adhesion of these cells to gp34 (OX40L), present on vascular endothelial cells. Both OX40 and gp34 have been shown to be constitutively expressed on the membrane of Tax-expressing T lymphocytes (15, 23).

In this report, we first show that T cells carrying proviral HTLV-1 were expressing high levels of vascular cell adhesion molecule 1 (VCAM-1–CD106), a 110-kDa member of the Ig gene superfamily, which has been found to be expressed mainly on endothelial cells, only when stimulated with inflammatory mediators (9, 40). We then investigate the role of Tax in *trans*-activating the expression of VCAM-1 by Jurkat clones stably expressing the *tax* gene and finally determine the regulatory elements in the VCAM-1 promoter that were responsive to Tax. We found that the two NF-κB sites present at positions −72 and −53 in the VCAM-1 gene promoter are absolutely required for the Tax-induced expression of this adhesion molecule.

MATERIALS AND METHODS

Antibodies. The mouse monoclonal antibodies (MAbs) directed against human VCAM-1–CD106 were clones 1G11 and 1.4C3 from Immunotech, Marseilles, France, and from Dako, Glostrup, Denmark, respectively. Mouse MAbs to CD25 (clone B1.49.9) and CD80 (clone 104) were purchased from Immunotech. IgG1, IgG2a (Dako), and IgG2b (Immunotech) were used as negative controls. The rabbit anti-Tax antiserum directed against a specific synthetic peptide was kindly provided by B. R. Cullen (Howard Hughes Institute, Durham, N.C.). Rabbit anti-actin antiserum was purchased from Sigma (St. Louis, Mo.).

Cells. The cells were cultured in RPMI 1640 medium (Gibco Laboratories, Grand Island, N.Y.) supplemented with 10% fetal calf serum (Dutscher, Brumath, France), 5 mM L-glutamine, and 50 μg of gentamicin per ml (both from Gibco). We used in this study (i) two HTLV-1-negative T-cell lines (Jurkat and CEM); (ii) two HTLV-1-transformed T-cell lines (MT2 [31] and C8166 [44]); (iii) three clones of Jurkat cells stably expressing Tax (28) (clones C11 and E12, which were obtained after cotransfection with pMTenvXLTR together with the SV40-Neo plasmid, and clone 50, which was obtained after cotransfection with plasmid pMTtax and a plasmid carrying the *neo* resistance gene; Tax expressed by the C11, E12, and 50 Jurkat clones was found to be functional for the *trans*-activation of the HTLV-1 LTR [28]); (iv) the JPX-9 cell line (kindly provided by K. Sugamura, Tohoku University, Sendai, Japan), which was generated by the stable introduction of a Tax expression plasmid under the control of the human metallothionein promoter (induction of Tax was performed in the presence of Cd^{2+} or Zn^{2+} [35]); and (v) the Tax-negative Jurkat C9 clone, which was obtained from Jurkat cells transfected with a plasmid carrying the *neo* resistance gene (28).

Freshly isolated T cells from one HAM/TSP patient were kindly provided by C. Blanc-Desgranges (INSERM U271, Lyon, France). In addition, T cells isolated from one normal blood donor and activated with phytohemagglutinin were then cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and 10 U of recombinant human interleukin-2 (IL-2) per ml (Boehringer, Mannheim, Germany).

Oligonucleotides and reagents. The cellular oligonucleotide primers used in this study were VCAM-3 (5′-CCAGA GATAC AACCG TCTTG-3′, nucleotides 2903 to 2922, sense), VCAM-4 (5′-GAGCA CGAGA AGCTC AGGAG-3′, nucleotides 3968 to 3987, antisense), TKI (5′-GAGTA CTCGG GTTCG TGAAC-3′, nucleotides 24 to 43, sense), and TKII (5′-GGTCA TGTGT GCAGA AGCTG-3′, nucleotides 246 to 265, antisense). The viral nucleotide primers were TRU 2 (5′-TGTTT GGAGA CTGTG TACAA GGCG-3′, nucleotides 7353 to 7376, sense) and Tax 2 (5′-CAGGC TGTTA GCGTG ACGG-3′, nucleotides 7968 to 7986, antisense). Oligonucleotides were purchased from Eurogentec (Seraing, Belgium). Pyrrolidine dithiocarbamate (PDTC) was from Sigma.

Plasmids and transfections. pMTenvXLTR, containing the coding sequences of *env*, *tax*, and *rex* together with the HTLV-1 3′ LTR, and MT-Tax, encoding only Tax, were under the control of the mouse metallothionein promoter (14). Another Tax expression plasmid (SV40-Tax) was kindly provided by P. Jalinot (Ecole Normale Supérieure, Lyon). The SV40-Neo plasmid carries the *neo* resistance gene under the control of the simian virus 40 (SV40) promoter. Three VCAM-1 reporter plasmids in which various fragments of the promoter/enhancer region of the human VCAM-1 gene were linked to bacterial chloramphenicol acetyltransferase (CAT) have been described previously (36) and were kindly provided by T. Collins (Harvard Medical School, Boston, Mass.). These deletion plasmids were Ff1-pCAT3 (−2167), F3-pCAT3 (−98), and F4-pCAT (−44). In addition, the two F3MA-pCAT3 and F3MB-pCAT3 constructs, both derived from the F3-pCAT3 plasmid, have point mutations in the 5′ and 3′ NF-κB motifs, respectively. Transfections were carried out by electroporation at 250 V and 1,500 μF with the Celljet electroporator (Eurogentec).

CAT assay. CEM cells were transiently transfected with the $LTR_{(HTLV-1)}$-CAT, Ff1-pCAT3 (−2167), F3pCAT3 (−98), or F4-pCAT3 (−44) construct (20 μg) or cotransfected with the same plasmids and the SV40-Tax construct (5 μg) by a previously published procedure (27). They were incubated at 37°C for 48 h and then harvested. After centrifugation at 300 × *g* for 3 min, the cell pellets were washed twice in phosphate-buffered saline (PBS). The cells were disintegrated by four rounds of freezing and thawing. After centrifugation, aliquots of the clear supernatant were tested for the CAT protein by a CAT antigen capture assay (Boehringer). An internal control was made available with the kit that allows quantification of the CAT enzyme contained in the extracts. In some experiments, JPX-9 cells transiently transfected with $LTR_{(HTLV-1)}$-CAT, Ff1-pCAT3 (−2167), F3pCAT3 (−98), or F4-pCAT3 (−44) construct (20 μg) were cultured for 48 h at 37°C in medium alone or in medium containing cadmium and the CAT assay was performed by the published method (37). Briefly, the method uses [^{3}H]acetyl coenzyme as the acetyl donor, and relies on the diffusion of labelled acetylchloramphenicol into a water-immiscible liquid scintillation cocktail. The quantity of [^{3}H]acetylchloramphenicol produced was measured directly by counting the samples at selected time intervals.

RT-PCR assay. PCR detection of retrotranscribed RNAs was performed by a previously published procedure with slight modifications (28). Briefly, total RNA was extracted in guanidinium thiocyanate from 4×10^6 cells and resuspended in 50 μl of H_2O–0.1% diethylpyrocarbonate. To reduce the amount of DNA originating from lysis, supernatants were treated with 10 U of RNase-free DNase (Boehringer) per μl for 30 min at 20°C and then for 5 min at 65°C. To 2 μg of RNA sample (10 μl) was added 200 ng of oligo(dT) primer (1 μl) for 10 min at 65°C. Each sample was made up with reaction buffer (50 mM Tris-HCl [pH 8.3], 30 mM KCl, 8 mM $MgCl_2$, 9 mM dithiothreitol (DTT), 320 nM deoxynucleoside triphosphate) to a final volume of 25 μl, supplemented with 20 U of RNase inhibitor (Boehringer) and 25 U of avian myeloblastosis virus (AMV) reverse transcriptase (Boehringer), and incubated for 90 min at 42°C. PCRs were carried out on 4 μl of the sample supplemented with an amplification mixture containing 20 pmol of each of the oligonucleotide primers and 2 U of *Taq* DNA polymerase. The amplification reaction was run in a PHC2 thermal cycler (Techne, Cambridge, United Kingdom). The amplified products were electrophoresed in a 1% agarose gel, blotted, and hybridized with a γ-^{32}P-labeled probe. Labeled DNA products were visualized by autoradiography.

Flow cytometry analysis. After two washes with PBS, 5×10^5 cells were suspended in 100 μl of PBS containing a saturating amount of unconjugated MAb and 5% goat serum (Gibco) and then incubated in ice for 45 min. After two washes with PBS, the cells were incubated for 30 min in ice with a 1:50 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig (Dako) in PBS with 5% goat serum. After an additional three washes in PBS, the cells were resuspended in 1% paraformaldehyde. A Becton Dickinson (Mountain View, Calif.) FACScan was used to identify fluorescence-labeled cells. Integrated fluorescence of the gated population was measured, and data were collected from at least 10,000 events. For analysis following PDTC treatment, the cells were labeled with 5 μg of propidium iodide (Sigma) per ml for 10 min at 4°C by the DNA-labeling technique. Dead cells labeled with propidium iodide were excluded from the analysis.

Western blot analysis. The cells were washed twice with ice-cold PBS, harvested in 1 ml of a sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer, scraped, and then boiled for 3 min. Equal amounts of lysates (30 μg) were subjected to electrophoresis on SDS–7.5% polyacrylamide gels. The fractionated proteins were transferred to nitrocellulose membranes (BA 85; Schleicher & Schüll, Dassel, Germany) with a semidry-blotting device (Bio-Rad, Hercules, Calif.). Membranes were then blocked with a 10% nonfat milk–0.1% Tween 20–PBS solution for 1 h at room temperature and probed with the appropriate antibody for 1 h. They were then rinsed with PBS–0.1% Tween 20 and incubated at room temperature with a secondary horseradish peroxidase-conjugated anti-mouse antibody for 1 h. Following four additional rinses in PBS–0.1% Tween 20, blots were developed by using an enhanced chemiluminescence detection system (Luminol reagent [CovalAb, Oullins, France]). The membranes were subjected to autoradiography with Hyperfilm MP (Amersham, Arlington Heights, Ill.).

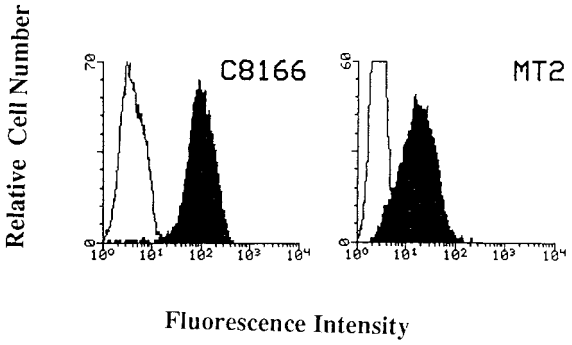

FIG. 1. Flow cytometry analysis of cell surface VCAM-1 expression by in vitro HTLV-1-transformed T cells. C8166 and MT2 cells were labeled with a MAb to VCAM-1 (1G11) for 30 min at 4°C and incubated with an FITC-conjugated anti-mouse Ig for an additional 30 min. Fluorescence profiles are shown as dark histograms after labeling with the VCAM-1 MAb and as light histograms after labeling with an IgG1 isotype control MAb. The results are representative of 3 to 10 separate experiments.

Electrophoretic mobility shift assay. Nuclear extracts were prepared by a published method (6). Briefly, cells (2×10^6) were extensively washed and harvested after a 16-h incubation at 37°C in culture medium (or medium supplemented with additives). The cells were washed with Tris-buffered saline (TBS; pH 7.8), transferred into 1.5-ml Eppendorf tubes, and microcentrifuged at 4°C for 15 s. The pellet was resuspended in 800 μl of buffer A (10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 4 μg of leupeptin per ml, 10 mM HEPES [pH 7.8]). After 15 min on ice, a 50-μl solution of 10% Nonidet P-40 was added to the sample and the cells were homogenized by vortexing and microcentrifuged at 4°C for 30 s. The pellets were resuspended in 100 μl of buffer B (50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 4 μg of leupeptin per ml, 10% glycerol, 50 mM HEPES [pH 7.8]). The nuclear extracts were microcentrifuged at 4°C for 5 min, and the supernatants were stored at −80°C until used.

The NF-κB mobility shift assays were performed with 2 μg of protein from nuclear extract, 10^5 cpm of radiolabeled double-stranded probe NF-κB (NF-κB sense strand only: 5′-CTGCCCTGGGTTTCCCCTTGAAGGATTTCCCTCCG CCTC-3′) in buffer C (100 mM KCl, 1 mM DTT, 1 mM $ZnSO_4$, 20% glycerol, 0.01% Nonidet P-40, 50 mM HEPES [pH 7.9]), supplemented with bovine serum albumin, tRNA, and poly(dI-dC) in a final volume of 20 μl. After 20 min at room temperature, the mixture was run at 120 V in a 10% polyacrylamide gel. The specificity of binding was determined by competition with 10-, 50-, and 100-fold molar excesses of the same unlabeled NF-κB double-stranded oligonucleotide sequence or unrelated Sp-1 oligonucleotide (Sp-1 sense strand only: 5′-GGA GGCGTGGCCTGGGCGGGACTGGGGAGTGGCGA-3′).

RESULTS

Expression of VCAM-1 by HTLV-1-infected human T cells and by Jurkat clones stably transformed with Tax expression plasmids. Analysis of the expression of adhesion molecules by HTLV-1-transformed T cells (C8166 and MT2) allowed us to show that these cells are expressing VCAM-1, as ascertained either by flow cytometry with MAb 1G11 or 1.4C3 to CD106 (Fig. 1) or by immunofluorescence (data not shown). Likewise, freshly isolated T cells from one HAM/TSP patient were found to express VCAM-1 together with CD25 (Fig. 2A). In contrast, VCAM-1 was not detected on T cells derived from one normal (HTLV-1-seronegative) blood donor, even when they were activated with phytohemagglutinin and cultivated in the presence of IL-2. As expected, these cells were expressing CD25 (Fig. 2B).

Since Tax is the only viral protein expressed by C8166 cells and it is expressed by T cells from HAM/TSP patients, the role played by this viral regulatory protein in the induction of VCAM-1 was next investigated by using Jurkat T cells that stably express Tax. Three clones of Jurkat cells, clones C11 and E12, which express both Tax and Rex, and clone 50, which expresses only Tax, were found to express VCAM-1 at a level comparable to that observed for HTLV-1-transformed T cells (Fig. 3A). Parental Jurkat cells as well as cells from a Tax-negative Jurkat clone, C9 (obtained under the same selection procedure as the Tax-positive clones), did not express VCAM-1 (Fig. 3A). Western blot analysis performed under nonreducing conditions with a blotting MAb directed against VCAM-1 revealed the presence of a protein of approximately 97 kDa in extracts of Tax-positive C11, E12, 50, and C8166 T cells (Fig. 3B, lanes 3 to 6, respectively). The molecular weight of this protein is consistent with the seven Ig-like domains of the VCAM-1 molecule. No such protein could be detected in extracts of parental Jurkat and C9 cells (lanes 1 and 2). These results clearly suggest that Tax expression in Jurkat cells correlates with the induction of VCAM-1 at the cell surface.

VCAM-1 expression is dependent on the Tax concentration in T cells. To further evaluate the effect of Tax on VCAM-1 gene expression, we next used JPX-9 cells, a subclone of Jurkat cells that was stably transformed with the pMAXRHneo-1 plasmid carrying the *tax* sequences under the control of the human metallothionein promoter responsive to heavy-metal ions. Previous experiments performed with JPX-9 cells transiently transfected with an $LTR_{(HTLV-1)}$-CAT construct and cultivated in a medium with increasing concentrations of cadmium chloride ($CdCl_2$) have indeed indicated a positive correlation between the expression of a functionally active Tax and the concentration of heavy-metal ions.

Expression of the VCAM-1 gene was assayed by RT-PCR performed on total mRNA extracted after a 24-h culture of cells either in medium alone or in medium containing 5 or 10 μM $CdCl_2$ with or without 100 μM PDTC, an antioxidant which has been shown to block the mobilization of NF-κB by Tax (45). In untreated cells, VCAM-1 mRNA was undetectable, whereas the constitutive expression of the TK gene, used as a control, was observed. After treatment of JPX-9 cells with $CdCl_2$, VCAM-1 mRNA was readily detected at both Cd^{2+} concentrations (Fig. 4).

The effect of Tax induction on the membrane expression of

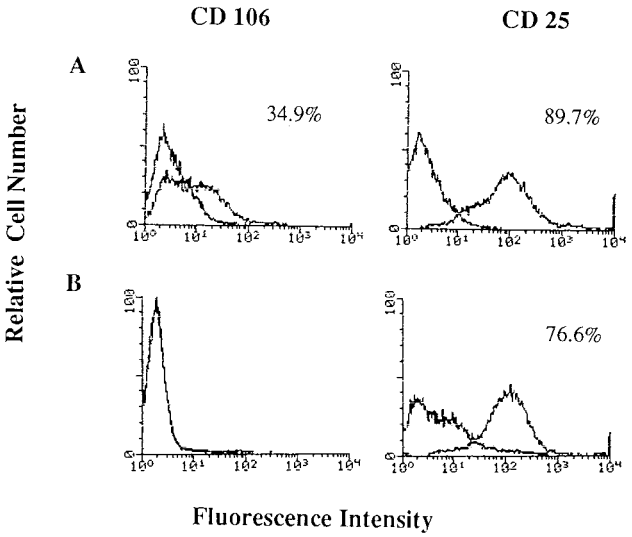

FIG. 2. Flow cytometry analysis of cell surface VCAM-1 expression by T cells from one HAM/TSP patient. Freshly isolated T cells from this patient (A) and T cells obtained from a normal blood donor, activated with phytohemagglutinin, and then cultured in growth medium supplemented with IL-2 (B) were labeled either with a MAb to VCAM-1 (1G11) or with a MAb to CD25 (B1.49.9) for 30 min at 4°C and incubated with an FITC-conjugated anti-mouse Ig for an additional 30 min. Fluorescence profiles of cells labeled with the appropriate IgG isotype are shown as controls. Where relevant, the percentage of positive cells is indicated.

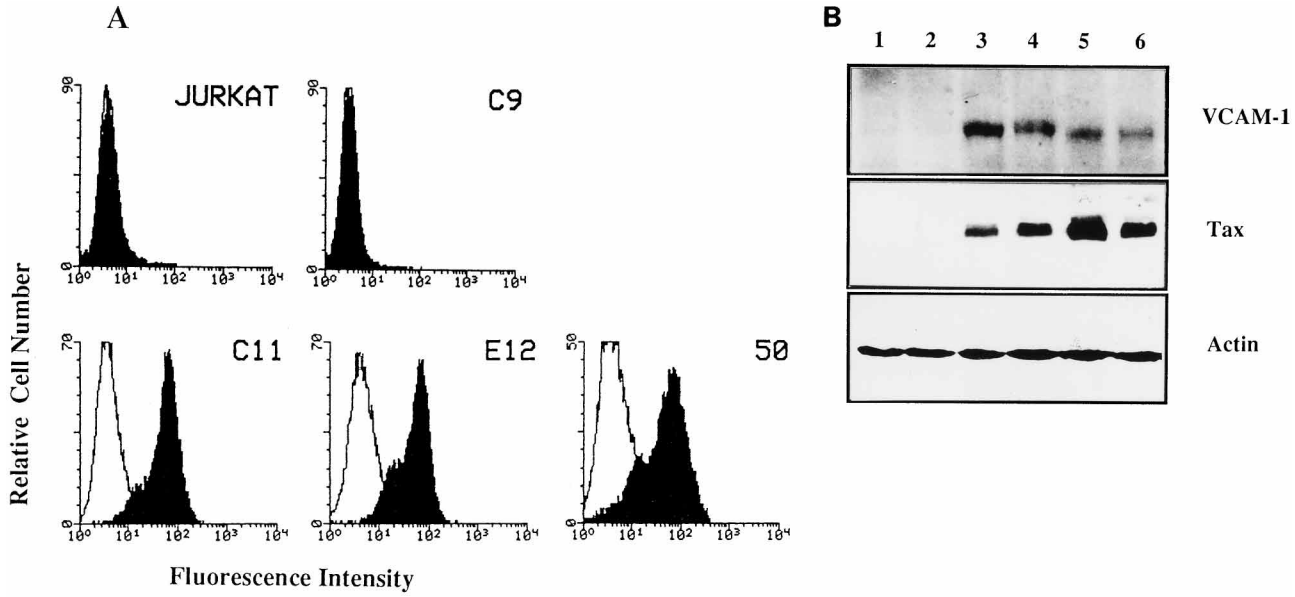

FIG. 3. Analysis of cell surface VCAM-1 expression by Tax-expressing cells. (A) For a flow cytometry analysis, Jurkat parental cells, a Tax-negative Jurkat clone (C9), and Tax-positive Jurkat clones (C11, E12, and clone 50) were labeled with a MAb to VCAM-1 (1G11) for 30 min at 4°C and incubated with an FITC-conjugated anti-mouse Ig for an additional 30 min. Fluorescence profiles are shown as dark histograms after labeling with the VCAM-1 MAb and as light histograms after labeling with an IgG1 isotype control. The results are representative of 3 to 10 separate experiments. (B) For an immunoblot analysis, lysates prepared from parental Jurkat and C9 cells (lanes 1 and 2) and from Tax-positive T cells, C11, E12, 50, and C8166 (lanes 3 to 6), were electrophoresed through an SDS–7.5% polyacrylamide gel under nonreducing conditions and then transferred onto a nitrocellulose membrane. The membrane was incubated first with a VCAM-1 MAb (1.4C3), then with a rabbit anti-Tax antiserum and with a rabbit anti-actin antiserum, and then with a horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit Ig antibody. Proteins were revealed by an enhanced chemiluminescence detection system and after exposure of the membrane to hyperfilms.

VCAM-1 was next analyzed by flow cytometry. In addition to that of VCAM-1 (CD106), the expression of CD4 and that of B7 (CD80), a Tax-inducible membrane antigen (used as a positive control), were determined on JPX-9 cells cultured for 48 h in medium alone or in medium containing either $ZnCl_2$ or $CdCl_2$. The same analysis (except for B7) performed with parental Jurkat T cells cultured in medium alone or in medium containing $ZnCl_2$ revealed that VCAM-1 could not be induced in these cells (Fig. 5A). In untreated JPX-9 cells, neither VCAM-1 nor B7 (CD80) was expressed (Fig. 5). Following Tax induction by $CdCl_2$ or $ZnCl_2$, JPX-9 cells expressing either VCAM-1 or B7 were detected, at a percentage about fourfold higher for cells treated with $ZnCl_2$ (Fig. 5B) than for those treated with $CdCl_2$ (Fig. 5C). No increase in the number of JPX-9 cells expressing CD4 was observed following Tax induction. These results, in agreement with data obtained by RT-PCR analysis, confirmed that Tax is involved in the induced expression of VCAM-1 in T cells.

Functional analysis of the VCAM-1 promoter for transactivation by Tax. To further ascertain the role of Tax in the transcriptional activation of the VCAM-1 gene, we next attempted to localize the Tax-responsive elements in the VCAM-1 promoter. To this end, three deletion constructs that contained portions of the VCAM-1 5′-flanking sequences, −2167 (Ff1), −98 (F3), and −44 (F4), linked to the promoterless CAT gene were transiently transfected by electroporation either into CEM lymphoblastoid T cells or into JPX-9 cells (Fig. 6A). To examine the effects of Tax, CEM cells were cotransfected with a Tax expression plasmid whereas JPX-9 cells were treated with $CdCl_2$. As shown in Fig. 6B, CAT assays performed 48 h after transfection revealed that Tax strongly induced the expression of the CAT reporter gene in CEM cells transfected with the Ff1 and F3 constructs, indicating that the sequences in the −2167 to −98 region are dispensable for Tax transactivation of the VCAM-1 promoter. However, with regard to JPX-9 cells transfected with the same constructs and treated with $CdCl_2$, CAT assays reveal that a responsive element appears to exist in the region extending from positions −2167 to −98 of the promoter (Fig. 6C). This discrepancy,

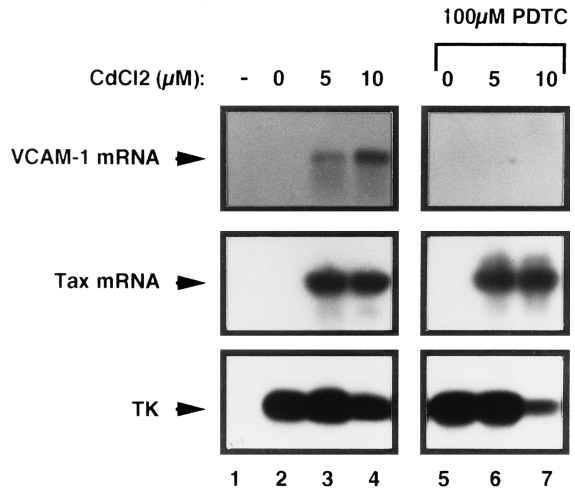

FIG. 4. VCAM-1 mRNA analysis in JPX-9 cells treated with cadmium. PCR analysis of retrotranscribed VCAM-1 mRNA in JPX-9 cells cultured in medium alone (lane 2) or medium containing 5 μM (lane 3) or 10 μM (lane 4) $CdCl_2$ was performed with the VCAM-1 oligonucleotide primer pair (see Materials and Methods). Amplified products were electrophoresed, blotted, and hybridized with an α-^{32}P-labelled VCAM-1 probe. Labelled DNA products were visualized by autoradiography. Autoradiograms of PCR amplification of retrotranscribed Tax mRNA and thymidine kinase (TK) mRNA are shown as controls. Lane 1 represents a control in which an RNA-free sample was treated like the extracted samples (lane 1). In addition, PCR analysis of retrotranscribed VCAM-1 mRNA in JPX-9 cells cultured in medium alone or medium containing 5 or 10 μM $CdCl_2$ and 100 μM PDTC is shown (lanes 5 to 7).

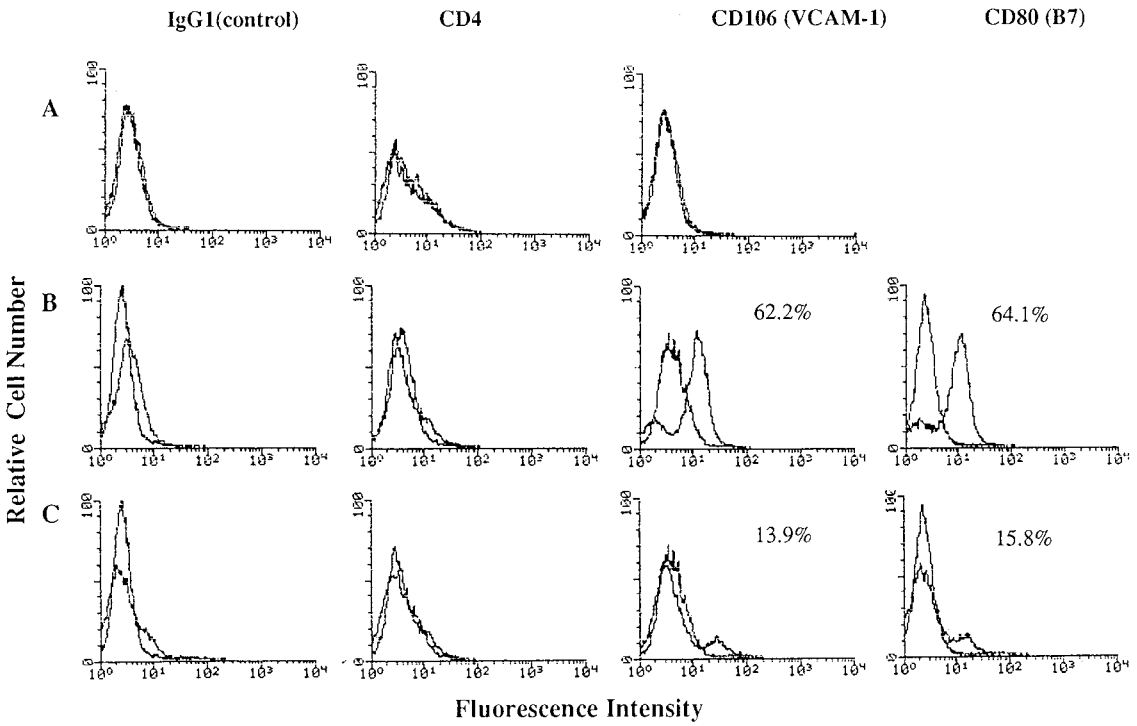

FIG. 5. Flow cytometry analysis of cell surface VCAM-1 expression by JPX9 cells and by parental Jurkat T cells. In addition to that of VCAM-1 (CD106), the expression of CD4 and that of B7 (CD80) were determined on Jurkat T cells cultured for 48 h in medium alone or medium containing $ZnCl_2$ (A) or on JPX-9 cells cultured for 48 h in medium alone or medium containing either $ZnCl_2$ (B) or $CdCl_2$ (C). Cells were labeled with an isotype control IgG1 MAb, with an anti-CD4 MAb (IOT4), with an anti-B7 MAb (104) (except for parental Jurkat T cells), and with an anti-VCAM-1 MAb (1G11) for 30 min at 4°C and incubated with an FITC-conjugated goat anti-mouse Ig for an additional 30 min. Fluorescence profiles before treatment are shown as controls. Where relevant, the percentage of positive cells is indicated.

which might be related to a difference in the efficiency of Tax either transiently or stably expressed, warrants further investigations. For both types of cells transfected with the F4 construct, no induction of CAT activity by Tax was observed, suggesting that the major Tax-responsive elements in the VCAM-1 promoter were found between −98 and −44. Interestingly, this region has been shown to contain two sequences, one at −57 and the other at −72, that each conform to consensus NF-κB elements (36). To investigate which of these elements is essential to Tax-induced VCAM-1 expression, we used two mutant constructs generated from the F3 deletion plasmid (F3mA and F3mB). Transfection experiments performed as described above indicated that with the upstream F3mA construct, no CAT activity was induced in presence of Tax. The same results were obtained with the F3mB mutant. These data clearly demonstrate that the Tax-induced expression of the VCAM-1 gene is mediated by the two NF-κB consensus binding sites present in the −98 to −44 region of the core promoter.

Tax induces VCAM-1 expression through the NF-κB pathway. Since Tax is known to intervene in the nuclear translocation of the NF-κB family of transcription factors, we next performed gel shift assays to demonstrate the specific binding of nuclear proteins to the two NF-κB sites. A labeled oligonucleotide encompassing these two elements was incubated with nuclear extracts prepared either from parental Jurkat cells or from JPX-9 cells, both cell types being either left uninduced or treated with 5 μM $CdCl_2$. As shown in Fig. 7, lane 4, a significant increase in the shift of the labeled oligonucleotide was observed when it was mixed with extracts of treated JPX-9 cells compared with extracts prepared either from untreated JPX-9 cells (lane 3) or from parental Jurkat cells that were untreated (lane 1) or treated with $CdCl_2$ (lane 2). The addition of a 10-, 50-, or 100-fold molecular excess of unlabeled NF-κB oligonucleotide decreased the formation of the Tax-inducible DNA-protein complex (lanes 7 to 9). In contrast, no such inhibition was observed after incubation of the nuclear proteins and the labeled oligonucleotide in the presence of a 100-fold excess of unlabeled Sp-1 oligonucleotide (lane 10). A significant reduction in the amount of the DNA-protein complex was also observed with nuclear extracts prepared from JPX-9 cells treated with $CdCl_2$ and with 100 μM PDTC (lane 3). Finally, treatment of Tax-expressing Jurkat T cells with increasing concentrations of PDTC led to a dose-dependent decrease in VCAM-1 expression. Thus, VCAM-1 expression was inhibited by more than 75% at the highest concentration of PDTC (100 μM). Such an effect did not correlate with a decreased functionality of Tax, since Tax was found to be still able to *trans*-activate the HTLV-1 LTR (data not shown).

Collectively, these results confirm the presence of nuclear proteins binding specifically to the two NF-κB consensus sites of the VCAM-1 gene promoter in Tax-expressing T cells. They further underline that these two sites are responsible for the Tax-mediated induction of the VCAM-1 expression on HTLV-1-infected T cells.

DISCUSSION

Infiltration of T lymphocytes carrying the HTLV-1 provirus into organs represents a crucial event in the evolution of lymphoproliferative and inflammatory diseases associated with HTLV-1 infection. Indeed, numerous studies have underlined

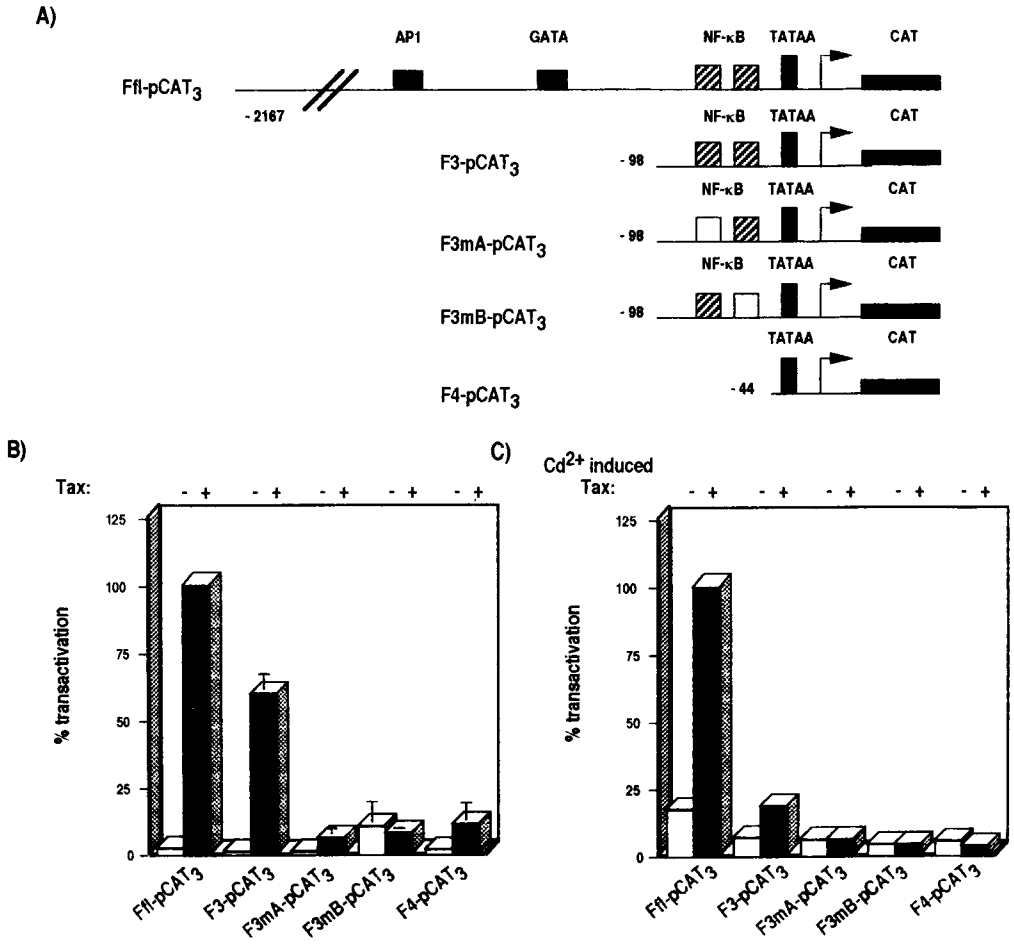

FIG. 6. Analysis of Tax-responsive elements in the VCAM-1 promoter. (A) Schematic diagram, adapted from reference 36, of the fusion plasmids containing portions of VCAM-1 5′-flanking sequence with or without deletion mutations in the regulatory regions and the CAT reporter gene. (B) CEM cells were transfected with VCAM-1 wild-type Ff1-pCAT3 construct, deletion plasmids F3-pCAT3 (−98) and F4-pCAT3 (−44), or mutant F3mA-pCAT3 or F3mB-pCAT3 construct or cotransfected with one of these constructs and a Tax expression vector, as indicated in Materials and Methods. Promoter transactivation was evaluated by measuring CAT antigen synthesis by a CAT antigen capture assay. Bars represent the average value of two independent experiments. (C) JPX-9 cells transfected with the same reporter plasmids as in panel B were cultured in medium supplemented or not supplemented with $CdCl_2$. Promoter transactivation was evaluated by measuring the [^{3}H]acetylchloramphenicol produced, as described in Materials and Methods. We provisionally considered the transactivation of VCAM-1 wild-type (Ff1) promoter by Tax to be 100% transactivation to allow comparison of panels A and B. Under similar experimental conditions, transactivation of $LTR_{(HTLV-1)}$-cCAT by Tax used as internal control was 107.5% ± 17.5%.

the role of adhesion molecules, not only in mediating cell-cell interactions but also in mediating tissue invasion (18, 20–22, 25, 50, 55). In the present report, we demonstrate that VCAM-1, an adhesion molecule that is a member of the Ig gene superfamily, is expressed on in vitro HTLV-1-transformed T cells, on T cells from one HAM/TSP patient, and on Jurkat T cells stably expressing the viral regulatory Tax protein. We also demonstrate that Tax activates VCAM-1 transcription mainly via the two NF-κB sites present in the promoter of the VCAM-1 gene.

Among the in vitro HTLV-1-transformed T cells examined in this study and which were found to express VCAM-1, C8166 cells were known to harbor a deleted HTLV-1 provirus. Because the only viral protein synthesized by these cells is Tax, we next investigated its role in the expression of this adhesion molecule by HTLV-1-infected T cells. Indeed, this regulatory protein has been shown to induce the transcriptional activation of cellular genes involved in the pathogenesis of HTLV-1 infection (10, 56). To this end, we have used three clones of the human Jurkat T-cell line which share the property to stably express Tax and found that VCAM-1 was present on these cells. Furthermore, induction of Tax in JPX-9 cells, a subclone of Jurkat cells stably transformed with a plasmid carrying the *tax* gene under the control of the inducible human metallothionein promoter, led to the expression of VCAM-1. These results clearly stress that Tax is responsible for the induction of VCAM-1 on HTLV-1-infected T cells. Indeed, with the exception of a subset of anergized autoreactive T cells bearing TCR-γδ (26), VCAM-1 has never been reported to be expressed by resting or activated T lymphocytes. As such, the present observations indicate a unique effect of Tax in the expression of an adhesion molecule, since other studies have shown that ICAM-1, LFA-3 and L-selectin expressed by normal T cells are upregulated by Tax on T cells carrying proviral HTLV-1 (50, 52). Finally, it should be underlined that HTLV-1-transformed T cells constitutively express both VCAM-1 and its ligand VLA-4 (8). The same is true for OX40 and its ligand gp34, which are both detected on the membrane of Tax-expressing T lymphocytes (23).

Deletion analysis of the 2.1-kb 5′-flanking region of the

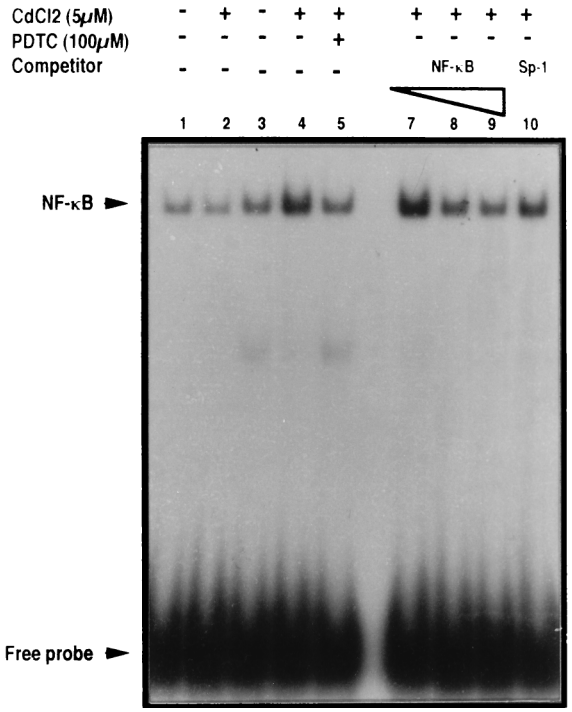

FIG. 7. Induction of NF-κB-binding proteins in JPX-9 cells after treatment with $CdCl_2$. Nuclear extracts prepared from JPX-9 cells cultured in medium alone (lane 3) or in medium containing 5 μM $CdCl_2$ (lane 4) or 5 μM $CdCl_2$ and 100 μM PDTC (lane 5) were reacted with a double-stranded oligonucleotide NF-κB probe. The specificity of binding of nuclear extracts prepared from JPX-9 cells cultured in medium containing 5 μM $CdCl_2$ to NF-κB probe was determined by competition with a 10-, 50-, and 100-fold molar excess of either the same unlabeled double-stranded NF-κB oligonucleotide sequence (lanes 7 to 9) or 100-fold unlabeled double-stranded Sp-1 oligonucleotide (lanes 10). Nuclear extracts prepared from Jurkat cells cultured in medium alone (lane 1) or in medium containing 5 μM $CdCl_2$ (lane 2) reacted with a double-stranded oligonucleotide NF-κB probe are shown as controls.

VCAM-1 gene, performed with the respective CAT reporter constructs transfected either in CEM cells in the presence of a Tax expression plasmid or in JPX-9 cells either induced or left uninduced, revealed that Tax was inducing VCAM-1 expression in a κB-dependent manner. Indeed, these experiments indicated that the −98 to −44 region of the VCAM-1 promoter, which contained two sequences that conform to consensus NF-κB elements, is indispensable for Tax-induced expression of VCAM-1. Next, transfection of two CAT constructs, each carrying the −98 to −44 region mutated in either NF-κB-like site, indicated that both of them were strictly required for Tax induction of this adhesion molecule. Furthermore, gel shift assays performed with nuclear extracts obtained from either induced or uninduced JPX-9 cells confirmed that Tax induction correlated with the formation of specific DNA-protein complexes in the presence of an oligonucleotide spanning these two NF-κB-like sequences. Finally, a decrease in the formation of these protein-DNA complexes was found to be linked to a decrease in the expression of VCAM-1 by induced JPX-9 cells treated with the antioxidant PDTC, which abolished oxidative processes involved in the activation of this transcription factor.

VCAM-1 was first identified as an adhesion molecule expressed on the surface of IL-1- or TNF-α-stimulated endothelial cells (9, 40). Previous studies have established that the two NF-κB sites of the VCAM-1 promoter were required for induction of VCAM-1 transcription by these inflammatory cytokines (36). Interestingly, these two sites were not responsive in Jurkat T cells treated with TNF-α, even though NF-κB elements present in other promoters were activated by TNF-α in these human T cells (19, 33, 36, 46). These observations therefore indicated that NF-κB subunits specific of endothelial cells were involved in the response of the VCAM-1 promoter to TNF-α. Experiments performed to ascertain whether IL-1 or IL-6 added to Jurkat T cells might be able to induce VCAM-1 expression consistently gave negative results (data not shown). Our present results indicate for the first time that these two NF-κB subunits are implicated in the induction of the VCAM-1 gene in Tax-positive T cells.

As underlined above, VCAM-1 was first identified as an adhesion molecule induced on endothelial cells by inflammatory cytokines. Indeed, quiescent endothelial cells normally exhibit a low affinity for circulating lymphocytes. The release of cytokines at sites of inflammation results in the upregulation of VCAM-1 on endothelial cells and the subsequent establishment of interactions of VCAM-1 with VLA-4 expressed on T lymphocytes, with this ligand-receptor pair allowing the attachment of lymphocytes to activated endothelium. Whether or not VCAM-1 expressed on HTLV-1-infected T lymphocytes is playing a major role in recruiting these cells from the peripheral blood and in mediating their passage into organs remains questionable, inasmuch as the α4 chain specifying the VLA-4 molecule was not found to be expressed by quiescent or stimulated endothelial cells. Although it might be postulated that an alternative counterreceptor for VCAM-1 on endothelial cells is promoting the adhesion and invasion of HTLV-1-infected T lymphocytes, VCAM-1 might rather favor the interaction of HTLV-1-infected T cells among themselves and with uninfected T cells expressing VLA-4. Such interactions may lead to cell fusion, transmission of HTLV-1, and/or immunological destruction of infected cells by cytotoxic T lymphocytes.

Recently VCAM-1 has been demonstrated to be involved as an accessory molecule or potential coreceptor for HTLV-1-induced cell fusion (16). Soon after its identification on activated endothelial cells, VCAM-1 was also found on nonendothelial cells, such as lymphoid dendritic cells, tissue macrophages, glial cells, muscle cells (1, 5, 30, 42, 43). In particular, VCAM-1 on myoblasts and myotubes and VLA-4 induced during myotube formation were shown to play a role in secondary myogenesis (43). A recent report indicates that HTLV-1-associated inflammatory polymyositis is not due to direct infection of the muscle fiber by the virus but, rather, to a T-cell-mediated and major histocompatibility complex class I-restricted immunopathological process such as myocytotoxicity (29). Thus, it is tempting to speculate that HTLV-1-infected T cells expressing VCAM-1 and infiltrating muscles may perturb the myogenic process at the origin of muscle disorders that have been described in some patients suffering from HAM/TSP.

The numerous studies devoted to the role of adhesion molecules have indicated that activation of leukocytes by various stimuli leads to different utilizations of adhesion pathways. In this perspective, the results presented here provide a novel example in which activation of an adhesion molecule such as VCAM-1 induced by a viral protein may represent a crucial event in the initiation and development of lesions associated with a retroviral infection in humans.

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