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Immunology

Adult T-cell leukemia-derived factor/thioredoxin, produced by both human T-lymphotropic virus type I- and Epstein–Barr virus-transformed lymphocytes, acts as an autocrine growth factor and synergizes with interleukin 1 and interleukin 2

(disulfide reducing enzyme/lymphocyte proliferation/interleukin 2 receptor inducer/competence factor/3B6 cell line)

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ABSTRACT **Interleukin 1 (IL-1) has been obtained from the Epstein–Barr virus-infected B-lymphoblastoid cell line 3B6 and shown to be involved in autocrine growth of 3B6 B cells. Independently, adult T-cell leukemia-derived factor (ADF) was purified from human T-lymphotropic virus I-infected leukemic T-cell line (ATL-2) and reported as an interleukin 2 (IL-2) receptor-inducing factor. We have previously reported the same molecular mass, pI, and NH_2-terminal amino acid sequence for both 3B6-derived IL-1 and ADF. cDNA cloning of ADF demonstrated high homology with the prokaryotic disulfide reducing enzyme thioredoxin. We show here that ADF and 3B6-derived IL-1 are identical. By RNA blot, 3B6 and ATL-2 cells were shown to contain high levels of 0.6-kilobase mRNA corresponding to ADF. Such message was not detected in resting peripheral blood lymphocytes but could be weakly induced by lymphocyte activation. Antibodies have been raised against synthetic peptides corresponding to the NH_2 terminus and the COOH terminus of ADF. Immunoblotting and sequential immunoprecipitation with these antibodies revealed the same 13-kDa protein in 3B6 and ATL-2 cells. Recombinant ADF could sustain growth of 3B6 and ATL-2 cells at low cellular concentration without fetal calf serum; ADF, thus, appears involved in their autocrine growth. Similarly, recombinant ADF could enhance growth of other B-cell lines, including the Epstein–Barr virus-negative Burkitt lymphoma line BL41 and the lymphoblastoid cell lines CRAG8, CRB95, and 1G8. Finally, recombinant ADF exhibits marked synergism with other cytokines, such as IL-1 and IL-2, allowing virally infected lymphocytes to respond to suboptimal amounts of a variety of growth factors.**

We have previously characterized subclone 3B6 of an Epstein–Barr virus (EBV)-containing B-cell line that constitutively produces high levels of interleukin 1 (IL-1)-like activity without any exogenous stimulation (1). This factor, referred to as 3B6-IL-1, showed biological similarities with IL-1s. Purification of 3B6-IL-1 led to a single 13.5-kDa protein with a pI value of 5.0. However, sequence of the 15 NH_2-terminal amino acids indicated that this molecule differed from either IL-1α or IL-1β. This difference suggested that 3B6-IL-1 could represent a separate molecular species (2). We also reported that purified 3B6-IL-1 could sustain growth of 3B6 cells at low cellular concentration without any fetal calf serum (FCS) and thus acted as an autocrine growth factor for 3B6 cells (3).

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Independently, Yodoi and colleagues (4) discovered adult T-cell leukemia-derived factor (ADF) produced by human T-lymphotropic virus type I (HTLV-I)-infected adult T leukemia cells (ATL lines) (5). HTLV-I virus is thought to be the causative agent for the disease. The p55 chain of the interleukin 2 receptor (IL-2R) (IL-2R/p55Tac) (6) is constitutively expressed on $CD4^+$ T cells transformed by HTLV-I (7). ADF was able to induce expression of both Tac antigen and high-affinity IL-2Rs on several cell lines (8). The molecular masses of the two purified molecules were similar (13 kDa), as well as their pI values ($\approx$5). Furthermore, we noticed the striking fact that the 15 NH_2-terminal amino acid sequences independently obtained by our two groups for 3B6-IL-1 and ADF were identical. For these reasons, we have suggested (3, 9) that the two factors could be the same.

Recently, cDNA clones of human ADF were obtained from a cDNA library of ATL-2 cells with oligonucleotide probes based on the amino acid sequence of human ADF (10). ADF was shown to be constitutively expressed in $HTLV\text{-}I^+$ T-cell lines and is inducible in normal human peripheral blood mononuclear cells (PBMCs) when activated. By using human ADF probe, murine ADF has subsequently been cloned. The most striking structural fact is that ADF is highly homologous to the disulfide reducing enzyme thioredoxin (11) found in prokaryotes as well as in mammalian cells. The homology seems significant because ADF has an identical sequence to the active enzymatic site of thioredoxin (-Cys-Gly-Pro-Cys-). Furthermore, recombinant ADF (rADF) does have thioredoxin activity measured by the degradation of insulin (33). We thus consider that ADF is actually a member of the thioredoxin family. We also assessed the biological activity of rADF obtained in COS-7 cells and demonstrated its IL-2R/Tac-inducing activity on YT cells and normal peripheral blood lymphocytes (10). We report here data showing that 3B6-IL-1 is, indeed, identical to ADF. We also show its essential role as an autocrine growth factor for 3B6 and ATL-2 cells, as well as its ability to synergize with other cytokines, such as IL-1 and IL-2.

MATERIALS AND METHODS

Cells. 3B6 cell line is an EBV-infected B-cell subclone obtained by limiting dilution from lymphoblastoid cell line

Abbreviations: IL-1 and -2, interleukin 1 and 2, respectively; ADF, adult T-cell leukemia-derived factor; HTLV-I, human T-lymphotropic virus type I; FCS, fetal calf serum; rADF, recombinant ADF; IL-2R, interleukin 2 receptor; PBMC, peripheral blood mononuclear cell; rIL-2, recombinant interleukin 2. 3B6-IL-1, IL-1 from 3B6 cells.

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721/84.5 and has been selected because of its high spontaneous release of a factor that has the biological properties of IL-1 (3). BL41 cell line is an EBV-negative Burkitt lymphoma cell line established at the International Agency for Research on Cancer (Lyon, France). CRB95 and CRAG8 are EBV-positive lymphoblastoid cell lines (gifts from A. B. Rickinson, University of Birmington Medical School, Birmingham, England). 1G8 cell line is another subclone of the 721/84.5 lymphoblastoid cell line, which expresses lower ADF mRNA levels than does 3B6 (established in our laboratory). ATL-2 is an IL-2-independent HTLV-I$^+$ leukemic T-cell line established from an adult T-cell leukemia patient and used for isolating ADF cDNA (12). These cells were routinely cultured in RPMI 1640 medium/10% FCS (Seromed, Berlin, West Germany)/2 mM L-glutamine/antibiotics. PBMCs were obtained from healthy donors by using Ficoll/Hypaque separation. The isolated PBMCs were cultured in 10% FCS/RPMI 1640 medium at a concentration of 1×10^6 cells per ml for 3 days before RNA extraction. Anti-CD3 monoclonal antibody (30 ng/ml) or phorbol 12-myristate 13-acetate (50 ng/ml) was used for stimulating lymphocytes.

^{125}I-Labeled IL-2 Binding and Scatchard Analysis. Recombinant IL-2 (rIL-2) (Takeda, Osaka) was labeled with ^{125}I by using the lactoperoxidase/glucose oxidase system (Enzymobeads, Bio-Rad). Specific activity of the radioiodinated IL-2 was estimated by calculating the dose of unlabeled rIL-2 necessary to inhibit by 50% labeled IL-2 binding to YT-C3 cells (natural killer-like leukemic cells with many IL-2Rs having intermediate affinity and inducible Tac-p55, established and used by Yodoi *et al.* for demonstrating biological activity of ADF) (8, 10). Specific activity of ^{125}I-labeled IL-2 was ≈30,000 cpm/ng. Serial dilutions of ^{125}I-labeled IL-2, ranging from 10 nM to 10 pM, were incubated together with extensively washed 3B6 cells (2×10^6) in a total volume of 200 μl of RPMI 1640/10% FCS at 37°C for 30 min. Cells were then centrifuged through a layer of 20% dioctyl phthalate/80% dibutyl phthalate oils (vol/vol). The tubes were cut off, and the radioactivities of the cell pellet and the medium were counted in a γ counter (LKB). Nonspecific binding of ^{125}I-labeled IL-2 was determined in 200-fold molar excess of unlabeled rIL-2. Specific binding was obtained by subtracting nonspecific binding from total binding. Scatchard plots were then analyzed by a nonlinear curve-fitting computer program with the Newton–Gauss elimination method (13).

RNA Blot Analysis with cDNA of ADF. Total RNAs of cells were extracted by the guanidium thiocyanate/cesium chloride method. Twenty micrograms of RNA from each sample was electrophoresed on 1.5% agarose-formaldehyde gel and was transferred to nitrocellulose membranes. Hybridization was performed with a cDNA probe for ADF radiolabeled with ^{32}P by nick-translation.

Antibodies. The peptides corresponding to either the NH_2 terminus (17 amino acids) or the COOH terminus (28 amino acids) of the protein sequence of ADF were synthesized and coupled with keyhole limpet hemocyanin. Rabbit antisera against ADF were raised by immunization with these synthetic peptides, and then serum IgG fractions were purified by ammonium sulfate precipitation and gel filtration by using protein A-Sepharose.

Immunoblot Analysis with Anti-ADF. 3B6 and ATL-2 cells (50×10^6) were washed twice with RPMI 1640 medium; solubilized by adding 0.5 ml of buffer containing 1% Nonidet P-40, 10 mM Tris·HCl (pH 7.6), 150 mM NaCl, 100 units of aprotinin, and 1 mM phenylmethylsulfonyl fluoride for 20 min on ice; and then centrifuged at 16,500 × *g* for 15 min. Sixty microliters of each sample was applied to SDS/PAGE (15%); then proteins were transferred to a nitrocellulose membrane for 6 hr. The membrane was washed and saturated with TBS (50 mM Tris/150 mM NaCl/0.02% sodium azide) containing 3% skim milk for 18 hr and then extensively washed; antisera against either the NH_2 terminus or the COOH terminus ADF moiety were added at 200-fold dilution and incubated overnight at 4°C; then the membrane was again washed three times. ^{125}I-labeled anti-rabbit immunoglobulin (Amersham) was used as second antibody at 10^6 cpm/ml for 5 hr at 4°C. After eight washings with TBS/0.1% Tween 20, the membrane was autoradiographed at −80°C for 4 hr.

Sequential Immunoprecipitation. 3B6 cells (40×10^6) were labeled with [^{35}S]methionine (1 mCi; 1 Ci = 37 GBq; Amersham) in methionine-free RPMI 1640 medium/10% dialyzed FCS. Labeled cells were solubilized in 800 μl of lysis buffer 10 mM NaH_2PO_4/1 mM EGTA/1 mM EDTA/1 mM NaF/0.5% deoxycholic acid/1% Triton X-100/100 units of aprotinin/1 mM phenylmethylsulfonyl fluoride at 4°C for 1 hr. Then the cells were centrifuged at 16,500 × *g* for 15 min. Preclearing was done by using *Staphylococcus* Cowan I strain and irrelevant IgG antibodies crosslinked to protein A-Sepharose. Specific immunoprecipitation used affinity-purified rabbit IgG from antisera against the synthetic peptides corresponding to either the NH_2 terminus or the COOH terminus of the ADF protein sequence. 3B6 cell lysate (150 μl corresponding to 7×10^6 cells) was incubated with 30 μl of rabbit IgG at 4°C overnight and then added to 50 μl of pellet protein A-Sepharose for 2 hr. Immunoprecipitations with the same antisera were repeated with the supernatant. The pellet was washed 3 times with lysis buffer containing either 10 mM EDTA, or 0.05% SDS, or 0.15 M NaCl. SDS/sample buffer was added and boiled at 100°C for 3 min. Sequential immunoprecipitations with alternative antisera were performed on the supernatants after three immunoprecipitations with the first antisera. The immunoprecipitates were analyzed by SDS/PAGE (15%) under reducing condition.

Proliferation Assays. 3B6 and ATL-2 cells were washed 3 times and suspended in RPMI 1640 medium without FCS and then adjusted to various cellular concentrations ranging from 5×10^3 cells per well to 5×10^4 cells per well and incubated for 3 days in flat-bottomed microculture plates, either with or without rADF or with rIL-1α or rIL-2. [^{3}H]Thymidine (1 μCi per well, Commissariat à l'Énergie Atomique, Gif-sur-Yvette, France) incorporation was measured after 72-hr culture. Similar experiments were completed with the other B-cell lines BL41, CRAG8, CRB95, and 1G8. These cells

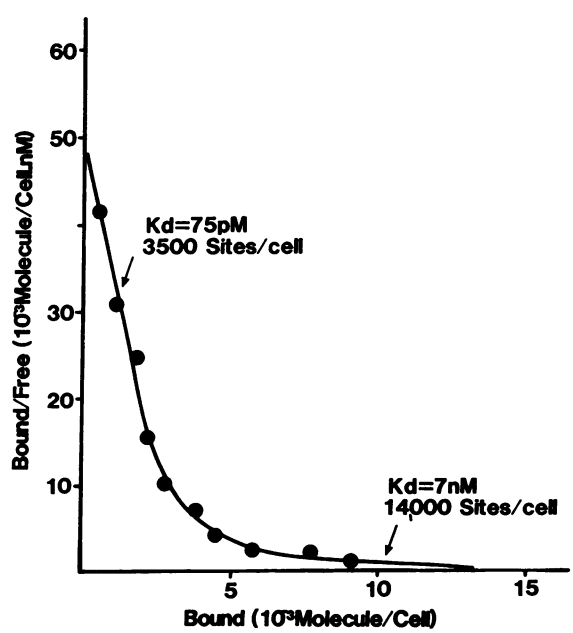

FIG. 1. Scatchard plot of ^{125}I-labeled IL-2 binding to 3B6 cells. Affinity and number of ^{125}I-labeled IL-2-binding receptor sites on 3B6 cells are known. Kd, K_d.

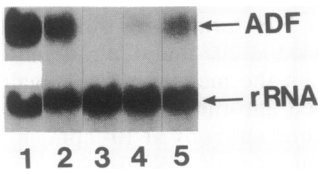

FIG. 2. Northern blot analysis with cDNA of ADF. Twenty micrograms of RNA from each sample was analyzed. Lanes: 1, ATL-2 cells; 2, 3B6 cells; 3, PBMC without stimulation; 4, PBMC stimulated with anti-CD3 monoclonal antibodies (30 ng/ml); 5, PBMC stimulated with anti-CD3 monoclonal antibodies (30 ng/ml) and phorbol 12-myristate 13-acetate (50 ng/ml).

were cultured with 2% FCS. rADF produced by *Escherichia coli* (1 mg/ml) was incubated with 10^{-3} M 2-mercaptoethanol overnight and dialyzed against 200-fold RPMI 1640 medium before use in the culture. rIL-1α (provided by Dainippon, Osaka) and rIL-2 (provided by Ajinomoto, Tokyo) were used, respectively, at 3 ng/ml and 100 international units per ml.

RESULTS

Expression of IL-2R on 3B6 Cells. The 3B6 B cells spontaneously expressed high amounts of Tac antigen (60–70% heavily labeled cells) by immunofluorescence. We examined expression of IL-2R on 3B6 cells by an ^{125}I-labeled IL-2-binding assay. Fig. 1 shows that 3B6 cells expressed unusually high amounts of IL-2R with both high (75 pM; 3500 sites per cell) and low (7 nM; 14,000 sites per cell) affinities. This high expression of IL-2R/p55Tac on 3B6 cells was confirmed by a crosslinking study of ^{125}I-labeled IL-2 and Northern blotting with cDNA for Tac (data not shown). These results led us to suspect the production of some IL-2R-inducing factor by 3B6 cells.

Expression of ADF mRNA. Northern blots of mRNA extracted from various cell lines and lymphocyte populations were hybridized with a cDNA probe for ADF (Fig. 2). Strong expression of a single species of 0.6-kilobase (kb) mRNA was seen in 3B6 cells (lane 2). High expression of ADF mRNA was also found in ATL-2 cells (lane 1). Normal resting PBMC did not express ADF mRNA (lane 3), and weak expression was seen in PBMC stimulated with anti-CD3 monoclonal antibody alone (lane 4). When PBMC were maximally stimulated with anti-CD3 plus phorbol 12-myristate 13-acetate, the expression of ADF mRNA markedly increased (lane 5), although remaining below levels seen in EBV$^+$ or HTLV-I$^+$ cell lines. We also analyzed several EBV-transformed B-cell lines and HTLV-I$^+$ T-cell lines other than 3B6 and ATL-2 cells. All of the infected cell lines tested expressed high levels of ADF mRNA, especially when compared with nonvirally infected T- and B-cell lines (data to be reported elsewhere).

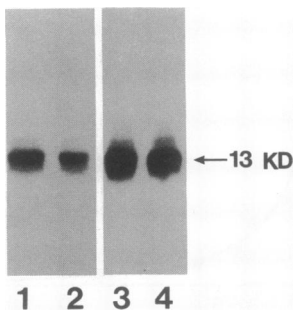

FIG. 3. Immunoblot analysis with anti-ADF. Similar pattern with a single band at 13 kDa was obtained in both ATL-2 (lanes 1 and 3) and 3B6 (lanes 2 and 4) cells with antisera either anti-NH_2 terminus (lane 1 and 2) or anti-COOH terminus (lanes 3 and 4). KD, kDa.

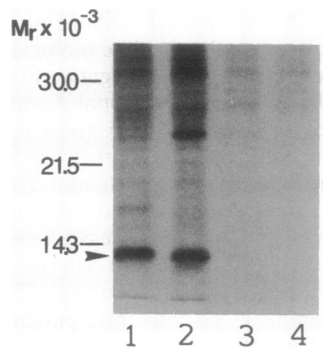

FIG. 4. Sequential immunoprecipitations for 3B6 cells. Immunoprecipitates were analyzed by SDS/PAGE (15%) under reducing conditions. Lanes: 1, with anti NH_2 terminus; 2, with anti-COOH terminus; 3, with anti-COOH terminus to the supernatant after immunoprecipitation with anti-NH_2 terminus; 4, with anti-NH_2 terminus to the supernatant after immunoprecipitation with anti-COOH terminus.

Immunoblot Analysis with Anti-ADF. Fig. 3 shows that both anti-ADF sera, not only anti-NH_2 terminus (lanes 1 and 2) but also anti-COOH terminus (lanes 3 and 4), reacted with a 13-kDa protein in 3B6 (lanes 2 and 4) as well as in ATL-2 cells (lanes 1 and 3).

Sequential Immunoprecipitations with Both Anti-ADF Sera. Fig. 4 shows that after immunoprecipitation with anti-COOH terminus antibodies, no protein can be further precipitated with anti-NH_2 terminus antibodies from 3B6 cells (lanes 2 and 4). The reciprocal experiments are also depicted in Fig. 4. They show that 3B6-IL-1, sharing the same NH_2-terminal sequence with ADF, also shares its COOH-terminal sequence with ADF and is actually the same 13-kDa molecule.

Effects of rADF on Growth of 3B6, Other B Cell Lines, and ATL-2 Cells. Because we had previously shown that purified 3B6-IL-1 sustained 3B6 cell growth in an autocrine manner, we analyzed the effect of rADF produced by *E. coli* on proliferation of 3B6 cells. Fig. 5 shows that rADF could promote the growth of 3B6 cells at low cellular concentra-

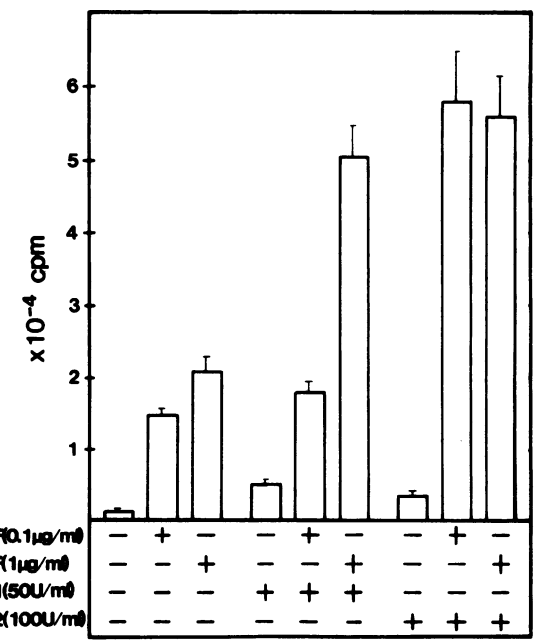

FIG. 5. Proliferation of 3B6 cells with or without rADF, IL-1α, and IL-2. The results are from experiments at 1×10^4 3B6 cells per well under serum-free conditions. A representative of five experiments is presented.

Table 1. Effect of ADF on growth of different B-cell lines

B-cell line	Origin	Cells/ml*	[^{3}H]Thymidine uptake,† cpm	
			−ADF	+ADF‡
BL41	BL(EBV$^-$)	50×10^4	2426 ± 720	23306 ± 1210
CRAG8	LCL	15×10^4	2785 ± 215	6658 ± 121
CRB95	LCL	15×10^4	22408 ± 1850	80110 ± 1958
1G8	LCL	10×10^4	4772 ± 824	30549 ± 5587

LCL, lymphoblastoid cell lines.
*Cellular concentration at initiation of experiment.
†Cell cultures were treated with [^{3}H]thymidine for the final 6 hr of 48-hr incubation. Values = mean ± SD of triplicates.
‡rADF (reduced) (3 μg/ml) was added at beginning of culture.

tions (1×10^5 cells per ml) and without FCS. This rADF effect was dose-dependent, and optimal concentrations ranged from 0.1 to 1 μg/ml. 2-Mercaptoethanol (10^{-3} M) alone dialyzed in the same condition did not induce 3B6 cell proliferation. At higher cellular concentration or with FCS, the effect of this exogenous rADF was very low, which suggests that in these conditions 3B6 cells are already producing and using sufficient amounts of their autocrine growth ADF for cell growth.

Table 1 shows that a similar effect of rADF on B-cell growth was obtained with BL41 (EBV$^-$ BL, nonproducer of ADF), CRB95, CRAG8, and 1G8 (low producer of ADF) cell lines, suggesting that ADF can act on other B cells as it does on 3B6.

The activity of rADF as an autocrine growth factor was also demonstrated in ATL-2 cells. Fig. 6 shows that rADF enhanced the growth of ATL-2 cells dose-dependently.

rADF Synergizes with IL-1 and IL-2 for 3B6 Cell Growth. Fig. 5 also shows that, whereas rIL-1 (50 units per ml) only marginally affects 3B6 proliferation, rADF addition can markedly enhance 3B6 cell growth, again with a dose-dependent effect of rADF. A similar synergistic effect was seen between rADF and IL-2. rIL-2 alone (100 international units per ml) exhibited little, if any, effect on 3B6 cell growth, but with rADF at 0.1–1 μg/ml, could efficiently support 3B6 cell proliferation without any FCS.

DISCUSSION

EBV is a 170-kb DNA virus belonging to the herpes virus family. EBV genome is not homologous with known c-*onc* genes. It has been speculated that some EBV-encoded proteins are acting as trans-activators to "switch on" transcription of a series of cellular genes. Some of these genes are involved in the physiological B-cell activation process and could govern the synthesis of lymphokines and growth factors as well as the expression of their receptors (14). We have previously observed that EBV-transformed 3B6 cells were widely responsive, not only to the autocrine 3B6-IL-1 but also to a variety of exogenous lymphokines or growth factors, such as IL-1α, IL-1β, IL-2, soluble CD23, and B-cell growth factors with low (12 kDa) and high (50 kDa) molecular mass. This wide susceptibility to a variety of growth factors has led to the hypothesis that 3B6 cells either exhibited dysregulation in expression of several growth factor receptor genes or produced a competence factor making the cells responsive to this large series of different lymphokines. As shown here, 3B6 cells express high amounts of IL-2R, whereas IL-2R expressed on B-cell line is usually with intermediate or low affinity, and the number of binding sites per cell is much lower than on 3B6 cells (15). These data led us to speculate that 3B6 cells may produce some IL-2R upregulating factor as ADF.

We report here data demonstrating that 3B6-IL-1 is, indeed, identical to ADF. Furthermore, a similar sequence has been reported from a cDNA library made from the 3B6 cells

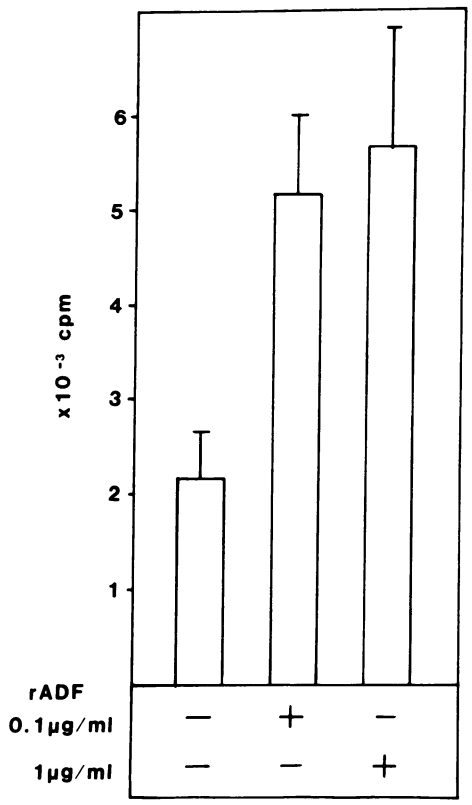

FIG. 6. Proliferation of ATL-2 cells with or without rADF. Maximal effects of rADF for ATL-2 cell growth were observed at 2×10^5 cells per ml without FCS. A representative of three experiments is presented.

(16). The fact that both HTLV-I-transformed T lymphocytes and EBV-immortalized B lymphocytes are constitutively producing the very same factor seems to imply that some underlying mechanisms of transformation could be common between the two viruses, which could possibly interact with similar cellular genes. High ADF expression was regularly seen in all tested cell lines infected by either HTLV-I or EBV.

Because we had shown (3) that purified 3B6-IL-1 sustained 3B6 cell growth in an autocrine manner, we analyzed the effect of rADF on 3B6 cell proliferation. Our data show that ADF is, indeed, an autocrine growth factor released by 3B6 cells. The effect of ADF on B-cell growth is not restricted to 3B6 cells because it could enhance cell proliferation in the other B-cell lines tested (Table 1). Furthermore, it can synergize with other lymphokines, which gives an attractive explanation for susceptibility of 3B6 cells to a wide variety of growth factors. ADF could, thus, act as a competence factor, allowing a cell to become sensitive to suboptimal amounts of other growth factors. The role of IL-1 as a putative autocrine growth factor for EBV-infected B cells (17) and HTLV-I$^+$ T cells (18) has already been suggested, although an actual autocrine loop could only be demonstrated in a few cases. On the other hand, involvement of the IL-2/IL-2R autocrine system in the development of adult T-cell leukemia by HTLV-I has been hypothesized. Although autocrine production of IL-2 by ATL cell lines is rare (19), some evidence for such an IL-2 autocrine loop has been demonstrated (20–22). We speculate that a series of factors act in synergy to support autocrine growth of infected cells. Our data suggest that ADF could be an autocrine growth factor *per se* and also potentiate the autocrine loop of other growth factors. Preliminary results suggest that rADF can synergize not only with IL-1 and IL-2 but also with interleukin 4 and interleukin 6. As already shown, ADF clearly appears involved in upregulation of IL-2R on PBMC and YT cells (8, 10). Also, ADF could

exert a similar effect on other receptors of lymphokines or growth factors. Interestingly, we have reported (23) that 3B6 also expressed strikingly high amounts of binding sites for IL-1. Whether the upregulating activity on IL-2R and the effects on cellular growth of ADF are mediated through reduction/oxidation reactions is important to know. Recombinant thioredoxin of *E. coli* in the presence of thioredoxin-reductase and NADPH can sustain 3B6 cell growth and also synergize with IL-1 and IL-2 (data not shown), which strongly suggests that ADF exerts its effects on cellular proliferation through its reducing activity. The role of reducing chemical agents on lymphoid cellular growth has already been widely described and discussed (24, 25). The positive effect of 2-mercaptoethanol on growth of murine cell lines (26) and its macrophage-replacing activity (27) have been known for many years. Thioredoxin activates the cytosolic glucocorticoid receptor (28) and insulin degradation (29). Interestingly, thioredoxin has a considerable homology with recently cloned phospholipase C (30). Activation of phospholipase C is a key event for signal transduction and cell activation. In phage T7, host *E. coli* thioredoxin is associated with T7 DNA polymerase (31) and essential for phage DNA replication (32). Thus, thioredoxin is required between host bacteria and bacteriophages in a "synbionism" mechanism. Release of an endogenous factor with redox potential might be considered as an original mechanism of "self-promotion" used by virus-infected lymphocytes, possibly in both HTLV-I- and EBV-infected lymphocytes.

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