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Human immunodeficiency virus induction of malignant transformation in human B lymphocytes

(c-*myc*/Epstein–Barr virus/B-cell lymphoma/trans-activation)

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ABSTRACT **Aggressive B-cell lymphomas are occurring with increasing incidence among individuals infected with human immunodeficiency virus (HIV). Several lines of evidence implicate both Epstein–Barr virus (EBV) and c-*myc* activation in the pathogenesis of a major subset of these tumors. These observations prompted our investigation of interactions among EBV, c-*myc*, and HIV in primary B cells. We show that nonimmortalized peripheral B lymphocytes from EBV-seropositive, HIV-seronegative donors can be infected by HIV and that a subset of these lymphocytes become transformed. Malignant transformation was documented by several criteria. These cells displayed altered growth properties, propagating in 1% serum and cloning in soft agar, and formed invasive tumors of Burkitt lymphoma phenotype after subcutaneous injection into severe combined immunodeficiency mice. Such cells revealed marked enhancement of EBV DNA and RNA and of endogenous c-*myc* transcripts and protein. HIV-1 infection of already immortalized B-cell lines led to a similar upregulation of EBV and c-*myc* transcripts. These data indicate that HIV has properties of a transforming retrovirus, as it mediates two events linked to B-cell neoplasia: deregulation of c-*myc* and activation of EBV. They also raise the possibility of a role for HIV, apart from induction of immune suppression, in the pathogenesis of B-cell lymphoma in the acquired immune deficiency syndrome.**

Human immunodeficiency virus (HIV)-infected individuals are at increased risk for development of fatal polyclonal B-cell lymphoproliferative disorders (1) and aggressive B-cell non-Hodgkin lymphomas (2). Serial biopsies of lymph nodes from HIV-seropositive patients indicate a progression from polyclonal B-cell proliferation to lymphoma (3), with both types of cell frequently carrying Epstein–Barr virus (EBV) genomes (4). c-*myc* deregulation is another manifestation of many AIDS-related clonal B-cell disorders (4). Neoplastic conversion of B cells may involve cooperative effects of these two phenomena. EBV can immortalize but not transform B lymphocytes (5). c-*myc* has a similar growth-perturbing but nontransforming effect in most systems (6), although it may directly lead to malignant transformation in certain hematopoietic cells (7). However, introduction of activated c-*myc* into EBV-infected B cells may lead to tumorigenicity as assessed by growth in agar, in low serum, and in nude mice (8).

HIV may be involved in these processes, either directly or through extracellular gene products. With this concern, we have investigated interactions among EBV, c-*myc*, and HIV in peripheral B cells as well as immortalized B-cell lines. Albeit only 5% at most of peripheral B lymphocytes express detectable levels of the high-affinity HIV receptor CD4 (9), most human B-cell lines can be infected with HIV-1 regardless of the presence of EBV genomes or CD4 expression (10, 11). This may relate to a very low level of membrane CD4, as infection may be blocked by anti-CD4 monoclonal antibodies in lines that lack detectable antigen (12). Alternatively, it may involve low-affinity receptors such as C3 and Fc (13).

We demonstrate here that circulating B lymphocytes are susceptible to HIV infection, similar to their immortalized counterparts. We also present evidence that HIV plays a direct role, at least *in vitro*, in initiating and maintaining transformation of B lymphocytes through a coupling of deregulation of c-*myc* expression with activation of EBV transforming proteins.

MATERIALS AND METHODS

Cells. Peripheral mononuclear cells were isolated from heparinized venous blood by Ficoll/Hypaque density-gradient centrifugation, monocytes were depleted by plastic adherence, and T cells were removed by rosetting with neuraminidase-treated sheep erythrocytes (14). When this procedure is used, the B-lymphocyte pool typically contains <1% $CD3^+$ T cells, <0.5% esterase-positive monocytes, and >95% surface immunoglobulin-positive cells.

Viral Infections. B lymphocytes (2×10^6 cells per well) were exposed to 1×10^5 transforming units of B95-8 EBV, or 1×10^4 50% tissue culture infectious doses (multiplicity of infection, 0.005) of HIV-1/IIIB for 2 hr at 37°C, followed by washing and resuspension in fresh culture medium.

Membrane Immunofluorescence Assays. Indirect immunofluorescence and cytofluorimetry were performed as described (15) using the following monoclonal antibodies (mAbs): anti-CD3 (mAb 454.3), anti-latent membrane protein (LMP) (kindly supplied by D. A. Thorley-Lawson, Tufts Univ. School of Medicine, Boston), anti-CD23 (mAb EBVCS2), and CD21 (B2, EBV receptor). Surface immunoglobulin was directly detected with fluoresceinated goat anti-human reagents.

Detection of EBV and HIV. *In situ* hybridization for EBV nucleic acid was performed with a cosmid clone, pM966/20 (16), containing ≈25 kilobases (kb) of EBV sequence, encompassing 5′ leader sequences and the gene for LMP. It was derivatized with digoxigenin-11-dUTP. Procedures for DNA probe labeling by the random-primer method, *p*-formaldehyde cell fixation, hybridization, and detection have been described elsewhere (17).

The PCR was performed as described elsewhere, using primer pairs SK68/69 (*env*) and SK38/39 (*gag*) (18). Reaction aliquots were run on a 1.5% agarose gel in the presence of ethidium bromide. Comparisons were made with amplified

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Abbreviations: EBV, Epstein–Barr virus; FBS, fetal bovine serum; HIV, human immunodeficiency virus; LMP, latent membrane protein; mAb, monoclonal antibody; *scid*, severe combined immunodeficiency.

DNA from U1.1A, a chronically infected promonocyte line containing two DNA copies of HIV per cell (17).

Analysis of c-*myc*. *In situ* hybridization was performed with a digoxigenin-labeled 1.5-kb fragment containing the third exon of human c-*myc* (19). Indirect immunofluorescence utilized a polyclonal rabbit anti-human Myc antiserum and a rhodamine-conjugated goat anti-rabbit immunoglobulin counterstain. An indication of relative amounts of c-myc RNA was obtained by using Quick-Blots (Schleicher & Schuell) (20), in which mRNA is immobilized onto nitrocellulose, followed by hybridization with the digoxigenin-labeled c-*myc* exon 3 probe (21). Controls for the amount of RNA bound to filters were performed on washed blots using ^{32}P-labeled oligo(dT).

Transformation Assays *in Vitro* and *in Vivo*. Growth in soft agar was assayed by embedding 5×10^3 cells in 0.5 ml of RPMI 1640 medium plus 20% fetal calf serum and 0.3% agarose (Difco), contained in polystyrene macrowells. Duplicate plates were scored for colonies 12 days after seeding.

A xenotransplantation model was used to evaluate malignant potential *in vivo* (22–26). Cells (10^7) in 0.2 ml of phosphate-buffered saline were injected subcutaneously into irradiated (4 Gy) 4- to 6-week-old female BALB/c athymic nude mice or 8- to 10-week-old female C.B.-17 severe combined immunodeficiency (*scid*) mice. All tumor masses were removed and examined blindly by a single hematopathologist.

RESULTS

Derivation of Cell Lines After Exposure of B Lymphocytes to EBV or HIV. B cells were isolated from two female HIV-seronegative, EBV-seropositive (32 and 37 years old) and two female HIV- and EBV-seronegative donors (6 and 15 years old). They were plated in macrowells at 2×10^6 cells per well in RPMI 1640 medium plus 10% fetal bovine serum (FBS) and treated under one of three separate conditions: exposure to buffer, exposure to HIV alone, or exposure to EBV. After cells from the EBV-seropositive individuals had been in culture for 21–28 days, immortalized lymphoblastoid lines emerged from B lymphocytes exposed to only HIV (growth in 3 of 10 wells) or to EBV (growth in all of 6 wells), while cells exposed to buffer alone failed to proliferate during a 45-day culture period. Lymphocytes from the two EBV-seronegative donors failed to yield immortalized cells after exposure to HIV.

Two representative lines from one donor were used for the majority of experiments reported here, designated B-EBV and B-HIV for the viruses to which they had been exposed exogenously. Immortalized lines were also derived from the second donor, although these did not grow in low serum and were not further characterized. All lines were negative for T-cell CD3 and positive for membrane IgM.

Both B-HIV and B-EBV lines expressed EBV-associated gene products LMP and CD23 and the EBV receptor (CD21). B-HIV cells expressed 3- to 5-fold higher levels of these antigens than did B-EBV. High LMP expression is associated with immortalization of B lymphocytes (5) and transformation of rat fibroblasts (27). CD23 is an autocrine growth receptor thought to be required for LMP expression and cell immortalization and is induced by the EBV nuclear protein antigen gene EBNA-2 (28).

All cell lines were maintained in culture medium plus 10% FBS for 3 months. Sublines from three different growth-positive macrowells were then prepared by growth of unselected B-HIV cells in 1% FBS over 4 weeks, with repetitive Ficoll/Hypaque density-gradient centrifugations to remove nonviable cells. One subline, derived from <100 original B-HIV cells of one donor, was extensively characterized. This B-HIV oligoclone was designated B-HIV1.

EBV and HIV Genomes. B-EBV cells contained EBV-specific transcripts and genome copy numbers below the sensitivity of our *in situ* assays (Fig. 1 *A* and *B*). In contrast, EBV genome number and gene expression were both markedly enhanced in all cells of the HIV immortalized population (Fig. 1 *C* and *D*).

By PCR, HIV proviral DNA was found solely in the B-HIV and B-HIV1 subline populations with, on average, one proviral copy per cell (Fig. 2).

HIV replication was not detected in tests of supernatant fluids for HIV p24 Gag protein (17) or particulate reverse transcriptase activity (14). However, thin-layer electron microscopy of these cells revealed few extracellular budding virions (data not shown).

Transformed Phenotype *in Vitro*. B-HIV1 cells grew logarithmically in medium containing 1% FBS (Fig. 3*A*). This growth curve, prepared by doing daily cell counts of cultures maintained without addition of fresh medium, was similar to one obtained with the EBV$^+$ Burkitt lymphoma line Raji (8). In contrast, B-EBV cells did not exhibit any significant growth under these conditions (Fig. 3*A*). Conditioned medium prepared from 3-day-old B-HIV1 cultures and used in concentrations as high as 75% (vol/vol) did not stimulate growth of B-EBV cells (Fig. 3*A*). Thus, the proliferative capacity of B-HIV1 cells appeared unrelated to lymphokine production or receptor shedding, factors that may recruit growth of nontransformed B cells in low serum (29, 30).

To compensate for nutrient depletion, growth curves were repeated with replacement of one-half of the culture medium with fresh 1% FBS every 2–3 days. B-HIV1 cells continued to proliferate (Fig. 3*B*) and have been in continuous culture in 1% serum for >40 weeks. B-HIV1 cells could also form colonies in soft agar, similar in size to those produced by Raji (data not shown). No colonies were formed by the B-EBV line. The cloning efficiency of B-HIV1 cells, ≥1.0%, is of the same order of magnitude as that of some Burkitt lymphoma cell lines (8).

Malignant Potential *in Vivo*. B-EBV cells failed to elicit masses in either nude (0/6) or *scid* (0/9) mice over an observation period of 8 weeks. Between 8 and 12 weeks, 0/6 nude but 7/9 *scid* mice grew observable tumors. In contrast, B-HIV1 was highly tumorigenic. With these cells, 2 of 4 *scid* mice developed masses >2 cm in diameter by 4 weeks, with one animal bearing a visible tumor by 11 days postinoculation (Fig. 4*A*). One of 6 nude mice developed a similar lesion.

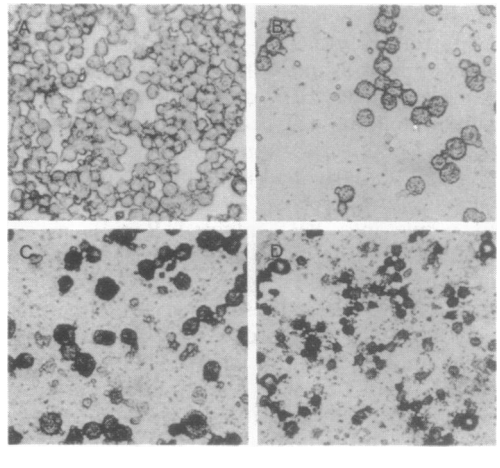

FIG. 1. Detection of EBV nucleic acids in B-EBV and B-HIV cells. *In situ* hybridization for the EBV transforming gene LMP was performed with B-EBV (*A* and *B*) and B-HIV (*C* and *D*) cells before (*A* and *C*) and after (*B* and *D*) RNase I treatment. Blue-black precipitates are formed at sites of probe–nucleic acid hybridization.

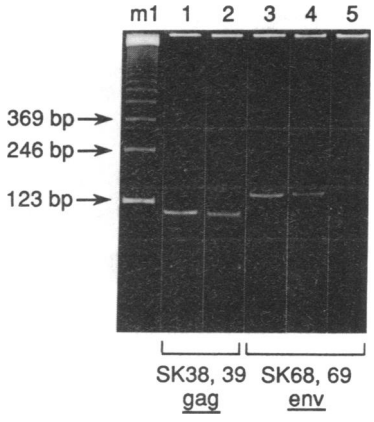

FIG. 2. Agarose gel electrophoresis of DNA extracted from various cell lines after PCR-directed DNA amplification. Twenty micrograms of each sample was electrophoresed through a 1.5% agarose minigel containing 0.5 mg of ethidium bromide per ml. Lanes: m1, size markers; 1 and 3, HIV-infected U937 cells (U1.1A) harboring two proviral copies of HIV-1 per cell; 2 and 4, B-HIV1 cells; 5, control U937 cells lacking HIV. The regions of the HIV-1 genome used for primer construction are listed below the appropriate lanes. bp, Base pairs.

Histologic examination of B-HIV1 *scid* tumors revealed a highly malignant lymphoma of immunoblastic phenotype (Fig. 4 *B* and *C*). These features included cells with multiple nucleoli and extensive invasion into muscle, endothelial vessels, and fat. In blinded fashion, they were diagnosed as "malignant lymphoma, probable Burkitt type." A partial karyotype of cells explanted from this tumor confirmed its human origin. In contrast, all tumors arising in four *scid* mice after inoculation with B-EBV cells were classified as "plasmacytomas."

HIV Activation of c-*myc* and EBV. Basal levels of c-*myc* transcripts (Fig. 5*A*, lane 1) and protein (data not shown) were below the limits of detection of our assays in the B-EBV line. In contrast, B-HIV1 cells contained *myc* transcripts at levels comparable to that of Burkitt lymphoma cell line Raji (compare lanes 2 and 3). Control hybridizations using ^{32}P-labeled oligo(dT) confirmed that mRNA concentrations were equivalent in all lanes (Fig. 5). In addition, B-HIV1 cells contained high levels of Myc nuclear protein (data not shown).

To determine whether this expression was a consequence of HIV infection, Raji cells were infected with HIV-1. Raji was chosen because of its elevated basal level of *myc* expression and because, despite having c-*myc* rearrangements, its *myc* promoters and >1 kb of upstream sequences are intact (31). Exposure of Raji to HIV-1 for 3 days led to detectable increases in *myc* transcription in 100% of cells by *in situ* hybridization (Fig. 6 *B* and *C*). Seven days postinfection an 8- to 16-fold enhancement in myc RNA expression was observed (Fig. 6*A*, compare lanes 1 and 2). At this point, culture supernatants contained >20 ng of HIV-1 p24 gag antigen per ml, an indication that cells were productively infected with HIV, consistent with the ability of other groups to infect these cells despite their lack of detectable membrane CD4 (10). Exposure of Raji to equivalent concentrations of heat-inactivated HIV did not lead to upregulation of *myc* levels (data not shown).

We also postulated that HIV infection of B cells harboring EBV directly induces expression of EBV transforming genes such as LMP. EBV transcription is dramatically upregulated in our B-HIV cell population, and EBV virion production in EBV$^+$ B cells superinfected with HIV is enhanced in most (10), albeit not all (32), systems. To further test this hypothesis, B-EBV cells were exposed to HIV-1 or to an identical

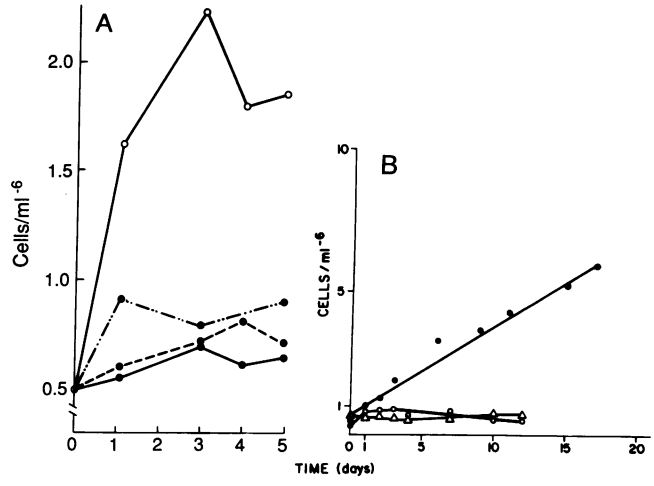

FIG. 3. Constitutive HIV and EBV expression decreases serum requirement for growth of immortalized cells. (*A*) Cell lines prepared from B lymphocytes exposed to HIV (○) or EBV (●) were resuspended at a concentration of 5×10^5 cells per ml in RPMI 1640 medium supplemented with 1% fetal bovine serum, and cell numbers were measured at daily intervals for 5 days. In addition, B-EBV cells were cultured in the presence of 20% (●---●) or 75% (●—●) conditioned medium, obtained from the cell-free supernatants of B-HIV1 cells seeded at 5×10^5 cells per ml and cultured for 3 days. (*B*) B-HIV1 (solid circle) and B-EBV (two preparations; open symbols) cells were plated in 1% serum-containing medium as described above with fresh medium added every 2–3 days.

amount of heat-inactivated virus. Both preparations induced equivalent cellular DNA synthetic responses as measured by [^{3}H]thymidine incorporation, a polyclonal mitogenic response initiated by viral envelope (33) and independent of productive viral infection. In contrast, transcription of a region of the EBV genome containing LMP was increased 3 days postinoculation with replication-competent HIV, but not after exposure to heat-inactivated virus (data not shown).

DISCUSSION

We document infection of nonimmortalized human B cells by HIV *in vitro* and malignant transformation of a subset of these cells. Albeit the mechanism of viral entry is unknown, a fraction of B cells express CD4 (9), and circulating B cells may contain HIV provirus at levels comparable to those of CD4$^+$ T cells (34).

Deregulation of c-*myc* together with expression of certain EBV proteins may be necessary and sufficient components for malignant transformation (8, 27). Our data indicate that HIV itself can initiate or potentiate both events, which then lead to tumorigenic growth patterns in B lymphocytes. The experiments demonstrating HIV induction of c-*myc* in B cells are consistent with the findings of others (35) that *myc* deregulation occurs after HIV infection of monocytic cells, with levels of viral RNA correlated to levels of *myc* expression. This effect may be cell lineage restricted, as upregulation of c-*myc* has not been reported in the few T-cell lines examined.

The ability of freshly isolated EBV$^+$ B-cell lines from normal individuals to proliferate in 1% serum, clone in agar, and rapidly form invasive tumors in *scid* mice is likely to be related to HIV infection in our cells. B-cell lines are usually not tumorigenic in *scid* mice (23). Large inocula of EBV$^+$ B-cell lines (24, 25, 36) or EBV$^+$ B lymphocytes (26) can cause tumors after variable incubation periods in *scid* mice, as also shown in our control group. However, in most of these instances the lymphoid masses do not fulfill the histopathologic and phenotypic criteria for malignancy, as noted here

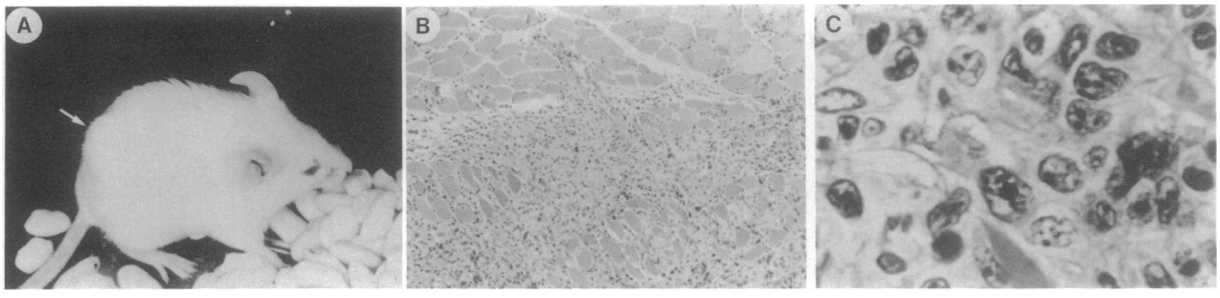

FIG. 4. Development of a xenotransplantation model for human HIV-infected B cells in mice. B-EBV or oligoclonal B-HIV1 cells (10^7 cells) were injected subcutaneously into the flanks of irradiated female C.B. 17-*scid* and athymic nude mice. (*A*) Gross tumors developed within 4 weeks in 1 of 6 nude mice and 2 of 4 *scid* mice; 1 of the latter is shown (arrow). Excised tissue was fixed in Bouin's solution. (*B* and *C*) After paraffin embedding, sectioning, and staining with hematoxylin and eosin, examination under a microscope revealed a malignant lymphoma of immunoblastic phenotype. (*B*, ×75; *C*, ×320.)

and recently shown in a direct comparison of groups of *scid* mice administered either EBV-infected peripheral lymphocytes, EBV-immortalized cell lines, or Burkitt lymphoma lines (24).

An important question is the relevance of this model to lymphomas found in AIDS patients. Up to 12% of HIV-seropositive individuals in the U.S. develop clinically aggressive B-cell non-Hodgkin lymphomas (37). A frequent role for c-*myc* activation in the pathogenesis of these lymphomas is documented by rearrangements of this protooncogene in many specimens (38–40). Both EBV expression and c-*myc* deregulation occur in at least 30% of these tumors. It is frequently hypothesized that these lymphomas arise as HIV-mediated immune suppression permits reactivation of latent EBV infection in B cells. This is thought to be followed by uncontrolled polyclonal proliferation, which increases the probability of a recombinase error and eventually leads to c-*myc* activation. However, development of these lymphomas may not correlate with changes in absolute $CD4^+$ T-cell count or CD4/CD8 T-cell ratios (41), which appear to be the best indicators of immune suppression in HIV infection. Indeed, 40% of all HIV-linked lymphomas occur in asymptomatic individuals (41). We speculate, based on our *in vitro* model, that HIV plays a role in initiating and maintaining transformation of B cells through a combination of c-*myc* deregulation with activation of EBV transforming proteins.

An ostensible limitation of this model is that HIV-1 sequences have not been found in biopsy samples of HIV-associated lymphomas by Southern analysis (4, 42, 43). PCR reveals levels of HIV only 10-fold greater than would be predicted from infiltrating T cells in the pathologic specimens (44, 45). This discrepancy may be explained in several ways.

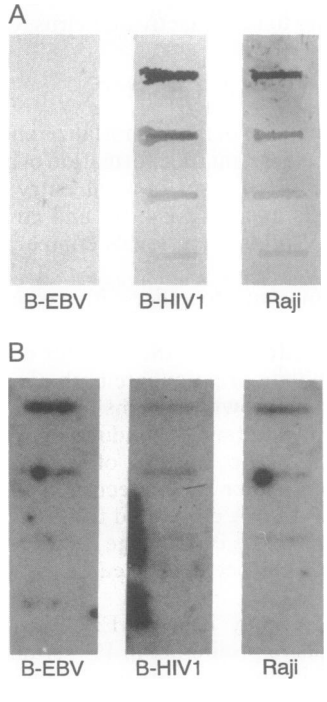

FIG. 5. Slot-blot hybridization for c-*myc* transcripts. Nucleic acids were extracted from 2.0×10^6 B-EBV, B-HIV1, and Raji cells, serial 5-fold dilutions were made, and mRNA was immobilized onto nitrocellulose by the Quick-Blot technique. Filters were probed with a digoxigenin-labeled c-*myc* construct (*A*) and then washed and probed with ^{32}P-labeled oligo(dT) to control for the amount of poly(A)-containing mRNA in the samples (*B*).

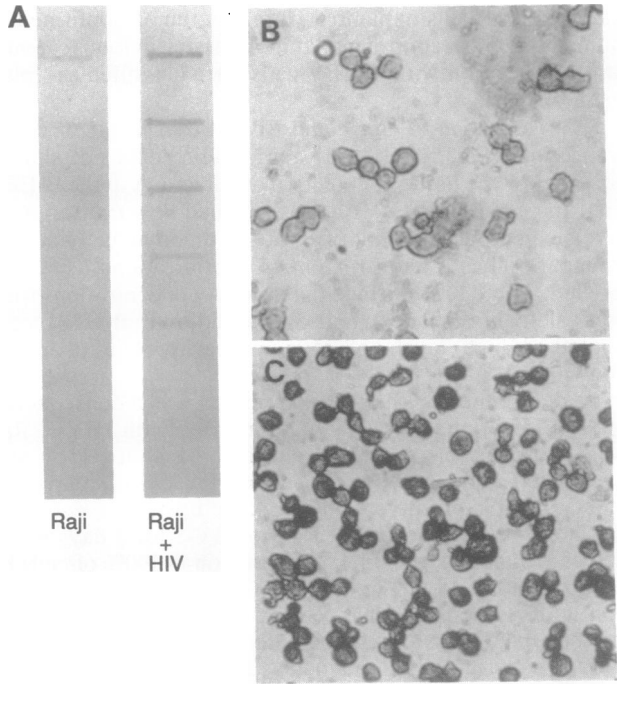

FIG. 6. Analysis of c-*myc* transcripts by slot-blot and *in situ* hybridization in Raji cells exposed to HIV. Raji cells were exposed to buffer or HIV-1 for 2 hr at 37°C, washed with phosphate-buffered saline, and then incubated in fresh culture medium at 37°C. *In situ* hybridization or slot blotting for c-*myc* with an exon 3 probe was then performed on days 3 and 7, respectively, after HIV exposure. (*A*) Nucleic acid was extracted from 1.2×10^6 cells, serial 2-fold dilutions were made, and mRNA was immobilized onto nitrocellulose by the Quick-Blot technique. (*B*) *In situ* hybridization of Raji exposed to buffer. (*C*) *In situ* hybridization of Raji exposed to HIV. Control hybridization with ^{32}P-labeled oligo(dT) revealed comparable amounts of poly(A)-containing mRNA in the upper slots of both samples (data not shown).

First, a limited number of tumors have been examined. Indeed, clonal cell lines derived from 2 of 2 AIDS-associated lymphomas maintained their transformed phenotype and c-*myc* rearrangements and expressed both EBV and HIV (‡, 46). Thus, there appears to be at least a subset of tumors *in vivo* that do contain HIV. Second, direct HIV infection may be irrelevant to the pathogenesis of certain of the lymphomas in AIDS, with such malignancies known to arise under diverse conditions of immune suppression unassociated with retroviral infection. Third, defective provirus undetectable by the methods used may be involved. In this regard, it is of interest that in transgenic mice expression of the HIV long terminal repeat, capable of interacting with numerous transcription factors, predominates in B lymphocytes over other cell types (47). Finally, biologically active factors important to cell transformation, secreted as cytokines generated by a few infected cells, might be directly taken up by neighboring B lymphocytes, obviating the requirement for maintenance of HIV in tumor tissue.

With respect to those possibilities directly involving HIV, we hypothesize that tumorigenicity may be mediated by *tat*, the HIV *trans*-activator of transcription. In preliminary experiments, we have explored a model in which Tat and B lymphocytes are used. Exposure of EBV immortalized B cells and certain types of transformed B-cell lines to a synthetic peptide representing the first 58 amino acids and all functional domains of Tat (48) resulted in upregulation of c-*myc* gene expression (unpublished data).

‡Suraiya, R., Yao, K. X. & Zhou, J. T., Fifth International Conference on AIDS, June 4–9, 1989, Montreal, Abstr. C.703.

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