Expression of an Exogenous Interleukin 6 Gene in Human Epstein Barr Virus B Cells Confers Growth Advantage and In Vivo Tumorigenicity

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Summary

The biological role of interleukin 6 (IL-6) molecules in human B cell tumorigenesis was studied by using an episomal expression vector, pHEBoSV-IL6, to introduce stably the human IL-6 gene into human Epstein Barr virus (EBV)-transformed B lymphoblasts. The gene was present in the IL-6-transfected cells in a high copy number and was efficiently expressed, resulting in the secretion of consistent levels of IL-6 molecules. The constitutive expression of the IL-6 gene led to an altered pattern of growth and to a malignant phenotype, as shown by clonogenicity in soft agar cultures and tumorigenicity in nude mice. These data suggest that the combined action of EBV, which exerts an immortalizing function, and of the growth-promoting activity of IL-6 molecules, can give rise to fully transformed B cell tumors in immunodeficient subjects.

EBV is a herpes virus that preferentially infects B lymphocytes and induces proliferation, Ig secretion, and immortalization, giving rise to long-term lymphoblastoid B cell lines (LCL)[1] (1). EBV infection appears to play a pathogenetic role in the development of endemic Burkitt lymphoma (eBL) and in the recently identified AIDS-associated Burkitt lymphoma (AIDS-BL) (2–4). The nontumorigenic capabilities of LCL suggest that the neoplastic EBV-infected clones present in eBL and AIDS-BL may have acquired a malignant phenotype through additional pathogenetic events, possibly mediated by a deregulated expression of oncogenes (5, 6). As shown for a variety of neoplastic cell types, the continuous proliferation of EBV-infected B cells appears to depend on autocrine secretion of cytokines, including IL-1 and B cell growth factor–like molecules (7–10). IL-6 is a glycoprotein secreted by a variety of cell types, e.g., monocytes, fibroblasts, certain T cell lines, and myeloma cells (11). Its biological action includes the growth stimulation of plasmacytomas and some EBV lymphoblastoid B cell lines (12, 13). To investigate whether EBV infection, which exerts a typical immortalizing function, cooperates with the B cell growth-promoting activity of IL-6 in inducing B cell tumorigenesis, we transfected ROHA-9 MC3 cells, an EBV B cell line (8, 9), with an expression vector that allows a constitutive expression of the human IL-6 cDNA. Our results indicate that hyper-expression of an exogenous IL-6 gene in EBV-immortalized B cells results in a dramatic decrease in the requirement for exogenous growth factors with the development of a fully tumorigenic phenotype.

Materials and Methods

Animals and Reagents. 4-wk-old Swiss female mice were purchased from Charles River Breeding Laboratories (Italy). LPS *Escherichia coli* 055:B55 was obtained from Difco Laboratories (Detroit, MI); Plasmid pHEBoSV and pUC9 $\beta_2$21 were obtained from R. Dalla Favera and P. B. Segal, respectively, hygromycin B was purchased from Boehringer Biochemica (Italy); F(ab)$_2$ fragments of goat antibodies to human or mouse Igs and Leu 16 mAb (anti-CD20) were obtained from Kallested (Austin, TX) and Becton-Dickinson (Italy), respectively. B6 mAb (anti CD23), W6/32 mAb (anti–human MHC class I), and OKDR (anti–human MHC class II) were purchased from Coulter Immunology (Hialeah, Florida), Seralab (Sussex, England), and Ortho Diagnostics (Raritan, NJ), respectively. rIL-6, specific activity 1×10^7 U/mg, was kindly provided by T. Kishimoto; goat polyclonal antibody to IL-6 (specific inhibitory activity 10^2 U/μl) was the gift of J. Van Damme.

Cells and Culture Conditions. The establishment and characterization of the EBV$^+$ ROHA-9 and ROHA-9MC3 cells have been previously reported (8, 9). Cells were cultured in DME supplemented with 10% vol/vol inactivated FCS (Flow Laboratory) or in HB101 medium (Hana Biologics, Berkeley, CA). Media were additioned with 3 mM glutamine and 10 mM Hepes buffer, pH 7.2 (Gibco Laboratories, Grand Island, NY). Where indicated, cells

[1] *Abbreviations used in this paper:* BL, Burkitt lymphoma; eBL, endemic BL; LCL, lymphoblastoid cell lines.

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were tested for reactivity with fluoresceinated $F(ab)_2$ fragments of goat antibodies to human or mouse Igs and for CD20, CD23, MHC class I and class II expression by flow cytometry (Ortho Spectrum Cytofluorography, Ortho Diagnostics, Italy). Human monocytes were isolated from PBMC by discontinuous Percoll gradients as described (14) with minor modifications.

Plasmid Construction and Transfection. To generate the pHEBoSV-IL6 plasmid, the human IL-6 cDNA was isolated from pUC9 $\beta_2$21 plasmid (15) by HindIII–SalI digestion and ligated to the HindIII–SalI sites of pHEBoSV plasmid (5) by standard techniques (16). Cells were transfected by electroporation (17). Cells were washed and resuspended in 0.8 ml of Ca^{2+} and Mg^{2+}–free PBS (D-PBS) at a concentration of 1.5×10^7/ml in the presence of 10 μg of intact pHEBoSV or pHEBoSV-IL6 plasmids. Cells were subjected to a single electrical pulse (0.2 kV, 960 μFD, 15 ms) using a Bio Rad apparatus, recovered, and cultured for 48 h in DME supplemented with 10% FCS before selection in 300 μg/ml of hygromycin B. Stable bulk cultures were obtained after 3 wk and subjected to limiting dilution cultures (0.2 cells/well) to obtain MC3–IL-6 subclones. Cells were routinely cultured in hygromycin B–supplemented medium.

DNA and RNA Analysis. Low molecular weight DNA was isolated according to Hirt (18), electrophoresed in 0.6% agarose gel, blotted on Gene Screen Plus filters (Du Pont, Italy), and analyzed by Southern blot using an IL-6 cDNA probe ^{32}P-labeled to a specific activity of $0.8–1.0 \times 10^9$ cpm/μg by random hexamer method (19). Hybridization was performed at 65°C in 1 M NaCl, 10% dextran sulfate, 1% SDS, 100 μg/ml denatured salmon sperm DNA. Washing was performed at 65°C as suggested by the manufacturer. Total RNA was extracted by the guanidine-isothiocyanate method, electrophoresed in 1% agarose/2.2 M formaldehyde gels, and then transferred to nylon filters (Gene Screen Plus) by Northern blot. Hybridization and washing were performed as described above. The amounts of RNA were calculated by absorbance at 260 nm and by equalization on agarose gel electrophoresis. After hybridization, the amounts of transferred RNA were checked by staining the filters with 0.04% methylene blue as described by Maniatis et al. (16).

Cell Growth Assay. To assay the secretion of IL-6 molecules by the transfected cells, supernatant fluids from control and IL-6-transfected cells were collected during the logarithmic growth of the cells, dialyzed extensively against DME, filtered (0.2 μm), and analyzed for their capacity to sustain the growth of the IL-6-dependent 7TD1 hybridoma cells. The assay was performed essentially as described (20), except that cold thymidine was omitted from the assay medium. Growth in soft agar was assayed by plating 10^3, 5×10^3, and 1×10^4 cells in 3 ml of medium supplemented with 20% FCS and 0.3% agar in 30-mm culture plates. Cultures were scored for colonies 14 d following seeding. Aggregates of at least 50 cells were considered colonies.

Tumorigenicity in Immunodeficient Mice. Tumorigenicity was assayed essentially as described (21). Briefly, 4-wk-old Swiss female nude mice (Charles River Breeding Laboratories) were injected subcutaneously with 5×10^6 cells in 0.2 ml of PBS. Tumor diagnosis was confirmed by standard histological examination; representative tumor samples were analyzed for B cell surface markers by flow cytometry.

Results and Discussion

To obtain a constitutive expression of human IL-6 cDNA in EBV-infected lymphoblasts, an appropriate plasmid was developed using the vector pHEBoSV, which carries the hygromycin B resistance gene together with the EBV origin of replication (oriP) (5, 22). The IL-6 cDNA coding sequences were excised from the pUC9 $\beta_2$21 plasmid (15) and inserted downstream to the SV40 promoter element of pHEBoSV plasmid (Fig. 1 *A*). The pHEBoSV-IL6 and pHEBoSV control vectors were transfected by electroporation into ROHA-9MC3 EBV-B cell line (hereafter referred to as MC3) (9). Following selection of the transfected cells in medium sup-

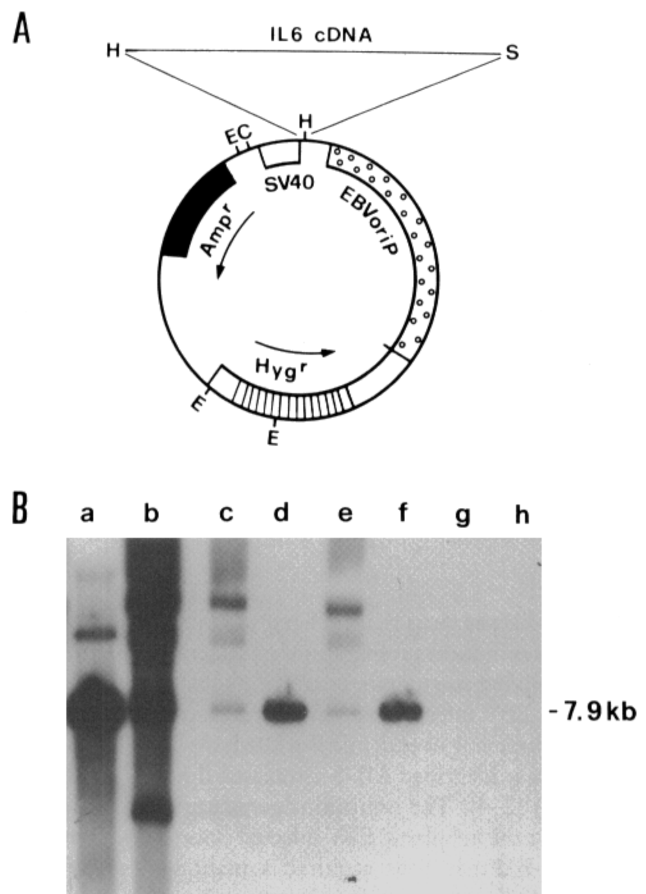

Figure 1. Schematic representation of the pHEBoSV-IL6 vector and evidence of its extrachromosomal copies in transfected cells. (*A*) Relevant regions of the pHEBoSV-IL-6 plasmid are indicated by boxes marked as follows: SV40, SV40 enhancer/promoter region; IL-6 cDNA, full length human IL6 cDNA; EBVorip, EBV origin of replication; Hyg^r, hygromycin B-resistance gene flanked by the thymidine kinase promoter and polyadenylation regions; Amp^r, ampicillin-resistance gene. Restriction sites: H, HindIII; S, SalI; E, EcoRI. (*B*) Southern blot analysis of transfected plasmids. Low molecular weight DNA was extracted from pHEBoSV-IL6 or pHEBoSV-transfected cells, transferred to nylon membrane and probed with a ^{32}P-labeled IL6 cDNA. (Lanes *a–b*) HindIII digested and undigested pHEBoSV-IL6 plasmid (1 ng); (lanes *c–d*) undigested and HindIII-digested plasmid DNA of MC3-IL6-9 cells; (lanes *e–f*) undigested and HindIII-digested plasmid DNA of MC3-IL6-11 cells; (lanes *g–h*) undigested and HindIII-digested plasmid DNA of MC3-pHEBoSV control cells. In the undigested samples the multiple bands correspond to the different circular forms of the transfected plasmid. Similar results were obtained in the case of control cells and other IL-6–transfected bulk cultures and clones (not shown).

plemented with hygromycin B (300 μg/ml), bulk cultures of IL-6-transfected MC3 cells (MC3-IL6) or MC3-pHEBoSV were obtained. The MC3-IL6 cells were then subjected to limiting dilution cultures (0.2 cells/well) to obtain cell clones. The MC3-IL6-9, MC3-IL6-11 bulk cultures and the MC3-IL6-2C4 and MC3-IL6-2A2 clones were randomly selected for the present study; MC3-pHEBoSV bulk cultures served as controls.

The presence of intact vectors in transfected cells was confirmed by Southern analysis of low molecular weight DNA. As shown in Fig. 1 *B*, the vectors were present as extrachromosomal plasmids with a copy number of ~5–10 per cell. No integrated copies of the plasmids were revealed by Southern analysis of genomic DNA (not shown). The expression of the transfected IL-6 cDNA was examined by Northern blots of total RNA from the MC3-IL6 cells by using an IL-6–specific probe. As shown in Fig. 2 *A*, all the transfected MC3-IL6 clones expressed high amounts of IL-6 mRNAs, whereas the MC3-pHEBoSV control cells showed low levels of specific IL-6 mRNA transcripts. Moreover, the MC3-IL6 cells secreted high levels of IL-6 activity, as assayed by the IL-6-dependent 7TD1 hybridoma cell line (20) (see Fig. 2 *B*). On a per cell basis, the transcription and secretion of IL-6 was higher in MC3-9, MC3-11, MC3-2C4, and MC3-2A2 cells than in LPS-activated monocytes, while the MC3-pHEBoSV control cells secreted very low amounts of IL-6.

To test whether the IL-6 expression may lead to an altered requirement for serum growth factors, the MC3-IL6 cells were cultured in serum-free medium at different cell concentrations as previously reported (9). As shown in Fig. 3 *A*, the MC3-IL6-9 cells were able to proliferate even when cultured at very low cell concentrations. Under the same culture conditions, the MC3-pHEBoSV control cells failed to elicit a significant proliferation unless the cultures were supplemented with a conditioned medium from MC3-IL6-9 cells. Similar results were obtained using the other IL-6-transfected cells (not shown). This indicated that the requirement for exogenous growth factors was decreased in the IL-6–expressing cells, suggesting that the IL-6 molecules could function as autocrine factors for the MC3-IL6 cells. In fact, IL-6 molecules were secreted by the cells and present in culture medium. This possibility was further tested in the experiments illustrated in Fig. 3 (*bottom panel*). Control and IL-6–transfected cells were cultured at 5 × 10^4/ml cell concentration in HB-101 serum–defined medium (9) and supplemented with optimal concentrations of IL-6. While the control cells retained the capacity to proliferate in response to IL-6, as previously reported for other EBV B cell lines (13), the IL-6–transfected

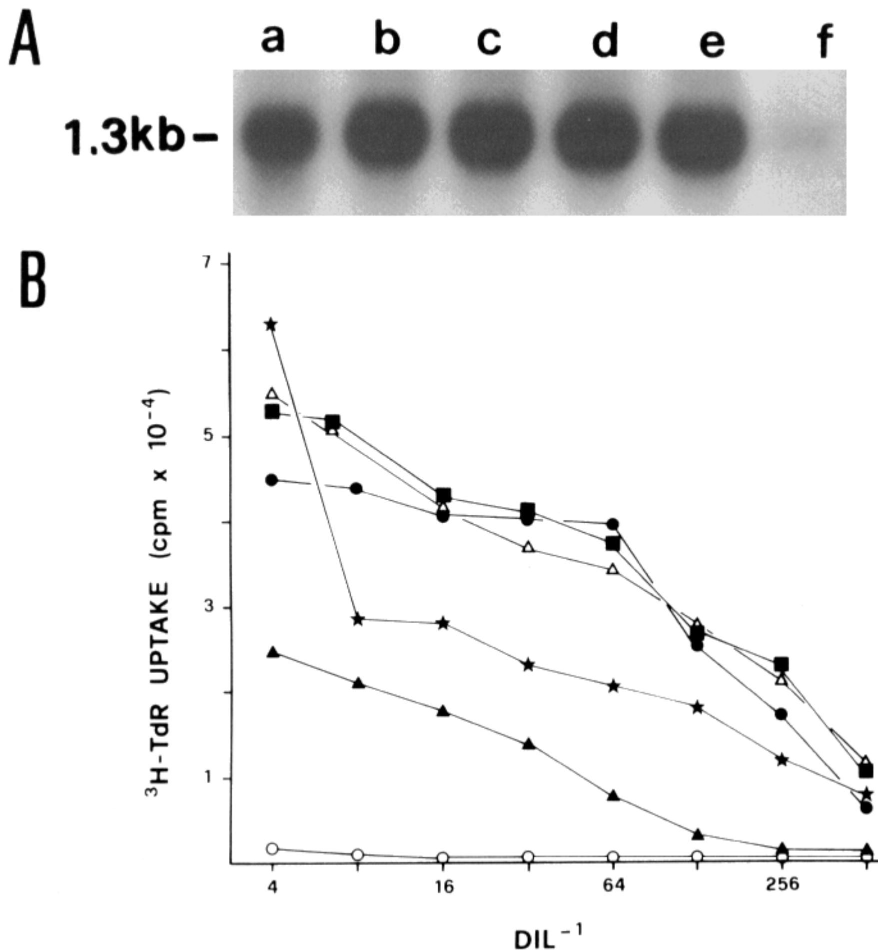

Figure 2. Northern blot analysis of IL-6 transcripts and IL-6 secretion in transfected MC3 cells. (*A*) Total cellular RNA was extracted from MC3 cells transfected with the pHEBoSV-IL6 or pHEBoSV control vector and from human adherent monocytes stimulated with LPS (1 μg/ml) for 6 h. RNA samples were separated on 1% denaturing agarose gel, transferred to nylon membranes, and hybridized as detailed in Materials and Methods. (Lane *a*) RNA from LPS-activated monocytes; (lanes *b* through *e*) RNA from MC3-IL6-9, 11, 2C4, and 2A2 cells; (lane *f*) RNA from MC3-pHEBoSV control cells. (*B*) The secretion of IL-6 molecules by the transfected cells was assayed by using the IL-6–dependent 7TD1 hybridoma cells (20). Data express the IL-6 activity secreted by 5 × 10^5 MC3 cells and human monocytes. (○), MC3-pHEBoSV; (*), MC3-IL6-2A2; (■), MC3-IL6-9; (●), MC3-IL6-11; (△), MC3-IL6-2C4; (▲), LPS-stimulated human monocytes.

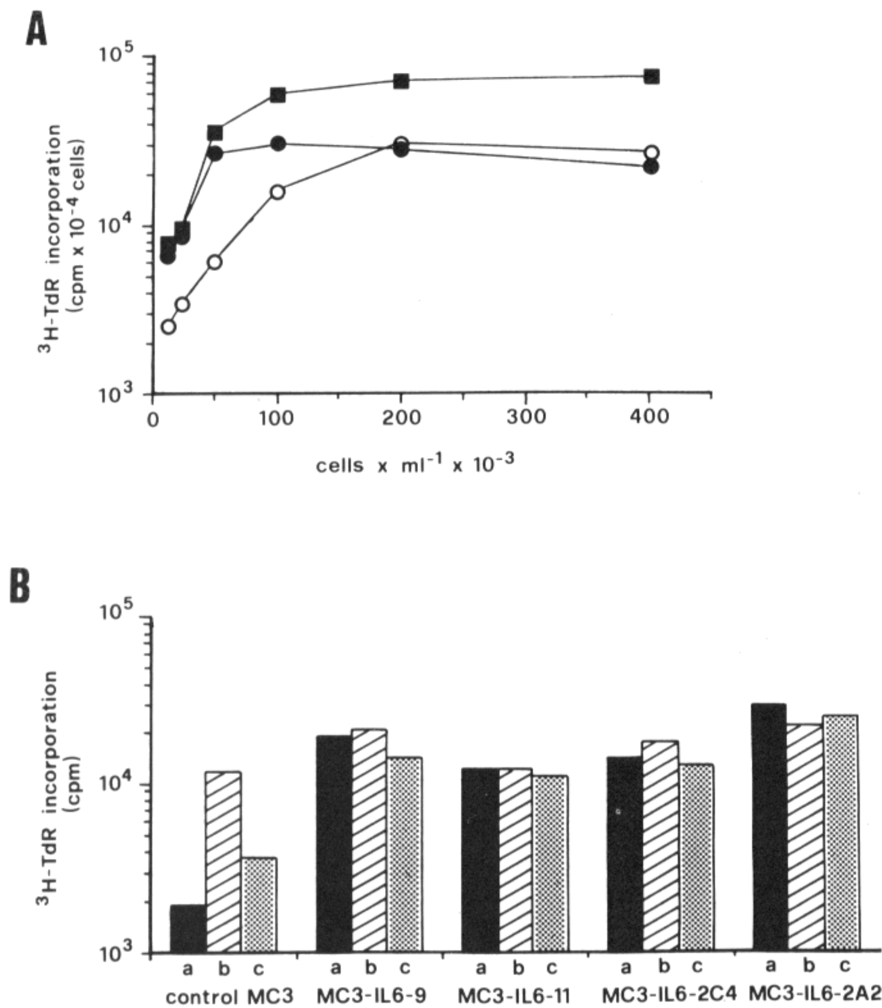

Figure 3. Constitutive expression of IL-6 gene alters the growth of the MC3-IL6 cells. (*A*) MC3-pHEBoSV (O) and MC3-IL6-9 (■) cells were cultured for 48 h at the indicated cell concentrations in HB-101 serum-defined medium as previously described (9). In parallel cultures (●), control MC3-pHEBoSV cells were cultured in presence of dialyzed conditioned medium obtained from MC3-IL6-9 cells cultured in HB-101 medium and tested at 25% final dilution. (*B*) The modulation of the growth response to exogenous IL-6 molecules was performed essentially as described (9). Briefly, cells were cultured in HB-101 medium at 5 × 10^3/well in 0.2 ml cultures for 48 h in presence of (*a*) control goat serum; (*b*) human rIL-6 (5 ng/ml, specific activity 1 × 10^7 U/mg), or (*c*) IL-6 and a goat antiserum to IL-6 (1:200 final dilution) (28). Cultures were pulsed for the last 5 h with 0.5 μCi of [^{3}H]thymidine before harvesting and analysis. Data are expressed as absolute cpm per 10^4 cells.

Table 1. *In Vitro Cloning Efficiency and In Vivo Tumorigenicity of Il-6-transfected MC3 Cells*

Cell line	Cloning efficiency*	Tumorigenicity in vivo‡	
		Number of mice with tumors/ number of inoculated mice	Average of tumors per mouse
MC3-pHEBoSV	0	0/6	0
MC3-IL6-9	120	4/4	3.5
MC3-IL6-11	80	4/4	2.5
MC3-IL6-2C4	78	3/4	3.0
MC3-IL6-2A2	148	5/5	4.0
BL (Daudi)§	166	5/5	4.0

* Growth in soft agar was assayed as detailed in Materials and Methods. Data are expressed as number of colonies per 10^{-4} cells and are the arithmetic mean of triplicate cultures. SEM were <10% of the mean values.
‡ Tumorigenicity was assayed by a single subcutaneous injection of 5 × 10^6 cells in 0.2 ml of PBS. Typically, tumors >5 mm in diameter developed after 2–3 wk of latency at the challenge site and in the regional lymph nodes. Data are the numbers of tumors recorded through 6 wk of observation.
§ Positive control.

cells showed a baseline increase in [^{3}H]thymidine uptake and were unresponsive to the exogenous IL-6 molecules. This may depend on a saturation of IL-6 surface receptors by the constitutively secreted IL-6 molecules. In parallel cultures, the addition of a goat polyclonal antibody to IL-6 molecules resulted in a partial decrease in the constitutive proliferation of the MC3–IL-6 cells, while the IL-6–induced proliferation of MC3-pHEBoSV cells was significantly inhibited. This again suggests a high level of occupancy of surface receptors by the secreted IL-6 molecules. Alternatively, the expression of the IL-6 gene in the transfected clones may have resulted in the constitutive activation of different pathways for B cell proliferation, e. g., receptor transduced or intracellular signals of activation. These latter mechanisms of proliferation would not be affected by antibodies to IL-6. Indeed, the gene coding for the IL-6 receptor was transcriptionally upregulated in MC3–IL-6 cells (Scala, G., work in progress).

The altered growth properties of the MC3-IL6 cells were also studied by examining their ability to form colonies in soft-agar cultures, a property common to many naturally occurring B cell neoplasias (23). As shown in Table 1, the MC3–IL-6 cells exhibited a significant increase in their clonogenic capabilities, while the control MC3-pHEBoSV cells did not form colonies under the same culture conditions, therefore behaving as a common EBV-infected B cell line. In parallel experiments, exogeneous IL-6 (up to 10^3 U/ml) only

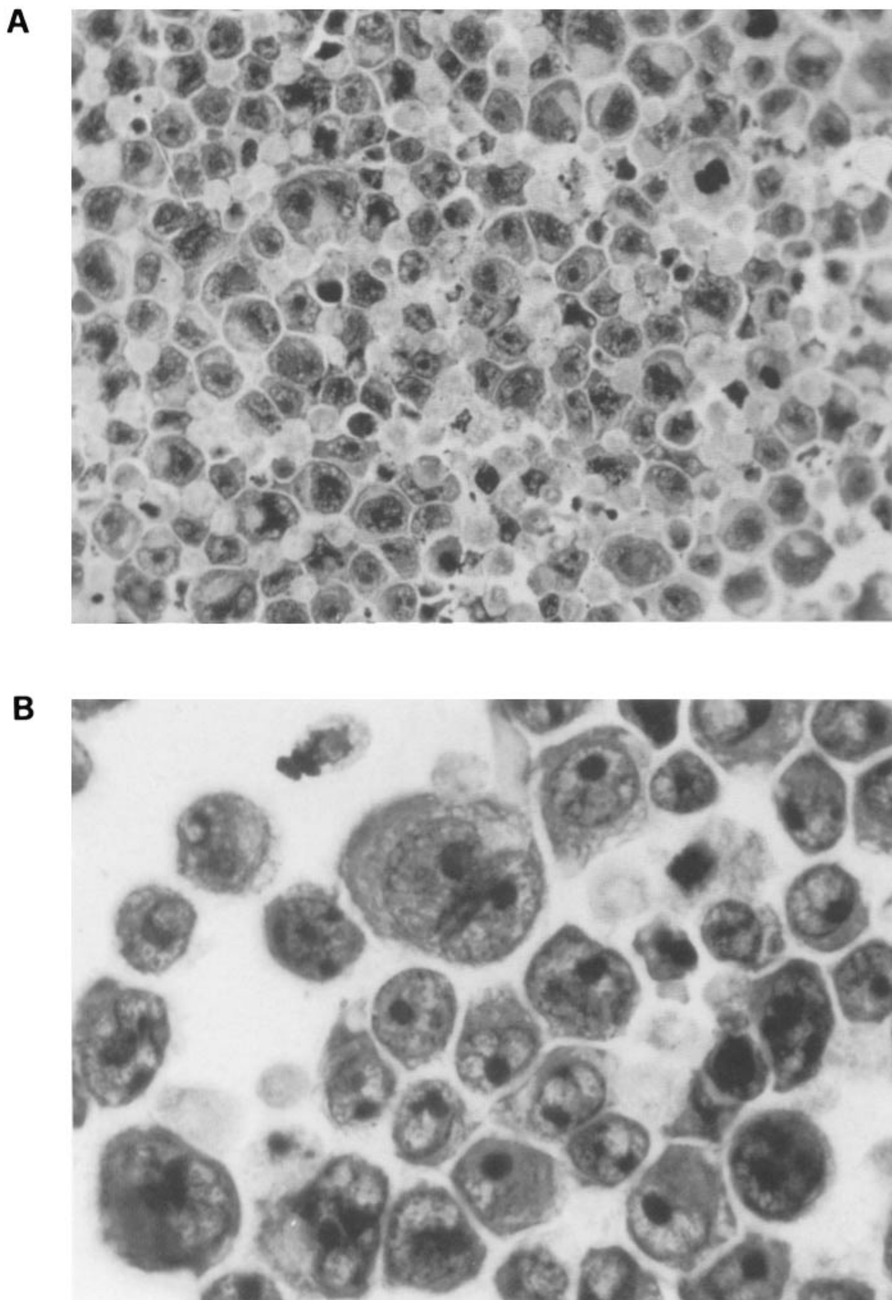

Figure 4. (*A*, *B*) Pathological findings in mice injected with MC3-IL6 cells. Cytological picture of tumor cells obtained after 3–4 wk from the injection site (*A*, Papanicolaou, ×430; *B*, Papanicolaou, ×1,060).

slightly increased the clonogenic properties of the MC3-pHEBoSV control cells and was uneffective on the growth of the IL-6–transfected MC3 and Daudi cells (not shown).

The clonogenic potential of transformed cells is often associated with a tumorigenic phenotype (23, 24). To test whether the MC3–IL-6 cells could have acquired a malignant phenotype, we subcutaneously injected various B cell populations into nude mice. As shown in Table 1, the MC3–IL-6 B cells gave rise to tumors in nude mice, whereas the control MC3-pHEBoSV cells were not tumorigenic. The tumors appeared subcutaneously 10–15 d after the injections, and showed a histological picture of homogeneous infiltrating B cell populations. These cells were characterized by eccentric nucleolated nuclei and large cytoplasms with peripheral condensation (Fig. 4, *A*, *B*). These immunoblastic-like cells were shown to infiltrate periglandular lymphonodes (Fig. 5, *A*, *B*). In the tumor-bearing mice, however, the secretion of IL-6 by MC3–IL-6 cells could have induced a polyclonal proliferation of mouse B cells that could account for the tumoral masses. To test this possibility, tumor cells from mice injected with MC3–IL-6 cells were examined for the expression of CD20, CD23, MHC class I and class II molecules, and for mouse and human surface Igs (μ, δ, γ, α specificities). In these experiments, both the original and tumor MC3–IL-6 cells showed a similar flow cytometry profile (data not shown). Moreover, analysis of the low molecular weight DNA

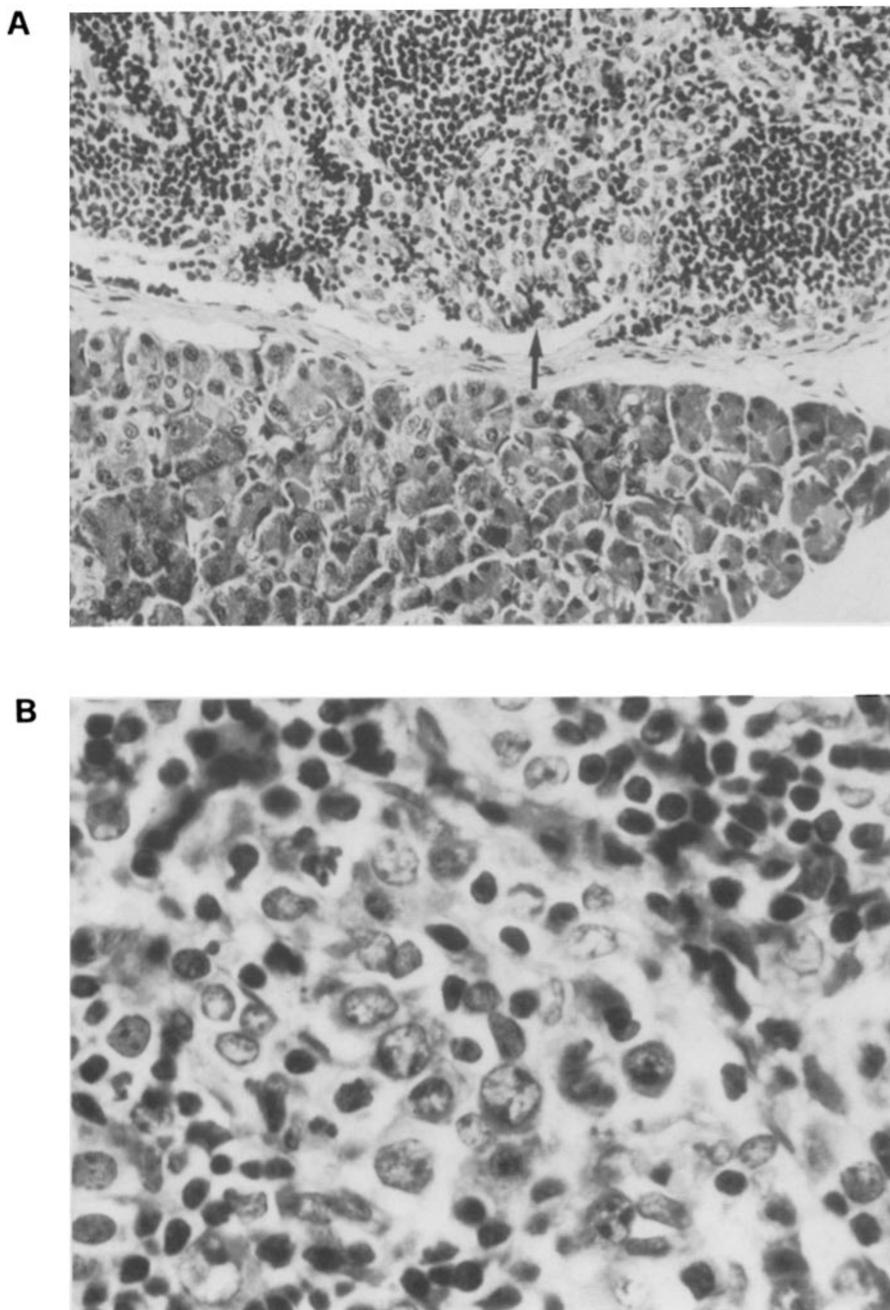

Figure 5. (*A*, *B*) Hematoxylin and eosin (*A*, ×270; *B*, ×1,060) of a periglandular lymph node showing a follicle infiltrated by immunoblastic cells (*arrow*).

of tumor cells showed the presence of pHEBoSV-IL6 plasmids (not shown).

The immortalizing function of EBV infection is not usually associated with tumorigenic capabilities that are often present in naturally occurring EBV+ B lymphomas, including eBL and the recently identified AIDS-BL (2–4). This indicated that other pathogenic events, in addition to EBV infection, may be involved in B cell tumorigenesis. Indeed, the nontumorigenic properties of in vitro infected EBV B cells suggest that additional pathogenic events have occurred in spontaneous EBV-related B lymphomas.

The growth of neoplastic lymphoid cells of both T and B phenotypes is often dependent upon interleukin-like growth factors that share most of the biochemical and biological properties of the factors secreted and used by normal lymphoid cells (9, 25). Among these growth factors, IL-6 has been recently identified as a growth and differentiation factor for both normal and transformed B cells (11). In this study, we attempted to drive an EBV B cell line, MC3, to a malignant phenotype by inserting a constitutively expressed IL-6 gene. Present in an episomal replicating plasmid, the gene was stably present in transfected cells with a high copy number and expressed high levels of the exogenous IL-6 gene. The resulting MC3–IL-6 cells showed a clonogenic capacity and were tumorigenic in nude mice. These data are consistent with and support the recent observations that transgenic mice carrying a human IL-6 gene develop plasmacytomas (26), that myeloma cells secrete and use IL-6 as autocrine growth factors (27), and they further suggest that IL-6 molecules play a role in the abnormal growth of neoplastic B cells.

The molecular events sustaining the acquisition of the malignant phenotype are at present unknown. It may be hypothesized that the deregulated expression of IL-6 may result in the activation of other pathogenic steps such as oncogene activation. Indeed, hyperexpression of the c-*myc* proto-oncogene in EBV B cells can induce altered growth and tumorigenic properties (5). These *myc*-transfected cells also secrete high levels of autocrine growth factors, including IL-6 molecules (Scala, G., manuscript in preparation). The in vivo tumorigenicity of the human IL-6–secreting MC3 cells could be expressed in immunodeficient hosts such as nude mice. In this regard, it is noteworthy that variable levels of immunodeficiency are a common feature of EBV-related malignancies, including eBL and AIDS-BL (2–4). Our data suggest that the concomitant presence of EBV, which acts as an immortalizing factor, and a deregulated secretion of a cytokine, acting as progression growth factor, can give rise to fully transformed B cell tumors in T cell–deficient subjects.

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