current activity from 7/14 (50%) cell-attached patches of normal B lymphoblasts exposed to 8-bromo–cAMP ($10^{-4}M$) (Fig. 3B). In contrast, Cl^- current activity was never seen in 13 (0%) of cell-attached patches of CF lymphoblasts after exposure of the cell to 8-bromo–cAMP ($10^{-4}M$). In two cases, including the example depicted in Fig. 3B, we were able to excise the patch after a 10-min recording period in the cell-attached configuration. In both cases, typical current activity was induced after patch excision and depolarization. Thus, at least in these two cases, Cl^- channels were present in the patches of CF cells but were unable to be activated by the cAMP-dependent pathway.

In a final protocol we compared the effect of 150 n*M* catalytic subunit of cAMP-dependent protein kinase plus 1 m*M* ATP on excised patches of normal versus CF-derived B lymphoblasts. The protocol was identical to that described for Jurkat T lymphocytes. Under these conditions Cl^- current activity was induced in 5/11 (45%) of normal B lymphoblast inside-out patches after exposure to catalytic subunit plus ATP (Fig. 3C). Activation occurred within a few seconds to 2 min after addition of catalytic subunit to the bath. In contrast, Cl^- current activity was never recorded in 22 (0%) inside-out patches of CF cells exposed to catalytic subunit plus ATP in an identical protocol (Fig. 3C).

In summary, we have presented evidence that a cAMP-regulated chloride channel similar to that described for airway epithelial cells (*3–6*) is present in both T and B lymphocyte cell lines. Importantly, the genetically programmed defect in the regulation by cAMP-dependent phosphorylation described for CF appears to be present in transformed lymphocytes as well as epithelial cells. The finding of CF-affected Cl^- channels in lymphocytes, which are easily accessible and abundant, may have important implications for the pathophysiology and for the future biochemical and molecular genetic analysis of the disease.

Note added in proof: While extending our studies we have noticed large variations in the percentage of lymphocyte patches with detectable Cl^- channel currents that may be due to different lots of fetal calf serum.

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Transformation and Plasmacytoid Differentiation of EBV-Infected Human B Lymphoblasts by *ras* Oncogenes

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The biological effects of *ras* oncogene activation in B cells were studied by using amphotropic retroviral vectors to introduce H- or N-*ras* oncogenes into human B lymphoblasts immortalized by Epstein-Barr virus. Expression of both H- and N-*ras* oncogenes led to malignant transformation of these cells, as shown by clonogenicity in semisolid media and tumorigenicity in immunodeficient mice. In addition, terminal differentiation into plasma cells was detectable as specific changes in morphology, immunoglobulin secretion, and cell surface antigen expression. This combined effect, promoting growth and differentiation in human lymphoblasts, represents a novel biological action of *ras* oncogenes and has implications for the pathogenesis of terminally differentiated B-lymphoid malignancies such as multiple myeloma.

ONCOGENE FUNCTION HAS BEEN mainly assessed by in vivo and in vitro studies whose endpoint is the production of a transformed phenotype in fibroblasts (*1*). Although of great value for our approaches to oncogenesis, these studies may not adequately reflect the complex alterations of both growth and differentiation that characterize the phenotype of naturally occurring malignancies in other tissue types. It is conceivable, and in part already proved, that distinct biological effects of different oncogenes and phenotypic changes specific for certain lineages or stages of differentiation may become apparent only by studying different tissue types.

The lymphoid tissues represent a particularly attractive model system for studying the biological effects of different oncogenes, since pathways controlling growth, lineage commitment, and differentiation of lymphoid cells can be dissected by use of a vast array of available molecular and immunological reagents. We established an in vitro transformation assay in which Epstein-Barr virus (EBV)–immortalized B lymphoblastoid cells (LCL) are used as targets for testing the biological activity of oncogenes (*2*). Using this system, we showed that the expression of c-*myc* oncogenes induces malignant transformation of these cells without relevant changes in their differentiation phenotype (*2*). We now describe the biological effects of activated H-*ras* and N-*ras* oncogenes (*3*) in the same target cells. These oncogenes also induced malignant transformation in LCL but in addition lead to dramatic phenotype changes involving terminal differentiation of lymphoblasts into plasmacytoid cells.

An activated H-*ras* allele derived from Harvey sarcoma virus (*4*), or a human N-*ras* allele, activated by a mutation at codon 12 (*5*), were introduced into LCL derived from normal cord blood (CB33 line) or from

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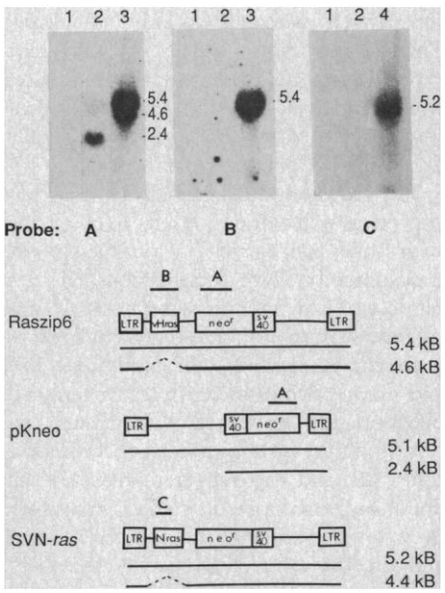

Fig. 1. Expression of retrovirus-encoded genes in infected LCL. RNA was extracted from CB33 cells (lanes 1), pKneo-infected CB33 cells (lanes 2), raszip6-infected CB33 cells (lanes 3), and SVN-*ras*–infected CB33 cells (lane 4), purified by the guanidine isothiocynate method, separated by electrophoresis in 0.9% agarose–2.2*M* formaldehyde gels, transferred to nitrocellulose filters, and hybridized with the indicated probes. Schematic representations of the retroviral vectors and of the expected hybridizing transcripts are also shown. LTR, long terminal repeat.

adult peripheral blood (UH3 line) by use of amphotropic retroviral vectors containing the G418 resistance (*neo*) gene as a selectable marker (Fig. 1) (*6*). A vector (pKneo) containing only the G418 resistance gene was used as a control. After infection, cells were selected in bulk for antibiotic resistance or, in order to ensure the selection of independently infected clones, were plated in limiting dilution and then individual clones were selected for antibiotic resistance. With either protocol, cell lines were established 2 to 3 weeks after infection, and the presence of clonally distinct viral integration sites was confirmed by DNA analysis. RNA hybridization analysis showed the presence of viral *ras* genes within the genome-size viral transcripts (Fig. 1).

Evidence of phenotypic changes in all cell populations carrying either H- or N-*ras* oncogenes was immediately detectable as increased cellular volume and increased tendency to adhere to plastic. Morphological examination by light microscopy of cells prepared by routine Giemsa cytochemical staining revealed that both parental and control infected CB33 and UH3 cells retained the typical morphology of EBV-immortalized lymphoblastoid cells (small cell volume, high nucleus-to-cytoplasm ratio, multiple prominent nucleoli). In contrast, a significant fraction (>80%) of cells carrying *ras* oncogenes showed morphological evidence of plasma cell differentiation, including an increase in cytoplasm, eccentric placement of the nucleus with the appearance of a single prominent nucleolus, and an increase in the number and size of cytoplasmic inclusions. The remaining cells displayed a relatively less mature, but still plasmacytoid phenotype.

We next sought evidence of plasma cell differentiation by analyzing immunoglobulin secretion and the expression of cell surface markers associated with terminal differentiation of B cells to plasma cells (*7*). In all cell populations carrying *ras* oncogenes, there was a marked increase in the secretion of immunoglobulin M (IgM), up to a 30-fold increase over control infected cells (Fig. 2A). This increase in IgM secretion was detectable in all oncogene-expressing cells, including those infected in bulk and those clonally infected. The cell surface expression of lineage-, differentiation-, and activation-specific antigens was studied with monoclonal antibodies (MAbs) in indirect immunofluorescence and flow cytometric analysis. Among many markers studied (*8*), the most significant and consistent changes were noted in the expression of the OKT10 (CD38) and PCA-1 antigens, as manifested by both an increase in the percentage of antibody-reactive cells and an increase in the antigen density of LCLs expressing either H- or N-*ras* oncogenes (Fig. 2B). Expression of both of these antigens is known to increase with plasma cell differentiation (*9*).

We next analyzed *ras* oncogene-expressing cells for growth properties associated with neoplastic transformation. First we assayed their ability to form colonies in soft agar. The expression of both H- and N-*ras* oncogenes appears to confer a modest, yet consistently detectable clonogenic capability to both CB33 and UH3 LCLs under conditions in which no colonies were formed by parental or control-infected lines (Table 1). As a second assay for transformed phenotype, we tested the ability of LCLs expressing *ras* oncogenes and control LCLs to form tumors in vivo when injected subcutaneously into athymic nude mice. LCLs expressing the mutated *ras* genes were tumorigenic, with subcutaneous tumors appearing 2 to 3 weeks after injection (Table 1). These results indicate that *ras* oncogenes can confer a rather weak, yet clearly and consistently identifiable transformed phenotype to LCLs.

We then investigated the relation between proliferation and terminal differentiation in LCLs containing *ras* oncogenes. Two scenarios could be envisioned. First, *ras* oncogenes could stimulate uncontrolled proliferation associated with stochastic entrance into a terminal differentiation pathway; such uncontrolled proliferation would result in a heterogeneous population of cells contain-

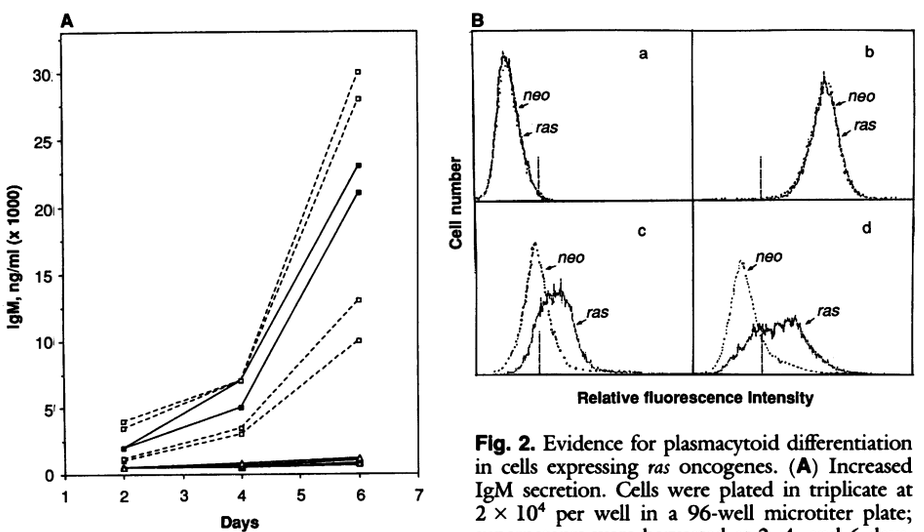

Fig. 2. Evidence for plasmacytoid differentiation in cells expressing *ras* oncogenes. (**A**) Increased IgM secretion. Cells were plated in triplicate at 2×10^4 per well in a 96-well microtiter plate; supernatants were harvested at 2, 4, and 6 days, and assayed for the presence of IgM by an enzyme-linked immunosorbent assay (ELISA) with alkaline phosphatase–linked antibody to human IgM. No IgA or IgG secretion was detected in any of the infected cell lines. Triangles indicate cell lines infected with the pKneo virus and squares indicate cell lines infected with the raszip6 virus. Each curve represents data from cell lines derived from distinct infection events. Solid lines represent data from cell lines derived from infection and selection in bulk and dashed lines represent data from cell lines that were the product of selection of clones. (**B**) Increased expression of plasma cell-specific antigens PCA-1 and OKT10. The pKneo-infected (dotted lines, *neo*) and raszip6-infected (solid lines, *ras*) CB33 LCL were labeled by indirect immunofluorescence and analyzed by cytofluorometry with the panel of MAbs described in (*8*). (a) MAb MOPC-21 (an isotype-identical negative control), (b) MAb TS-1-22 [used as a control with specificity for an antigen, LFA-1 (CD11a), whose expression is not changed], (c) MAb PCA-1, and (d) MAb OKT10. Analogous results were obtained when LCLs expressing N-*ras* oncogenes were analyzed.

ing both self-renewing and terminally differentiating proliferatively quiescent subsets. Alternatively, *ras* oncogene expression could confer proliferative capacity to terminally differentiated cells, and would result in a phenotypically homogeneous population composed of mature, proliferating elements. To distinguish between these two possibilities, we studied DNA replication at the single cell level by autoradiographic determination of [^{3}H]thymidine uptake. Cells expressing the *ras* oncogene displayed a heterogeneous pattern, thymidine incorporation being limited to the smaller, more immature elements and virtually no evidence of DNA replication in the more mature larger elements (Fig. 3). Although these findings appear to correspond to the first scenario described here, the possibility remained that the observed heterogeneity was due to clonal variations of cells expressing different levels of *ras* oncogene. To address this issue, we tested whether clonogenic cells—that is, cells recovered from colonies in semisolid medium (Table 1)—were restricted to self-renewal or whether they were also able to generate differentiated progeny. When replated in semisolid medium, these cells did not show any increased clonogenic ability, whereas they rapidly generated mature progeny when grown in liquid culture. These data indicate that *ras* oncogene expression in LCL stimulates both self-renewal and stochastically determined entrance into a pathway leading to terminally differentiated, quiescent plasma cells.

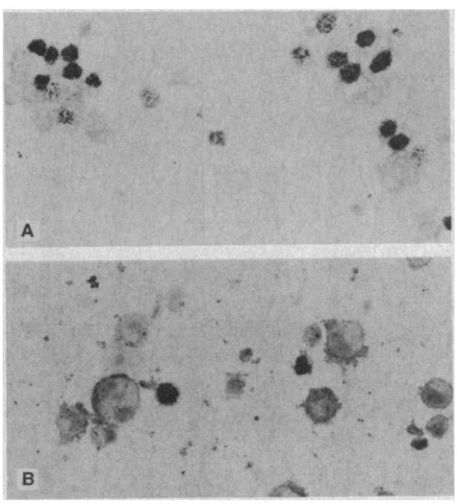

Fig. 3. Analysis of DNA replication in cells expressing *ras* oncogenes. [^{3}H]Thymidine (1 μCi/ml) was added to growth medium of (**A**) pKneo-infected and (**B**) raszip6-infected UH3 LCL, which were then harvested at 24 hours and transferred to glass slides by cytocentrifugation. Slides were coated with NTB-2 emulsion (Kodak), exposed for 12 days at 4°C, developed, counterstained with Giemsa reagent, and assessed for thymidine incorporation. Two hundred cells from this experiment were counted, and showed 61% positive (>5 granules per nucleus) cells in pKneo-infected UH3 LCL and 22% positive cells in raszip6-infected UH3 LCL. Without exception, raszip6-infected UH3 LCL that were positive for thymidine incorporation were in the population of cells whose nuclear diameter represented 80% or more of the total cell diameter—that is, the morphologically less mature subpopulation. Analogous experiments performed in CB33 LCL showed 60% and 39% positive cells for pKneo-infected and raszip6-infected LCL, respectively.

Table 1. In vitro cloning efficiency and in vivo tumorigenicity of LCL infected with retroviral constructs. Cloning efficiency was assessed by examining colonies 14 days after cells were seeded in quadruplicate at 1×10^3, 5×10^3, and 1×10^4 per 30-mm culture dish in 1 ml of growth medium containing 20% fetal calf serum and 0.3% agar. Data are the average of at least three experiments. When 0% cloning efficiency is reported, no colonies were obtained for at least 5×10^5 cells plated. SEs were less than 10% of the mean value. In vivo tumorigenicity was assessed by injecting 4-week-old athymic Swiss nude mice with 5×10^6 cells in 0.2 ml of phosphate-buffered saline in two distinct sites. Data are the number of tumors observed after 4 weeks.

Cell line	Cloning efficiency (%)	Tumorigenicity	
		Tumors per injection	Latency (weeks)
BL (P3HR1)*	24	4/4	2–3
CB33pHEBOSV*myc*2,3†	2.2	4/6	3
CB33	0	0/12	
CB33 *neo*	0	0/12	
CB33 H-ras II‡	0.31	6/6	2–3
CB33 H-ras III‡	0.28	5/6	2–3
CB33 H-ras IV.1§	0.35	6/6	2–3
CB33 H-ras IV.2§	0.25	5/6	2–3
CB33 N-ras I‡	0.22	4/6	2–3
UH3	0	0/6	
UH3 *neo*	0	0/6	
UH3 H-ras I§	0.07	5/6	2–3
UH3 H-ras II§	0.09	4/6	2–3
UH3 N-ras I§	0.10	4/6	2–3

*Burkitt Lymphoma cell line used as a positive control. (2). ‡LCL derived from infection and selection in bulk. †CB33 LCL transformed by a c-*myc* oncogene §LCL clones derived from selection in limiting dilution.

The growth stimulatory effects of *ras* oncogenes as well as their capacity to cooperate with other oncogenes in producing cell transformation have been established in a number of experimental systems (*3*). Examples also exist of *ras*-induced growth arrest as in certain rat embryo fibroblast lines (*10*) and murine Schwann cells (*11*) or terminal differentiation associated with growth arrest, as in the case of murine pheochromocytoma (*12*) and thyroid carcinoma (*13*) cell lines. In hematopoietic tissues, transitory growth followed by terminal differentiation and growth arrest has been observed in murine myeloid and erythroid precursors infected by retroviruses carrying *ras* oncogenes (*14*). However, in these cells the effect of *ras* oncogenes has been interpreted as promoting growth without interfering with the program of terminal differentiation that normally occurs in in vitro colony-forming assays. In contrast with all these findings, our results indicate that *ras* oncogenes can both stimulate sustained growth by cooperating with EBV in transforming LCL and trigger terminal differentiation that is normally blocked in EBV-infected B cells. Differentiation appears to represent a specific effect of *ras* oncogene expression rather than a consequence of increased proliferation, since expression of c-*myc* oncogenes in LCL causes analogous alterations of growth, yet no differentiation is observed (*2*). Furthermore, we recently observed that *ras* oncogenes act dominantly to induce differentiation of LCL that have been previously transformed by *myc* oncogenes (*15*).

The mechanisms involved in the observed biological effects remain unknown because of our limited knowledge of the function of *ras* genes. Whether these effects are specific to the lymphoid lineage or to EBV-infected lymphoid cells is also unknown. However, phenotypic changes induced by *ras* oncogenes in LCL are strikingly similar, in terms of differentiation induction, to the ones observed after phorbol ester treatment of human B cell lymphoma lines (*16*). This would suggest that *ras* affects a biochemical pathway involving protein kinase C (*17*).

Regardless of the mechanism, the observed biological effects of *ras* oncogenes in LCL may have implications for the pathogenesis of certain hematopoietic malignancies. Whereas in most types of tumors malignant transformation is clearly associated with a block of maturation, examples exist of malignancies, such as multiple myeloma, in

which transformation and increased proliferation are associated with a fully differentiated phenotype. In fact, the phenotype of *ras*-transformed LCL we have described is highly reminiscent of multiple myeloma, since in this malignancy there is overproduction of terminally differentiated plasma cells derived from more immature B cell precursors (*18*). We have recently determined that the more immature B cell malignancies such as lymphoma and chronic lymphocytic leukemia do not contain activated *ras* genes, whereas multiple myeloma is associated with N-*ras* activation in approximately 30% of the cases studied (*19*). These observations suggest that N-*ras* activation may be a late event during the development of at least some myeloma cases, leading to the acquisition of both a fully transformed and terminally differentiated phenotype.

In general, our observations underscore the notion that oncogenes may have distinct biological effects in different tissues and that the careful dissection of these effects will contribute to understanding both the function of proto-oncogenes and the role of their respective activated counterparts in the pathogenesis of different types of malignancies. The transformation assay based on EBV-infected human lymphoblasts represents a model system for understanding the mechanisms through which known oncogenes contribute to B cell lymphomagenesis, as well as for identifying novel tissue-specific oncogenes.

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Modulation of Calcium Channels in Cardiac and Neuronal Cells by an Endogenous Peptide

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Calcium channels mediate the generation of action potentials, pacemaking, excitation-contraction coupling, and secretion and signal integration in muscle, secretory, and neuronal cells. The physiological regulation of the L-type calcium channel is thought to be mediated primarily by guanine nucleotide–binding proteins (G proteins). A low molecular weight endogenous peptide has been isolated and purified from rat brain. This peptide regulates up and down the cardiac and neuronal calcium channels, respectively. In cardiac myocytes, the peptide-induced enhancement of the L-type calcium current had a slow onset (half-time ≈75 seconds), occurred via a G protein–independent mechanism, and could not be inhibited by α_1-adrenergic, β-adrenergic, or angiotensin II blockers. In neuronal cells, on the other hand, the negative effect had a rapid onset (half-time <500 milliseconds) and was observed on both T-type and L-type calcium channels.

CALCIUM CHANNELS ARE WIDELY distributed in excitable cells and mediate many biological processes. Cardiac L-type Ca^{2+} channels are primarily up-regulated by adenosine 3′,5′-monophosphate (cAMP)–dependent phosphorylation (*1–3*). Neuronal Ca^{2+} channels are inhibited by a variety of adrenergic and peptidergic receptors (*4–9*). L-type channels have been identified in vitro by their specific binding site to dihydropyridines (*10*). Because these stereospecific sites can be up- or down-regulated experimentally (*11, 12*), they may be regulated by putative endogenous ligands. A low molecular weight material isolated from the rat brain inhibits the nitrendipine-binding sites and decreases veratridine-induced ^{45}Ca uptake in rat brain synaptosomes (*13, 14*). We now show that this endogenous compound is an acidic peptide with a molecular size of about 1 kD, which interacts with Ca^{2+} channels, enhancing their activity in isolated cardiac myocytes and suppressing activity in the central neurons.

The nitrendipine-displacing material was extracted from rat brain homogenates prepared with trichloroacetic acid at 95°C. The supernatant was retained on silica Sep-pak cartridge (Waters, Milford, Massachusetts) (*13, 14*), and final purification was obtained by high-performance liquid chromatography (HPLC) with a Partisil-10 silica column (Whatman, Clifton, New Jersey) and a linear gradient from 5 to 45% methanol in methylene chloride for 40 min. The nitrendipine-displacing activity eluted in one single peak from a Partisil-10 column with a retention time (RT) of about 23 min. When this eluate was reinjected and eluted with the same gradient conditions, one major peak (RT, 22.90 min) and several smaller peaks (RT, 21.38 and 25.22 min) were obtained. The active material eluting in the peak with an RT of 22.90 min was resolved into two peaks by ^{252}Cf plasma desorption mass spectrometry (*15*). The major peak had the mass number of 948, whereas that of the smaller one was 1022 (Fig. 1).

The purified material inhibited in a concentration-dependent manner [^{3}H]nitrendi-

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