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Introduction of an Activated N-*ras* Oncogene Alters the Growth Characteristics of the Interleukin 6-dependent Myeloma Cell Line ANBL6[1]

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ABSTRACT

Multiple myeloma (MM) is a late-stage B-cell cancer with an unknown etiology. Activating mutations of the N-*ras* and K-*ras* oncogenes occur with a high frequency in myeloma and, therefore, may play a role in the pathogenesis of the disease. To study the role of N-*ras*-activating mutations in the regulation of myeloma tumor growth, we introduced a constitutively active N-*ras* cDNA containing a glutamine to arginine (CAA-CGA) amino acid substitution at codon 61 into the interleukin 6 (IL-6)-dependent myeloma cell line ANBL6. Expression of the mutant N-*ras* cDNA resulted in significant IL-6-independent growth, as well as augmentation of growth at suboptimal concentrations of IL-6. The IL-6-independent growth pattern was not the result of activation of autocrine IL-6 production in the mutant N-*ras*-expressing population because neutralizing antibodies to the IL-6 receptor and to IL-6 had no effect on the rate of DNA synthesis in the absence of IL-6. Furthermore, mutant N-*ras* expression decreased the percentage of cells undergoing apoptosis in the absence of IL-6. These data suggest that activating mutations of the *ras* oncogenes may result in growth factor independence accompanied by a suppression of apoptosis in MM. Therefore, the use of therapies designed to block IL-6 action in MM may have less of an impact on tumors bearing activated *ras* mutations.

INTRODUCTION

Malignant transformation is thought to occur as a result of a series of genetic insults involving mutational activation of normal cellular proto-oncogenes and inactivation of tumor suppressor genes (1). Activating mutations of the ras family of GTP-binding proteins (H-, K-, and N-) are found frequently in a variety of cancers, thereby implicating them in the initiation or progression of malignant growth (2). Genetic and biochemical analyses have demonstrated that ras proteins transduce growth and/or differentiation signals from receptor and nonreceptor tyrosine kinases by switching from the inactive GDP-bound state to the active GTP-bound state (3). It has been shown recently that growth of normal hematopoietic cells requires N-*ras* gene expression because antisense oligonucleotides can inhibit colony formation *in vitro* (4). Activating point mutations in *ras* genes occur at codons 12, 13, and 61 and result in an amino acid substitution at these critical positions that leads to a constitutively active GTP-bound state. Many early-lineage hematological tumors, such as acute lymphoblastic leukemia and acute myelogenous leukemia, exhibit activating *ras* mutations, whereas later stage tumors, such as chronic lymphocytic leukemia, hairy cell leukemia, and non-Hodgkin's lymphoma, do not (5–7). However, in MM,[3] activating mutations of the K-*ras* and N-*ras* genes are observed with a frequency as high as 40–50% (8–11).[4]

MM is a late-stage B-cell cancer of unknown etiology that is characterized by the clonal expansion of the plasma cell compartment, as determined by mAb production and clonal immunoglobulin gene rearrangements. In a recent study of benign and malignant plasma cell disorders, *ras* mutations were found to be restricted to stage III MM and plasma cell leukemia (11). It is of interest that no *ras* mutations were observed in stage I or II MM or in the benign plasma cell disorders, smoldering myeloma and monoclonal gammopathy of undetermined significance (11). These observations suggest that activating *ras* mutations may impart a more aggressive phenotype to the myeloma tumor population and, unlike other models of tumor formation, *ras* may play a greater role in tumor progression than in tumor initiation. However, a study in which EBV-transformed lymphoblastoid cell lines were used showed that the introduction of a mutant *ras* gene resulted in malignant transformation and differentiation into a more terminally differentiated plasma cell stage (12). Therefore, the role of activating *ras* mutations in myeloma is unclear, although the high frequency of mutations and their association with more aggressive disease suggest that mutant *ras* may significantly alter the growth properties of the transformed plasma cell.

IL-6 appears to be one major growth factor involved in the proliferation of the myeloma tumor population (13). This cytokine imparts differentiation signals during normal B-cell ontogeny (14) and may act through either an autocrine or paracrine fashion in the growth potentiation of a myeloma cell (15–19). Ligand interaction with the IL-6R and subsequent dimerization with the signaling partner gp130 result in the activation of a variety of cytoplasmic and nuclear factors (20). It has been shown previously that there is an accumulation of active Ras-GTP after signaling through a number of different growth factor and IL receptors, including IL-2, IL-3, IL-6, and granulocyte-macrophage colony-stimulating factor (21, 22). It is significant that two proteins that play an important role in facilitating Ras activation, Shc and the adapter protein Grb2, become phosphorylated after stimulation with IL-6 (23). These data suggest that IL-6 signaling results in activation of Ras. Therefore, it is conceivable that an activated *ras* gene may alter the growth characteristics of the myeloma clone by either augmenting its response to IL-6 or making it IL-6 independent.

To determine whether Ras activation can contribute to alterations in the growth characteristics of myeloma cells, we have taken advantage of the previously characterized myeloma cell line ANBL6 (19). This cell line requires exogenous IL-6 for growth *in vitro*. It is significant that the parental cell line ANBL6 does not harbor any activating *ras* mutations.[5] To determine whether an activating *ras* mutation alters the growth properties of myeloma cells, ANBL6 cells were transformed with a retroviral vector containing an activated N-*ras* cDNA and a *neo*-selectable marker. The activated N-*ras* cDNA contains a glutamine (CAA) to arginine (CGA) substitution at codon 61. We show that, in this system, an activated N-*ras* gene leads to: (*a*) augmented growth in suboptimal IL-6 concentrations; (*b*) IL-6-independent

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[3] The abbreviations used are: MM, multiple myeloma; IL-6, interleukin 6; IL-6R, IL-6 receptor; PI, propidium iodide; PCLI, plasma cell labeling index.

[4] B. Van Ness, unpublished observations.

[5] D. Billadeau, unpublished observations.

growth; and (*c*) decreased apoptotic cell death. Our data suggest that an activated *ras* mutation in MM may result in a more aggressive proliferation of the tumor by allowing significant IL-6-independent growth, as well as conferring heightened sensitivity and survival to suboptimal concentrations of IL-6.

MATERIALS AND METHODS

RNA Isolation and Analysis. Total cellular RNA was prepared as described previously (24). Twenty μg of total cellular RNA were treated with glyoxal, separated on a 1.0% agarose gel, and transferred onto Zetabind (Cuno Laboratory Products, Meriden, CT). The membrane was probed under stringent conditions with a ^{32}P-labeled pRas61 probe and exposed to X-ray film.

Vector Design and Virus Packaging. To obtain a full-length N-*ras* cDNA, ANBL6 RNA was reverse transcribed using an oligonucleotide 3′ of the coding region and then amplified using gene-specific primers (25) and PCR. The reverse transcription reaction contained the following in a 20-μl final volume: 200 μM deoxynucleotide triphosphate, 5 μg RNA, 2.5 mM Mg^{2+}, 50 mM KCl, 20 mM Tris (pH 8.4), 200 units Moloney murine leukemia virus-reverse transcriptase (GIBCO-BRL, Gaithersburg, MD), and 1 μM reverse transcription primer (5′-CTACTGAGAGCTGGGGAAGT-3′). Reverse transcription was carried out at 42°C for 1 h, followed by a 7-min incubation at 95°C. Five μl of this reaction were used in a subsequent 50-μl PCR amplification containing the following: 200 mM deoxynucleotide triphosphate, 1.5 mM Mg^{2+}, 50 mM KCl, 20 mM Tris (pH 8.4), 1.25 units Taq polymerase (Perkin Elmer Cetus, Branchburg, NJ), 50 μl mineral oil, and 1 μM of a gene-specific 5′ N-*ras* (CAGAGGCAGTGGAG**CTCGAG**GTTCTTGCTG) and 3′ N-*ras* (GGAATACTTCTCC**TCGAG**GAAGTCAGGACC) primer (sequences in bold indicate the unique *Xho*I sites that were engineered into the primers). This reaction was subjected to 30 cycles of 15 s denaturation at 92°C, 15 s extension at 60°C, and 25 s annealing at 74°C. The resultant amplified product was separated on a 1.5% agarose gel, isolated in low melting point agarose, and cloned by standard molecular techniques using the unique *Xho*I sites in the primers to insert the amplified cDNA into the retroviral expression vector pLXSN (26). To obtain the codon 61 mutant N-*ras* cDNA, we used overlapping PCR mutagenesis. Two homologous primers that spanned codon 61 [5′-Ras61(5′-ACAGCTGGA**CGA**GAAGAGTAC-3′) and 3′-Ras61(5′-GTACTCTTC**TCG**TCCAGCTGT-3′)] contained a point mutation at the second base of codon 61; there would be a glutamine to arginine substitution at this position. PCR amplification of the cloned N-*ras* cDNA was carried out as described above, except that the mutant 5′ primer was paired with the 3′ N-*ras* primer, and the mutant 3′ primer was paired with the 5′ N-*ras* primer. One-tenth of the two products were gel purified, then mixed together, and amplified with the use of the external 5′ and 3′ N-*ras* primers as described above. The resultant mutant N-*ras* 61 cDNA was then digested with *Xho*I and subcloned into pLXSN by standard molecular biology techniques. Clones were sequenced as described previously (24) to ensure that the only mutation in the coding region was at codon 61, and that no additional mutations were obtained during PCR amplification.

CsCl gradient-purified pRAS61 plasmid was used to transiently transfect 5×10^5 cells of the ecotropic retrovirus packaging cell line GP+E86 by the calcium phosphate precipitation method (26, 27). The transiently produced virus was used to infect the amphotropic retrovirus packaging cell line PA317 to produce stable vector-producing cell lines as described previously (26).

Cell Culture and Infection/Selection. ANBL6 is a previously described myeloma cell line that demonstrates a strict IL-6 requirement for growth (19). For these studies, the cell lines ANBL6, ANBL6/LSXN, and ANBL6/Ras were maintained in RPMI 1640 containing 10% FCS, L-glutamine, penicillin-streptomycin, and 0.5 ng/ml IL-6 (R&D Systems, Minneapolis, MN). To obtain the ANBL6/Ras and ANBL6/LXSN populations, 5×10^6 ANBL6 cells were infected with high titer amphotropic pRas61 or pLXSN virus supernatant, 8 μg/ml Polybrene (Sigma Chemical Co., St. Louis, MO), and 1.0 ng/ml IL-6 for 48 h. The cells were washed and then plated in RPMI containing 400 μg/ml G418 for selection of clones expressing neomycin resistance. Neomycin-resistant populations arose after approximately 1 month of selection.

In experiments to determine cell growth and cell death, viability was determined using trypan blue exclusion as a measure of cell viability. All growth/doubling assays were performed in triplicate.

[^{3}H]Thymidine Incorporation Assays. A total of 5×10^5 cells were washed three times in PBS and then incubated in 2 ml of medium that either lacked IL-6 or contained IL-6 at the indicated concentrations for 6 days. The cells were then removed from the wells, washed, and counted, and 3×10^4 viable cells were plated per well. One μCi of [^{3}H]thymidine (5.0 Ci/mmol; Amersham, Arlington Heights, IL) was present in the last 18 h of the assay. The cells were harvested onto glass fiber filter paper, and [^{3}H]thymidine counts were determined by liquid scintillation spectroscopy. Assays involving neutralizing IL-6 antibody and blocking antibody to the IL-6R (Biosource, Camarillo, CA) were performed as described previously (19).

Analysis of Apoptosis and Cell Cycle Distributions. Cells (1×10^6) were washed three times with PBS and then cultured in 3 ml of medium without IL-6 or in the presence of IL-6 at the indicated concentrations. Cells were fed with new medium and IL-6 on days 4 and 8 of the 2-week experiment. Cells were harvested at the indicated time points and analyzed for apoptosis and cell cycle using the method of Nicoletti *et al.* (28). In brief, a cell pellet of 1×10^5 cells was gently resuspended in 500 μl of hypotonic solution (50 μg/ml PI in 0.1% sodium citrate plus 0.1% Triton X-100) and incubated overnight at 4°C in the dark. Analysis of the nuclei was preformed using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Chromatin degradation, a characteristic of apoptosis, was detected as a heterogeneous subdiploid population to the left of intact nuclei in G_0-G_1. For the analysis of chromatin degradation, total genomic DNA was isolated as described previously (28) from ANBL6 and ANBL6/Ras after a 2-week incubation in the presence (1 ng/ml) or absence of IL-6. Fifteen μg of total DNA were separated on a 2% agarose gel containing 1 μg/ml of ethidium bromide in 1 × Tris-acetate EDTA buffer.

RESULTS

Construction of a Mutant Retroviral *ras* Vector and Characterization of the Transformed ANBL6/Ras Population. The ANBL6 cell line initially consisted of a near diploid and near tetraploid population (19). However, the ANBL6, ANBL6/LXSN, and ANBL6/Ras cell lines we used consist of the near tetraploid population only, as determined by flow cytometry (data not shown). The N-*ras* gene is located on chromosome 1, and in the near tetraploid population, only a diploid equivalent of chromosome 1 is present (19). RNA from the IL-6-dependent myeloma cell line ANBL6 was reverse transcribed using a gene-specific primer (25), and the entire coding region of the N-*ras* cDNA was amplified using gene-specific primers. PCR mutagenesis was used to generate the mutant N-*ras* cDNA retroviral expression vector pRas61 (Fig. 1*A*), which was subsequently used to infect ANBL6 by standard infection protocols (see "Materials and Methods"). The N-*ras* codon 61 mutation was chosen because it is the most prevalent of the activating mutations in MM (11).[4] A neomycin-resistant population was obtained after selection in G418 and was designated ANBL6/Ras. There are, on average, two integrated copies of the mutant N-*ras* cDNA within the ANBL6/Ras population, as determined by Southern blotting (data not shown). As a control, the LXSN vector alone was used in an infection to establish the control cell population ANBL6/LXSN. A Northern blot of the three cell lines ANBL6, ANBL6/Ras, and ANBL6/LXSN probed with pRas61 (Fig. 1*B*) shows a 3.9-kb retroviral *ras* transcript initiated from the retroviral long terminal repeat in the ANBL6/Ras population along with a Neo transcript of approximately 1.7 kb (see Fig. 1*A*). The ANBL6/LXSN population expresses the expected Neo transcripts only (Fig. 1, *A* and *B*).

Mutant *ras* Expression Augments Growth and Relieves the IL-6 Requirement of ANBL6. The IL-6 dependence of the ANBL6 cell line in culture has been described previously (19). The optimal concentration of IL-6 was 0.1–1.0 ng/ml, as determined by growth curves and [^{3}H]thymidine uptake assays. To test the IL-6 requirement of the newly established ANBL6/Ras population, we plated ANBL6, ANBL6/LXSN, and ANBL6/Ras at an initial concentration of 5×10^4

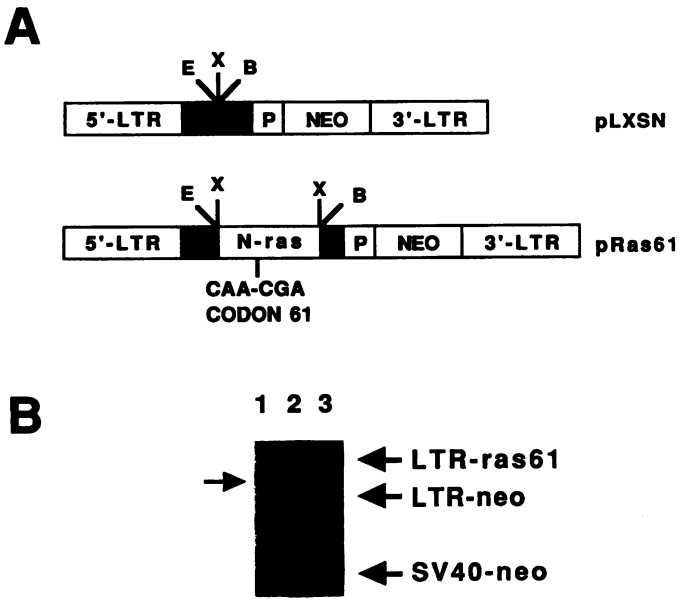

Fig. 1. Characterization of the ANBL6/Ras population. *A*, structure of the two retroviral vectors. The mutant N-*ras* cDNA was cloned into the unique *Xho*I site (*X*) and expression is driven off the 5′ long terminal repeat (*LTR*). In the LXSN vector, expression of the Neomycin (*NEO*) resistance gene arises from both the 5′ long terminal repeat and SV40 promoter (*P*). Two other unique restriction sites within the pLXSN cloning site are indicated *Bam*HI (*B*) and *Eco*RI (*E*). *B*, Northern analysis of 20 μg of total RNA from ANBL6, ANBL6/LXSN, and ANBL6/Ras (*Lanes 1*, *2*, and *3*, respectively) probed with an [α-^{32}P]dCTP-labeled pRas61 plasmid. The 3.3- and 1.7-kb transcripts observed in *Lane 2* represent the two NEO transcripts driven from the 5′ long terminal repeat and SV40 promoters, respectively. The 3.9-kb transcript in *Lane 3* represents the N-*ras* containing transcript initiating from the 5′ long terminal repeat. *Unlabeled arrow*, nonspecific cross hybridizing transcript that is observed in all three samples.

cells/ml in the absence or presence of varying concentrations of IL-6. After a 2-week incubation, the growth of the three cell populations was examined and is expressed as the number of cell doublings (Fig. 2*A*). The parent cell line and the ANBL6/Ras population were found to grow equally well in the presence of 1.0 and 0.1 ng/ml IL-6 (Fig. 2*A*), although some augmentation of growth was reproducible in the ANBL6/Ras population. It is interesting that only the ANBL6/Ras population demonstrated a significant and reproducible growth enhancement in suboptimal IL-6 concentrations (0.01 and 0.001 ng/ml, respectively) when compared with the ANBL6 parent line or the vector control ANBL6/LXSN. Furthermore, in contrast with the parent cell line and vector control, the mutant ANBL6/Ras population grew even in the absence of IL-6 (Fig. 2*A*).

The number of viable cells was also monitored over the 2-week period. Fig. 2*B* shows IL-6 dose dependence on cell viability in the ANBL6 cell line. At suboptimal IL-6 concentrations or in the absence of IL-6, there was a slow decrease in the number of viable cells. Only approximately 30% of the input cells from the parent cell line were viable at the end of the assay in the wells containing no IL-6 and in those containing 0.001 ng/ml IL-6 (Fig. 2*B*). It is of interest that approximately 80% of the cells remained viable in the parent cell population containing 0.01 ng/ml IL-6, suggesting that at this concentration of IL-6, the cell death process was slowed when compared with ANBL6 grown in the absence of IL-6; however, there did not appear to be any net growth. IL-6 dependency and cell viability of ANBL6/LXSN were identical to those of ANBL6 (data not shown). These data are in contrast with those observed in the ANBL6/Ras population; at all concentrations of IL-6, ANBL6/Ras cells showed an increase in viable cells (Fig. 2*C*). Therefore, at each corresponding IL-6 concentration, ANBL6/Ras grew significantly better than did the parent cell line (Fig. 2, compare *B* and *C*), and notably, the number of viable cells increased even in the absence of IL-6 (Fig. 2*C*). These data indicate that expression of mutant *ras* allows proliferation of this myeloma cell line in the absence and at suboptimal concentrations of IL-6.

Mutant *ras* Augments DNA Synthesis in ANBL6/Ras at Suboptimal Concentrations of IL-6. To define further the growth characteristics of the ANBL6/Ras population, we performed DNA synthesis assays in the presence of varying concentrations of IL-6 and in the absence of IL-6. As shown in Fig. 3*A*, the ANBL6/Ras population demonstrated an enhanced rate of DNA synthesis compared with the parent cell line and the vector only control at all concentrations of IL-6 tested. Furthermore, in the absence of IL-6, the ANBL6/Ras population had a greater than 5-fold increase in the amount of [^{3}H]thymidine incorporated compared with the parent cell line and the vector only control (Fig. 3*A*). The ANBL6/LXSN population and the parent cell line behaved identically, demonstrating that the vector alone was not contributing to the increase in DNA synthesis.

It remained possible that the increase in DNA synthesis of the

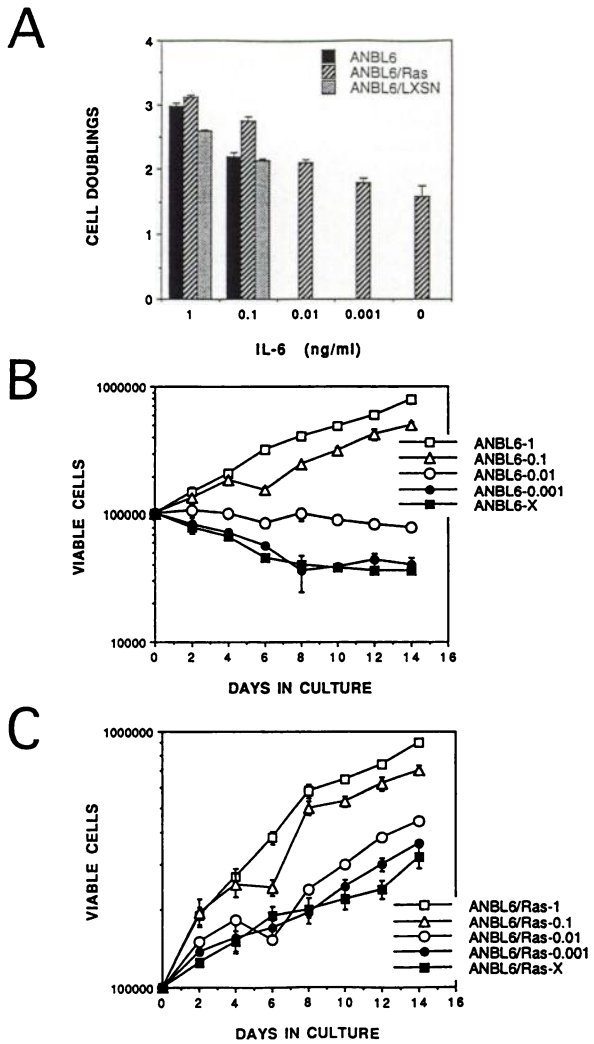

Fig. 2. Mutant *ras* promotes growth of the IL-6-dependent cell line ANBL6 in the absence of and at suboptimal IL-6 concentrations. *A*, cell lines (5×10^4/ml) were plated in triplicate at the indicated concentrations of IL-6 and fed on day 7. After a 2-week incubation, viable cells were counted using trypan blue exclusion, and cell growth was calculated as the number of doublings. *B* and *C*, the ANBL6 cell line (*B*) and the ANBL6/Ras population (*C*) were cultured as described above, and the number of viable cells was counted at 2-day intervals using trypan blue exclusion as a measure of cell viability. This is a representative example of three experiments set up in triplicate. *Columns and points*, mean; *bars*, SEM; in most cases, these were within the symbol dimensions used to plot the data.

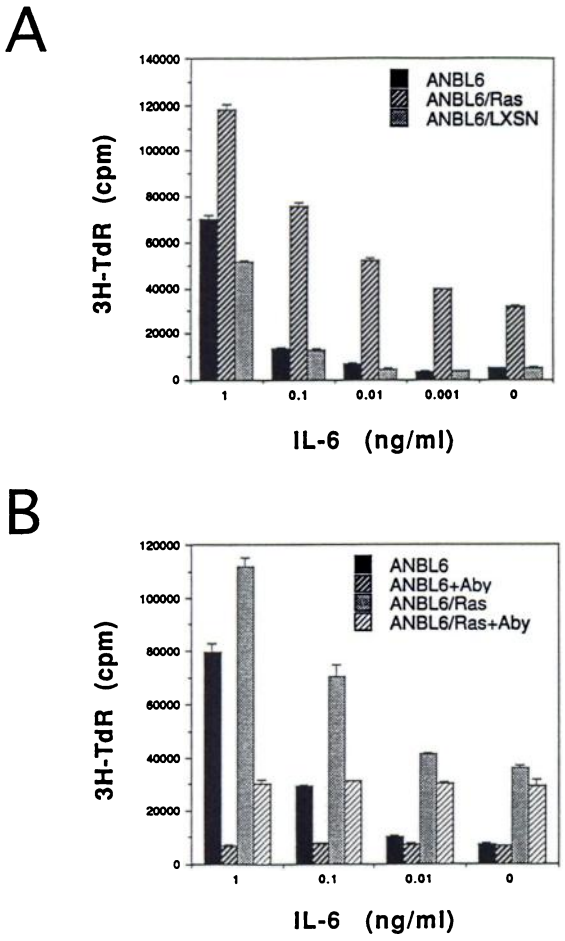

Fig. 3. Mutant *ras* expression augments DNA synthesis at suboptimal IL-6 concentrations. *A*, indicated cell lines (3×10^4) were grown in the absence and presence of varying IL-6 concentrations, and the amount of DNA synthesis occurring after 1-week experiment was measured using [^{3}H]thymidine, as described in "Materials and Methods." The results shown are a representative example of three experiments performed in quintuplet. *B*, indicated cell lines were assayed as described above. However, neutralizing antibodies to IL-6 and a blocking antibody to the IL-6R were added where indicated (*ABY*). *Columns*, mean; *bars*, SEM.

ANBL6/Ras population in the low IL-6 concentrations and in the absence of IL-6 was caused by an autocrine production of IL-6 induced by the activated N-*ras* oncogene. To test this possibility, we measured DNA synthesis in the presence of both an IL-6-neutralizing and an IL-6R-blocking antibody. As shown in Fig. 3*B*, incubation of the parent cell line at the various IL-6 concentrations with the antibodies resulted in a decrease in [^{3}H]thymidine incorporation identical to the background level of DNA synthesis observed in the absence of IL-6. Similar results were observed in the ANBL6/Ras population when the antibodies were used to block IL-6-induced DNA synthesis (Fig. 3*B*). However, the increased level of DNA synthesis in the ANBL6/Ras population in the absence of IL-6 was not decreased further by the presence of the antibodies (Fig. 3*B*). This demonstrates that the augmented rate of DNA synthesis in ANBL6/Ras is not the result of an autocrine production of IL-6. Furthermore, when ANBL6 and ANBL6/LXSN were cultured in ANBL6/Ras-conditioned media, there was no change in growth rate or DNA synthesis, further suggesting that the augmented growth of ANBL6/Ras in the absence of IL-6 did not result from the production of some other soluble growth factor (data not shown).

Mutant *ras* Expression Provides Resistance to Apoptosis Induced by IL-6 Withdrawal. The observations described above demonstrated that expression of the mutant *ras* cDNA conferred IL-6-independent growth to the ANBL6/Ras population. To understand in greater detail the effect of mutant *ras* expression on the growth/survival properties of the ANBL6/Ras population in the presence and absence of IL-6, apoptotic and cell cycle distributions were analyzed by flow cytometry. The analysis of PI-stained cells or intact nuclei has been shown to be a rapid and quantitative measure of apoptotic death within a cell population (28–30). As a result of nucleosomal degradation and DNA loss from apoptotic nuclei, cells become hypodiploid and can be analyzed by flow cytometry after PI staining (28). Hypodiploid nuclei are observed as a peak arising to the left of the G_0-G_1 peak. In the presence of 1 ng/ml IL-6, ANBL6 and the ANBL6/Ras population demonstrate equal proportions of cells undergoing a slow apoptotic death over the 2-week time course of the experiment (14 and 13%, respectively; Fig. 4 and Table 1). Although there is an accumulation in the number of cells that have undergone apoptosis, a majority of the cells are in G_0-G_1 or in the growth phase of the cell cycle. In the absence of IL-6, the ANBL6 cell line shows a dramatic increase in the number of apoptotic nuclei analyzed over the 2-week period, reaching a total of 50% (Fig. 4 and Table 1). In addition, DNA from this cell population shows the characteristic DNA laddering often associated with apoptosis (Fig. 4, photo *inset*). Along with this accumulation of apoptotic nuclei over the 2-week period is an initial increase in the number of cells arrested in G_0-G_1 and a decrease in the number of cells in G_2-M, followed by an overall decrease in both populations (Fig. 4, compare days 0 and 14). In contrast, in the absence of IL-6, the ANBL6/Ras population contains a significant reduction in the percentage of apoptotic cells when compared with the parent line (Fig. 4; 30 *versus* 50%). Table 1 shows the results of three independent experiments and demonstrates that the expression of mutant *ras* can suppress the number of cells entering the apoptotic pathway in the absence of IL-6. Moreover, the number of ANBL6/Ras nuclei in the G_2-M portion at the end of 2 weeks is significantly greater than that observed in the parent cell line in the absence of IL-6. Therefore, these data indicate that mutant *ras* expression in ANBL6 permits growth in the absence and at suboptimal concentrations of IL-6 by stimulating progression of the cells through G_0-G_1 and decreasing the number of cells undergoing apoptosis. The survival factor Bcl-2 has been shown to be an inhibitor of apoptotic cell death (31–33). Our observed suppression of apoptosis in the ANBL6/Ras population in the absence of IL-6 was not caused by a difference in the amount of Bcl-2 present, inasmuch as equivalent amounts of the protein were found in the parent cell line and in the ANBL6/Ras population in the presence and absence of IL-6 (data not shown).

DISCUSSION

MM is an incurable B-cell cancer with an unknown etiology. Previous studies have demonstrated that IL-6 plays an important role in the growth of the malignant clone (13, 34, 35). The observation that the *ras* gene product accumulates in the active GTP-bound state after IL-6 stimulation (22), and the frequency of mutation of the N-*ras* and K-*ras* proto-oncogenes in MM (8–11)[4] suggest a role for *ras* in the proliferation of the tumor cells. Herein, we have used the IL-6-dependent MM cell line ANBL6 to show that expression of mutant *ras* can stimulate growth at suboptimal concentrations of IL-6 and can even lead to growth in the absence of IL-6. Furthermore, mutant *ras* expression results in a decrease in the number of tumor cells undergoing apoptosis in the absence and at suboptimal concentrations of IL-6.

Ras-GTP-binding proteins have been suggested to play a key role during cellular growth and differentiation in many cells (3). Previous studies involving the introduction of an oncogenic H-*ras* or K-*ras* gene into mouse IL-3-dependent myeloid and mast cell lines have

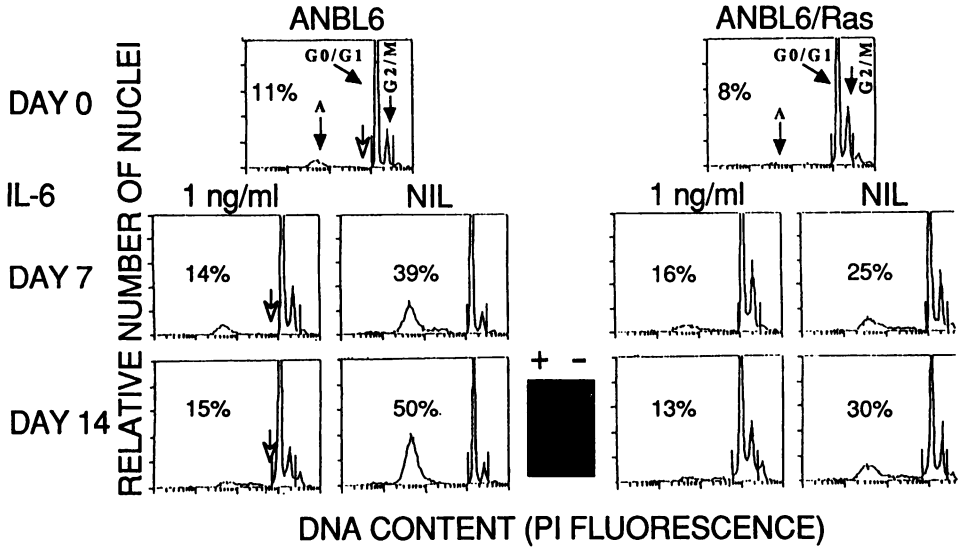

Fig. 4. In the absence of IL-6, the expression of mutant *ras* results in a decrease in the number of cells undergoing apoptosis. Indicated cell lines were cultured in the presence or absence of IL-6, as described in "Materials and Methods." At the indicated time points, 1×10^5 cells were removed, and the nuclei were stained with PI. The nuclei were then analyzed by flow cytometry, and the results are expressed as the number of nuclei *versus* DNA content. A vertical marker to the *left* of the G_0-G_1 peak separates the apoptotic nuclei from those nuclei in the cell cycle. This marker set was used to calculate the values given in Table 1. *Filled arrows*, relative positions of nuclei in cycle (G_0-G_1 and G_2-M) or apoptotic (*A*). *Open arrow*, the diploid G_0-G_1 peak from peripheral blood mononuclear cells. The percentage of hypodiploid nuclei is given within the individual plots. The analysis shown is a representative example of three independent experiments (see Table 1). The *photo inset* shows DNA laddering from the ANBL6 cell line after 2 weeks in the presence (+) or absence (−) of IL-6.

Table 1 *Effect of ras mutation on apoptosis in ANBL6*[a]

		Day		
		0	7	14
Expt. 1	ANBL6	19/19[b]	40/50	46/69
	ANBL6/Ras	14/14	37/24	43/45
Expt. 2	ANBL6	7/7	16/24	25/43
	ANBL6/Ras	6/6	10/11	17/12
Expt. 3	ANBL6	11/11	14/39	15/50
	ANBL6/Ras	8/8	16/25	13/30

[a] ANBL6 and ANBL6/Ras were grown in the presence (1 ng/ml) or absence of IL-6, and the percentages of apoptotic nuclei were determined as described in the text.
[b] Percentage of apopotic cells grown in the presence/absence of IL-6.

yielded insights into *ras* function in tumor progression (36–38). The activated *ras* genes were found to augment growth at low concentrations of IL-3 (36, 38) and, in some instances, to lead to growth factor independence as a result of the production of an autocrine growth loop (37, 38). It has been shown previously that the introduction of an activated H-*ras* or N-*ras* into an EBV-transformed B-lymphoblastoid cell line results in differentiation to the plasma cell stage and tumorigenicity (12). These data suggest that *ras* plays a role in the process of B-cell maturation and, furthermore, that *ras* may be involved in tumor initiation, because the parent EBV-transformed line was nontumorigenic in athymic nude mice. However, recent observations have suggested the possibility that mutant *ras* may be involved in tumor progression because nonmalignant plasma cell dyscrasias and stage I and II MM do not harbor activating *ras* mutations, whereas more aggressive stage III MM and plasma cell leukemias do (11).

Not all myeloma tumors contain *ras* mutations; however, mutations in *p53* and *Rb* have been documented and are primarily associated with aggressive disease (10, 39–41). Furthermore, it has been observed that very aggressive late-stage myeloma tumors contain both activating *ras* oncogene mutations and mutations leading to inactivation of tumor suppressor genes (10, 40). Notably, the ANBL6 cell line is hemizygous at the *p53* locus; the remaining allele contains a mutation at codon 241 in exon 7.[6] Therefore, the combination of an activated *ras* and an inactivated *p53* may be permissive for IL-6-independent growth. Indeed, wild-type *p53* has been shown to inhibit transformation induced by activated *ras* and other nuclear oncogenes (42, 43).

The proliferative capacity of myeloma tumor populations can be measured by the incorporation of bromodeoxyuridine into DNA and is expressed as the PCLI. In general, a high PCLI correlates with more aggressive tumors (44). It is interesting to note that, although the ANBL6/Ras population demonstrates an increased rate of DNA synthesis at all concentrations of IL-6 tested compared with the parent cell line (Fig. 1*A*), growth of the parent and the ANBL6/Ras population are nearly equivalent at the two highest IL-6 concentrations (Fig. 2*A*). We have analyzed 183 myelomas for *ras* mutations and PCLI. In our study, we found a *ras* mutation frequency of approximately 50% and found no correlation with high PCLI.[7] This is in agreement with a previous analysis in which seven myelomas containing *ras* mutations showed no correlation with high PCLI (11). These data suggest that when concentrations of IL-6 are optimal, myeloma tumors harboring a *ras* mutation would not display a growth advantage over tumors harboring a normal *ras* gene. In contrast, in the absence or presence of suboptimal concentrations of IL-6, a myeloma cell harboring a mutant *ras* gene would have a significant proliferative advantage over a cell that is strictly dependent on IL-6.

It has been shown that bone marrow stromal cells produce IL-6 and can support the growth of myeloma cell lines and fresh cells (45, 46). We have found that ANBL6 can also be sustained on bone marrow stromal cell cultures without the addition of exogenous IL-6.[8] Because an activating *ras* mutation can lead to IL-6-independent growth, this may facilitate mobilization from the bone marrow to the peripheral blood. Therefore, although clonally related tumor cells are commonly found in the peripheral blood (47), their growth potential leading to late-stage disease may be augmented by a *ras* mutation. Alternatively, the growth advantage provided by a mutant *ras* gene could lead to a significant increase in bone marrow tumor load, with subsequent mobilization to the peripheral blood occurring in late-stage disease.

The most interesting effect that expression of mutant *ras* had on ANBL6 was an ability to limit the number of cells undergoing apoptosis in the absence of IL-6. A similar increase in survival in the absence of IL-3 was observed in the mutant K-*ras*-expressing FDC-P1 cells (38). Bcl-2 has been shown to be a potent inhibitor of apoptosis after the withdrawal of growth factor from factor-dependent cell lines (32). We did not observe any change in the amount of Bcl-2 protein expressed in the parent cell line or the ANBL6/Ras population in the presence or absence of IL-6 (data not shown). Similarly, no change in the amount of Bcl-2

[6] D. F. Jelinek, unpublished observations.

[7] B. Van Ness and P. Greipp, unpublished observations.
[8] D. Billadeau and B. Van Ness, unpublished observations.

protein was observed in the v-*raf*-expressing clones after withdrawal of IL-3 (48) or in a recent analysis of IL-5-mediated apoptosis in freshly isolated chronic lymphocytic leukemia cells (30). It is of interest that, in the absence of IL-6, there is a slow but steady accumulation of apoptotic cells in the parent ANBL6 cell line over a 2-week period. This slow onset of apoptosis in myeloma cells after the withdrawal of IL-6 may be an inherent attribute associated with terminally differentiated plasma cells that can survive for weeks *in vivo* (49).

In conclusion, we have shown that mutant *ras* expression can affect the growth/survival characteristics of the myeloma cell line ANBL6 by promoting growth in the absence and at suboptimal concentrations of IL-6, and can decrease cell progression into an apoptotic pathway. These observations suggest that MM tumors containing *ras* mutations could remain viable *in vivo* at suboptimal concentrations of IL-6 and could also result in an increased tumor load. Furthermore, therapeutic trials designed to kill the tumor by removing IL-6 (50) or by using antibodies to block the signaling subunit of the IL-6R (51) may have less of an effect on tumors containing *ras* mutations.

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Introduction of an Activated N-*ras* Oncogene Alters the Growth Characteristics of the Interleukin 6-dependent Myeloma Cell Line ANBL6

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