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Early Pre-B-Cell Transformation Induced by the v-*fms* Oncogene in Long-Term Mouse Bone Marrow Cultures

GARY V. BORZILLO AND CHARLES J. SHERR*

Howard Hughes Medical Institute, Department of Tumor Cell Biology, St. Jude Children's Research Hospital, Memphis, Tennessee 38105

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Murine long-term bone marrow cultures that support B-lymphoid-cell development were infected with a helper-free retrovirus containing the v-*fms* oncogene. Infection of B-lymphoid cultures resulted in the rapid clonal outgrowth of early pre-B cells, which grew to high cell densities on stromal cell feeder layers, expressed v-*fms*-coded glycoproteins, and underwent immunoglobulin heavy-chain gene rearrangements. Late-passage cultures gave rise to factor-independent variants that proliferated in the absence of feeder layers, developed resistance to hydrocortisone, and became tumorigenic in syngeneic mice. The v-*fms* oncogene therefore recapitulates known effects of the v-*abl* and *bcr-abl* oncogenes on B-lineage cells. The ability of v-*fms* to induce transformation of early pre-B cells in vitro underscores the capacity of oncogenic mutants of the colony-stimulating factor-1 receptor to function outside the mononuclear phagocyte lineage.

The v-*fms* oncogene of the Susan McDonough (SM) strain of feline sarcoma virus (FeSV) is derived from the gene encoding the cell surface receptor for the macrophage colony-stimulating factor CSF-1 (46). Both the CSF-1 receptor (CSF-1R) and v-*fms* oncogene product consist of an extracellular ligand-binding domain joined through a single membrane-spanning segment to an intracellular tyrosine kinase domain (11, 18). Whereas the tyrosine kinase activity of CSF-1R is strictly regulated by ligand binding, the v-*fms*-coded glycoprotein acts constitutively as a kinase and provides persistent enzymatic signals for cell growth in the absence of CSF-1 (15, 30, 44, 48, 49). The latter properties are primarily due to an activating mutation(s) in the extracellular domain of CSF-1R that affects its conformation and induces the receptor kinase (40, 41, 57). In addition, genetic alterations involving the CSF-1R carboxyl terminus, which do not confer transforming ability on their own, can nonetheless enhance the transforming efficiency of receptors bearing activating mutations in their extracellular domains (42). Because the activating mutations do not disrupt the high-affinity ligand-binding site in CSF-1R, the kinase activity of the feline v-*fms* or mutant human c-*fms* gene product can be further up-regulated in response to exogenous CSF-1 (26, 40, 41). This may account for the propensity of SM-FeSV to selectively induce malignant tumors of CSF-1-producing cells (fibroblasts) in its natural host, the domestic cat.

Because the v-*fms* gene is derived from a receptor that normally functions in supporting the proliferation, differentiation, and survival of mononuclear phagocytes, we tested the ability of the gene to transform hematopoietic cells. Introduction of the v-*fms* gene into simian virus 40-immortalized, CSF-1-dependent murine macrophages induced factor independence and tumorigenicity (49). Since mouse CSF-1 does not bind to the feline v-*fms* gene with high affinity and was not produced in detectable quantities by v-*fms*-transformed macrophages, we concluded that the mechanism of transformation was nonautocrine. Analogous results were obtained when v-*fms* was expressed in a murine interleukin-3 (IL-3)-dependent myeloid cell line, suggesting that v-*fms* could also transform less mature myeloid progenitors (48). To assay the transforming functions of the v-*fms*-encoded tyrosine kinase in an in vivo setting, mouse bone marrow cells were infected with helper-free SM-FeSV, and the infected cells were used to reconstitute the hematopoietic compartment of lethally irradiated mice. Myeloproliferative disorders and histiocytic sarcoma were observed in some primary recipients, whereas transplantation of provirus-positive spleen cells into secondary irradiated recipients resulted in the appearance of clonal erythroleukemias and B-cell lymphomas (19). Thus, although CSF-1R is normally restricted in its expression to mononuclear phagocytes, the v-*fms* gene has a broader target cell specificity and can contribute to the transformation of hematopoietic cells that do not express c-*fms*-encoded kinase activity.

The only retroviral oncogene known to consistently transform B-lymphoid cells in vitro and in vivo is v-*abl* (for reviews, see references 39, 51, and 55). The use of long-term bone marrow cultures that support the growth of immature B cells has been instrumental in studying the effects of the v-*abl* kinase on cell proliferation and differentiation (50, 52–54). More recently, chimeric *bcr-abl* cDNAs resulting from translocations observed in human chronic myelogenous leukemia and in some acute lymphoid leukemias of childhood have been evaluated for transforming activity by this method (29, 58). To determine whether similar techniques might also be useful in defining in vitro B-lymphoid targets of v-*fms* transformation, we infected long-term bone marrow cultures with SM-FeSV. We demonstrate that v-*fms* recapitulates known effects of v-*abl* on immature B-lineage cells, leading to the clonal high-density outgrowth of early pre-B cells, which eventually evolve to a tumorigenic phenotype.

MATERIALS AND METHODS

Establishment and maintenance of long-term B-lymphoid cultures. Long-term murine bone marrow cultures specific for the growth of B-lymphoid cells were established from 4- to 6-week-old BALB/c mice (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) (52). Bone marrow cells explanted from femurs and tibias were spun down and resuspended at 10^6 cells per ml. Each mouse provided about

* Corresponding author.

4×10^7 bone marrow cells. B-lymphoid cultures (established with 5×10^6 cells per 60-mm-diameter dish) were grown at 37°C (6% CO_2) in RPMI 1640 medium supplemented with 5% fetal calf serum (FCS; GIBCO Laboratories, Grand Island, N.Y.), 10 mM L-glutamine, and 5×10^{-5} M 2-mercaptoethanol. The cultures were fed biweekly as described elsewhere (52). Different lots of FCS were prescreened for the ability to support long-term marrow cultures as compared with preselected FCS samples provided by Judy Young and Owen Witte (Howard Hughes Medical Institute, University of California, Los Angeles, School of Medicine).

SM-FeSV virus stocks and infection. SM-FeSV proviral DNA (12) was transfected into the ψ2 packaging cell line (28), and clones of virus-producing cells were derived by limiting dilution in 96-well plates (49). A ψ2 subclone producing helper-free virus in excess of 5×10^6 focus-forming units per ml of culture medium was previously used to infect NIH 3T3 fibroblasts, several myeloid cell lines, and BALB/c bone marrow cells (19, 42, 48, 49). In these experiments, the infected cells expressed the v-*fms* products but did not produce infectious virus, as determined by a supernatant reverse transcriptase assay.

Unless otherwise indicated, the lymphoid cultures were infected 4 to 6 weeks after establishment. During this initial period, an adherent stromal feeder layer developed, consisting primarily of macrophages, endothelial cells, and fibroblasts. After formation of a stromal layer (3 to 5 weeks postexplantation), hematopoietic cells appeared in overlying clusters. Two days before infection, SM-FeSV-producing ψ2 cells were seeded subconfluently into 75-cm^2 flasks in Dulbecco modified Eagle medium containing 10% FCS. The next day, the medium was replaced with 8 ml of fresh B-lymphoid cell culture medium, and virus was collected for 24 h. On the day of infection, ~75% of the medium in the long-term cultures was replaced with the same amount of filtered virus supernatant. Cells removed from the long-term cultures during infection were spun down and returned to the original cultures.

Freshly explanted BALB/c marrow cells were also infected before being placed in long-term culture. In these experiments, bone marrow cells were infected by 24-h cocultivation on irradiated (2,500 rads; 842 rads/min) SM-FeSV-producing ψ2 cells in the appropriate culture medium. After cocultivation, the bone marrow cells were removed, washed, and placed in culture as described above.

Protein and nucleic acid analyses. High-molecular-weight DNA (5 to 10 μg) was prepared and analyzed by Southern blotting on Nytran filters (Schleicher & Schuell, Inc., Keene, N.H.) as described previously (6). Probes used for hybridization were restriction fragments radiolabeled by nick translation. Probes and restriction maps are described and illustrated in the figure legends and figures. Filters were stripped with 0.2 N NaOH before rehybridization. For Southern blots of DNA from control lymphoid cultures, DNA was isolated from adherent as well as nonadherent cells.

Preparation of cell lysates, immunoprecipitation, immune complex kinase assays, and electrophoresis of proteins in gels containing sodium dodecyl sulfate were performed as described previously (3, 43). Procedures for immunofluorescence staining and flow cytometry, using antisera to either cell surface immunoglobulin or v-*fms*-encoded epitopes, are also described in detail elsewhere (4, 43). Antibodies to v-*fms*-encoded epitopes specifically react with the feline viral oncogene product and do not cross-react with the murine CSF-1 receptor (2, 16, 46).

Growth parameters and tumorigenicity of SM-FeSV-infected hematopoietic cells. Nonadherent cells from SM-FeSV-infected long-term B-lymphoid cultures were seeded at 10^5 cells per ml in 24-well plates (Costar, Cambridge, Mass.) supplemented with various proportions of medium conditioned by normal B-lymphoid cultures. Cells were counted after 72 h, and viability was determined by trypan blue exclusion. To assay growth on stromal layers, uninfected low-density long-term marrow cultures were established as described previously (52), and lymphoid cells were plated at 5×10^5 cells per ml in the presence or absence of 10^{-7} M hydrocortisone. Cells were counted every 3 days for 2 weeks. Growth in soft agar was assayed by plating 10^3 or 10^4 nonadherent cells in 35-mm-diameter culture dishes in the presence or absence of 25% conditioned medium. SM-FeSV-infected cells were also plated in agar over uninfected low-density feeder layers as described previously (54). Macroscopic colonies were first observed at day 6 and were enumerated at day 12.

To test for in vivo tumorigenicity, cells from early (8 week)- and late (15 week)-passage SM-FeSV-infected lymphoid cultures were injected intraperitoneally into 4- to 8-week-old BALB/c mice. The animals were observed over a course of 20 weeks (early-passage cultures) or 16 weeks (late-passage cultures) for tumors at the injection sites or in lymphoid organs. Complete blood counts with differentials were performed at the Animal Resource Center, St. Jude Children's Research Hospital, and diseased animals were subjected to necropsy (19).

RESULTS

SM-FeSV infection of long-term B-lymphoid cultures results in increased cell growth. To study the effects of v-*fms* expression on different hematopoietic lineages, B-lymphoid long-term bone marrow cultures were infected with SM-FeSV. The nonadherent fraction contains B-lineage cells at various stages of development that express B-cell antigens, exhibit immunoglobulin gene rearrangements, and can restore immunity to immunodeficient CBA/N and SCID mice (13, 14, 25, 39, 51, 52, 56).

Several approaches were taken to pinpoint efficient methods for infecting long-term B-lymphoid cultures with SM-FeSV. In initial experiments, freshly explanted bone marrow was infected by overnight cocultivation on irradiated SM-FeSV-producing ψ2 cells and then plated in long-term cultures. Of the 25 cultures established in this manner, most grew poorly, rarely generating typical numbers (1×10^5 to 2×10^5/ml) of nonadherent lymphoid elements. The stromal cell fraction appeared to have been depleted during the cocultivation step, either from attachment during cocultivation or perhaps as a direct result of SM-FeSV infection. A second method of infection was to add filtered SM-FeSV-containing supernatants directly to the cultures after establishment, namely, at 3 to 5 weeks postinitiation, when clusters of rapidly dividing hematopoietic cells were first observed over the adherent layer (Fig. 1A). Each of the 15 B-lymphoid cultures infected in this manner was observed to contain increased numbers of nonadherent cells by 6 days postinfection. By 3 weeks postinfection, the nonadherent fraction of the SM-FeSV cultures contained 20- to 30-fold more cells than did control or mock-infected cultures, often yielding $>3 \times 10^6$ cells per ml. The medium in the SM-FeSV-infected cultures became rapidly acidified; therefore, the feeding regimen had to be changed from weekly demidepopulation (mock-infected cultures) to biweekly demi-

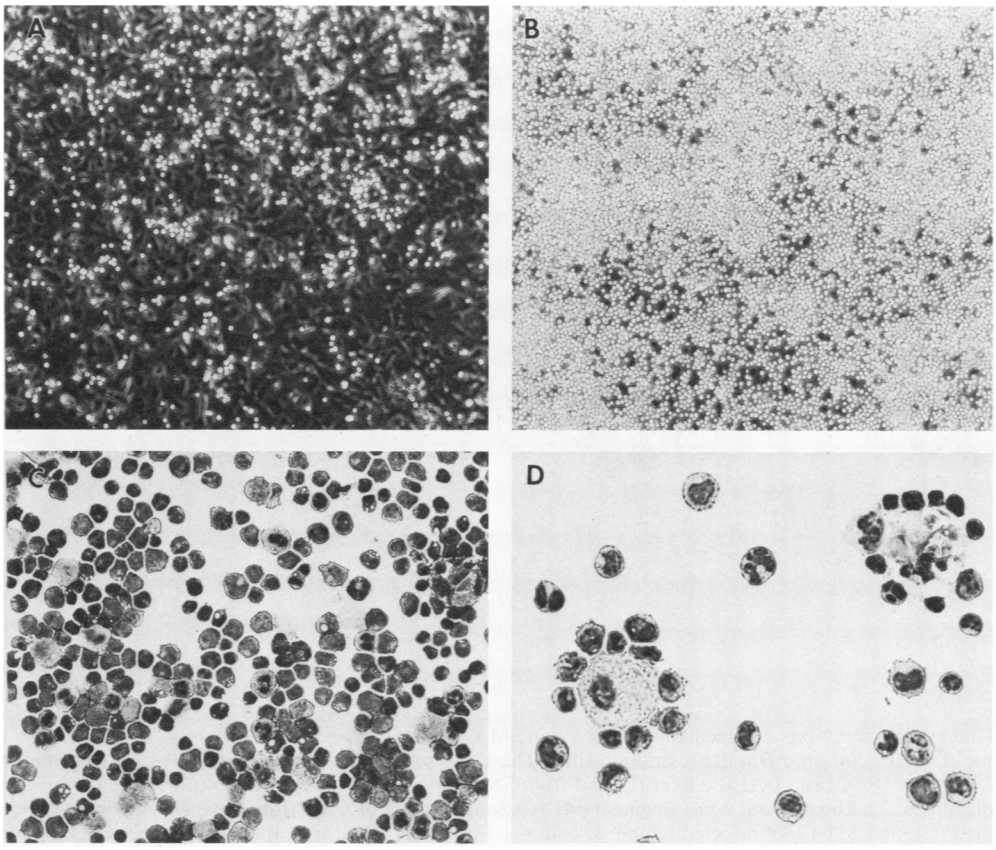

FIG. 1. Lymphoid bone marrow cultures infected with SM-FeSV. Shown are phase-contrast micrographs of mock-infected (A) and SM-FeSV-infected (B) cultures at 8 weeks postinfection and Wright-Giemsa-stained cytocentrifuge preparations exhibiting lymphocytes (C) and macrophage-lymphoid rosettes (D) from an SM-FeSV-infected lymphoid culture. Magnifications: A and B, ×38; C, ×400; D, ×500.

population (SM-FeSV-infected cultures). By 15 weeks postinfection, SM-FeSV-infected cultures had to be fed three times per week and continued to produce $>3 \times 10^6$ cells per ml. In most of the infected cultures, the feeder layer was completely obscured by refractile, nonadherent lymphoid cells (Fig. 1B). Wright-Giemsa staining of the nonadherent fraction of SM-FeSV-infected cultures revealed an atypical population of lymphocytes with many mitotic figures (Fig. 1C). At 2 weeks postinfection, the nonadherent fraction also contained rosettes composed of macrophages containing ingested cells and ringed by lymphoid blasts (Fig. 1D).

SM-FeSV-infected cultures consist of clonal populations expressing the v-*fms*-encoded kinase. To determine whether the rapidly proliferating SM-FeSV-infected cells in the B-lymphoid cultures contained the integrated SM-FeSV provirus, high-molecular-weight DNA from the nonadherent cells from six cultures was digested with *Cla*I and *Bam*HI, and Southern blots were hybridized with a v-*fms* probe. Since each of these enzymes recognizes single restriction sites within the SM-FeSV provirus (12; Fig. 2A), codigestion was expected to yield an internal 2.6-kilobase-pair (kbp) restriction fragment corresponding to the v-*fms* probe. DNA from each of the infected cultures yielded the expected 2.6-kbp v-*fms* fragment (Fig. 2B). Background bands, also detected in an uninfected lymphoid culture and in mouse liver (lane L), probably correspond to c-*fms* proto-oncogene sequences present in all normal cells. The fact that the hybridization signal intensity of the 2.6-kbp v-*fms* fragment was significantly greater than those observed for c-*fms* sequences was not unexpected, since the mouse cellular CSF-1R-coding sequences are interrupted by introns, and the probe and SM-FeSV sequences were each of feline origin. Moreover, individual cells in the population might also have contained more than one copy of the SM-FeSV provirus per haploid genome, particularly since the cells were infected at a relatively high virus multiplicity.

To determine whether the cells represented clonal populations, the same DNA samples were digested with *Hin*dIII, which recognizes a single internal restriction site 3′ to the v-*fms* gene in the SM-FeSV provirus (Fig. 2A). Each copy of integrated SM-FeSV should yield a single v-*fms*-containing fragment whose length would be determined by adventitious *Hin*dIII sites in host cellular DNA flanking the 5′ end of the integrated provirus. The ability to visualize discrete junction fragments rather than a smear of hybridizing bands depends on the frequency at which particular integration events are represented in the total population (21). The DNA from each culture yielded discrete hybridizable bands, none of which were detected in mock-infected control cultures (Fig. 2C, lane C) or in normal BALB/c liver DNA (lane L). An analogous pattern of multiple proviral integrations was observed after *Bam*HI digestion (not shown). The apparent oligoclonal nature of SM-FeSV integration events in these cell populations indicated that among the B-lymphoid cells initially present in the culture, a limited number of infected clones had acquired a growth advantage by the time of blot analysis (i.e., by 12 weeks postinfection).

To verify that the v-*fms*-encoded glycoprotein was expressed in the SM-FeSV-infected cultures, lysates prepared from nonadherent cells were incubated with an antiserum to

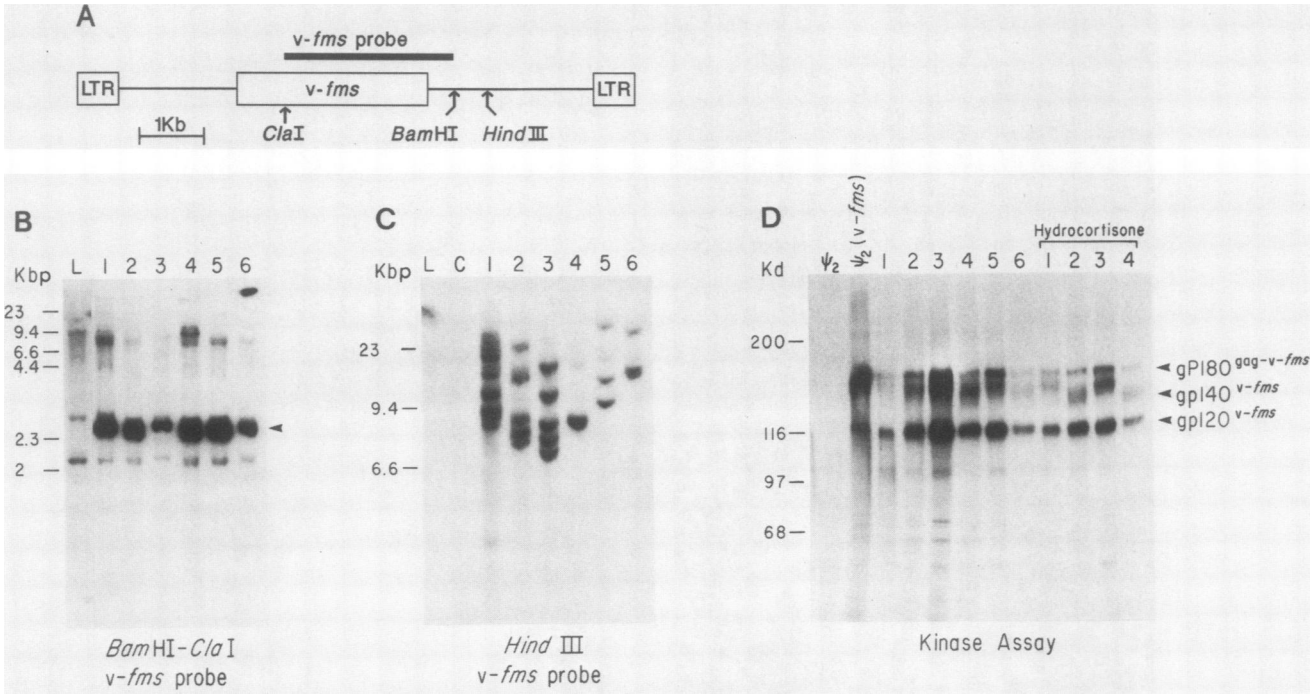

FIG. 2. Proviral integration and v-*fms*-encoded kinase activity in SM-FeSV-infected lymphoid cultures. (A) Restriction map of SM-FeSV provirus (12) showing *Bam*HI, *Cla*I, and *Hin*dIII restriction sites. The v-*fms* probe was a 2.6-kbp *Bam*HI-*Cla*I fragment. (B) Southern blot of *Bam*HI and *Cla*I-digested DNA from BALB/c liver (L) and from SM-FeSV-infected lymphoid cultures 1 to 6 hybridized with the v-*fms* probe showing the diagnostic 2.6-kbp proviral v-*fms* fragment (◀). (C) Southern blot of *Hin*dIII-digested DNA from BALB/c liver (L), control mock-infected cultures (C), and SM-FeSV-infected lymphoid cultures 1 to 6 hybridized with the v-*fms* probe. Background bands in panels B and C probably represent murine endogenous c-*fms* proto-oncogene sequences. (D) Immune complex kinase assays performed with lysates of ψ2 cells (lane ψ2), SM-FeSV-producing ψ2 cells [lane ψ2 (v-*fms*)], and different SM-FeSV-infected cultures (nonadherent cells only; lanes 1 to 6). Data with SM-FeSV-infected cultures grown in the presence of 10^{-7} M hydrocortisone are shown in lanes 1 to 4 at the extreme right. Positions of $gP180^{gag-v\text{-}fms}$ (the primary SM-FeSV translation product) and of the immature ($gp120^{v\text{-}fms}$) and mature ($gp140^{v\text{-}fms}$) glycoproteins (3) are indicated on the right. Molecular size markers in kilobase pairs or kilodaltons (kd) are shown to the left of each panel.

the v-*fms* gene product (16), and immune complexes were assayed for protein kinase activity. The three characteristic forms of the v-*fms*-encoded glycoprotein (3) were phosphorylated in immune complexes prepared from SM-FeSV-infected cultures but not in those from uninfected cultures (Fig. 2D). Therefore, introduction of the v-*fms* gene into B cells resulted in the clonal outgrowth of high-density cell populations that expressed the v-*fms*-encoded kinase.

Nonadherent cells in SM-FeSV-infected lymphoid cultures exhibit an early pre-B-cell phenotype. To investigate the lineage and stage of differentiation of the rapidly dividing blast cells in SM-FeSV-infected B-lymphoid cultures, immunoglobulin heavy-chain (IgH) and light-chain (IgL; κ and λ) and T-cell receptor β-chain rearrangements were analyzed by Southern blotting. Figures 3A and B show the results of Southern blots of *Xba*I-digested DNA samples hybridized sequentially with a J_H and a 5′ D-region probe (1). Since *Xba*I sites flank the murine J_H region (Fig. 3A, top), V_HDJ_H or DJ_H joining will be manifested by the loss of the 5′ *Xba*I site and the presence of a rearranged (non-germ line) J_H band on a Southern blot. J_H rearrangements are characteristic of committed B-lineage cells but can occasionally be found in T cells (24). That subset of J_H rearrangements resulting from DJ_H joining events would also be expected to hybridize to a 5′ D-region probe. By contrast, J_H rearrangements resulting from complete V_HDJ_H joining events should lack 5′ D regions and therefore not hybridize to any 5′ D-region probes. Thus, 5′ D-region probes can be used to distinguish DJ_H from VDJ_H rearrangements. The two 5′ D-region probes used in this study (5′ $D_{sp\text{-}2}$ [Fig. 3B, top] and 5′ D_{FL16} [not shown]) are thought to detect most of the known D segments (1, 23, 24).

When *Xba*I-digested DNAs from SM-FeSV-infected lymphoid cultures were analyzed with the J_H probe, two to six J_H rearrangements were observed in each culture (Fig. 3A). The absence of a detectable germ line J_H band in any of the infected cultures indicated that most of the cells in each SM-FeSV-infected population had undergone J_H rearrangements on both IgH alleles. Thus, by these criteria, most cultures consisted of several dominant clones, consistent with results obtained with the v-*fms* probe (Fig. 2C). When the filter was stripped and rehybridized with the 5′ $D_{sp\text{-}2}$ probe (Fig. 3B), a single J_H rearrangement in culture 4 (arrowheads in Fig. 3A and B) was again detected, indicative of a DJ_H joining event. Deletions of 5′ $D_{sp\text{-}2}$ regions consistent with VDJ_H rearrangements were observed in cultures 2 and 5. 5′ D_{FL16} regions were also completely deleted in these lanes, but a single D_{FL16} rearrangement was identified in culture 4 (data not shown). The remaining J_H rearrangements observed in Fig. 3A therefore reflect undetected DJ_H rearrangements or complete V_HDJ_H rearrangements. The presence of both DJ_H and V_HDJ_H joining events is consistent with different stages of B-cell maturation.

The status of the IgL (κ and λ) loci was also investigated with *Hin*dIII-digested DNA (J_κ probe [20]; Fig. 3C) and *Eco*RI-digested DNA (C_λ probe [5]; not shown). The κ and λ loci were in germ line configuration in all six cultures tested. Moreover, the T-cell receptor β-chain locus (27; analyzed

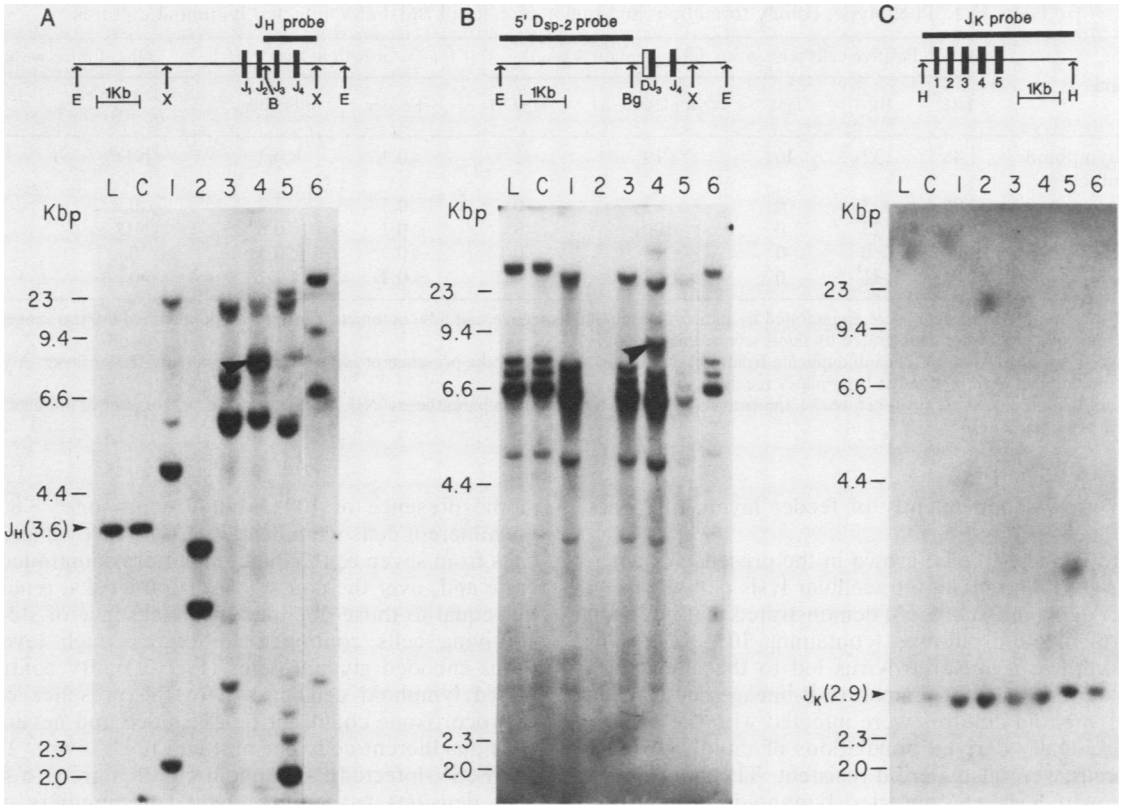

FIG. 3. Demonstration that SM-FeSV-infected lymphoid cultures contain IgH VDJ and DJ rearrangements. (A) Restriction map of the J_H-containing 3.6-kbp *Xba*I fragment of mouse genomic DNA (adapted from reference 1). The J_H probe was a 1.2-kbp *Bam*HI-*Xba*I fragment (heavy line). DNA isolated from BALB/c liver (L), control lymphoid cultures (C), and SM-FeSV-infected cultures 1 to 6 was digested with *Xba*I, and the blot was hybridized to the J_H probe. Restriction enzymes: X, *Xba*I; B, *Bam*HI; E, *Eco*RI; Bg, *Bgl*II. (B) Restriction map of a DNA segment containing the 5'-flanking sequence of a $D_{sp2.3}$-J_{H3} joint (1). The 5' D_{sp-2}-family probe was a 2.7-kbp *Eco*RI-*Bgl*II fragment (heavy line). The Southern blot shown in panel A was hybridized with the 5' D_{sp-2} probe. The J_H rearrangement in culture 4, which comigrated with the 5' D_{sp-2}-region rearrangement, represents a $D_{sp-2}J_H$ joint and is indicated by an arrowhead in panel A; the corresponding D_{sp-2} rearrangement in panel B is similarly indicated. (C) Restriction map of a DNA segment spanning the murine J_κ region. The J_κ probe was a 2.9 kbp *Hin*dIII (H) fragment (20). DNA isolated from SM-FeSV-infected cultures 1 to 6 and control DNA was digested with *Hin*dIII, and the blot was hybridized to the J_κ probe. The positions of the germ line band and molecular size markers are shown to the left of each panel.

with *Hin*dIII [not shown]) was unrearranged in all of the infected cultures. The presence of IgH rearrangements together with germ line organizations of Igκ and Igλ loci and the T-cell receptor β gene suggested that the nonadherent cells in the SM-FeSV-infected cultures conformed to a pre-B (IgH μ^+ LC^-) or early pre-B (μ^- LC^-) stage of development. To further distinguish between these stages, two-color fluorescence microscopy of the cell surface B220 antigen (detected by fluorescein-conjugated monoclonal antibody 14.8 [25]) and intracellular μ chains (detected by rhodamine-conjugated goat anti-mouse IgM) was used. The cells were also stained with monoclonal antibodies MAC-1, which detects an epitope on mature monocytic and granulocytic cells (47), and BP-1, which recognizes a cell surface epitope on early B-lineage cells (10). The majority of nonadherent cells in six of the seven SM-FeSV-infected cultures were $B220^+$ μ^- LC^- (Table 1), corresponding to early pre-B cells in phenotype. Culture 2, by contrast, was populated by a nearly pure population of cytoplasmic μ^+ pre-B cells.

SM-FeSV-infected early pre-B cells rapidly develop steroid resistance but initially remain growth factor dependent. SM-FeSV-infected nonadherent cells in B-lymphoid cultures grew to densities 20- to 30-fold greater than those in mock-infected cultures. To determine whether the adherent stromal cell feeder layer still contributed to their growth, nonadherent cells from infected cultures 1, 7, and 8 were removed at 4 weeks postinfection, and their growth was analyzed on uninfected low-density feeder layers, in feeder layer-conditioned medium, or in unconditioned long-term B-lymphoid culture medium. On uninfected low-density feeder layers, nonadherent cells from SM-FeSV-infected cultures grew rapidly, and the cultures were soon indistinguishable from parental cultures. Conditioned medium from feeder layers from either infected or uninfected cultures supported the growth of infected early pre-B cells (Fig. 4), albeit at slower rates than those obtained on feeder layers. By contrast, the infected cells failed to proliferate extensively when plated in the absence of a feeder layer in unconditioned medium. Thus, although early-passage SM-FeSV-infected cells were clonal and proliferated to abnormally high cell densities, they remained dependent on a factor produced by the feeder layer. Measurement of [^{3}H]thymidine uptake by the infected cells in proliferation assays confirmed the presence of a growth-promoting activity in medium conditioned by feeder layers. A survey suggested that the factor could not be replaced by CSF-1, granulocyte-CSF, granulocyte-macrophage-CSF, IL-3, IL-4, or IL-6 (data not shown). Late (15 week)-passage cultures exhibited decreased factor responsiveness and could be

TABLE 1. Phenotype, colony formation, and tumorigenicity of SM-FeSV-infected lymphoid cultures

Culture	% Positive cells with given differentiation marker[a]					% Growth in agar[b]		No. of mice with tumors[c]	
	14.8	BP-1	IgM	Cytoplasmic μ	MAC-1	−Feeder	+Feeder	8-Wk cultures (n = 3)	15-Wk cultures (n = 5)
Uninfected lymphoid	48	32	46	19	1	<0.1	<0.1	ND	ND
SM-FeSV infected									
1	99	3	0	7	0	<0.1	2.2	0	2 (35–45)
2	86	23	0	100	0	<0.1	0.6	ND	ND
6	100	0	0	13	0	<0.1	0.5	0	5 (24–52)
7	86	11	0	3	0	<0.1	3.2	0	0

[a] 14.8$^+$, BP-1$^+$, and MAC-1$^+$ cells were enumerated by indirect immunofluorescence and flow cytometry, using isotype-matched control reagents. IgM and cytoplasmic μ-positive cells were enumerated by fluorescence microscopy.

[b] Determined by plating 10^3 or 10^4 cells in duplicate from each culture in soft agar in the presence or absence of a low-density feeder layer. A value of <0.1 indicates that no colonies were generated from plates receiving 10^3 cells.

[c] The range of the latent period (days) before the appearance of tumors is indicated in parentheses. ND, Not determined. n, Number of animals injected with cells from each lymphoid culture.

adapted to grow independently of feeder layers (data not shown).

Immature B-lineage cells, grown in the presence of corticosteroids, are killed by an intracellular lysis pathway (17). However, Young and Witte (58) demonstrated that infection of long-term myeloid cultures (containing 10^{-7} M hydrocortisone) with a *bcr-abl* retrovirus led to the clonal outgrowth of steroid-resistant, immature B-lineage cells. When long-term lymphoid cultures were infected with the *bcr-abl* retrovirus, clonally derived populations of rapidly dividing B-lineage cells were also steroid resistent. The steroid sensitivity of eight SM-FeSV-infected lymphoid cultures was therefore tested by seeding infected cells onto uninfected low-density feeder layers in the presence or absence of 10^{-7} M hydrocortisone. In the absence of steroids, the infected early pre-B cells rapidly overgrew the feeder layer (Fig. 5); in the presence of 10^{-7} M hydrocortisone, ~80% of the nonadherent cells were dead 48 h later. However, surviving cells from seven of the infected cultures continued to proliferate and, over the course of several weeks, reached densities equal to those obtained in the absence of steroids. The surviving cells continued to express high levels of the v-*fms*-encoded glycoproteins (Fig. 2D). By contrast, uninfected lymphoid cells grown in the presence of 10^{-7} M hydrocortisone could not be expanded and never exceeded 10^4 nonadherent cells per ml (data not shown). Thus, most SM-FeSV-infected B-lymphoid cultures grew to sufficiently high densities to acquire significant numbers of steroid-resistant variants.

SM-FeSV-infected lymphoid cultures exhibit limited transformed characteristics. We examined the ability of the nonadherent cells from the infected lymphoid cultures to form

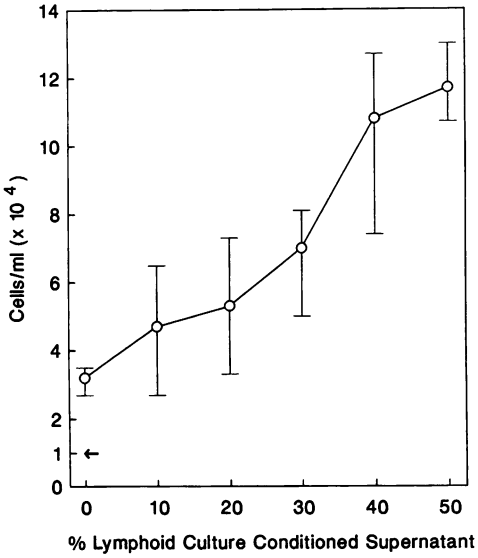

FIG. 4. Growth of SM-FeSV-infected early B-lymphoid cells in conditioned medium from lymphoid cultures. SM-FeSV-infected nonadherent cells (2×10^4 per culture from cultures 1, 7, and 8) 4 weeks postinfection were incubated in 2 ml of medium supplemented with increasing proportions of conditioned medium obtained from uninfected cultures. After 72 h, duplicate cultures were harvested and counted by trypan blue exclusion. Cell counts represent the averages from three cultures. ←, Number of cells seeded at the start of the experiment.

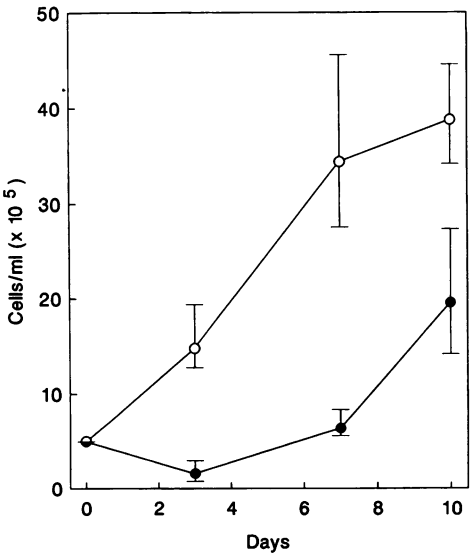

FIG. 5. Acquisition by SM-FeSV-infected early pre-B cells of resistance to steroids. Nonadherent cells from eight independently derived SM-FeSV-infected long-term B-lymphoid cultures were plated onto uninfected low-density feeder layers in the presence (●) or absence (○) of 10^{-7} M hydrocortisone. Every 3 days, cells were counted and viability was determined by trypan blue exclusion. Data averaged from three cultures are shown. After 2 weeks, hydrocortisone-containing cultures were fed by the usual regimen with lymphoid medium plus 10^{-7} M hydrocortisone.

colonies when plated in semisolid medium at 2 months postinfection. The cells were unable to form colonies in standard long-term lymphoid medium; however, all cultures yielded colonies when plated over a feeder layer (Table 1). Substitution of a feeder layer with 25% lymphoid culture conditioned medium did not support significant colony formation.

To test the ability of the v-*fms*-expressing cells to form tumors in syngeneic animals, 5×10^6 cells from early (8 week)- and late (15 week)-passage cultures were injected intraperitoneally into BALB/c mice. None of the mice injected with early-passage cells developed tumors during 20 weeks of observation. By contrast, late-passage cells were tumorigenic, producing tumors in 7 of 15 animals tested after latent periods as short as 24 days (Table 1).

DISCUSSION

Introduction of the CSF-1R can render naive cells CSF-1 responsive, implying that downstream components of the intracellular mitogenic pathway can engage and interpret receptor-mediated signals for cell growth (42, 45a). The v-*fms* oncogene encodes an analog of CSF-1R that retains a functional ligand-binding domain but, because of mutations, acts constitutively as a tyrosine kinase. The fact that relevant physiologic substrates for the CSF-1R kinase are expressed more ubiquitously than CSF-1R itself implies that the v-*fms* oncogene can transform hematopoietic target cells other than those of the mononuclear phagocyte lineage. Indeed, when SM-FeSV was introduced into mouse bone marrow progenitors and the infected cells were used to reconstitute irradiated mice, expression of the v-*fms* kinase contributed through a multistep mechanism to a variety of hematopoietic disorders, including clonal B-cell lymphomas (19).

SM-FeSV infection of lymphoid cultures resulted in the high-density outgrowth of immature B lineage cells that were oligoclonal and expressed v-*fms*-encoded glycoproteins. Analyses of differentiation markers and immunoglobulin gene rearrangements demonstrated that the infected clones represented cells at early pre-B (μ^- LC^-) and pre-B (μ^+ LC^-) stages of development. Although infected B-lymphoid cells grew to densities 20- to 30-fold higher than those in uninfected cultures, early (<8 week)-passage cells could not proliferate in the absence of a feeder layer and did not induce tumors in syngeneic mice. With prolonged passage in vitro, however, these cultures gave rise to cells that grew in the absence of feeder layers and were tumorigenic. Thus, as previously observed in vivo (19), the v-*fms* oncogene initiated a chain of events involving expansion of pre-B progenitors and clonal evolution, ultimately resulting in the emergence of tumorigenic B cells.

We have not identified the nature of the lymphoid growth-promoting activity present in conditioned medium from either uninfected or infected lymphoid cultures. Hunt and co-workers (22) previously pointed to the presence of a pre-B-cell growth factor (distinct from IL-1, IL-2, IL-3, and IL-4) in conditioned supernatants of a stromal cell line. It would be of interest to determine whether a unique growth factor, possibly IL-7 (32, 33), could abrogate the feeder layer requirements of SM-FeSV-infected, early-passage pre-B cells.

This study demonstrates that v-*fms*- and *abl*-containing retroviruses can transform similar lymphoid targets in long-term marrow cultures. Specifically, SM-FeSV, different strains of Abelson murine leukemia viruses (A-MuLV), and *bcr-abl* retrovirus infection all result in the rapid outgrowth of immature B-lineage cells that contain IgH but not IgL gene rearrangements and express their respective kinases (58; reviewed in reference 55). Other oncogenes have also been shown to transform immature B-lineage cells derived from long-term bone marrow cultures (v-*myc*/v-Ha-*ras* [45]) and in soft agar (v-*fes* [35]). We have not tested whether viral *gag* sequences, required for lymphoid (but not fibroblast) transformation by A-MuLV (37), are also required by SM-FeSV, nor is it known whether rare subpopulations of SM-FeSV-infected lymphocytes (like some A-MuLV-infected lines) retain the capacity to undergo IgL gene rearrangement and isotype switching in culture (7, 53). Therefore, although additional experiments are necessary to directly compare the growth and transformation parameters of SM-FeSV- and A-MuLV-infected lymphocytes, many aspects of v-*fms*- and v-*abl*-mediated lymphoid transformation and tumor induction appear to be similar.

SM-FeSV and A-MuLV can also transform NIH 3T3 fibroblasts as well as hematopoietic targets distinct from pre-B cells. Thymic injection of A-MuLV into mice induced T-cell malignancies (8), whereas plasmacytoma and macrophage cell lines have been generated by A-MuLV infection of pristane-primed mice (31, 38). Insertion of v-*abl* into IL-3-dependent myeloid cell lines abrogated their growth factor dependence and rendered them tumorigenic (9, 34, 36). Similarly, v-*fms* can affect the growth factor requirements of myeloid cell lines in culture (48, 49) and can induce a variety of hematopoietic malignancies in vivo (19). An unanswered question concerns the extent to which both of these oncogene-encoded kinases interact with the same substrates and whether their signal transduction pathways are invariably represented in different cell types. Analogously, the ability of the v-*fms* gene to stimulate growth of early pre-B cells raises the possibility that infection of lymphoid cells with a c-*fms*-containing virus might enable early pre-B cells to be clonally expanded in CSF-1.

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