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Differentiation In Vitro of a Leukemia Virus-Induced B-Cell Lymphoma into Macrophages

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Cells of the hemopoietic system arise by proliferation and differentiation of progenitor cells. This process begins with multipotential stem cells which can self-renew and also undergo progressive differentiation to progenitor cells committed to particular lineages, ultimately yielding mature blood cells (D. Metcalf and M. A. S. Moore, *Haematopoietic Cells*, 1971). Early commitment of lymphoid progenitors is generally believed to separate the lymphoid lineage from the myeloid and erythroid lineages, whose progenitors are separated late in differentiation (Metcalf and Moore, 1971). We recently developed a derivative of Moloney murine leukemia virus (M-MuLV) in which the enhancer sequences from simian virus 40 were substituted into the M-MuLV long terminal repeat. This recombinant virus (ΔMo+SV M-MuLV) induces pre-B and B lymphoid leukemia with long latency after inoculation of 2-day-old NIH Swiss mice (R. Hanecak, P. K. Pattengale, and H. Fan, J. Virol. 62:2427–2436, 1988). In this report, we describe the derivation of a permanent, virus-producing cell line with the phenotypic characteristics of mature macrophages from a B-cell-derived lymphoblastic lymphoma induced by ΔMo+SV M-MuLV. Comparison studies of immunoglobulin heavy-chain gene rearrangements and also ΔMo+SV M-MuLV proviral integration sites confirmed that the macrophage cell line was derived from the original B-lymphoblastic lymphoma. Moreover, inoculation of the macrophage cell line into animals resulted in histiocytic sarcomas of the macrophage type, thus reflecting stable conversion of B-lymphoid tumor cells to the macrophage phenotype. These results suggest a closer relationship between lymphoid and myeloid cells than previously believed.

Inoculation of ΔMo+SV Moloney murine leukemia virus (M-MuLV) into neonatal NIH Swiss mice results in B-lineage lymphoblastic lymphomas with a mean latency of 17 months (8). The tumors show rearrangements of the immunoglobulin heavy-chain (μ) genes, and in some cases immunoglobulin light-chain (κ) genes as well; the tumors do not show rearrangements of the T-cell receptor beta genes. Thus, the ΔMo+SV M-MuLV-induced tumors have been classified as B lineage derived (pre-B or B lymphoid). One such lymphoblastic lymphoma from a leukemic cervical lymph node was established in tissue culture. Histopathology of the primary tumor (Fig. 1B) demonstrated lymphoblastic lymphoma with small-to-intermediate lymphoid cells with monomorphic nuclei, scant cytoplasm, and primitive, dispersed nuclear chromatin. Positive immunoperoxidase staining of tumor tissue sections with the monoclonal antibody RA3-6B2 specific for the B220 antigen (a B-cell-lineage-specific marker [14]) indicated that the lymphoid tumor was B cell derived (data not shown). In addition, molecular analyses of high-molecular-weight DNA prepared from the tumor showed both immunoglobulin heavy- and light-chain gene rearrangements (8).

The ΔMo+SV M-MuLV-induced lymphoblastic lymphoma described above was established in culture by finely mincing the leukemic cervical lymph node and plating the tissue in Dulbecco modified Eagle medium supplemented with 15% fetal calf serum, 4.0 mM L-glutamine, nonessential amino acids (GIBCO), and 5×10^{-5} M 2-mercaptoethanol. After 8 weeks in culture, a permanent cell line was obtained. Interestingly, the morphology of the cells was distinct from those of the primary tumor. In comparison to the typical lymphoblastic leukemia cells in the ΔMo+SV M-MuLV-induced lymphoma (Fig. 1B), the 85-2M cells exhibited a morphology typical of macrophages on cytocentrifuge preparations (Fig. 1A). The cells demonstrated conspicuous vacuolated cytoplasm with eccentric nuclei and were irregular in shape.

The 85-2M cells were also tested for the presence of surface antigens found associated with lymphoid cells or myeloid cells. 85-2M cell suspensions (1.5×10^6 cells) were incubated separately at 4°C with antibodies directed against Thy1.2 or B220 (T- and B-cell surface markers, respectively [5, 12]) and also with antibodies against Mac-1 or Mac-2, surface markers characteristic of myeloid cells and not generally found associated with lymphoid cells (9, 16). Monoclonal antibodies directed against B220, Mac-1, and Mac-2 antigens were not directly conjugated to fluorescein isothiocyanate and therefore were indirectly labeled by a second reaction with goat anti-rat fluorescein isothiocyanate-conjugated second antibody. Unstained 85-2M cells and 85-2M cells incubated with the second antibody alone were used to control for nonspecific fluorescence. The results of flow cytometry are shown in Fig. 2. The 85-2M cells, while negative for Thy1.2 and B220 surface antigens, were positive for both Mac-1 and Mac-2, a cell surface phenotype which is typical of mature macrophages. In addition, the 85-2M cells possessed the ability to phagocytose cellular debris when plated on a stromal cell layer (6). Thus, by both morphological and surface antigen characteristics, as well as by their ability to phagocytose, the 85-2M cells were classified as macrophagelike.

The 85-2M cells were tumorigenic when inoculated into sublethally irradiated adult NIH Swiss mice. Adult mice (4 weeks old) were irradiated (450 R X-ray), and 2×10^7 cells were inoculated into the tail vein. Grossly evident tumors developed in the spleen and liver 2 to 10 weeks postinocu-

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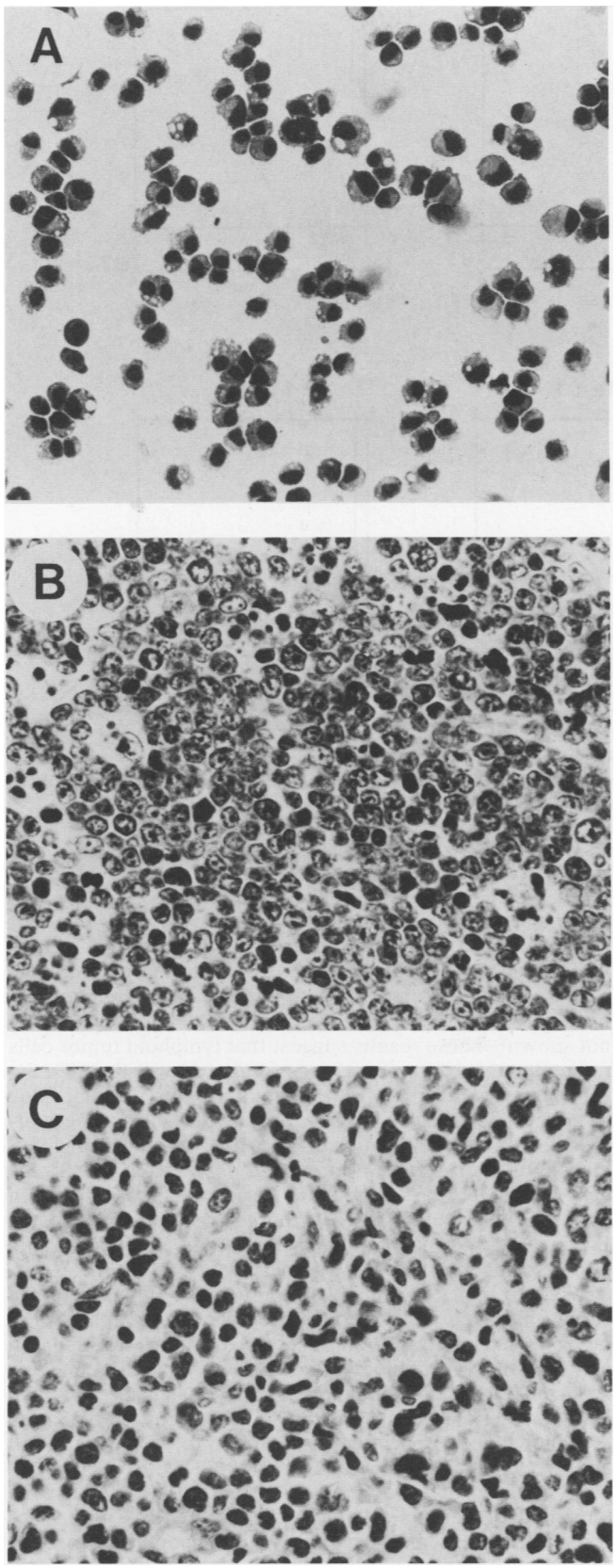

lation. Histopathologically, the tumors induced by the 85-2M cells were diagnosed as histiocyte (macrophage) sarcomas (true nonlymphoid histiocytic sarcomas [Fig. 1C]). In contrast to the primary B-lineage lymphoblastic lymphoma cells which were monomorphic and exhibited scant cytoplasm (Fig. 1B), the neoplastic macrophages were pleomorphic and contained abundant cytoplasm and mature, condensed nuclear chromatin (Fig. 1C).

It was important to determine if the 85-2M cells were related to the majority of cells in the primary lymphoid tumor. This was accomplished by two approaches. First, rearrangements in the immunoglobulin heavy-chain genes were studied. High-molecular-weight DNA from the primary tumor as well as from 85-2M cells was analyzed by Southern blot hybridization, using a radioactively labeled probe specific for the 3′ J_H region of the mouse immunoglobulin heavy-chain μ gene (18). The cells in the primary tumor showed rearrangement of the immunoglobulin heavy-chain gene, a result typical of other pre-B and B lymphoid tumors induced by ΔMo+SV M-MuLV (8). Importantly, high-molecular-weight DNA from the 85-2M cell line showed the identical immunoglobulin heavy-chain gene rearrangement pattern as that in the primary tumor, strongly supporting the hypothesis that the 85-2M cells were derived from the cells of the lymphoid tumor (Fig. 3A). Hybridization of an identical filter with two radioactive probes specific for conserved sequences present 5′ of the immunoglobulin heavy-chain D segments showed virtual elimination of the D segments, indicating that the tumor cells and also the 85-2M cells contained a V_HDJ_H rearrangement (1) (Fig. 3B). Lack of strong hybridization of germ line D-containing DNA fragments in the tumor (and the cell line) to 5′ D-specific probes also indicated that the great majority of cells in the tumor mass were leukemic cells.

In light of the surprising finding that the myeloid 85-2M cells appeared to be derived from a primary B-lymphoid tumor, an independent test of their relation was applied. Since the original tumor was induced by ΔMo+SV M-MuLV, it was possible to examine the tumor cells for patterns of proviral integrations. For this experiment, tumor and cell line DNAs were digested with *Sal*I plus *Eco*RI and analyzed by Southern blot hybridization using a labeled simian virus 40 (SV40) probe. Each integrated ΔMo+SV M-MuLV provirus will give two restriction fragments, whose sizes reflect the relative location of *Eco*RI sites in the adjacent cell DNA; the pattern of junction fragments are characteristic for a particular infected cell. Use of the SV40 probe also was advantageous, since it did not hybridize to the multiple copies of endogenous MuLV-related sequences present in all mouse cells (Fig. 4, lane NS). The primary lymphoid tumor showed a pattern of ΔMo+SV M-MuLV-specific junction fragments (Fig. 4), indicating multiple proviral integrations. These proviral integrations were apparently all in the same tumor cells, since the results of Fig. 3 indicated that the primary tumor mass was quite homoge-

FIG. 1. Morphology of 85-2M myeloid cells and histopathologies of the original ΔMo+SV M-MuLV-induced tumor and neoplastic tissue from an irradiated mouse inoculated with the 85-2M cells. (A) Cytocentrifuge preparation of 85-2M cells. Cells are stained with Wright-Giemsa. Magnification, ×250. (B) ΔMo+SV M-MuLV-induced lymphoblastic lymphoma. Hematoxylin and eosin staining of the lymph node neoplasm is shown. Magnification, ×600. (C) A characteristic histiocyte (macrophage) sarcoma induced in NIH Swiss mice by 85-2M cells. Hematoxylin and eosin stain of liver tissue is shown. Magnification, ×550.

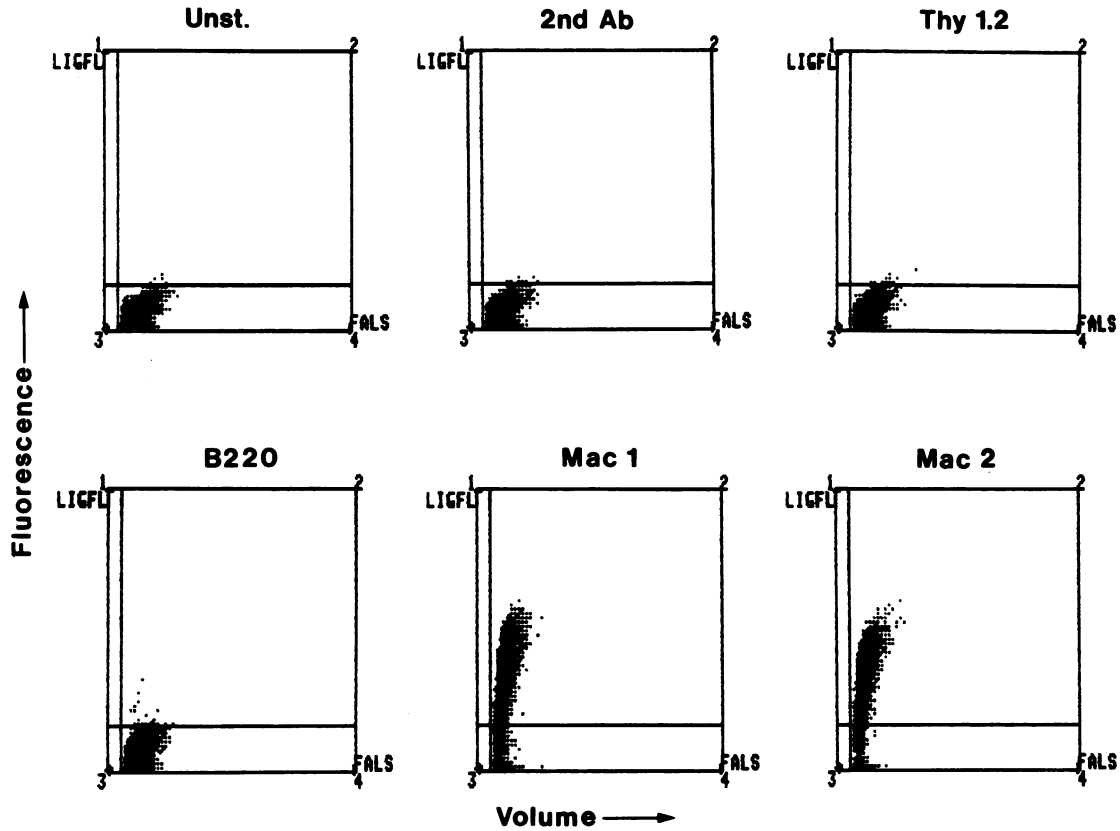

FIG. 2. Immunophenotypic characterization of the 85-2M cell line. Samples were analyzed by multiparameter flow cytometry (fluorescence-activated cell sorter, Coulter epics 2). Results are displayed as a plot of fluorescence versus cell volume. Abbreviations: Unst., unstained; 2nd Ab, second antibody; LIGFL, log integrated green fluorescence; FALS, forward-angle light scatter.

neous. Importantly, all of the same junction fragments of the primary tumor cells were also present in the 85-2M cells. (Two additional junction fragments present in the 85-2M cells presumably represented reinfection, resulting in an additional ΔMo+SV M-MuLV provirus during establishment in tissue culture. Junction fragments smaller than 3.7 kilobases in size may represent ΔMo+SV M-MuLVs which, as a result of recombination, carry mink cell focus-forming virus envelope sequences.) Thus, by this independent criterion, the myeloid 85-2M cells were derived from the initial lymphoid tumor. In addition, tumor tissue from six animals inoculated with 85-2M cells all showed identical junction fragment patterns to those of the 85-2M cells. This confirmed that (i) the 85-2M cells gave rise to histiocyte (macrophage) tumors and (ii) the complex pattern of junction fragments represented multiple proviral integrations within the same cell.

In summary, we have described the derivation of a cell line with characteristics of macrophages from a lymphoid tumor. The original tumor was classified as lymphoid on the basis of histopathology, expression of the B-cell-specific marker B220 and also rearrangement of immunoglobulin heavy and light chains. In addition, immunoperoxidase staining of the tumor tissue to detect the presence of Mac-1 antigen, a myeloid cell marker, proved negative. This tumor was indistinguishable from other pre-B or B-lymphoid tumors induced by the same virus which we have previously characterized (8). The 85-2M cells were classified as myeloid by four criteria: (i) morphology, (ii) presence of Mac-1 and Mac-2 antigens at the cell surface, (iii) histopathology of tumors resulting from transplantation, and (iv) phagocytosis when plated on a stromal cell culture. Two independent molecular analyses (immunoglobulin heavy-chain gene rearrangement and ΔMo+SV M-MuLV integration sites) confirmed that the 85-2M cells were indeed derived from the original lymphoid tumor. In addition, the 85-2M myeloid cell line expressed the lymphoid cell surface antigen Lyt-1 (data not shown). These results suggest that lymphoid tumor cells redirected their differentiation program towards that of macrophages spontaneously during in vitro culture, perhaps due to environmental signals, such as growth factors. The morphology, surface antigen characteristics, and immunoglobulin heavy-chain gene rearrangements observed for the 85-2M cell line have remained stable during further passage in culture. These findings suggest that the relationship between B-lymphoid and macrophage or monocytic myeloid cells is closer than conventionally believed.

Work by other investigators could also be interpreted as supporting a closer relationship between B-lymphoid and myeloid cells. Human chronic myelogenous leukemia, a leukemia of the granulocytic branch of the myeloid lineage, occasionally develops into pre-B lymphoblastic crisis (2, 17). Hematopoietic cells transformed in vitro by retroviruses containing the *ras* oncogene were found to coexpress B-cell and myeloid cell surface antigens (10). Davidson et al. (7) have reported that v-Ha-*ras*-transformed Lyt-1 positive B-lymphoid progenitor cell lines will differentiate into macrophagelike cells after treatment with lipopolysaccharide. Murine macrophagelike cell lines have been derived from lymphoid tumors induced by Abelson virus (which carries

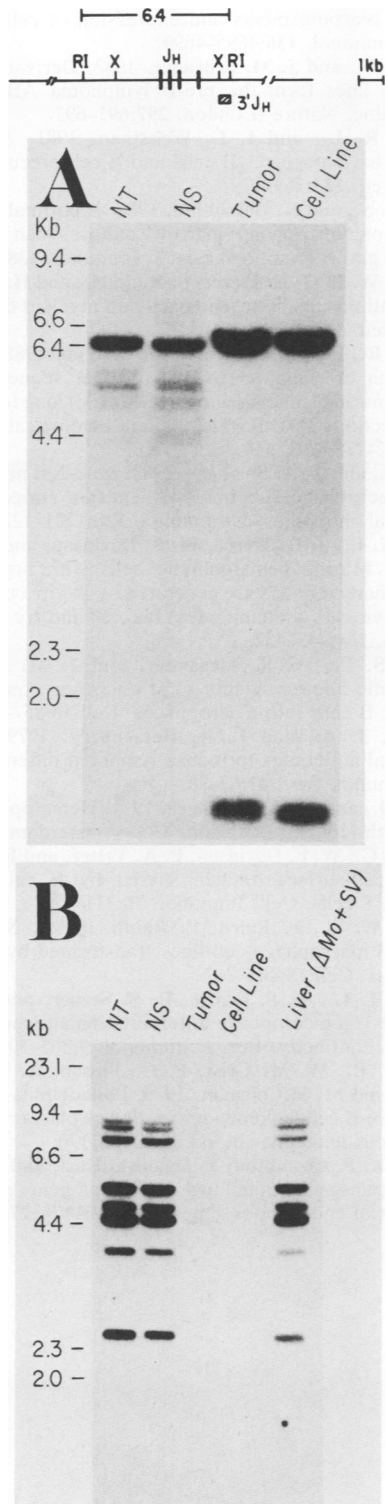

FIG. 3. Immunoglobulin heavy-chain gene rearrangements in ΔMo+SV M-MuLV-induced tumor DNA and 85-2M cellular DNA. (A) High-molecular-weight DNA prepared from the cervical lymph node tumor and 85-2M cells was digested with *Eco*RI and analyzed by Southern blot hybridization using a 3′ J_H probe. The line drawing above the gel shows the structure of the immunoglobulin heavy-chain μ region, and sequences hybridizing to the 3′ J_H probe are shown (RI, *Eco*RI; X, *Xba*I). Lanes showing DNA prepared from a normal mouse thymus (NT) and normal mouse spleen (NS) digested with *Eco*RI illustrate the germ line configuration of the immunoglobulin heavy-chain μ gene. Positions (in kilobases [kb]) of *Hin*dIII-digested lambda DNA fragments are shown. (B) V_HDJ_H gene rearrangements in the ΔMo+SV M-MuLV-induced lymphoid tumor and the tumor-derived 85-2M cell line. The filter was hybridized with a 1:1 mixture of two probes ($D_{38B9\text{-}7.1}$ and $D_{40E4\text{-}2\text{-}P\text{-}5.2}$). These two probes, together, detect the majority of known genomic D segments. The assay used to distinguish J_H-associated rearrangements has been described previously (1). Lanes are as described above with normal thymus (NT) and normal spleen (NS) lanes demonstrating the germ line pattern of D segment-containing *Eco*RI DNA fragments. Nonneoplastic liver DNA prepared from the ΔMo+SV M-MuLV mouse from which the original tumor was derived is also shown.

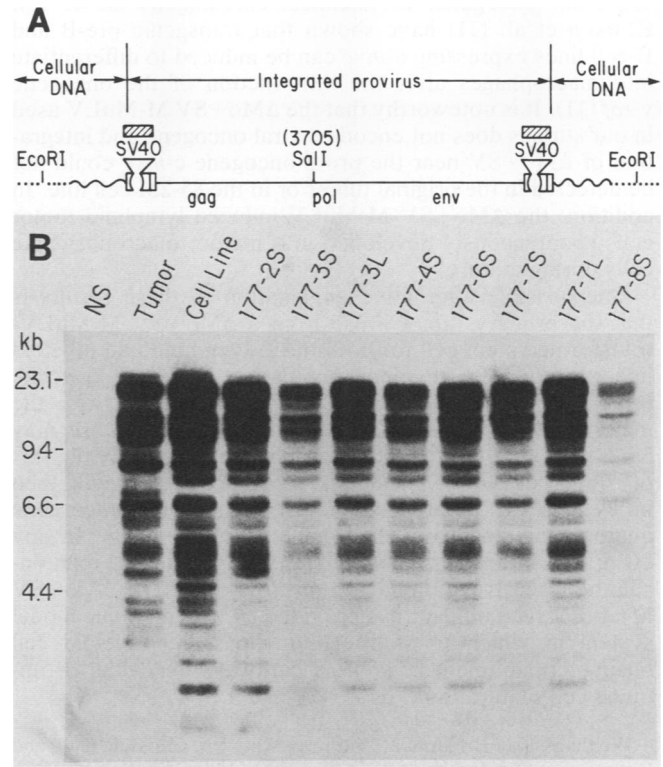

FIG. 4. ΔMo+SV M-MuLV proviral integration patterns in the original tumor, the 85-2M cell line, and several irradiated syngeneic host mice inoculated with 85-2M cells. (A) ΔMo+SV proviral DNA integrated into the cellular genome. The SV40 enhancers (72- and 21-base-pair repeats) were substituted into the U3 region of the long terminal repeat with the remainder of the genome unaltered and identical to that for wild-type M-MuLV (8). The unique *Sal*I site present within the M-MuLV genome is shown. *Eco*RI sites are located within the flanking cellular DNA sequences. Sequences within ΔMo+SV M-MuLV which hybridize to the SV40-specific probe (▨) used in our study are shown. (B) High-molecular-weight DNA preparations were digested with *Eco*RI and *Sal*I, and Southern blots were hybridized with an SV40 sequence-specific probe. Lane (NS) shows normal spleen DNA digested with *Eco*RI and *Sal*I. DNA samples from the ΔMo+SV M-MuLV-induced tumor and 85-2M cells are also indicated. The 177 series of spleen (S) and liver (L) tumor DNAs were prepared from irradiated mice inoculated intravenously with the 85-2M cell line. The positions (in kilobases [kb]) of *Hin*dIII-digested lambda DNA markers are shown to the left of the autoradiogram.

the v-*abl* oncogene) or chemical carcinogens (3, 4, 15). Klinken et al. (11) have shown that transgenic pre-B and B-cell lines expressing c-*myc* can be induced to differentiate into macrophages after the introduction of the oncogene v-*raf* (11). It is noteworthy that the ΔMo+SV M-MuLV used in our studies does not encode a viral oncogene and integration of ΔMo+SV near the proto-oncogene c-*myc* could not be detected in the original tumor or in the 85-2M cell line. In addition, the ΔMo+SV M-MuLV-induced lymphoid tumor cells spontaneously developed into mature macrophagelike cells during culture.

One possible alternative explanation to these results is that the primary tumor arose from a ΔMo+SV M-MuLV-transformed stem cell for both the B-lymphoid and myeloid lineages and that a minor population of macrophagelike tumor cells existed in the primary tumor along with the majority B-lymphoid cells. Growth in tissue culture may have then selected for the macrophagelike cells. While this possibility cannot be excluded, the data of Fig. 3 would then imply that the putative stem cell had already undergone immunoglobulin heavy-chain gene rearrangements. In any event, this interpretation would also support a close relationship between B-lymphoid and myeloid cells. The ΔMo+SV M-MuLV recombinant has provided us with a unique model system in which to study B-lymphoid and myeloid cell lineage commitment as well as pathways available for immune cell maturation.

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