MOLECULAR AND CELLULAR BIOLOGY, Sept. 1991, p. 4710–4716
0270-7306/91/094710-07$02.00/0

Vol. 11, No. 9

Differences in Oncogenic Potency but Not Target Cell Specificity Distinguish the Two Forms of the *BCR/ABL* Oncogene

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Received 6 May 1991/Accepted 24 June 1991

Two forms of activated *BCR/ABL* proteins, P210 and P185, that differ in *BCR*-derived sequences, are associated with Philadelphia chromosome-positive leukemias. One of these diseases is chronic myelogenous leukemia, an indolent disease arising in hematopoietic stem cells that is almost always associated with the P210 form of *BCR/ABL*. Acute lymphocytic leukemia, a more aggressive malignancy, can be associated with both forms of *BCR/ABL*. While it is virtually certain that *BCR/ABL* plays a central role in both of these diseases, the features that determine the association of a particular form with a given disease have not been elucidated. We have used the bone marrow reconstitution leukemogenesis model to test the hypothesis that *BCR* sequences influence the ability of activated *ABL* to transform different types of hematopoietic cells. Our studies reveal that both P185 and P210 induce a similar spectrum of hematological diseases, including granulocytic, myelomonocytic, and lymphocytic leukemias. Despite the similarity of the disease patterns, animals given P185-infected marrow developed a more aggressive disease after a shorter latent period than those given P210-infected marrow. These data demonstrate that the structure of the *BCR/ABL* oncoprotein does not affect the type of disease induced by each form of the oncogene but does control the potency of the oncogenic signal.

The Philadelphia chromosome is a pathognomonic marker associated with chronic myelogenous leukemias (CMLs), a significant number of acute lymphocytic leukemias, and rare cases of acute myelogenous leukemia (reviewed in reference 6). As a consequence of the translocation that generates this marker, *ABL* proto-oncogene sequences are fused to *BCR* sequences (13, 19), and an activated protein tyrosine kinase, the *BCR/ABL* protein, is produced (30). This molecule exists in two forms, reflecting the fact that some translocations occur in the first *BCR* intron while others occur within the originally designated breakpoint cluster region of *BCR* (15, 19, 25). Both *BCR/ABL* proteins contain identical *ABL*-derived sequences, but one form, called P210, contains 927 *BCR*-derived amino acids, while the second, P185, contains only the first 436 of these *BCR*-specified residues (16, 20, 25, 40). The presence of these latter sequences is sufficient to activate the transforming potential of the *BCR/ABL* protein and the tyrosine kinase activity of the molecule (39, 41). Direct comparisons of P185 and P210 suggest that the additional residues present in P210 moderate both in vitro transforming potential (34, 38) and protein tyrosine kinase activity (34, 39, 41).

Although both $P185^{BCR/ABL}$ and $P210^{BCR/ABL}$ transform similar types of cells in tissue culture systems (34, 38), the human leukemias associated with *BCR/ABL* proteins are distinct in that they arise in different cell types and have different clinical courses (reviewed in references 2 and 45). Analyses of large numbers of patient samples have left the impression that the differences in *BCR*-derived sequence between P210 and P185 either preferentially target the oncogene to specific hematopoietic cell types or affect the potency of the oncogenic signal or both (reviewed in references 6 and 49). For example, CML, almost always associated with the P210 form (19, 28, 29), is initially an indolent disease of hematopoietic stem cells characterized by the overproliferation and differentiation of myeloid lineage cells (2, 18). The P185 form is most commonly associated with acute lymphocytic leukemia (3, 7, 31, 53) and acute myelogenous leukemia (4, 32), aggressive malignancies of early lymphoid and myeloid precursors, respectively. However, rare cases of P185-positive CML have been documented (50, 52), and perhaps as many as half of all Philadelphia chromosome-positive acute lymphocytic leukemias express P210 (3, 15, 48, 51).

The issues of lineage specificity and potency of transforming signal can best be addressed by direct comparison of P185 and P210 in an experimental system that recapitulates features of the human *BCR/ABL*-associated leukemias. In the work presented here, we have used such a system, the murine bone marrow reconstitution leukemogenesis model (12, 14, 23, 26, 27). Our analysis of mice whose depleted bone marrow was reconstituted with stem cell populations infected with either P185- or P210-expressing retrovirus vectors reveals a similar spectrum of disease, including granulocytic, myelomonocytic, and lymphocytic leukemias. However, the animals given $P185^{BCR/ABL}$-infected progenitors develop a more aggressive disease after a shorter latent period than animals treated with P210-infected cells. These data demonstrate that the differences in *BCR* sequences that distinguish P210 and P185 control the oncogenic potential of these two proteins but do not directly determine the types of hematopoietic cells that can be transformed by *BCR/ABL*.

MATERIALS AND METHODS

Cells and viruses. Bone marrow cells were prepared from mice treated with 5-fluorouracil (5-FU), infected, and used

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TABLE 1. Disease induction by $P185^{BCR/ABL}$ and $P210^{BCR/ABL}$[a]

Form (no. of animals)	Disease	No. with disease[b]	Leukocytes/μl		Avg differential[c]			Other tumors[d]		
			Range	Avg	%L	%M	%G	Mac	Pre-B	Both
P185(26)	Granulocytic leukemia	5	12,500–192,000	54,340	15	11	75	4/5	0/5	1/5
	Myelomonocytic leukemia	3	10,000–28,000	16,800	18	55	26	0/3	1/3	2/3
	Lymphocytic leukemia	4	13,200–44,000	27,900	80	<1	17	0/4	3/4	0/4
	Pre-B lymphoma	11	1,600–17,000	6,600	61	15	24	0/11	NA[e]	0/11
	Macrophage tumor	2	3,600–8,250	5,900	45	2	54	NA	1/2	0/2
P210(23)	Granulocytic leukemia	4	17,500–84,000	51,400	9	9	82	0/4	1/4	1/4
	Myelomonocytic leukemia	2	11,800–25,000	18,400	36	38	26	0/2	1/2	0/2
	Lymphocytic leukemia	3	12,500–18,500	14,500	71	9	19	0/3	2/3	0/3
	Pre-B lymphoma	13	3,000–7,000	4,800	67	8	26	1/13	NA	1/13

[a] Lethally irradiated mice were given bone marrow from 5-FU-treated mice that had been infected with retrovirus stocks expressing $P185^{BCR/ABL}$ or $P210^{BCR/ABL}$ cDNAs. Animals were killed when signs of tumor were evident. Data for eight of the animals included in the P210 series were reported previously (27). Diagnosis was based on examination of the blood data and other pathology in hematologic tissues.

[b] Number of animals with the particular disease of the total number of animals (26 or 23) available for evaluation. Five animals given $P185^{BCR/ABL}$-infected marrow failed to survive the 2-week postreconstitution period and were excluded from the analysis; eight animals given P210-infected cells and seven given P185-infected cells died during the experiment but were not recovered in a condition suitable for examination. One animal given P210-infected cells was lost, and one died from an infection during the course of the study. Data for these animals were also excluded from the tabulation.

[c] Differential counts were performed on smears stained with Wright-Giemsa stain: %L, percent lymphocytes; %M, percent monocytes and macrophages; %G, percent granulocytes. Leukocyte types not listed in the table were present in the samples at a frequency of 1% or less.

[d] Some animals contained multiple tumor types. Mac, macrophage tumors; pre-B, pre-B lymphomas.

[e] NA, not applicable.

for reconstitution as described previously (27). The cells were infected with stocks of either −447P185/M, a retrovirus containing a $P185^{BCR/ABL}$ cDNA (38), or −447P210/M, a retrovirus containing a $P210^{BCR/ABL}$ cDNA (11). Both stocks contained Moloney murine leukemia virus. Mock-infected animals and animals infected with Moloney virus have been evaluated earlier in our laboratory (27). As described previously (38, 41), the titer of the *BCR/ABL* retrovirus stocks was monitored by Southern analysis of NIH 3T3 or RAT-1 cells that had been infected for the same period of time as the bone marrow cultures with equal volumes of the two virus stocks. The relative intensity of the internal *Eco*RI fragment from the P210 and P185 proviruses was revealed by probing Southern blots with an *ABL* probe (40).

Evaluation of mice. Complete peripheral blood cell counts were performed on the marrow-reconstituted mice biweekly, beginning 2 weeks postreconstitution. Animals were monitored daily and killed when signs of disease, such as splenomegaly, lymphadenopathy, cachexia, ruffled fur, or other evidence of ill health, were noted. Gross pathologic observations were recorded, and blood, spleen, marrow, liver, lymph node, and any other tissue showing enlargement or macroscopic evidence of tumor was processed for histologic examination. Leukocytes were purified from the blood samples by Ficoll (Pharmacia Chemical Co., Piscataway, N.J.) gradient centrifugation and frozen for subsequent analysis; samples of other tissues were frozen directly.

DNA analysis. DNAs were prepared from frozen tissues by grinding the material into a fine powder with a mortar and pestle in a dry-ice bath. Lysis buffer (10 mM NaCl, 10 mM Tris [pH 7.5], 25 mM EDTA, 1% sodium dodecyl sulfate) was added, and the material was treated with 625 μg of pronase per ml for at least 2 h at 37°C. Purified leukocyte pellets were lysed directly as described above. DNAs were extracted, digested with appropriate restriction enzymes, and analyzed by Southern blotting as described previously (44). The Nytran membrane (Schleicher & Schuell, Keene, N.H.) blots were hybridized to DNA probes labeled by the random priming method (17) according to the manufacturer's suggestions. The 1-kb *Pst*I fragment from pJWp5 (40) was used as an *ABL* probe; the 1.9-kb *Cla*I fragment from pMV6TKneo (35) was used as a *neo* probe; the 2-kb *Bam*HI-*Eco*RI fragment from pJ11 was used as an immunoglobulin heavy-chain gene probe (37); the 2.7-kb *Hin*dIII fragment from $pC_{\beta 2}$ (36) was used to probe the T-cell receptor β gene; and the 1.4-kb *Eco*RI-*Sal*I fragment from pY2 was used as a Y chromosome probe (33).

RESULTS

Animals given P185- or P210-infected 5-FU-treated marrow develop an identical disease spectrum. Lethally irradiated animals were given bone marrow from 5-FU-treated mice that had been infected with matched retrovirus stocks expressing either $P185^{BCR/ABL}$ or $P210^{BCR/ABL}$ and were then monitored for disease. Autopsy and histologic analyses revealed that 26% of the animals given $P210^{BCR/ABL}$-infected marrow and 39% of those treated with $P185^{BCR/ABL}$-infected cells developed diseases involving cells of the myeloid lineage (Table 1). Included in this group are animals that showed total peripheral leukocyte counts of greater than 10,000 cells per μl and differential counts demonstrating the presence of abnormal numbers of mature granulocytes or macrophages. Most of the animals given P185-infected bone marrow in this group displayed marked splenomegaly, primarily reflecting the presence of large macrophage tumors in this organ (Fig. 1A and B), a feature that was less prominent in the P210-infected animals with granulocytic disease. Marked granulocytic proliferation and trilineage proliferation and differentiation, hallmarks of the granulocytic disease induced by $P210^{BCR/ABL}$ (12, 27), were also prominent in the spleens of these mice (Fig. 1C). Animals in both groups also showed evidence of lymphoblastoid tumor cells in the peripheral lymph nodes. In addition to diseases with an obvious leukemic component, a small number of animals in both groups developed splenic tumors containing macrophages either as the only obvious pathology or coincident with pre-B lymphoma.

A significant number of animals developed diseases in which the dominant pathology involved lymphoid cells. While a disease identical to typical Abelson virus-induced pre-B lymphoma (46) was observed in many of these ani-

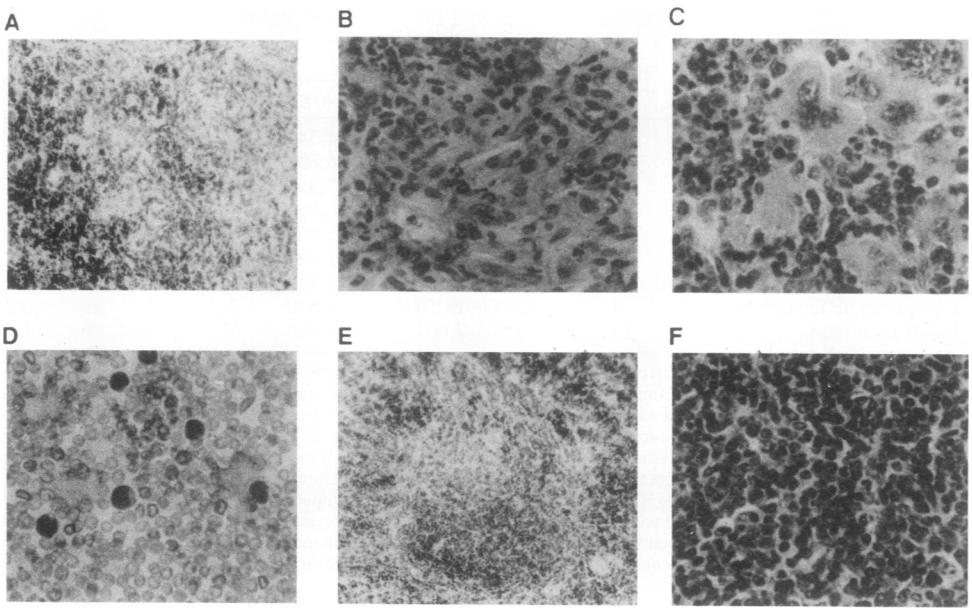

FIG. 1. Histopathological examination. (A) Representative hematoxylin-eosin-stained sections from a mouse given P185$^{BCR/ABL}$-infected bone marrow that developed granulocytic leukemia illustrate the expansion of the red pulp and the presence of a tumor mass. (B) The morphology of macrophages in the tumor mass and (C) the extensive trilineage expansion and differentiation observed in animals with granulocytic and myelomonocytic leukemia, demonstrated by the presence of large numbers of mature granulocytes, megakaryocytes, and erythroid cells. (D) Representative Wright-Giemsa-stained peripheral blood smear and (E) a hematoxylin-eosin-stained spleen section showing expanded white pulp, reflecting lymphoblastoid cell proliferation (F) from an animal with lymphocytic leukemia. Magnification: (A and E) ×100; (B, C, D, F) ×400.

mals, some animals in both groups developed lymphocytic leukemias (Table 1). Unlike typical pre-B lymphomas induced by activated *ABL* genes, in which tumor cells are rarely observed in the circulation, animals with this disease had elevated numbers of lymphoblasts in the peripheral blood (Fig. 1D). Histologic examination of tissues from most of these mice revealed a picture similar to that observed in pre-B lymphoma, with large numbers of lymphoblasts in the spleen, lymph node, and bone marrow (Fig. 1E and F and not shown). The thymus was spared in all cases. Consistent with this picture, analysis of DNA prepared from the leukemic cells with probes for the immunoglobulin heavy-chain locus and the T-cell receptor β locus indicated that they were related to the B-lymphocyte lineage (data not shown).

The pathologic examinations revealed that both forms of *BCR/ABL* induced the same spectrum of hematologic diseases (Table 1). Although classically associated with leukemias involving lymphoid precursors (45), the P185 form of *BCR/ABL* actually induced a slightly higher frequency of myeloid disease than the P210 form in the group of animals studied. In addition, the pattern of lymphoid disease observed in animals given P210- and P185-infected bone marrow was similar, with pre-B lymphomas and lymphocytic leukemias occurring in both groups. Thus, the sequence differences that distinguish P185 and P210 do not modulate the type of disease each oncogene induces when they are tested in an identical setting.

P185$^{BCR/ABL}$ induces more rapid and aggressive leukemias than P210. Although the disease spectrum observed in the P210- and P185-reconstituted animals is similar, the latent period required for evidence of disease was not identical. Comparison of animals inoculated with matched stocks of the P210 and P185 retroviruses revealed that animals given P185$^{BCR/ABL}$-infected marrow exhibited leukocytosis about 4 weeks earlier than those given P210-infected cells (data not shown). Most of these mice also developed disease sooner than the P210-treated animals (Fig. 2A), with 50% of the animals succumbing by day 49 postreconstitution, compared with 71 days for the P210-reconstituted animals. The difference in latent period was particularly striking for the granulocytic leukemias, which developed during a much narrower time window than the other diseases in both sets of mice (Fig. 2B). Because the P210 and P185 stocks are matched for titer by assays that measure infectivity in rodent fibroblast cells, we also evaluated animals given bone marrow that had been infected with a 10-fold dilution of the P185 retrovirus stock. Even under these circumstances, the P185-treated mice developed disease more rapidly than those treated with P210-infected bone marrow (Fig. 2A).

Histologic examinations of tissues revealed a second difference between animals given P210- and P185-infected bone marrow. The animals given P185$^{BCR/ABL}$-infected bone marrow developed more invasive tumors. This difference was particularly evident in the liver, where extensive granulocyte and macrophage infiltration was observed in 10 of 11 of the P185-treated animals with myeloid disease (Fig. 3A and B). In contrast, infiltration was observed in only one of six of the P210-treated animals with myeloid disease, and most animals had little evidence of tumor cells in the liver (Fig. 3C and D). These data, and those derived from the mortality curve, are consistent with the idea that P185$^{BCR/ABL}$ delivers a more potent transforming signal than the P210 form of the oncogene.

P185$^{BCR/ABL}$, like P210, induces clonal tumors that can arise from hematopoietic progenitor cells. The histologic and latent period assessments suggest that P185$^{BCR/ABL}$ delivers a more potent oncogenic signal than P210. However, this interpretation is based on the assumption that the P185 provirus is

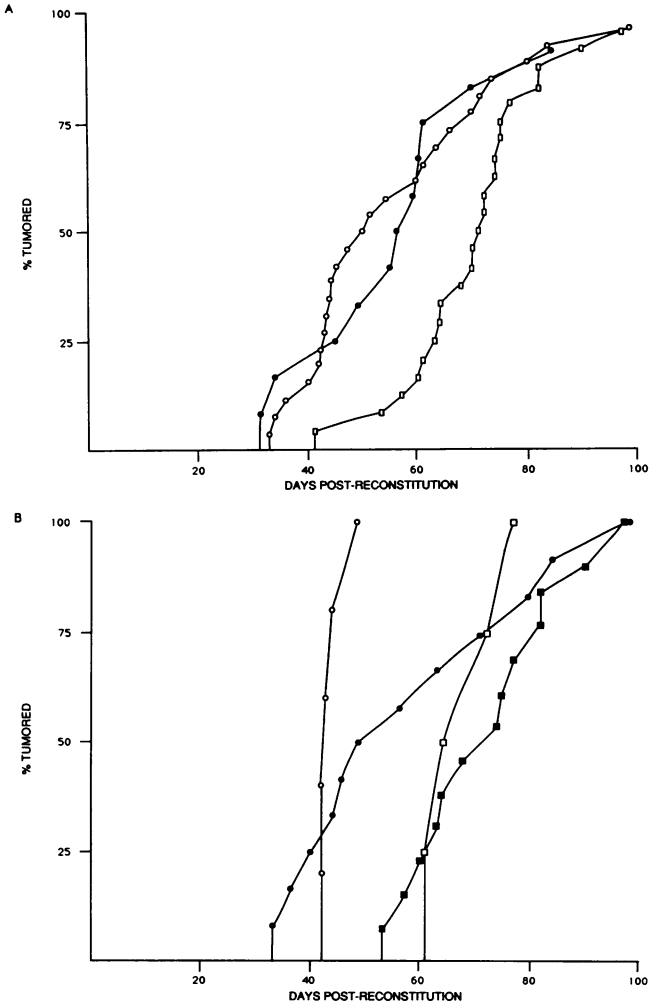

FIG. 2. P185$^{BCR/ABL}$-treated mice develop tumors more rapidly than P210-treated mice. Lethally irradiated adult BALB/c mice were given 10^5 cells infected with matched stocks of P185 (○) and P210 (□) retroviruses or a 10-fold dilution of the P185 stock (●). Animals were monitored daily and killed when disease was evident. (A) Each point represents a single animal, and the percent tumored is calculated from the total number of animals that could be evaluated in each experiment, as detailed in Table 1, footnote *b*. Including data for mice found dead after the initial reconstitution phase does not alter the shape of the curves (not shown). (B) Only animals diagnosed with granulocytic leukemias (P185 [○] or P210 [□] and pre-B lymphomas (P185 [●] or P210 [■]) are represented. The data are pooled from six separate experiments with undiluted P185 virus, four separate experiments with P210 virus, and two separate experiments with diluted P185 virus.

present in all of the infiltrating cells and that the P185-induced tumors, like those induced by P210 (12, 14, 27), are clonal or oligoclonal. To test the first assumption, DNAs from involved tissues were analyzed by Southern blotting of *Eco*RI-digested DNAs with an *ABL* probe (40). With this strategy, the provirus was visualized as a single internal fragment in all of the tumor tissue and leukemic peripheral blood samples that were tested (Fig. 4A). Analysis of selected tissues with a Y chromosome-specific probe (33) confirmed the presence of male and therefore donor cells in the tumor tissue (data not shown).

The clonality of the P185-induced tumors was assessed by

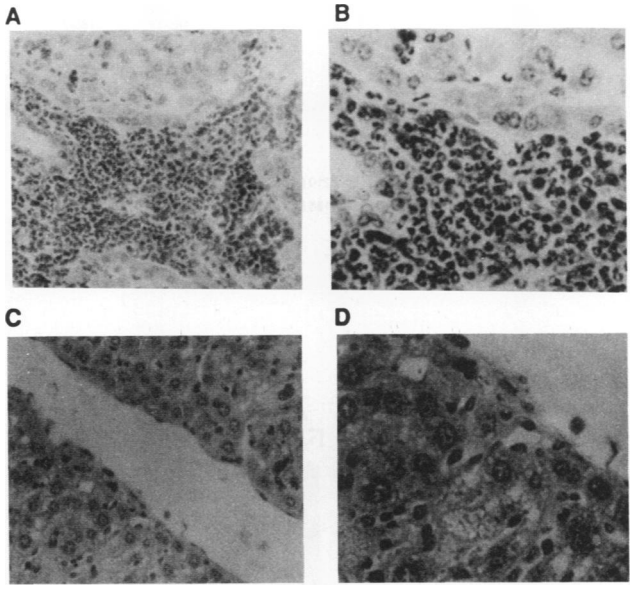

FIG. 3. P185$^{BCR/ABL}$-treated mice develop a more invasive disease. Hematoxylin and eosin stains of sections of liver tissue from (A and B) P185-treated and (C and D) P210-treated animals, showing marked granulocytic infiltration in the sample from the P185$^{BCR/ABL}$-treated mouse. Magnification: (A and C) ×100; (B and D) ×400.

Southern blot analysis of *Eco*RI-digested DNAs with a *neo* probe (35), which reveals the provirus in the context of flanking cellular sequences. In addition, the intensity of the proviral bands was compared with a standard dilution series to give an indication of the frequency of cells in the tumor sample that contained the provirus. These studies, similar to those reported for animals given P210$^{BCR/ABL}$-infected marrow (27), revealed that all four samples of peripheral blood from animals with myeloid leukemias contained a dominant fragment of an intensity similar to that observed for one copy of *BCR/ABL* in a standard cell line (Fig. 4B). Analysis of DNAs from tumor tissue and peripheral blood revealed that the tumor cells present at different sites often arose from a single clone of cells (Fig. 4C). In several of these cases, samples from different tissues contained different types of hematopoietic tumor cells. For example, in two cases of granulocytic leukemia, peripheral blood samples contained predominantly granulocytes, while macrophage tumors were present in the liver and spleen. Analysis of the proviral integration sites in these tissues revealed that all of the tumor tissue and the peripheral blood contained the same single dominant *BCR/ABL*-infected clone (Fig. 4C). Thus, these two distinct tumor types arose from an infected cell that, at a minimum, could differentiate into macrophages and granulocytes.

DISCUSSION

Our work represents the first instance in which the oncogenic potential of P210$^{BCR/ABL}$ and P185$^{BCR/ABL}$ has been compared directly under conditions that favor development of a broad range of hematologic malignancies. Because both forms induce a similar spectrum of disease in the bone marrow reconstitution leukemogenesis model, the *BCR* sequences that distinguish them do not control the selection of particular types of hematopoietic cells. The idea that the precise structure of activated *abl* genes does not control the

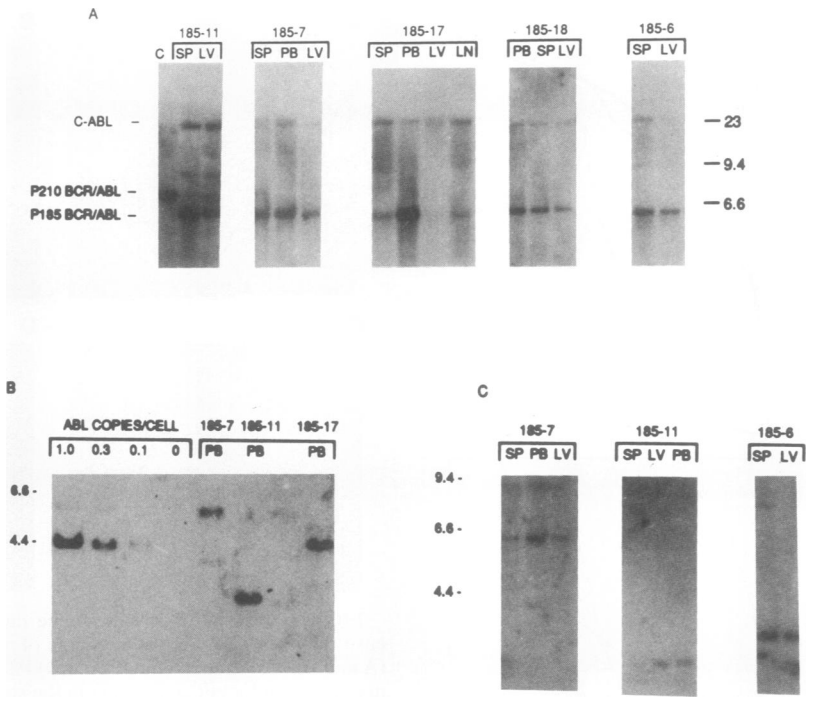

FIG. 4. Tumors contain the P185$^{BCR/ABL}$ provirus and are clonal. (A) *Eco*RI-digested DNAs from peripheral blood (PB), spleen (SP), liver (LV), and lymph node (LN) from five different animals, as indicated by the numbers above the lanes, were analyzed by Southern blotting with an *ABL* probe (40). Lane C contains control DNA from a P210$^{BCR/ABL}$-transformed pre-B-cell line. Sizes (in kilobases) are indicated to the right. (B and C) The clonality of the tumor cells was examined with a *neo* probe (35). A dilution series prepared from a standard cell line transformed by P210$^{BCR/ABL}$ was used to estimate the frequency of infected cells in the tumor tissue. The number above each panel identifies the mouse, and the letters designate the tissue from which the DNA was extracted. Sizes (in kilobases) are indicated to the left.

outcome of the cell-virus interaction is supported by a wide variety of in vitro experiments. For example, virtually all forms of activated *ABL* genes tested transform pre-B lymphoid cells in vitro (1, 38, 47) and abrogate the requirement for certain interleukins in other tissue culture cells (8–10, 21, 42, 43). In addition, infection of bone marrow from 5-FU-treated mice with Abelson virus or retroviruses expressing either form of *BCR/ABL* induces growth of both lymphoid colonies and granulocyte-macrophage colonies in vitro (27a). This parallel may extend to the v-*abl* form of the oncogene in the reconstitution model, where diseases with a presentation similar to those observed with *BCR/ABL* have been documented (27). However, tests for v-*abl* provirus integrations in peripheral blood granulocytes have not yet been conducted, and the granulocytosis observed may result from indirect effects of the disease process (14). Indeed, both the *fms* and *src* oncogenes induce multiple hematologic malignancies in the reconstitution leukemogenesis model (23, 26). However, only those associated with *fms* appear to be similar to the spectrum of disease associated with *BCR/ABL*.

The human malignancies associated with *BCR/ABL* expression are distinct in clinical course and probably involve different target cells. P185 expression is almost always associated with human leukemias involving proliferation of early hematopoietic progenitors that fail to differentiate (45). In contrast, P210 is most commonly found in a chronic leukemia initially characterized by quasi-normal differentiation (2). However, our results demonstrate that both P210 and P185 expression can stimulate maturation of clonal populations of myeloid cells. Furthermore, both oncogenes can also induce proliferation of B-lineage precursors that are probably arrested in their differentiation. A likely explanation for these apparently contrasting results is that the impact of *BCR/ABL* expression on growth and differentiation depends in large part on the cell in which the oncogene is expressed. The ability of v-*abl* to stimulate either malignant transformation or normal differentiation, depending upon the cell type in which it is expressed (54), lends experimental support to this point of view, as do results obtained with transgenic animals expressing various forms of *ABL* under the control of different promoters (22, 24).

The exact target cells involved in the diseases studied here remain a subject of intense investigation. In some animals with multiple tumor types, analyses of provirus integration patterns suggest that the lymphoid malignancies arise from clones distinct from those contributing to myeloid disease (unpublished data). The same analyses reveal that the CML-like disease arises in a cell that is at least a bipotential myeloid progenitor. Indeed, the fact that the trilineage proliferation and differentiation seen in some animals involve a single clone of *BCR/ABL*-infected cells suggests that an even earlier precursor gives rise to the malignant clone in some cases. Such an interpretation is consistent with several lines of indirect evidence supporting infection of multipotential cells in the bone marrow reconstitution leukemogenesis model (5, 12, 27).

P185$^{BCR/ABL}$ is most commonly associated with highly aggressive, acute leukemias in humans (6). Consistent with this association, the animals given P185-infected bone marrow developed disease after a shorter latent period than those given P210-infected cells. This difference is particularly striking for the granulocytic leukemias that resemble

chronic-phase CML. The results of the dilution experiment demonstrate that this difference is not a function of the titer of the infecting virus stock. Therefore, the P185 protein must deliver a more potent transforming signal to the cell. This interpretation is consistent with the ability of P185 to transform cells more readily in a variety of in vitro culture systems (34, 38). This property of $P185^{BCR/ABL}$ may stem from its having a higher protein tyrosine kinase activity than P210 (34). Alternatively, P185 and P210 may differ in their ability to interact effectively with important cellular substrates. Either of these features might increase the probability that cells stimulated with P185 would continue to cycle, providing additional opportunities for secondary events that are important in the tumorigenic process.

ACKNOWLEDGMENTS

We thank John Coffin and David Schenkein and the members of our laboratories for suggestions on the manuscript and Michael Thursby for preparing the figures.

This work was supported by Public Health Service grants CA 33771 (N.R.) and CA 27507 (O.N.W.) from the National Institutes of Health. O.N.W. is an Investigator of the Howard Hughes Medical Institute.

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