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bcr-abl, the hallmark of chronic myeloid leukaemia in man, induces multiple haemopoietic neoplasms in mice

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The chromosome translocation forming the hybrid *bcr-abl* gene is thought to be the initiating event in chronic myeloid leukaemia (CML) and some cases of acute lymphoblastic leukaemia. To assess the impact of *bcr-abl* upon haemopoiesis, lethally irradiated mice were reconstituted with bone marrow cells enriched for cycling stem cells and infected with a *bcr-abl* bearing retrovirus. The mice developed several fatal diseases with abnormal accumulations of macrophage, erythroid, mast and lymphoid cells, and marked strain differences in disease distribution and kinetics. Some mice exhibited more than one neoplastic cell type and, in some instances, these were clonally related, indicating that a progenitor or stem cell had been transformed. While classical CML was not observed, the macrophage tumours were accompanied by a mild CML-like syndrome, probably due to myeloid growth factor production by tumour cells. The erythroid and mast cell diseases were rarely transplantable, in contrast to the macrophage tumours and lymphomas, but all disease types displayed limited clonality. These results establish that *bcr-abl* confers a proliferative advantage on diverse haemopoietic cells but complete transformation probably involves additional genetic changes.

Key words: bcr-abl gene/bone marrow/chronic myeloid leukaemia/haemopoietic neoplasia/oncogene/retroviral vector

Introduction

Chronic myeloid leukaemia (CML) is a clonal malignancy characterized by an accumulation of myeloid progenitors in the bone marrow and of granulocytes and their precursors in the peripheral blood (reviewed by Champlin and Golde, 1985). Its hallmark is the Philadelphia (Ph^1) chromosome (Nowell and Hungerford, 1960), a shortened chromosome 22 created by a reciprocal 9;22 translocation (Rowley, 1973). Since the Ph^1 chromosome is found in both the myeloid and lymphoid populations of a CML patient, the translocation must occur in a multipotential stem cell (Fialkow *et al.*, 1977; Martin *et al.*, 1982). It is generated by recombination between a gene of unknown function, *bcr*, on chromosome 22 and the tyrosine kinase-encoding c-*abl* proto-oncogene on chromosome 9 (reviewed by Groffen and Heisterkamp, 1987). The hybrid (p210) polypeptide derived from the *bcr-abl* gene lacks the N terminus of the normal *abl* product, as does the *gag-abl* fusion protein of Abelson murine leukaemia virus, and both proteins have heightened tyrosine kinase activity (Konopka and Witte, 1985).

After an initial chronic phase of 3-5 years, the disease in CML patients typically accelerates and immature cells start to predominate. The terminal phase is an acute leukaemia, usually dominated by myeloblasts (65% of cases) or pre-B lymphoblasts (30%) (Champlin and Golde, 1985) but sometimes by erythroid or megakaryocytic blasts (Rosenthal *et al.*, 1977; Breton-Gorius *et al.*, 1978). T lymphoid involvement in either the chronic or acute phase is rare (reviewed by Kuriyama *et al.*, 1989), perhaps because the long-lived peripheral T cell population largely arose prior to the translocation, or because translocation can occur in a stem cell which is not totipotent. Blast crisis is presumably precipitated by additional genetic change(s) in a single cell of the Ph^1-positive clone, with outgrowth of progressively more immature subclones that frequently exhibit non-random karyotypic abnormalities (reviewed by Kantarjian *et al.*, 1987). Some *de novo* acute lymphoblastic leukaemias and rare acute myeloid leukaemias exhibit a Ph^1 chromosome encoding a (p190) *bcr-abl* protein with a smaller *bcr* moiety (reviewed by Kurzrock *et al.*, 1988).

The universal association of the *bcr-abl* hybrid gene with the earliest detectable stages of CML implies that it has an initiating role. Attempts to mimic CML *in vitro* by infecting long-term murine bone marrow cultures with a *bcr-abl* bearing retrovirus have yielded transformed pre-B cell lines (McLaughlin *et al.*, 1987) but with no prior myeloid overproliferation, even in cultures designed to favour myeloid cell growth (Young and Witte, 1988). Introducing a *bcr-abl* gene into immature haemopoietic cell lines rendered them growth factor-independent and tumorigenic (Daley and Baltimore, 1988; Hariharan *et al.*, 1988) and induced the expression of interleukin 3 (IL-3), albeit at very low levels, suggesting a possible role for unscheduled growth factor production in CML (Hariharan *et al.*, 1988).

Endeavouring to establish a CML model *in vivo*, we generated transgenic mice carrying *bcr-v-abl* constructs (Hariharan *et al.*, 1989). The mice yielded lymphoid tumours (both pre-B and T) but no myeloid abnormalities, even with a construct bearing control sequences from the myeloproliferative sarcoma virus (MPSV) long terminal repeat (LTR), which functions particularly effectively in myeloid cells (Bowtell *et al.*, 1988). However, since *bcr-abl* expression was detectable only in tumour tissues, the apparent lymphoid predilection may have reflected more frequent activation of the transgene in lymphocytes rather than a basic bias in oncogenic potential.

We here report our results with an alternative *in vivo* system: lethally irradiated mice reconstituted with bone marrow cells infected with helper virus-free *bcr-abl* retrovirus. These mice developed at high frequency macrophage, erythroid, mast cell, pre-B lymphoid and T lymphoid tumours, the incidence of the various neoplasms varying with genetic background. The mixed tumours observed in some

mice appeared to reflect transformation of a multipotential progenitor. The results establish that *bcr−abl* confers a marked proliferative advantage on diverse haemopoietic cells, and that malignant transformation probably requires additional genetic change.

Results

bcr−abl virus infection of bone marrow cells induces a spectrum of fatal haemopoietic diseases

The *bcr−abl* retrovirus (Figure 1A) comprises a human *bcr−abl* cDNA (Mes-Masson *et al.*, 1986) in the vector pMPZen (Hariharan *et al.*, 1988), which exploits the enhancer from the MPSV to augment expression in myeloid cells (Bowtell *et al.*, 1988) and facilitate long-term expression *in vivo* (Bowtell *et al.*, 1987). To characterize the virus, two cell lines were derived by infecting day 13 fetal liver cells, one an erythroid (FL 42) and the other a mast cell line (FL 37). Analysis of DNA and RNA by blot hybridization demonstrated that both lines contained provirus with an intact (7 kb) *bcr−abl* insert (Figure 1B) and expressed viral RNA of the expected size at levels comparable to that in the human CML line K562, which carries an amplified *bcr−abl* gene (Collins and Groudine, 1983) (Figure 1C). Moreover, immunoprecipitation with an *abl* antiserum in the presence of [γ-^{32}P]ATP confirmed that the infected cells expressed an *abl*-related protein with autokinase activity of the size and abundance of the K562 product (Figure 1D). These experiments established that the MPZen(*bcr−abl*) virus is efficiently expressed *in vitro* in both the erythroid and mast cell lineages and generates the full length p210 *bcr−abl* tyrosine kinase.

To investigate the impact of *bcr−abl* on haemopoiesis, bone marrow cells from DBA/2 or C57BL/6 mice injected 4 days previously with 5-fluorouracil (5-FU), which increases the proportion of cycling primitive cells (Hodgson and Bradley, 1979), were co-cultivated with Ψ-2 fibroblasts (Mann *et al.*, 1983) secreting helper virus-free *bcr−abl* virus, and the infected marrow cells injected into lethally irradiated syngeneic recipients. Control mice received bone marrow cells co-cultivated with the parental Ψ-2 line. To check the efficiency of infection of stem cells by the *bcr−abl* virus, d13 spleen colonies were dissected from two batches of DBA/2 mice receiving 5×10^4 cells and analysed for the presence of provirus by Southern blot analysis. On average, 25% of colonies were positive. The remaining mice received 10^6 cells and, from a total of nine experiments, >90% recovered from irradiation, irrespective of strain. Significantly, all 26 reconstituted DBA/2 mice and 22 of the 32 C57BL/6 mice (69%) eventually succumbed to *bcr−abl*-induced disease, while control animals remained healthy. As documented below, the *bcr−abl*-induced tumours all proved to be haemopoietic in origin but were diverse in phenotype, involving the macrophage, erythroid, mast cell and lymphoid lineages.

Intriguingly, the two strains differed in their susceptibility to the various diseases (Table I). Whereas DBA/2 mice usually developed only reticulum cell sarcomas (RCS) of macrophage origin (see below), erythroleukaemia predominated in C57BL/6 mice. Among the lymphoid tumours, which arose in ~12% of mice of each strain, DBA/2 mice developed only pre-B lymphomas, whereas C57BL/6 mice developed T lymphomas. The two strains also differed in

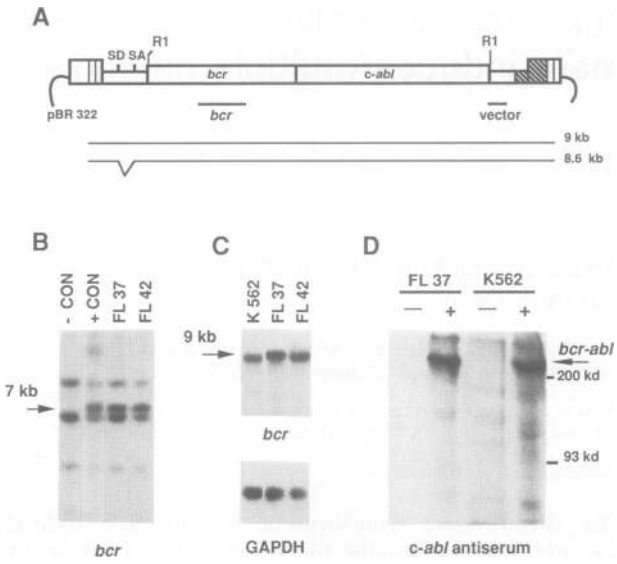

Fig. 1. Characterization of the MPZen(*bcr−abl*) provirus. (**A**) Structure of the MPZen(*bcr−abl*) provirus (Hariharan *et al.*, 1988) and its genomic and spliced transcripts. The vector sequences derive from Moloney leukaemia virus except for the hatched region, which is from the myeloproliferative sarcoma virus. SD and SA denote splice donor and acceptor sites respectively and RI indicates *Eco*RI restriction sites created by insertion of *Eco*RI linkers at the *Xho*I site of MPZen. Lines below the provirus indicate the location of the *bcr* and vector probes (see Materials and methods). (**B**) Southern blot analysis with the *bcr* probe of *Eco*RI digested DNA (15 μg) from two independently derived MPZen(*bcr−abl*) virus-infected fetal liver cell lines (FL37 and FL 42) (see Materials and methods) and, as a control, from normal mouse liver with and without addition of 75 pg of pMPZen(*bcr−abl*) plasmid (+CON, −CON). The 7 kb *bcr−abl* fragment liberated from the provirus is indicated by an arrow; other bands correspond to endogenous *bcr* sequences. (**C**) Northern blot analysis of poly(A)$^+$ RNA (4 μg) from FL 37, FL 42 and the CML blast crisis cell line K562, first using the *bcr* probe and then a probe for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The ~9 kb band comprising the co-migrating genomic and spliced *bcr−abl* transcripts is indicated. (**D**) Immunoprecipitation and *in vitro* autophosphorylation of cell extracts from FL 37 and K562, performed essentially as described by Konopka *et al.* (1984). Lanes marked + were immunoprecipitated with c-*abl* antiserum, αpEX-5 (Konopka *et al.*, 1984), whilst those marked − were immunoprecipitated with normal rabbit serum. The 210 kd *bcr−abl* protein is indicated, as are the positions of mol. wt markers.

disease latency, with a mean of 15 weeks for the DBA/2 mice and 22 weeks for the C57BL/6 animals (Figure 2). This primarily reflected the slower onset of the RCS disease and lymphomas in the C57BL/6 mice, most erythroleukaemias occurring relatively early in both strains (Table I).

Macrophage origin of bcr−abl induced RCS

The RCS of DBA/2 mice was very characteristic. The principal tumour mass was usually in the mesenteric lymph node and adjacent ascending colon, and white tumour nodules were apparent in the liver (Figure 3A). The spleen sometimes contained tumour foci (20% of cases) but was invariably enlarged 2- to 4-fold by increased numbers of myeloid and erythroid cells (see below). In C57BL/6 mice, colon and lymph node involvement was less pronounced, but tumour foci were common in the liver, and the lungs were often extensively consolidated by tumour cells and mature granulocytes. The bone marrow was a prominent site of RCS in both strains while the kidneys, heart, thymus,

Table I. Haemopoietic diseases induced by *bcr–abl*

	DBA/2		C57BL/6	
	incidence (%)[a]	latency[b]	incidence (%)[a]	latency[b]
RCS (macrophage)	21 (80)	115 ± 23	5 (16)	243 ± 69
Lymphoma[c]				
pre-B	3 (12)	61 ± 17	0	
T cell	0		4 (13)	147 ± 19
Erythroid tumour	0		2 (6)	50 ± 1
+ RCS	1 (4)	21	9 (28)	88 ± 68
+ RCS/mastocytosis	1 (4)	112	1 (3)	323
Mastocytosis + RCS	0		1 (3)	344
Total	26 (100)	105 ± 32	22 (69)[d]	153 ± 103

[a]Only mice autopsied because of illness are included. Percentage in parentheses is related to the number of reconstituted animals.
[b]Mean ± SD in days.
[c]RCS also present in variable amounts in five out of seven mice.
[d]Of the other 10 reconstituted animals, three died from radiation-induced thymomas between 142 and 194 days (see text), and one autopsied at 70 days had no detectable tumour. Of the six remaining mice autopsied healthy at 350 days, five had minor foci of tumour detected either histologically or by DNA analysis and the sixth had no disease.

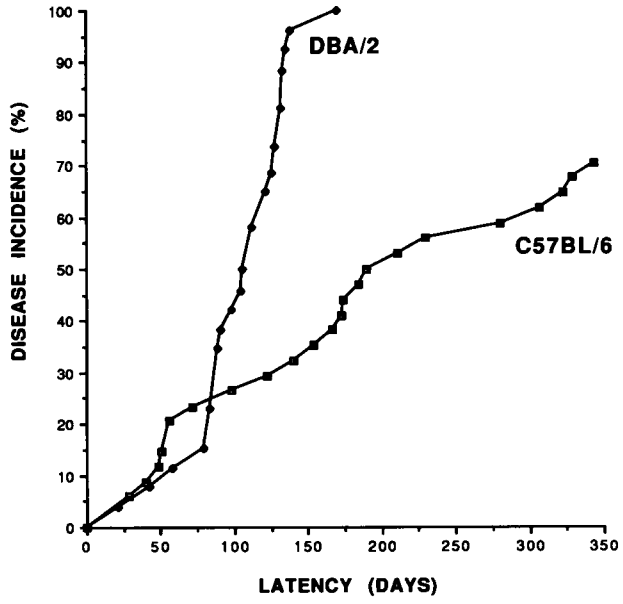

Fig. 2. Cumulative incidence of haematologic disease in mice reconstituted with MPZen(*bcr–abl*) virus-infected bone marrow cells. A total of 26 DBA/2 and 32 C57BL/6 mice were followed for 12 months. Only animals which on autopsy had tumours subsequently shown to harbour *bcr–abl* provirus have been included in the analysis.

muscle and extradural space were sometimes invaded.

Histological analysis showed that the tumour tissue comprised cohesive sheets or nodules of large, pleomorphic cells with abundant eosinophilic cytoplasm and occasional multinucleate giant cells (Figure 3B and C). Nuclei were large and open with clumped heterochromatin and mitoses were rare. The morphology of this tumour closely resembles that of RCS, Type A (Dunn, 1954), a disseminated tumour which occurs spontaneously at low frequency in old mice (Frith *et al.*, 1980). Spontaneous RCS is commonly erythrophagocytic, but the *bcr–abl* tumours were not.

All tissues identified microscopically as heavily infiltrated by RCS contained *bcr–abl* provirus. *Eco*RI digested DNA yielded the expected 7 kb internal viral fragment at a level commensurate with 1–2 copies of provirus per tumour cell, as is apparent for the mesenteric lymph node, lung and colon samples from two mice with RCS (46.2 and 45.8) analysed in Figure 4A. Abundant viral *bcr–abl* RNA was also evident by Northern blot analysis (see colon tumours from 46.3 and 45.7 in Figure 5). Tumour clonality was assessed by rehybridization with a vector probe (see Figure 1A) which detects the 3′ provirus–host DNA junction and is therefore insertion site-specific. Monoclonal tumours were clearly present in five of 20 DBA/2 mice evaluated (25%) and six of 12 C57BL/6 mice (50%), since a single proviral band was detected in every positive tissue analysed. The tumour tissue in at least another nine DBA/2 mice (43%) and four C57BL/6 animals (33%) was judged to be oligoclonal, because several bands of different intensity were obtained from a single tissue (e.g. the colon sample from mouse 45.8, Figure 4B), or because foci from different sites yielded distinct bands. Surprisingly, two animals had a clonal tumour in one location and polyclonal disease elsewhere. The tumour infiltrating the lymph node of mouse 46.2, for example, had a single proviral insert, but no clonal bands were discernible in its lungs, despite the high concentration of proviral DNA (compare Figure 4A and B).

The RCS were almost certainly macrophage in nature, as initially suggested by their morphology. Cell surface marker analysis was precluded because it proved impossible to prepare viable cell suspensions from the extremely cohesive tumours. However, RNA analysis revealed gene expression characteristic of macrophages: lysozyme, M-CSF receptor (*c-fms*), and the myeloid growth factors G-, M- and GM-CSF (Figure 5, mouse 46.3, mouse 45.7). As expected of myeloid cells, no rearrangement of either the immunoglobulin heavy chain or T cell receptor γ loci was detected (data not shown). Since the protocol selected against adherent cells (see Materials and methods), the macrophage tumours were presumably descended from infected immature precursors.

RCS are transplantable and induce a secondary myeloproliferation

Most animals with RCS tumours also overproduced other myeloid cells. This effect was most evident in DBA/2 mice, which had excess mature neutrophils in the peripheral blood (Table II) and the lungs (16/21), whilst a range of mature and developing granulocytes were present in the bone marrow (20/21) and spleen (17/21). Half these mice (11/21) also had mast cells in the bone marrow, a phenomenon not apparent in normal mice (McCarthy *et al.*, 1980). Eosinophils were frequently associated with RCS tumour masses, especially in C57BL/6 mice. Immunophenotyping confirmed that the bone marrow and spleen contained elevated proportions of myeloid (Mac-1$^+$) cells and agar colony assays revealed that myeloid progenitors were elevated 60-fold in the spleen (Table II). From the sum of the myeloid components of the blood, spleen and bone marrow, the myeloid compartment appears to be expanded by about two thirds over control DBA/2 mice (Table II).

The myeloid overproduction appeared to be a secondary effect. Proviral DNA could not be detected in DNA prepared from intact spleens of RCS animals lacking any other *bcr–abl* neoplasm, or from suspensions of their bone

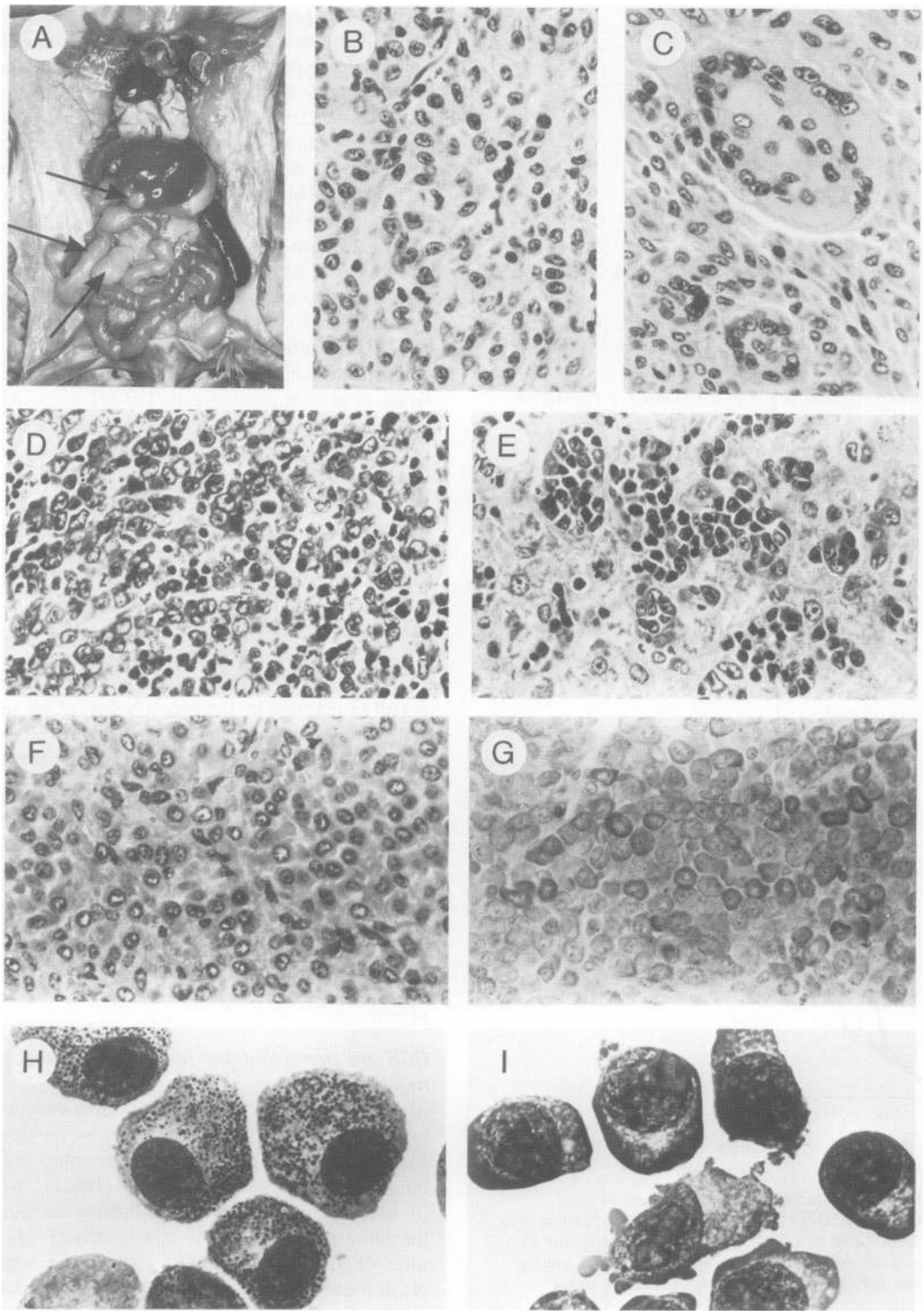

Fig. 3. Pathology of *bcr–abl*-induced disease. (**A**) Dissection of a DBA/2 mouse demonstrating typical RCS with tumour nodules in the liver (upper arrow), extensive colonic infiltration (middle arrow), mesenteric lymph node enlargement (lower arrow) and moderate splenomegaly. (**B**) Histology of RCS infiltrating an inguinal lymph node; (**C**) prominent giant cell formation was evident in some fields. (**D**) Erythroid tumour replacing the splenic red pulp (mouse 45.14) and (**E**) infiltrating through the hepatic sinusoids. (**F**) Area of mast cell tumour from mouse 34.4 (see text) stained with haematoxylin and eosin or (**G**) Alcian Blue–Safranin, showing heavy cytoplasmic granulation in all cells. All other sections were stained with haematoxylin and eosin. Magnification ×450. Cytocentrifuge preparations from cell line derived from subcutaneous tumour of 45.14 tertiary recipient (see text) at (**H**) mast cell and (**I**) erythroblast stages (stained with May-Grunwald-Giemsa; magnification ×1125).

marrow cells (e.g. mouse 46.2 and mouse 45.8, Figure 4A), which lack significant numbers of RCS cells either because they adhered to the femur or fragmented on pipetting. Thus the excess granulocytes in these tissues did not derive from virus-infected precursors. The mild CML-like syndrome probably results from secretion of myeloid growth factors

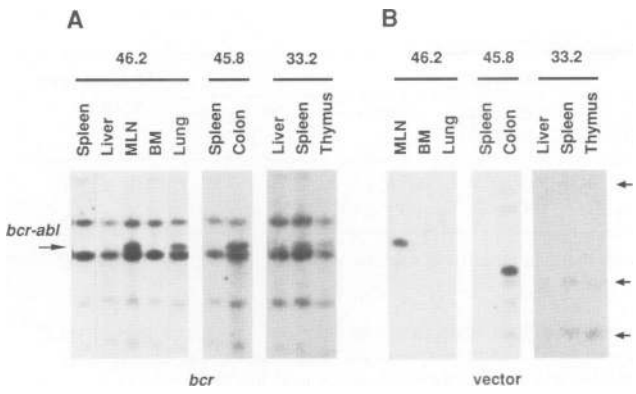

Fig. 4. Location and clonal analysis of *bcr−abl* provirus in tissues of reconstituted mice. **(A)** Southern blot analysis with the *bcr* probe (see Figure 1A) of *Eco*RI digested DNA (15 μg) from tissues of animals with RCS (mouse 46.2, 45.8) and erythroid tumour (mouse 33.2). BM is bone marrow; MLN is mesenteric lymph node. The position of the 7 kb proviral-specific *bcr−abl* fragment is indicated. **(B)** The same filters rehybridized with the insertion site-specific vector probe (see Figure 1A). Weakly hybridizing clonal bands in tissue from mouse 33.2 are indicated by arrows.

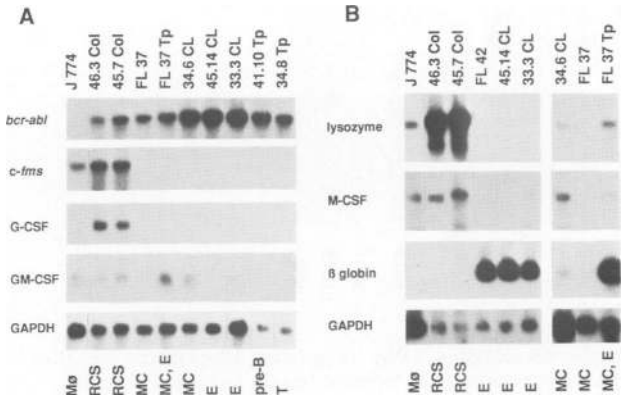

Fig. 5. Northern blot analysis of RNA from tissues and cell lines harbouring *bcr−abl* provirus. Polyadenylated RNA samples (4 μg) bound to two filters (**A** and **B**) were sequentially hybridized with the indicated probes (see Materials and methods). In (B), 2 h exposures were used for the lysozyme and β-globin for all samples except the last three, which required 2 days for lysozyme and 16 h for globin; with the other probes, the same exposure was used for all samples. The RNAs were from: J774, macrophage cell line (control); 46.3, 45.7 Col, colonic tumour; FL 37, FL 37 Tp, primary and transplant-derived fetal liver cell lines; 34.6 CL, bone marrow derived cell line; 45.14 CL, cell line derived from subcutaneous tumour of tertiary recipient of 45.14 spleen tumour; 33.3 CL, bone marrow derived cell line; 41.10 Tp, lymph node tumour, transplanted from 41.10 pre-B tumour; 34.8 Tp, T cell tumour transplanted from 34.8 thymoma; FL 42, primary fetal liver cell line. The predominant cell type constituting each sample is shown below each track: Mϕ, macrophage; RCS, reticulum cell sarcoma; MC, mast cell; E, erythroid; pre-B, pre-B lymphoid; T, T lymphoid. No IL-3 RNA could be detected in any of these RNAs.

by the macrophage tumours. Bioassay for IL-3 and GM-CSF revealed only traces of GM-CSF in the serum of one of eight mice, but G-CSF activity was readily apparent in two of eight ($16-32 \times 10^3$ U/ml).

To test for transplantability, minced tissue from seven RCS tumours was injected intraperitoneally and subcutaneously into sublethally irradiated syngeneic recipients. To date, six of the seven primary tumours have produced a fatal RCS in 11 of 19 recipients, after a mean latency of 208 days. Thus the RCS tumours induced by expression of *bcr−abl* are transplantable but grow very slowly. Consistent with the hypothesis that these tumours induce myeloid expansion, the recipients also exhibited increased numbers of mature granulocytes and their precursors in the bone marrow, spleen and lungs.

The lymphomas are clonal and transplantable

The major sites of pre-B tumour growth in DBA/2 mice were the spleen, lymph nodes and bone marrow. The animals did not have elevated peripheral blood white cell counts but immature lymphoblasts were apparent on the blood film (8−26% of leukocytes). In two of the three cases, the central nervous system was affected, with intracranial lymphoma in one instance and extradural spinal cord compression in the other. The $B220^+$, sIg^- lymphomas each carried a single *bcr−abl* provirus and exhibited a unique pattern of one or two J_H rearrangements (data not shown). Thus they were monoclonal and of independent origin. Curiously, however, all three arose in a cohort of 10 mice transplanted with one batch of infected 5-FU marrow cells, perhaps indicating that it had been enriched in lymphoid targets. Two of the pre-B lymphomas were transplantable and cultured cell lines were readily established in the absence of added B cell growth factors. The transplanted tumours and derived cell lines displayed the proviral inserts and J_H rearrangements of the parent tumours.

The four C57BL/6 mice with T lymphomas presented with a dominant thymic mass, usually accompanied by generalized lymphadenopathy. One mouse was leukaemic, with a white cell count of 117×10^6/ml, almost entirely due to lymphoblasts. Each lymphoma was monoclonal as judged by proviral insertion data and/or rearrangement of T cell receptor γ or β genes (data not shown). All were highly malignant on transplantation, but attempts to develop cell lines from them (in the absence of added IL-2) were unsuccessful. Three other C57BL/6 mice (excluded from the incidence and kinetics data in Table I and Figure 2) also developed T lymphomas within a similar period but lacked detectable provirus and presumably were of recipient origin, induced by radiation (Kaplan and Brown, 1952).

None of the pre-B or T lymphomas tested showed any evidence of myeloid growth factor production, as assessed by RNA analysis (Figure 5), bioassay of serum or conditioned medium, or histologic determination of myeloid cell mass in transplant recipients.

Erythroleukaemias bear bcr−abl provirus but few are transplantable

The dominant disease in C57BL/6 mice was erythroleukaemia, seen in 12 of the 22 sick mice (Table I). While two exhibited only erythroid disease, the others also had variable amounts of RCS and one mouse (33.3) displayed a marked increase in mast cells as well. The two erythroleukaemias seen in DBA/2 mice were also accompanied by RCS and, in one case, a mast cell tumour (Table I). The key pathological feature was splenomegaly caused by erythroblasts expanding the splenic red pulp (Figure 3D). The hepatic sinusoids were also replete with erythroblasts (Figure 3E), but the bone marrow, curiously, was not infiltrated. In most animals (60%), the peripheral blood

Table II. Myeloid cells in RCS-bearing DBA/2 mice

Mice[a]	Peripheral blood		Spleen			Bone marrow			Myeloid mass[c] ($\times 10^{-6}$)
	WCC[b] ($\times 10^{-6}$/ml)	neutrophils (%)	total cells ($\times 10^{-6}$)	Mac-1$^+$ (%)	CFC ($\times 10^{-3}$)	cells/femur ($\times 10^{-6}$)	Mac-1$^+$ (%)	CFC/femur ($\times 10^{-3}$)	
bcr–abl	24 ± 11	71 ± 15	390 ± 250	27 ± 16	246 ± 59	6 ± 3	74 ± 13	28 ± 13	210 ± 80
control	11 ± 1	21 ± 3	110 ± 10	2 ± 1	4 ± 2	14 ± 2	49 ± 9	12 ± 4	120 ± 40

[a]Data for 21 *bcr–abl* mice bearing RCS tumours and two mice reconstituted with mock-infected bone marrow cells except for CFC, where six RCS-bearing mice were assayed.
[b]White cell count.
[c]Sum of blood neutrophils, splenic and bone marrow myeloid cells. The total number of blood neutrophils was calculated by multiplying the WCC by its % neutrophil content and the blood volume (estimated at 2.5 ml). The myeloid cell content of spleen and bone marrow was calculated by multiplying the % Mac-1$^+$ cells by the total number of cells in each organ, assuming one femur equals 6% of the total marrow.

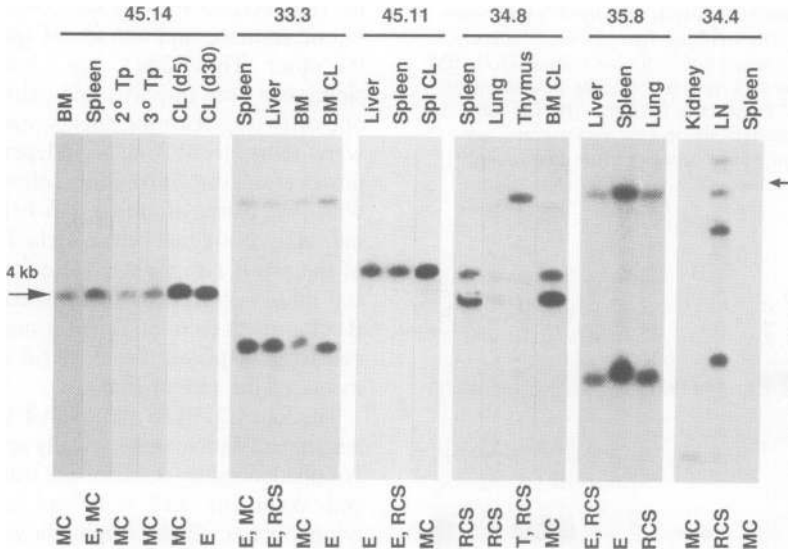

Fig. 6. Clonal relationship of multiple diseases induced *in vivo* by *bcr–abl*. Southern blot analysis of DNA (15 μg) from tissues or cell lines (CL) derived from mice with multiple tumour types. The dominant cell type(s) identified morphologically are indicated beneath each track of *Eco*RI digested DNA hybridized to the vector probe. The arrow on the right indicates the faint proviral band in the spleen of mouse 34.4. BM, bone marrow; 2° Tp, 3° Tp, subcutaneous tumour masses from secondary and tertiary recipients of 45.14 spleen tumour; CL (day 5), CL (day 30), cell lines derived from tertiary recipient of 45.14 tumour, harvested after 5 and 30 days in culture; Spl CL, spleen derived cell line; LN, lymph node. Other abbreviations are as in Figures 4 and 5.

contained small numbers of nucleated erythroid cells (1–29 per 100 leukocytes). The proliferating erythroblasts derived from *bcr–abl* virus-infected cells, since high levels of proviral DNA were found in the spleens of all five C57BL/6 mice with a purely erythroid tumour in this organ (e.g. mouse 33.2 in Figure 4A). Moreover, cell lines established from two mice (45.14 and 33.3) expressed globin mRNA (Figure 5) and harboured *bcr–abl* provirus, with insertion patterns identical to those found in the primary tumours (Figure 6). Since the integration-specific probe usually revealed only one to four proviral bands (examples in Figures 4B and 6), the erythroleukaemias were dominated by the progeny of one or a very few infected cells.

In contrast to the RCS tumours and lymphomas, *bcr–abl* induced erythroleukaemia was rarely transplantable. Bone marrow and/or spleen cells from nine of the primary mice were injected into sublethally irradiated recipients, but in only one case (45.14) did tumours arise. However, the erythroblast cell line established from the bone marrow of another mouse (33.3) proved to be highly malignant, as was the fetal liver erythroid line FL 42 (Figure 1), all recipients developing a disseminated erythroleukaemia within 3–5 weeks. GM-CSF and M-CSF RNA was detected at low level in one erythroid cell line (33.3) but not another (45.14) (Figure 5).

Aggressive and benign mast cell diseases

Mast cell tumour tissue was prominent in the spleen, bone marrow and lymph nodes of one DBA/2 (45.14) and two C57BL/6 (33.3 and 34.4) mice. The cytoplasmic granules characteristic of mast cells were easily visible on Alcian Blue–Safranin staining (compare Figures 3F and 3G). Erythroleukaemia also contributed to the tumour burden in two of these mice (33.3 and 45.14) and RCS was found in all three. The *bcr–abl* provirus was readily detectable in tissues solely infiltrated by mast cells (e.g. in Figure 6, bone marrow of mouse 33.3; spleen and kidney of mouse 34.4; bone marrow of mouse 45.14), implying that the mast cell tumours arose as a direct consequence of proviral expression rather than as a secondary response by the animal to the other tumour elements. The mast cell tumour from both mice tested was readily transplantable.

Expression of *bcr–abl* by mast cells was not, however, invariably associated with overt mastocytosis. Provirus-containing mast cell lines evolved from cultures of bone marrow from six other mice in which mast cells were not demonstrable histologically. Five of these lines derived from mice with erythroleukaemia (with or without RCS), whilst the sixth arose from the bone marrow of an animal (34.8) that presented with a thymoma and RCS (see below). Five lines grew only slowly in culture, and the only example tested was not tumorigenic. The final line, 34.6, behaved somewhat differently and is discussed further below.

Some tumours may arise from transformed multipotential precursors

The co-existence of multiple tumour types within one animal (Table I) naturally raises the question of their clonal relationship. We addressed this issue by comparing the proviral insertion pattern in individual tissues with the relative burden of different tumour types determined histologically.

Erythroid and mast cell tumours can share a common precursor. Histological analysis of mouse 45.14 revealed an erythroid tumour in the splenic red pulp of mouse 45.14 (Figure 3D) and mast cell tumour in its splenic white pulp and bone marrow. Mice transplanted with these spleen cells succumbed in 3 weeks to disseminated erythroid and mast cell tumours, which in turn rapidly yielded mixed tumours on further transplantation. Increased myeloid elements were apparent in the spleen and bone marrow of the 2° and 3° recipients and GM-CSF activity was detectable in the serum of one of two animals tested. DNA from 1°, 2° or 3° mice revealed only a single (4 kb) provirus-bearing fragment (Figure 6). Six cell lines derived from 2° and 3° tumours initially contained only mast cells (Figure 3H) and medium conditioned by these cells had high levels of IL-3 activity (60–400 bone marrow U/ml) as assessed by FD and 32D cell bioassay (see Materials and methods). However, after 4–6 weeks of culture, factor production waned, erythroblasts and basophilic normoblasts predominated (Figure 3I), and Northern blot analysis revealed abundant globin mRNA (Figure 5B). Irrespective of cellular morphology at the time of harvest, every 45.14 derived line yielded only the 4 kb proviral fragment (e.g. Figure 6) and all produced erythroid and mast cell tumours on transplantation. Clearly this mixed disease emanated from a single *bcr–abl* virus-infected cell and significant levels of myeloid growth factor were produced by the mast cell component.

The erythroid and mast cell tumours in mouse 33.3 also shared a common precursor, since DNA from the erythroid cell line yielded the same pair of proviral bands as the bone marrow, which was dominated by mast cell tumour, and the spleen, which harboured both tumours (Figure 6). A third instructive example was provided by mouse 45.11, which displayed no obvious mastocytosis, but yielded a slowly growing mast cell line with the same single proviral insert as the erythroleukaemia in its liver and spleen (Figure 6). Finally, the cloned *bcr–abl* virus-infected fetal liver line FL 37 (Figure 1) had a mast cell phenotype and lacked detectable β-globin mRNA expression but produced tumours containing both erythroid and mast cell elements upon transplantation. Moreover, a cell line established from one of the FL 37 transplant recipients exhibited erythroid (as well as mast cell) elements and produced globin mRNA (Figure 5). Intriguingly, the re-established line also expressed GM-CSF and M-CSF RNA, unlike the parental line (Figure 5).

We conclude that the cell transformed by *bcr–abl* virus in each of these examples was a progenitor or stem cell with capacity to differentiate into both erythroid and mast cell lineages, perhaps the self-renewing bipotential erythroid/mast cell progenitor characterized by Wendling *et al.* (1985).

RCS tumours can derive from a progenitor which also displays erythroid and/or mast cell potential. In two of the mice just described, 33.3 and 45.11, certain tissues contained substantial levels of RCS as well as erythroid/mast cell tumour, but the only discernible proviral bands were those ascribed to the latter by analysis of tissues and/or cell lines lacking RCS (Figure 6). These data imply that all three components arose from a common precursor. Although our inability to culture the RCS tumours *in vitro* hampered attempts to confirm this hypothesis, a cell line from mouse 34.6 has provided persuasive evidence. This line, which carries a single proviral insert, initially had a typical mature mast cell morphology but became progressively less differentiated with time. When analysed after 14 weeks in culture the cells expressed low levels of mRNA for β-globin, lysozyme, M- and GM-CSF (Figure 5). All six animals transplanted with this clonal line (after 103 days in culture) developed tumours within 41–73 days. Significantly, mast cell, erythroid and RCS tumour components were all seen histologically, although their relative distribution varied greatly between recipients. An erythroid cell line rescued from one recipient carried the 6 kb proviral fragment of the parent cell line (data not shown).

Two other mice support a shared origin for RCS and erythroid or mast cell tumours (Figure 6). In mouse 34.8, the RCS-dominated spleen and lung showed the same proviral pattern as the bone marrow derived mast cell line, while in 35.8 the erythroleukaemic liver and spleen displayed the same proviral pattern as the lung, which contained only RCS. In contrast, the mast cell clones infiltrating the spleen and kidney of mouse 34.4 derived from precursors different from those generating the RCS tumour in the abdominal lymph node (Figure 6).

In one of the two animals where DNA samples were available from both lymphoid and non-lymphoid tumour components, the lymphoid tumour clearly was of independent origin (mouse 34.8, Figure 6). The data from mouse 33.2, however, were suggestive of a common T cell and erythroid precursor, since the same (weak) proviral integration pattern was seen in the erythroblast-dominated spleen and the thymus (Figure 4B), judged histologically to harbour a developing T lymphoma.

Discussion

Previous studies have documented the lymphomagenic potential of the *bcr–abl* gene (see Introduction). We show here that mice reconstituted with *bcr–abl* virus infected bone marrow cells develop not only lymphomas but also macrophage, erythroid and mast cell tumours. Proviral RNA and DNA was evident in the affected tissues and derived cell lines. There were prominent strain differences in disease susceptibility and latency, DBA/2 mice usually succumbing

to disseminated macrophage tumours and C57BL/6 animals to erythroleukaemia (Table I). The restriction of pre-B lymphomas to DBA/2 mice and T lymphomas to C57BL/6 mice is also intriguing. Such differences may reflect variation in response to the *bcr−abl* oncogene, in numbers of progenitor cells available for retroviral infection, or in factors affecting expression of regulatory sequences in the recombinant retroviral vector.

Many mice exhibited hyperplasia involving cells of more than one haemopoietic lineage. Proviral insertion analysis established that, in at least some cases, the different neoplastic components derived from a common *bcr−abl* virus-infected precursor. Clonally related erythroid and mast cell lesions were relatively frequent, and macrophage/erythroid or macrophage/mast cell clones were also detected. Furthermore, two clonal tumours (33.3 and 45.11) and one transplantable clonal cell line (34.6) yielded erythroid, mast cell and macrophage components. The contribution of different cell types to the mixed clone was variable and may therefore have been influenced by the progenitor cell, the growth milieu (*in vitro* or *in vivo*) and/or the chance occurrence of somatic mutations capable of synergizing with *bcr−abl* (see below). Cell lines with mixed differentiation potential have also been established from patients in CML blast crisis. K562 and LAMA-84 exhibit erythroid/megakaryocytic characteristics (see Keating, 1987), while KU 812 shows evidence of erythroid, mast cell and macrophage differentiation (Fukuda *et al.*, 1987; Nakazawa *et al.*, 1989).

No overt CML was observed, even though the protocol used allows infection of stem cells capable of long-term reconstitution (Bowtell *et al.*, 1987). Moreover, in the present experiments, a significant proportion of day 13 spleen colonies bore the *bcr−abl* provirus and certain tumours were shown to derive from progenitor cells that were at least bi- or tri-potential for the erythroid/mast cell/macrophage lineages. The absence of observable granulopoiesis from virus-infected cells could mean that *bcr−abl* expression by murine stem or progenitor cells biases proliferation and differentiation toward other lineages. This would imply a major difference in the control of human and murine haemopoiesis. Alternatively, we may simply have failed to infect a stem cell with potential to differentiate into granulocytes.

Intriguingly, a mild CML-like syndrome was apparent in animals harbouring RCS, a macrophage tumour. Since the provirus could not be detected in tissues which displayed excess granulocytes but lacked RCS, we ascribe the increase in mature myeloid cells and their progenitors to a bystander effect—probably excess growth factor production, since the serum of certain RCS-bearing animals contained significant levels of G-CSF, and RCS tumours expressed readily detectable G-CSF RNA, as well as M- and GM-CSF RNA.

These results suggest that excess myeloid growth factor production may partly account for the exaggerated myeloid representation seen in CML. An expanded basophil/mast cell compartment is a key feature of CML and absolute monocyte numbers are also frequently raised (Shepherd *et al.*, 1987). Both these cell types produce myeloid growth factors if activated (Nicola, 1989; Plaut *et al.*, 1989; Wodnar-Filipowicz *et al.*, 1989) or if they harbour a *bcr−abl* or v-*abl* gene (this paper; Mori *et al.*, 1987; Humphries *et al.*, 1988). Moreover, *bcr−abl* may induce unscheduled growth factor production in other haemopoietic cell types, as it did in FDC-P1 cells (Hariharan *et al.*, 1988). CML cells remain dependent on growth factors (Metcalf *et al.*, 1974), so any increased local factor production, be it paracrine or autocrine, could significantly boost granulopoiesis. Significantly, CML patients often exhibit elevated serum CSFs (Metcalf *et al.*, 1974; Metcalf, 1977) and a profound myeloid imbalance can be induced in mice by enforced expression of GM-CSF, G-CSF or IL-3 (Lang *et al.*, 1987; Chang *et al.*, 1989a,b; Johnson *et al.*, 1989; Wong *et al.*, 1989).

Is *bcr−abl* expression the only factor responsible for the haemopoietic syndromes observed in the reconstituted mice? In the case of the lymphomas, all tumours were clonal, suggesting that additional somatic changes, presumably genetic, had conferred a selective growth advantage on one cell within the susceptible population. In this respect, *bcr−abl* lymphoid disease is analogous to the lymphoblastic crisis of CML. Of the three pre-B lymphomas, only two were tumorigenic in secondary recipients and yielded (factor-independent) cell lines in culture. Thus evolution of a fully transformed phenotype appears to require several steps. The latency of *bcr−abl* induced thymomas (of donor origin) in C57BL/6 mice was the same as irradiation-induced thymomas of host origin, suggesting that progression to full tumorigenicity is of comparable complexity in the two diseases. Transgenic mice bearing the *bcr−abl* analogue *bcr−*v-*abl* also developed pre-B and T lymphomas (Hariharan *et al.*, 1989). It is clear from these results that *bcr−abl* can contribute to tumorigenesis within both lymphoid lineages, and hence the rarity of T lymphoblastic leukaemia in CML patients probably simply reflects the low frequency of Ph^1-positive T lymphocytes.

The development of macrophage tumours may also have involved additional genetic change, since all transplantable tumours were monoclonal and the lesions in most 1° mice were dominated by one or very few clones. While there were two instances of an apparently polyclonal infiltrate, one in lung and the other in the peritoneal cavity, these may have been the progeny of a 'pre-neoplastic' pool of *bcr−abl* virus-infected macrophage precursors which seeded these sites at the time of reconstitution.

The *bcr−abl*-bearing erythroleukaemias were of two types. The predominant type was not transplantable, despite its vigorous proliferative activity and restricted clonality in the primary reconstituted animal. No erythroid cell lines could be established from these animals but slow-growing non-transplantable mast cell lines, which bore the provirus, grew out from cultures of their bone marrow cells. In at least one case (45.11), the mast cell line had the same proviral insert as the erythroid clone. These results establish that *bcr−abl* expression in erythroid and mast cells or their precursors provides a marked proliferative stimulus, often sufficient to overwhelm the primary mouse, but is not fully transforming. This syndrome is analogous to the chronic or accelerated phase of CML, although involving a different lineage.

The second type of erythroid disease, observed in two mice (45.14 and 33.3), was a disseminated transplantable tumour which could be maintained continuously *in vitro* in the absence of added growth factor. Both examples arose after a much longer latency (112 and 323 days) than the non-transplantable erythroleukaemias (mean 65 days) and each was accompanied by a malignant mast cell clone harbouring

the same provirus. Infection of day 13 (CBA) fetal liver cells with *bcr−abl* virus *in vitro* also produced a transplantable erythroid line (FL 42) and a line with mast cell morphology (FL 37) which proved on transplantation to be bipotential, producing both mast cell and erythroid tumours. Taken together, these results suggest that many of the erythroleukaemias stemmed from a *bcr−abl* virus-infected progenitor cell with potential to differentiate down both mast cell and erythroid lineages, but only following acquisition of additional mutation(s) within the progenitor cell did transplantable bi-lineage tumours emerge.

To date, no clues have emerged regarding mutation(s) apparently synergizing with *bcr−abl* expression to produce the various tumours. No altered *bcr−abl* transcripts were observed which could encode a further alteration of the tyrosine kinase. Nor has any rearrangement yet been detected of p53, the putative anti-oncogene recently reported to be altered in many CML patients in blast crisis (Ahuja *et al.*, 1989; Kelman *et al.*, 1989).

Certain aspects of MPZen(*bcr−abl*) virus-induced tumorigenicity resemble that provoked by Abelson virus. However, Abelson virus typically produces pre-B or T lymphomas; mast cell and macrophage tumours are very infrequent (Whitlock and Witte, 1985). Moreover, erythroleukaemia has never been obtained *in vivo* with Abelson virus, and the factor-independent erythroid colonies that it generates from fetal liver cells cannot be established as permanent cell lines (Waneck and Rosenberg, 1981). While these differences may partly reflect the different LTRs of the two viruses and the different infection protocols, which would influence target cell accessibility, it seems likely that the *bcr−abl* and *gag−abl* genes have distinctive oncogenic potential.

The effects of two other tyrosine kinase oncogenes on murine haemopoiesis have been evaluated with protocols similar to ours. Reconstitution with v-*src* bearing bone marrow cells induced a severe myeloproliferative disorder, with increased myeloid progenitor cells in the spleen and peripheral blood and a decrease in the bone marrow, but no transformation was detected, either *in vivo* or *in vitro* (Keller and Wagner, 1989). Intriguingly, v-*fms* induced B lymphomas, erythroleukaemia and a myeloproliferative disorder (Heard *et al.*, 1987) in reconstituted mice, but it is difficult to compare their relative frequency to those induced by *bcr−abl* because a different, non-inbred strain was utilized (NIH/Swiss) and fewer mice were followed for a shorter time period.

In summary, our results have clearly demonstrated that *bcr−abl* is an effective oncogene *in vivo* for diverse haemopoietic cell types, including some multipotential progenitors, although full tumorigenicity appeared to require additional genetic change. Since the absence of overt CML may have reflected the very low abundance of appropriate stem cell targets, we are attempting to infect purified stem cells (Spangrude *et al.*, 1988) and, in view of the marked genetic background effects observed here, to determine whether CML can be more readily induced in a different inbred mouse strain.

Materials and methods

Mice

Mice used were 2−3-month-old DBA/2JWehi and C57BL/6JWehi stocks raised under specific pathogen-free conditions and 12−13 day fetal CBA/CaHWehi mice. For transplantation studies, animals were γ-irradiated with 9.0−9.5 Gy for a lethal dose, 4.5 Gy for a sublethal dose and injected with cells within 3 h.

Infection of haemopoietic cells

The origin of MPZen(*bcr−abl*) virus-producing transfected Ψ-2 cell clone 210.16, which renders ~10% of the FDC-P1 cells factor-independent following a 3 day co-cultivation, has been described elsewhere (Hariharan *et al.*, 1988), as has the protocol for infection of bone marrow cells (Bowtell *et al.*, 1987; Johnson *et al.*, 1989). Briefly, day 4 post 5-FU-treated bone marrow cells (two femur equivalents per 10 cm dish) were co-cultivated in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal calf serum (FCS), 0.05 mM 2-mercaptoethanol (2-ME) and 10% pokeweed mitogen-stimulated spleen conditioned medium (PWM-SCM, a source of IL-3 and GM-CSF) for 5 days with virus-producing Ψ-2 cells plated 24 h previously at 5×10^4 cells per 10 cm dish. Non-adherent cells were then harvested, replated in fresh dishes for 3 h to remove any remaining adherent cells and then injected into irradiated mice.

Day 12−13 fetal liver cells (7.5×10^6) were infected by co-cultivation with irradiated (35 Gy) virus-producing Ψ-2 cells (1×10^6) for 2 days in 8 ml DMEM containing 20% FCS, 0.05 mM 2-ME and 0.33% conditioned medium from WEHI-3B cells (W3CM) as a source of IL-3. Non-adherent cells were then harvested, cultivated for a further 3 days in medium plus W3CM and then continuously grown in the absence of W3CM. The line designated FL 37 was obtained by cloning in methyl cellulose, whilst FL 42 represents the monoclonal product of prolonged culture in the absence of factor.

Haemopoietic assays

GM-CSF and IL-3 were assayed using FDC-P1 cells (responsive to GM-CSF and IL-3) and 32Dcl.23 cells (responsive to IL-3) as described previously (Metcalf, 1985). Levels of CSF were converted to bone marrow units by use in each assay of purified preparations of GM-CSF and IL-3 of known activity. G-CSF was assayed by its ability to induce differentiation in colonies of the murine myelomonocytic cell line WEHI-3B D+ (Nicola *et al.*, 1983). Colony forming cells (CFC) were assayed by culturing bone marrow (5×10^4 cells/ml) and spleen (1×10^5 cells/ml) in triplicate 1 ml agar cultures, with and without addition of 0.1 ml PWM-SCM, for 7 days at 37°C as described previously (Bowtell *et al.*, 1987).

Establishment of cell lines in vitro

Bone marrow, spleen or lymph node cells from tumour bearing mice were seeded at $1-2 \times 10^6$ cells/ml in 10 ml DMEM containing 20% FCS and 0.05 mM 2-ME. After 5−7 days, 5−10 ml fresh medium was added. When a non-adherent cell fraction was established, this was passaged into a fresh flask. Thereafter half the medium was changed every 5−7 days, or more frequently, depending on the rate of cell growth.

Analysis of DNA and RNA

Nucleic acid isolation and fractionation was as described previously (Bowtell *et al.*, 1987). The probes used for Northern and Southern blot analysis were the following: *bcr*, the 1.4 kb *Bam*HI fragment of clone *bcr*-29 (corresponding to residues 1016−2430 in Hariharan and Adams, 1987); vector, a 0.4 kb *Xho*I−*Hin*cII fragment of pZip-Neo SV[X]1 (Cepko *et al.*, 1984), which derives from the p15E region of Moloney virus; v-*fms*, a 1.4 kb *Pst*I fragment corresponding to the 3′ region of the v-*fms* gene of SM-FeSV (see Figure 3, Donner *et al.*, 1982); lysozyme, a 0.78 kb *Eco*RI fragment of the mouse lysozyme M cDNA clone λmLc8[a] (Cross *et al.*, 1988); GM-CSF, a 0.48 kb *Xho*I fragment of GM-CSF cDNA (see Johnson *et al.*, 1989); G-CSF, a 0.7 kb *Eco*RI−*Bgl*II G-CSF cDNA (see Chang *et al.*, 1989b); M-CSF, a 2.1 kb *Eco*RI fragment of M-CSF cDNA, clone J6/4 (DeLamarter *et al.*, 1987); β-globin, a 1 kb *Pst*I fragment of β^{maj}-globin corresponding to fragment B in Hofer and Darnell (1981); and GAPDH, a 1.1 kb *Pst*I fragment of rat G3PDH cDNA (a kind gift from Dr M.Piechaczyk). Probes were labelled with [α-^{32}P]dATP using a random hexamer priming kit (Bresatec Limited, Adelaide, South Australia).

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