Variability of the Molecular Defects Corresponding to the Presence of a Philadelphia Chromosome in Human Hematologic Malignancies


By analyzing a total of 107 patients affected by chronic myelogenous leukemia (CML; chronic and blast crisis) or lymphoid and myeloid Philadelphia chromosome (Ph') positive acute leukemias, we have investigated the relationship between the molecular defect on the Ph' chromosome and the associated hematologic phenotype. As expected, approximately half of the Ph' positive acute leukemias showed a breakpoint on chromosome 22 falling outside the “breakpoint cluster region” (bcr) known to be involved in CML. Surprisingly, seven of 80 CML cases in chronic phase also showed rearrangements falling outside the bcr region. In two of these cases the breakpoint on chromosome 22 was mapped between 9 and 12 kb upstream to the bcr region. In another case, the breakpoint was located approximately 16 kb downstream to bcr. In the remaining four cases, the precise position of the rearrangement could not be localized with the available bcr probes. DNAs from patients with CML blast crises showed classical bcr rearrangements. No molecular changes were observed during the progression of the disease in six patients whose DNA from both a chronic and acute phase was available. Our results seem to indicate a greater degree of variability of chromosome 22 breakpoints in CML than previously observed, and the lack of additional rearrangements on the Ph' chromosome in CML blast crises with respect to chronic phase.

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THE PHILADELPHIA CHROMOSOME (Ph'), a shortened chromosome 22 derived from a reciprocal translocation with chromosome 9, t (9;22)(q34;q11) (1), is considered the cytogenetic hallmark of chronic myelogenous leukemia (CML). As a consequence of this translocation, the c-abl protooncogene moves from chromosome 9 to chromosome 22. While the breakpoint on chromosome 9 falls between the first large intron of the c-abl protooncogene or one further upstream, the breakpoint on chromosome 22 occurs in a very short region breakpoint cluster region (bcr) of a transcribed gene, also called bcr. The translocation generates a hybrid bcr/c-abl locus that is transcribed in a new mRNA species, containing both bcr and c-abl sequences. A chimaeric P210 c-abl protein with an increased tyrosine-kinase activity, has been identified in CML leukemic cells and represents the translation product of the fused bcr/c-abl transcript. The biological function of this protein (as well as that of the normal c-abl product) are unknown. The involvement of P210 with CML is strongly suggested by its strict association with the disease, both in chronic and acute phases. However, it appears that an additional event must occur for the progression to blast crisis. In approximately 20% of the cases, this phase mimicks an acute lymphoblastic leukemia (ALL) phenotype.

Approximately 15% of ALLs and sporadic cases of AML also carry the Ph' chromosome. However, while approximately half of Ph' positive ALLs show a molecular defect similar to the one present in CML, the other half lacks a rearrangement within the bcr region and expresses an abnormal c-abl protein, P190, which also shows an increased tyrosine kinase activity. P190 is the product of a new hybrid mRNA arising from the junction of the first bcr exon alone to the c-abl gene. A rearrangement occurring within the first intron of the bcr gene has been recently documented in two of these ALLs. For P210 and CML, the question of whether P190 may be directly responsible for the ALL phenotype still remains open.

This study was carried out to further investigate the relationship between the molecular defect on Ph' chromosome and the associated leukemic phenotype, and to verify whether the type of rearrangement on chromosome 22 can play a direct role in the transition from a chronic to acute phase of CML. Therefore, we have characterized the breakpoint on chromosome 22 in a large series of Ph' positive CML patients. For a number of them, we analyzed DNAs obtained at different phases of the disease. We also studied patients affected by Ph' positive ALLs and AMLs. In CML, we have found a greater degree of variability of chromosome 22 breakpoints than previously observed. On the other hand, we did not observe any additional rearrangements on the Ph' chromosome in CML blast crises with respect to chronic phase.

MATERIALS AND METHODS

Patients and DNA samples. DNA from 107 patients was analyzed. Seventy-four patients had CML in chronic phase; six in accelerated phase. Eight patients had ALL, two had AML, nine had lymphoid, and eight had myeloid blast crises.

All patients with acute leukemia were adults (age ranging between 35 and 67 years). None had manifestation of a preceding chronic phase.
In the chronic and accelerated phases of CML, the DNA was extracted directly from peripheral blood leucocytes, while in acute leukemias and in blast crises, immature cells were purified from the more mature elements by Ficoll-Hypaque gradient separation. In six blast crises, DNA from the preceding chronic phase was available.

The cellular phenotype of the acute leukemias and blast crises was determined by routine cytochemical methods, by testing their capacity to form E rosettes, to express surface and cytoplasmic immunoglobulins (SmIg, CyIg) and nuclear terminal transferase (TdT) and determined by routine cytochemical methods, by testing their capacity to react with a panel of standard monoclonal antibodies, as detailed elsewhere. In addition, the blasts lymphoid or myeloid lineage was confirmed by molecular study of the immunoglobulin and T-cell receptor gene organization, as previously described.

Chromosome analysis. Chromosome spreads from leukemic cells were prepared and subjected to G-banding, as previously reported.

Southern blotting. DNAs were digested with restriction enzymes, run on a 0.8% agarose gel, and subjected to the Southern blotting procedure as previously described. Each sample was digested with BglII and BclI and additional digestions (EcoRI, BamHI, HindIII, KpnI) were performed in cases showing unusual patterns.

The probes used in this study were (a) a 1.2 kb HindIII-BglII genomic fragment corresponding to the 3' end of the bcr region (probe A in Fig 1, reference 4); (b) a 450 base pair (bp) EcoRI-PstI cDNA fragment corresponding to bcr exons mapping 5' to the position where the breakpoint on chromosome 22 normally occurs (probe B in Fig 1, reference 6); (c) a 162 bp Sau 3A fragment obtained from the 5' part of the bcr cDNA (probe C in Fig 1, reference 23); and (d) a 1.5 kb BamHI cDNA fragment corresponding to bcr exon 1 and subsequent exons (probe D in Fig 1, reference 24).

By using probe A, the breakpoint on chromosome 22 can be precisely localized if it falls within the region usually rearranged in CML. However, in the majority of cases, this probe only visualizes the rearrangement on chromosome 22, which is involved in the pathogenesis of the leukemic process. Therefore, all filters were rehybridized with a cDNA probe (probe B in Fig 1) containing bcr exons mapping immediately 5' to the bcr region, and thus, left on the Ph' chromosome by the t(9;22) translocation. Bcr probes C and D were used in cases in which the breakpoint could not be localized with either probe A or B.

RNA preparation and Northern blot analysis. Total RNA was obtained from leukemic cells by the guanidine-HCl extraction method and 10 μg were run on a 7% formaldehyde-1% agarose denaturing gel, blotted onto nitrocellulose filters, and hybridized to a nick translated c-abl cDNA probe. Hybridization was carried out at 42°C in 50% formamide and 10% dextrane sulfate. After stringent washing, the filters were exposed to x-ray films for various periods of time.

RESULTS

Table 1 summarizes the molecular data obtained.

CML in chronic and accelerated phase. Sixty-six of 80 cases (82%) presented rearrangements within the classic bcr region (defined as major breakpoint cluster region [mbcr]), as detected by probes A and B. The remaining cases differed in several ways.

1) In seven samples, the rearrangement was detected with probe B (specific for bcr sequences on the Ph' chromosome) and not with probe A (specific for bcr sequences on the Ph chromosome) or probe A or B.

Table 1. Summary of the Southern Blot Findings Obtained in Different Ph' Positive Hematologic Malignancies

<table>
<thead>
<tr>
<th>Ph' positive CML — chronic and accelerated phases (n = 80)</th>
</tr>
</thead>
<tbody>
<tr>
<td>66 cases show a rearrangement occurring in the mbcr region detectable with both probes A and B (Fig 1)</td>
</tr>
<tr>
<td>7 cases show a rearrangement occurring in the mbcr region detectable only with probe B (deletions of bcr sequences on the derivative chromosome 9q+)</td>
</tr>
<tr>
<td>2 cases show breakpoints on chromosome 22 mapping between 9 and 12 kb upstream to the mbcr region (segments 1 and 2, Fig 1)</td>
</tr>
<tr>
<td>1 case shows a breakpoint on chromosome 22 mapping at least 16 kb downstream to the mbcr region (segment 4, Fig 1)</td>
</tr>
<tr>
<td>4 cases still lack a precise definition of the breakpoint position on chromosome 22</td>
</tr>
</tbody>
</table>

CML in Ph' positive blast crises of CML (n = 17)

8 are myeloid cases
9 are lymphoid blast crises

All cases show a rearrangement occurring within the mbcr region.

Ph' positive Acute Leukemias

ALLs (n = 8)

4 cases show a rearrangement occurring within the mbcr region
1 case shows a breakpoint on chromosome 22 mapping at least 2 kb upstream the mbcr region (segment 3, Fig 1)
3 cases show breakpoints on chromosome 22 outside the mbcr region

AMLs (n = 2)

1 case shows a rearrangement occurring within the mbcr region
1 case shows a breakpoint position on chromosome 22 mapping outside the mbcr region
BCR REARRANGEMENTS IN PH' POSITIVE LEUKEMIAS

Fig 2. Southern blotting analysis of the bcr locus showing (lanes 1) DNA from a CML patient showing the typical rearrangement occurring within the mbcr region and detected by probe A and B (abnormal bands indicated by arrows); (lanes 2) a case showing abnormal bands only with probe B, but not with probe A. The enzyme used is BclI and the probes used are indicated below the lanes. The same pattern was observed with different restriction enzymes, excluding a possible comigration between normal and abnormal bands. The intensity of the hybridization signal with probe A is compatible with a deletion of the sequences corresponding to the probe.

translocated to chromosome 9) (Fig 2). This finding could be due to the loss of mbcr sequences translocated to chromosome 9q+, or to the loss of the entire chromosome. This is known to occur in the K562 cell line26 and in other CMLs.27 In two of our cases, a 9q+ derivative chromosome was clearly detectable, suggesting the presence of a deletion. By contrast, all rearrangements detected with probe A could also be seen with probe B. This allows us to conclude that probes corresponding to bcr sequences remaining on chromosome 22 are more suitable for molecular analysis of CML.

(2) In two cases, the breakpoints on chromosome 22 were located upstream to the mbcr region. One case (Table 2, patient B.F. for clinical parameters) showed normal BglII, HindIII, BamHI, KpnI, and BclI fragments with both probe A and B, whereas an abnormal EcoRI fragment was clearly detectable with both probes (Fig 3, data not shown). This suggests the presence of a rearrangement between the EcoRI and the BclI restriction sites, respectively 12 and 10 kb 5' to the mbcr area (segment 1 in Fig 1). The presence of a polymorphic EcoRI site could be excluded because the abnormal EcoRI fragment was absent from the DNA of a Ph’ negative lymphoblastoid cell line derived from the B lymphocytes of the same patient by Epstein-Barr virus (EBV) infection.

In the second case (Table 2, patient C.R. for clinical parameters), abnormal fragments were visible with EcoRI and BclI, but not with the other restriction enzymes (Fig 3, data not shown). This suggests that the rearrangement occurred within the BclI-BamHI fragment located 8 kb 5' to the mbcr region (segment 2 in Fig 1). Therefore, in these two cases the fused bcr/c-abl genes must lack five bcr exons normally present in classically rearranged CMLs.

(3) Five CML cases (6% of the whole series: G.M., C.V., M.L., B.G., and S.I. in Table 2), were negative for rearrangements with either probe A or B. Based on the intensity of the hybridization signals, the occurrence of deletions involving probe A- and B-homologous sequences seemed unlikely. Therefore, we decided to study these cases with two cDNA probes corresponding to other portions of the bcr gene. One short cDNA probe covered part of two small bcr exons located approximately 16 kb 3' to the mbcr region (probe C, Fig 1). These exons belong to a region of bcr that has a high degree of homology with three other bcr-related genes (bcr 2, bcr 3, and bcr 4) located on chromosome 22.23 Using this cDNA probe, four HindIII fragments of different molecular size are visible on Southern

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>Splen (cm)</th>
<th>WBC (x 10^9/L)</th>
<th>Platelets (x 10^9/L)</th>
<th>Response to Therapy</th>
<th>Survival (mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B.F.</td>
<td>M</td>
<td>51</td>
<td>3</td>
<td>97</td>
<td>340</td>
<td>Complete</td>
<td>18†</td>
</tr>
<tr>
<td>C.R.</td>
<td>F</td>
<td>42</td>
<td>2</td>
<td>180</td>
<td>818</td>
<td>Partial</td>
<td>28#</td>
</tr>
<tr>
<td>G.M.</td>
<td>F</td>
<td>40</td>
<td>15</td>
<td>120</td>
<td>480</td>
<td>Complete</td>
<td>36†</td>
</tr>
<tr>
<td>C.V.</td>
<td>M</td>
<td>48</td>
<td>8</td>
<td>40</td>
<td>600</td>
<td>Complete</td>
<td>86†</td>
</tr>
<tr>
<td>M.L.</td>
<td>F</td>
<td>74</td>
<td>4</td>
<td>28</td>
<td>1,020</td>
<td>Complete</td>
<td>36†</td>
</tr>
<tr>
<td>B.G.</td>
<td>M</td>
<td>64</td>
<td>ND</td>
<td>50</td>
<td>168</td>
<td>Complete</td>
<td>26#</td>
</tr>
<tr>
<td>S.I.</td>
<td>F</td>
<td>37</td>
<td>10</td>
<td>180</td>
<td>154</td>
<td>Complete</td>
<td>42††</td>
</tr>
</tbody>
</table>

*Below left costal margin.
†WBC at diagnosis.
‡Considered in terms of hematological remission.
§Months from diagnosis.
||Case with a bcr rearrangement 5' to the mbcr region.
||Patient deceased.
#Patient alive on April 15, 1988.
**Case without detected bcr rearrangements.
††Case with a bcr rearrangement 3' to the mbcr region.
‡‡Patient treated by allogeneic bone marrow transplantation.

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blots, the smallest one (9 kb) corresponding to bcr 1. One of the five cases (M.L., Table 2), showed a rearrangement with this probe (Fig 4, lane 4), and consequently, a lower hybridization signal of the bcr 1-specific 9 kb HindIII fragment. Therefore, in this case the breakpoint is located approximately 16 kb downstream to the mbcr region (segment 4, Fig 1). Another case (S.I., Table 2) also presented a 9 kb HindIII fragment of low intensity (lane 2, Fig 4). However, in this one an abnormal fragment could not be detected. Digestion with EcoRI again confirmed rearrangement in patient M.L., but provided no evidence of rearrangement in any other case (data not shown).

The second cDNA probe (probe D, Fig 1) covers the 5' part of the bcr gene. No rearrangements were seen with this probe (data not shown). However, this does not exclude the presence of a rearrangement in the first intron of the bcr 1 gene, which is very large and almost unexplored by cDNA probes because of its size and of a peculiar clustering of the most common restriction sites around the flanking exons.

Expression studies were performed on two of these patients (M.L. and B.G., Table 2). Patient M.L., who has a rearrangement in the 3' end of the bcr gene, reveals an abnormal c-abl transcript similar in size to that expressed by the K562 cell line (data not shown). Since the 3' bcr breakpoint would determine the addition of only two small exons to the bcr/c-abl coding region, such a small size difference in the mRNA could not be detected by Northern blotting. By comparison, patient B.G., whose breakpoint position is still undefined, apparently expresses only the two normal c-abl transcripts (data not shown).

It was interesting to investigate whether the clinical behavior of the variant cases (although low in number and heterogeneous) differed from that of the CML patients displaying the classical molecular defect. Their clinical features (Table 2) and prognosis did not appear worse compared with those generally observed in CML.

Ph' positive acute leukemias. With respect to chronic phase CMLs, a higher percentage of Ph' positive acute leukemias presented chromosome 22 breakpoints outside the mbcr region. One of the two AMLs and three of the eight ALLs studied lacked any detectable bcr rearrangement, and one of the remaining ALLs showed a rearrangement occurring between the KpnI and the BclI sites located, respectively, 4.50 kb and 2.50 kb upstream to the BglII site delimiting the mbcr region (segment 3, Fig 1). This case is very similar to another previously described. All other cases were similar to classical CML.

CML blast crisis. All blast crisis CMLs revealed a breakpoint on chromosome 22 within the mbcr region, as observed in the majority of the chronic phase cases. In six CML cases evolved in myeloid blast crisis, DNA from both phases were analyzed. No variation in the DNA restriction...
patterns was observed during the progression of the disease on chromosome 22 or on chromosome 9 (data not shown).

**DISCUSSION**

The original aim of our study was to test the possibility that a switch in the molecular defect on the Ph' chromosome could cooperate to the progression of CML into blast crisis. To this purpose, we focused our attention on the type of bcr/c-abl rearrangement present on chromosome 22. Since Ph' positive ALLs with a variant breakpoint on chromosome 22 express an abnormal P190 c-abl protein associated with the acute leukemia phenotype,15-18 the progression from chronic to acute phase in CML could be due to the occurrence of this variant defect, as a second event superimposed on the classical bcr/c-abl rearrangement. Our data on the bcr/c-abl rearrangement present in different phases of CML do not support this hypothesis, although they do not rule it out definitively. In fact, no variations in the bcr/c-abl restriction patterns were observed in six cases studied during chronic phase and blast crisis, where the type of rearrangement was found to be of the classical bcr type. Nevertheless, we should keep in mind that the hybrid bcr/c-abl locus can only be partially explored with the probes currently available.

Molecular changes, if present, could be restricted to small mutations with more functional than structural consequences, or determine alternative RNA splicings. Recent data on the structure of the normal c-abl locus and its transcripts implicate alternative RNA splicings. Recent studies on the c-abl transcripts and protein expression in blast crisis could help to verify this hypothesis.

In addition, it must be pointed out that the study of a large series of CMLs and of other Ph' positive hematologic malignancies has revealed the existence of new and interesting types of rearrangements.

While the heterogeneity of the breakpoints on chromosome 22 in Ph' positive ALLs has already been reported by several groups,15-13 the fact that one of two Ph' positive AMLs studied by us lacked a bcr rearrangement, confirms a recent report24 and suggests that bcr rearrangements outside the mbcr region, rather than being specific for ALL, may also occur in myeloid Ph' positive acute leukemias.

Perhaps the most surprising result of our study was the observation that the degree of variability of the molecular defect present on the Ph' chromosome in CML is higher than previously reported.33 Three CML cases showed a rearrangement upstream (two) and downstream (one) to the mbcr region, whereas in four others the breakpoint on chromosome 22 still needs to be localized. Recently, Ph' positive CML cases lacking a mbcr rearrangement have also been reported by other groups.24 Thus, although with a minor incidence, a heterogeneity similar to that present in Ph' positive acute leukemias seems to occur also in CML. Apparently, the CML patients with the variant molecular defects on chromosome 22 do not show a different clinical course, compared with those bearing the classical bcr/c-abl rearrangement. This molecular heterogeneity further complicates the relationship existing between the clinical phenotype, the type of bcr/c-abl rearrangement present on chromosome 22, and possibly, the type of abnormal c-abl protein expressed.

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Variability of the molecular defects corresponding to the presence of a Philadelphia chromosome in human hematologic malignancies

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