Detection of Major bcr-abl Gene Expression at a Very Low Level in Blood Cells of Some Healthy Individuals

By C. Biernaux, M. Loos, A. Sels, G. Huez, and P. Stryckmans

The major bcr-abl fusion gene is presently seen as the hallmark of chronic myeloid leukemia (CML) and presumably as the cause of its development. Accordingly, long-term disappearance of bcr-abl after intensive therapy is considered to be a probable cure of CML. The nested reverse transcriptase-polymerase chain reaction (RT-PCR) provides a powerful tool for minimal residual CML detection. The RT-PCR was optimized by (1) increasing the amount of total RNA involved in the reverse transcription reaction to correspond to total RNA extracted from 10⁶ cells, (2) using a specific abl primer in this reverse reaction, and (3) reamplifying 10% of the RT-PCR product in nested amplification. This optimized RT-PCR permitted us to detect up to 1 copy of RNA bcr-abl synthesized in vitro, mixed with yeast RNA in an equivalent quantity to 10⁶ white blood cells (WBCs). Using this highly sensitive RT-PCR during the follow-up of CML patients, a signal was unexpectedly found in healthy controls. Therefore, a systematic study of the possible expression of bcr-abl RNA in the WBCs of healthy adults and children and in umbilical cord blood was undertaken. It showed the presence of bcr-abl transcript in the blood of 22 of 73 healthy adults and in the blood of 1 of 22 children but not in 22 samples of umbilical cord blood.

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THE PHILADELPHIA (Ph) chromosome characteristic for chronic myeloid leukemia (CML) can also be seen in acute lymphoblastic leukemia (ALL). In CML, it is found in marrow cells of approximately 95% of patients. Among ALL patients, its frequency is estimated to be 3% to 5% in children and around 15% to 25% in adults. This Ph chromosome derives from a reciprocal translocation between chromosome 9 and chromosome 22, ie, (t(9;22)(q34;q11). It has never been shown in normal subjects. In all Ph+ CML and in approximately one third of Ph+ ALL, the breakpoint on the chromosome 22 is kb, the major breakpoint cluster region (Mbcr). In two thirds of Ph+ ALL, the breakpoint on the chromosome 22 is also situated in the bcr gene but 5' to the Mbcr. It is therefore, by opposition, called the minor breakpoint region (mbcr).

The M bcr-abl mRNA transcripts are formed by the junction of bcr exon b2 and/or b3 to abl exon 2 (b2a2 and b3a2, respectively). This junction results in a bcr-abl fusion protein of 210 kD molecular weight (P210) implicated in the development of hematologic disorders, including leukemias similar to human CML or ALL, in mice carrying the human Ph+ CML. Allogeneic bone marrow transplantation (BMT), presently the most effective curative treatment of CML, may ultimately result in permanent eradication of the bcr-abl rearranged cell clone and cure of CML. Conversely, the appearance of bcr-abl or its long-term persistence after BMT is often followed by disease recurrence. Therefore, several techniques, such as reverse transcriptase-polymerase chain reaction (RT-PCR), were used to detect the presence of residual bcr-abl fusion messenger RNA. Several groups using a nested RT-PCR technique capable of detecting from 1 copy/10⁶ cells up to 1 copy/10⁷ cells showed, after BMT, the persistence of bcr-abl expression in blood and/or marrow of some CML patients in hematologic and cytogenetic remission, a situation usually evolving into an hematologic relapse. To improve the follow-up of such patients, we decided to increase as much as possible the sensitivity of the RT-PCR. Using the optimized nested RT-PCR described below, we observed, as had other investigators, sporadic residual expression of bcr-abl mRNA in blood cells of BMT patients in complete cytogenetic remission (data not shown). During the follow-up of CML patients who were treated using BMT, an RNA sample from the white blood cells (WBCs) of a healthy subject was included as a control factor. This publication was supported in part by “Oeuvre Belge du Cancer” training grant. M.L. received financial help from 7.45.12.91 TELEVIE. RNA extraction. Total cellular RNAs extracted from peripheral blood cells from patients and healthy individuals were prepared using a modification of the method previously described. cDNA synthesis. cDNA synthesis was performed using an optimized protocol described by Perkin Elmer/Cetus GeneAmp RNA PCR Kit (Emeryville, CA). Four to 10 μg of total RNA with 40
pmol of 3′ specific primer (c-ab1 a3) were used in the reverse transcriptase reaction. In certain cases, the amount of total RNA involved in the reverse transcription reaction was increased to be consistent with the total RNA extracted from 10⁶ cells (equivalent to approximately 100 mL of blood).

Polymerase chain reaction (PCR) amplification procedures. Because the highly sensitive PCR procedure is prone to false-positive results due to sample contamination, very strict conditions and controls were used to perform all experiments. The guidelines recommended and adopted by the Molecular Biology/BMT Study Group were adhered to.¹⁵,²⁰,³¹

The total amount of cDNA obtained from the reverse transcriptase reaction was submitted to the PCR amplification in a final volume of 100 μL using the sets of primers described in Table 1. The PCR run consisted of 35 cycles, each composed of 1 minute at 94°C, 1 minute at 62°C, and 1 minute at 72°C, preceded by a hot start of 2 minutes at 94°C and followed by 5 minutes at 72°C. For nested amplification, 10% of the first PCR reaction product was involved into the second PCR run, as described above. As a control of the quality of RNAs, cDNA synthesis and PCR were performed in parallel with primers detecting the abl RNA (Table 1).

Estimation of the minimal level of detection of the nested PCR. The minimal level of detection of the nested PCR was estimated using as a reference a bcr-abl RNA synthesized in vitro. A 395-bp bcr-abl PCR product characterized by the presence of exon 3 of the bcr gene obtained from a CML patient in chronic phase was cloned in the EcoRV site of the plasmid bluescript. Thirteen copies of the bcr-abl 395-bp fragment corresponding to the bcr-abl junction was transcribed from this plasmid using T7 polymerase (GIBCO BRL, Grand Island, NY). A decreasing quantity of this RNA was diluted in yeast RNA (negative control) and submitted to nested RT-PCR, as described under Fig 1.

The nested RT-PCR products corresponding to these different dilutions of RNA bcr-abl were hybridized with radioactive complementary bcr-abl RNA. With their respective counted radioactivity, a chart was made that showed an exponential shape that is linear in semilogarithmic form for low concentrations from 1 to 50 copies. In parallel, samples from healthy individuals were submitted to the same cycles of amplification. The amount of amplified DNA obtained in these different samples was estimated by counting the radioactivity in each band after Southern blotting and hybridization with a specific radioactively labeled bcr-abl probe. By reference to the amount of radioactivity obtained in different concentrations of our internal control and the chart, the number of bcr-abl RNA copies in the samples could be estimated. In that way, the relative amount of the bcr-abl mRNA present in total RNA from WBCs could be estimated.

Detection of amplified DNA. Aliquots (10 μL) of the reaction products of RT-PCR were electrophoresed in Tris/borate/EDTA (TBE) buffer, at 100 V for 1 hour at 4°C on 2% agarose gel staining with ethidium bromide.

Statistical methods. The Fisher exact test (two-tailed), χ² for linear trend (χ²) and the overall χ² (χ²) tests were used.

RESULTS

In the experimental conditions described above, RT-PCR showed the equivalent of 1 copy of the bcr-abl RNA in 10⁶ cells within the internal control reference (Fig 1). The analysis of blood samples from healthy individuals with this sensitive method showed in some of them a low, but clear, expression of bcr-abl mRNA.

To assess the frequency of M-bcr-abl expression in healthy individuals, a total of 95 blood samples from normal subjects (regrouped according to age) and 22 samples of UCB were examined and analyzed at random by sets of experiments. As shown in Table 2, no expression of bcr-abl RNA was detected in UCB. In the children's WBCs, only 1 sample was positive when 4 μg of total RNA was involved in the RT-PCR reaction. For adults, a low expression of bcr-abl was found in 22 of the 73 cases examined. These results were obtained with either 4 and/or 10 μg samples of total RNA (Fig 2). A significant difference was seen when comparing 44 samples from UCB and children with 73 samples from adults (P = .001; Table 3). A significant difference was still seen (P = .002) when making the same type of

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Table 1. Oligonucleotide Primers and Probes

<table>
<thead>
<tr>
<th>Code</th>
<th>Sequence From 5' to 3'</th>
<th>Use</th>
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<tbody>
<tr>
<td>c-ab1 a3</td>
<td>5' TGGTATTAGTACCGACGAGG 3'</td>
<td>3' Primer reverse run</td>
</tr>
<tr>
<td>mcr b2</td>
<td>5' GAAGAAGTGGTTGCAGAGCTTCC 3'</td>
<td>5' Primer PCR first run</td>
</tr>
<tr>
<td>c-ab1 a2 bis</td>
<td>5' GGCCCAACATATACGGTCACAGC 3'</td>
<td>3' Primer PCR second run</td>
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<td>mcr b2 bis</td>
<td>5' TGAAACCTGACGTGTCACAGCAT 3'</td>
<td>5' Primer PCR second run</td>
</tr>
<tr>
<td>c-ab1 a1</td>
<td>5' GGCTGCAAATCCAGAAGGGCCTG 3'</td>
<td>3' Primer PCR abl control</td>
</tr>
</tbody>
</table>

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Fig 1. Estimation of the minimal level of detection of the nested PCR using as a reference a bcr-abl RNA synthesized in vitro. Lane 1, 10⁶ copies; lane 2, 10⁵ copies; lane 3, 10⁴ copies; lane 4, 75; lane 5, 50; lane 6, 25; lane 7, 20; lane 8, 10; lane 9, 5; lane 10, 1; lane 11, 0.1; lane 12, negative control (yeast RNA); lane 13, RNA 10⁶ copies without reverse transcriptase; lane 14, negative control of the two steps of RT-PCR, no RNA; lane 15, 1 μg of 1-kb ladder GIBCO products.
comparison but taking in to consideration only the 103 cases tested at 4 mg (P = .002) or the 31 cases tested at 10 mg of total RNA (P = .028). When considering all age subgroups, the χ² for linearity trend was also significant (P = .017), indicating a tendency for the anomaly to increase with age.

In 18 of these 23 positive cases, the bcr-abl PCR product observed had the b3a2 junction corresponding to the residual signal found by others in the majority of CML patients who received transplants (for 1 individual, this product was sequenced and shown a typical b3a2 junction). An amplification product corresponding to the smallest bcr-abl mRNA b2a2 lacking the exon 3 of bcr was found in 3 positive individuals, including the positive child sample. In 4 cases, the expression of both types of M-bcr-abl mRNAs was observed. The relative amount of the bcr-abl mRNA present in the WBCs of healthy individuals generally ranged from 5 to 20 copies/5 x 10⁶ to 10⁸ WBCs or greater.

**DISCUSSION**

The present observation that normal individuals may present very small quantities of bcr-abl RNA in their blood cells is most probably linked to the use of a very sensitive method. To detect the equivalent of 1 copy of bcr-abl in 10⁸ cells in such an RT-PCR experiment, the following specific steps were followed: (1) the amount of total RNA involved in the reverse transcriptase reaction was increased from 1 μg (the usual maximum amount used by most investigators) to 4 or 10 μg (this step seems to be the major cause for augmenting the method sensitivity), (2) a specific abl primer was used in the reverse transcriptase reaction, (3) the whole product of this reaction was submitted to PCR amplification, and (4) 10% of the product of the RT-PCR product was reamplified in a specific nested amplification, a quantity much higher than that usually taken by other groups.

Because a highly sensitive PCR procedure is prone to false-positive results caused by contamination of the sample, very strict conditions and controls were used to perform all experiments. (1) We adhered to the guidelines recommended and adopted by the Molecular Biology/BMT Study Group. (2) Several specific procedures were followed: samples were manipulated in a specific laminar flow oven, RNA extraction and RT-PCR were performed in a different room of the building, and all locations in which manipulations were performed were exposed to UV light between each experiment for a period of at least 20 hours. (3) RNA extraction and nested RT-PCR were repeated with a complete set of new reaction products in another laboratory elsewhere in Belgium in which CML RNA samples had never been manipulated before. (4) The RT-PCR products obtained from healthy individuals have been sequenced directly on the amplified DNA according to the method described in the protocol of DYNABEADS M-280 Streptavidin (DYNAL AS, Oslo, Norway). It showed that this amplified DNA product corresponded to a typical bcr-abl fragment with a clear sequence junction between bcr exon 3 and abl exon 2 (junction b3a2). (5) To rule out a possible error of the reverse transcriptase caused by the formation of artificial heteroduplexes between the bcr and the abl mRNAs that could serve as a template for the reverse transcriptase, the RNA preparations were heated at 90°C for 5 minutes before retrotranscription. (6) The same RNA samples were also retro-transcribed with a thermostable reverse transcriptase working at 72°C after heating the RNA sample at 90°C (RTTh DNA polymerase; Perkin Elmer Cetus, Norwalk, CT). It should be noted that the different samples were manipulated at random in all steps of the analysis; nevertheless, positive and negative bcr-
abl samples were found side by side in a same experiment, thus ruling out a possible intersample contamination. Despite the use of these stringent conditions, the presence of bcr-abl expression was observed in all samples from healthy individuals that had initially been found to be positive.

The systematic study of blood samples from 117 normal subjects disclosed 23 positives cases among them. This observation appears to be age-dependent; indeed, in subjects from the 0 to 12 years of age group, only 1 of 44 was positive, significantly less than the 22 of 73 in the 20 to 80 years of age group \( (P = .001) \). The significant difference between newborn and children as against adults not only raises an intriguing biologic question but also brings an additional strong argument against the hypothesis of all the positive cases being only artefacts due to the high sensitivity of the method. Finally, it can be seen that the difference between newborns and children group and the adults group remains highly significant when considering either only the results obtained with 4-\( \mu \)g RNA samples \( (P = .002) \) or even the few obtained with 10 \( \mu \)g \( (P = .028) \). This finding rules out the possibility that the low level of positivity in the 0 to 13 years of age group was due to blood samples smaller than those of adults because all technical conditions were similar for children and adults.

Some of the negative and positive samples have been reanalyzed using an identical procedure in two independent laboratories. Eleven samples of RNA of normal adults were manipulated at random using exactly the same procedure previously described. The results confirm the presence of bcr-abl RNA in 4 of 11 samples of normal adult individuals.

From the use of this highly sensitive method, it is concluded that the bcr-abl fusion gene may occur in healthy individuals at a frequency much higher than would be expected if it merely represented early detection of CML or ALL. However, the observation that the age of healthy subjects influences the probability of bcr-abl positivity is in keeping with the observation (1) that Ph CML, extremely rare in children, is seen almost exclusively in adults; and (2) that Ph ALL with M-bcr-abl representing less than 3% of childhood ALL reaches 10% to 20% in adult ALL,\(^4\) with a tendency to increase with adult age. These parallels might suggest a putative link with the pathogenesis of Ph\(^+\) leukemias.

The high frequency of healthy showing the presence of the bcr-abl in RNA in their blood cells is surprisingly reminiscent of the recent observation that t(14:18) \( (q32;q21) \) chromosomal translocation or Bcl2 mutation is commonly present in the peripheral blood cells of patients without lymphoma and even normal individuals (up to 50% of them) and increase in frequency with age.\(^2,3\). The effects of age for bcr-abl as for Bcl2 could be explained by the fact that, in adults, there have been more cell divisions and therefore a greater chance of accumulating genetic lesions.

By analogy with CML and Ph\(^+\) ALL, it is hypothesized that the bcr-abl gene in healthy subjects could occur in 1 hematopoietic cell able to generate a clone of cells, the magnitude and the survival of which must depend on the self-renewal capacity (stemcellness) and the degree of differentiation of the original translocated cell. With the bcr-abl clones detected in healthy subjects obviously being small, it is deduced that the first progenitor in which the \( (9;22) \) translocation appeared probably represents a more differentiated one than in CML or ALL and consequently that the clone it generates might be prone to die out by normal terminal differentiation. This also represents a likely explanation for the disappearance of the signal in 2 cases observed so far.

These results could have important implications for the use of PCR method to monitor minimal residual disease in CML.

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