Immunologic Characterization of the Tumor-Specific \textit{bcr-}abl Junction in Philadelphia Chromosome-Positive Acute Lymphoblastic Leukemia

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Philadelphia (Ph')-positive acute lymphoblastic leukemia (ALL) is highly associated with two forms of chimeric \textit{bcr-}abl proteins: \textit{P190bc'-ab'} and \textit{P210bc'-ab'}. Whereas \textit{P210bc'-abl} also occurs in chronic myeloid leukemia, \textit{P190bc'-abl} is uniquely expressed in Ph'-positive ALL. As a consequence, \textit{P190bc'-abl} is preemminently a tumor-specific marker in leukemic cells of ALL patients. Because \textit{P190bc'-abl} is composed of the normal \textit{bcr} and \textit{abl} proteins, the major part of the \textit{P190bc'-abl} molecule comprises nontumor-specific determinants. The joining region between \textit{bcr} and \textit{abl}, newly generated during the Ph' translocation, is exclusively a tumor-specific epitope on the \textit{P190bc'-abl} molecule. Therefore, only antibodies against the \textit{bcr-}abl joining region will detect the tumor-specificity of \textit{P190bc'-abl}. In this study a polyclonal antiseraum, termed BP-ALL, was raised against a synthetic peptide corresponding to the \textit{bcr-}abl junction in \textit{P190bc'-abl}. The reactivity of BP-ALL with native \textit{P190bc'-abl} derived from a Ph'-positive ALL cell line (TOM-1) was tested using immunoprecipitation analysis. BP-ALL reacted highly specifically with \textit{P190bc'-abl} but not with \textit{P210bc'-abl} isolated from chronic myeloid leukemia cell lines. Peptide inhibition studies further confirmed the fine specificity of BP-ALL. Our data indicate that the tumor-specific \textit{bcr-}abl junction domain is exposed in an antigenic fashion on the \textit{P190bc'-abl} molecule.

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The Philadelphia (Ph') chromosome, a minute chromosome 22, is found in 90% of all patients with chronic myeloid leukemia (CML). Therefore, this chromosome is regarded as the cytogenetic hallmark of CML. However, the Ph' chromosome has also been reported to occur in some patients with other types of leukemia. Thus, in the leukemic cells of 2% to 3% of all patients with acute myeloid leukemia, the Ph' chromosome can be detected. Moreover, in childhood acute lymphoblastic leukemia (ALL), a 2% to 10% incidence is reported, whereas in adult ALL the Ph' chromosome is found to be the most frequent chromosomal aberration, with an incidence of 20% to 30%.

Although the Ph' chromosomes in CML and acute leukemias are cytogenetically indistinguishable, molecular analyses have shown important differences. In all cases the Ph' chromosome is the result of a reciprocal translocation between chromosomes 9 and 22. Here, the \textit{abl} oncogene has moved from chromosome 9 into the \textit{bcr} gene on chromosome 22. On the \textit{bcr} gene two regions are defined in which breakpoints occur. These regions are termed major breakpoint cluster region 1 (M-\textit{bcr}-1) and minor breakpoint cluster region 1 (\textit{m-bcr}-1). In all Ph'-positive CML patients the \textit{abl} oncogene has moved from chromosome 9 into M-\textit{bcr}-1, localized in the middle of the \textit{bcr} gene on chromosome 22. The result of this particular translocation is a chimeric \textit{bcr-}abl gene, encoding a 8.5-kilobase (kb) messenger RNA (mRNA) that is translated into a 210-Kd protein, P210\textit{bc'-abl}.

With respect to the Ph' translocation process, Ph'-positive ALL patients are heterogeneous: 50% of adults and 10% of children with Ph'-positive ALL show the same \textit{bcr-}abl rearrangement as found in CML. Accordingly, these patients express P210\textit{bc'-abl}. In contrast, a P190\textit{bc'-abl} protein is demonstrated in the other group of ALL patients, including almost all cases of childhood ALL. We have previously shown a different \textit{bcr-}abl rearrangement in such patients. Here, breakpoints were noticed in the first intron of the \textit{bcr} gene, termed \textit{m-bcr}-1. The first \textit{bcr} exon (\textit{e1}) is then spliced to exon 2 of the \textit{abl} gene (termed \textit{a2}), resulting in a 7.0-kb \textit{bcr-}abl mRNA. Therefore, in the chimeric P190\textit{bc'-abl} protein the e1-a2 \textit{bcr-}abl junction is present.

Because P210\textit{bc'-abl} as well as P190\textit{bc'-abl} are expressed exclusively in malignant cells in CML and ALL, these proteins are by definition tumor-specific. However, P210\textit{bc'-abl} and P190\textit{bc'-abl} are composed of parts of \textit{bcr} and \textit{abl} molecules, which are, as such, normal cellular proteins. The only tumor-specific determinant on the chimeric proteins is formed just in the joining region between \textit{bcr} and \textit{abl}. Recently we have shown that the \textit{bcr-}abl junction in P210\textit{bc'-abl} is antigenically exposed on this protein.

In this study we investigate the expression of the e1-a2 \textit{bcr-}abl junction in P190\textit{bc'-abl} in cell lines and in leukemic cells of a Ph'-positive ALL patient. Our data also indicate that the \textit{bcr-}abl junction in P190\textit{bc'-abl} can be recognized by antibodies. These experiments indicate that the tumor-specific joining regions in the \textit{bcr-}abl fusion proteins are new immunologic markers for CML and ALL diagnosis.

MATERIALS AND METHODS

Cell Lines

TOM-1 is a cell line derived from a Ph'-positive ALL patient. The cell line was kindly provided by Dr M. Okabe (Sapporo, Japan). K562 and BV173 are cell lines derived from patients during blast crisis of CML. Cells were cultured in RPMI medium supplemented with 10% fetal calf serum, 100 \(\mu\)g/mL penicillin, and 60 \(\mu\)g/mL streptomycin.
Patients

Patient L. is a 52-year-old and patient K. is a 44-year-old male ALL patient. Both patients exhibited a standard Philadelphia translocation, t(9,22)(q34;q11). In both patients the diagnosis precursor B-ALL was based on immunologic and hematologic criteria obtained at presentation. Blast cells were isolated from peripheral blood by Ficoll-Hypaque centrifugation. After cryopreservation using a controlled freezing apparatus (Planer Biomed, Sunbury-on-Thames, UK), the cells were stored under liquid nitrogen.

Peptide Synthesis, Purification, and Conjugation

Peptides were synthesized using solid-phase synthesis as described previously. Purification of the peptides was performed by gel filtration and reverse phase high performance liquid chromatography. A cysteine residue was added to the amino terminus during synthesis. Peptides were crosslinked through free sulfhydryl groups to the carrier protein chicken γ globulin (CGG) using m-maleimidobenzoyl-sulfosuccinimide-ester (MBS).

Antisera, and Purification of Immunoglobulins (Igs)

Antiserum BP-ALL. Flemish rabbits (MBL-TNO, Rijswijk, The Netherlands) were primed intracutaneously with 250 μg protein complex consisting of peptide a1-a2 coupled via MBS to CGG, emulsified in an equal volume of complete Freund adjuvans (CFA). Rabbits were boosted twice after intervals of 4 weeks; the first time with 250 μg of protein in CFA, the second time with the same dose in incomplete Freund adjuvans. Fourteen days after the last boost, rabbits were bled and sera were collected.

Anti-bcr antiserum. Anti-bcr antiserum is a polyclonal rabbit antiserum directed against the amino terminal part of the bcr protein. This antiserum was prepared as described previously.

The Ig fraction of both antisera was purified by precipitation with 16% Na2SO4 (final concentration). The precipitates were dissolved in phosphate-buffered saline and desalted using Sephadex G-25M columns (Pharmacy, Uppsala, Sweden).

Immunoprecipitation and Protein Kinase Reaction

Immunoprecipitation and protein kinase reaction were performed as described previously, except that, in the present study, the tyrosine kinase reaction was performed for 10 minutes at 30°C instead of 10 minutes at 37°C.

Amplification of cDNA by the Polymerase Chain Reaction (PCR) Method

Total RNA was extracted according to the LiCl method followed by cDNA synthesis by reverse transcriptase. The cDNA amplification was performed using the PCR as described previously. For the amplification, bcr primers from exon e1 and b2 and an abl primer from exon a3 were used. In Fig 1 the primers on the respective cDNA molecules are shown. Twenty-four cycles of the PCR were performed using Taq polymerase. The samples were electrophorized, and transferred to nylon filter (zeta probe). Filters were hybridized to 32P end-labeled oligonucleotides, spanning the e1-a2, and b2-a2 b3-a2 junctions. After hybridization the blots were washed and exposed to Fuji XR films (Tokyo, Japan).

RESULTS

Peptides Corresponding to the bcr-abl Junction in P190bcr-abl

Based on the previously published nucleotide sequence, a peptide was synthesized corresponding to the bcr-abl junction in P190bcr-abl. The peptide sequence was chosen in such a way that a short peptide, probably one antigenic determinant, with optimal hydrophilicity pattern was selected. This peptide, termed SP e1-a2, consists of the following amino acids:

\[
\text{cys-ala-phe-his-asp-ala-glu-ala-leu-gln-}
\]

As a control peptide b3-a2 (SP b3-a2) was constructed corresponding to the b3-a2 joining region in the CML-specific protein P210bcr-abl. SP b3-a2 has the following amino acid sequence:

\[
\text{cys-lys-gln-ser-ser-lys-ala-leu-gln-arg-pro}
\]

Amino-terminal cysteine residues were added to both peptides to provide a coupling site for carrier molecules.

Because fusion of the bcr and abl genes in both P190bcr-abl and P210bcr-abl occurs in a coding triplet, the glu in SP e1-a2 and the second lys in SP b3-a2 are newly generated by the translocation process. Figure 1 schematically shows the respective chimeric bcr-abl RNAs and proteins as occurring in ALL and CML. All fusion proteins share the abl carboxy terminal amino acids, but differ at the amino terminal bcr sequence. As a consequence the synthetic peptides also differ at their amino terminus and have the same abl terminal amino acids.
cDNA Analysis of the bcr-abl Joining in TOM-1 Cells

TOM-1 cells were used as a source of P190bcr-abl protein. First, presence of P190bcr-abl mRNA comprising the e1-a2 bcr-abl junction in our TOM-1 cells had to be confirmed. To this purpose the highly sensitive PCR technique was performed. The bcr-abl cDNA was amplified and hybridized to oligonucleotides specific for the e1-a2, b2-a2, and the b3-a2 joining in the chimeric cDNA.

As shown in Fig 2, a 298-base pair (bp) fragment, specific for the e1-a2 joining, was amplified in the TOM-1 cells (lane 3, e1-a2 probe). As expected, this fragment was not amplified in both CML cell lines K562 and BV173. However, a 394-bp fragment specific for the b3-a2 joining was found to be present after amplification in K562 cells (Fig 2, lane 1, b3-a2 probe), while a 319-bp fragment, specific for a b2-a2 joining, was detected in BV173 cells (Fig 2, lane 2, b2-a2 probe).

Antibody Binding to bcr-abl Chimeric Proteins

Antiserum BP-ALL was raised against SP e1-a2. Reactivity of BP-ALL with native bcr-abl chimeric proteins P190bcr-abl and P210bcr-abl in different cell lines was tested with an immunoprecipitation assay followed by an autophosphorylation reaction. As shown in Fig 2, TOM-1 cells contain mRNA comprising the e1-a2 bcr-abl junction. However, to confirm that this mRNA was indeed translated into protein, we searched for the presence of P190bcr-abl in TOM-1 cells. Fig 3 shows that P190bcr-abl can be precipitated from these cells using an antiserum directed against the amino terminal side of the bcr protein (lane 2). Two CML cell lines, K562 and BV173, showed presence of P210bcr-abl after immunoprecipitation with the same anti-bcr antisemur (Fig 3, lanes 4 and 6).

Next, we tested the reactivity of antiserum BP-ALL with P190bcr-abl. Incubation of BP-ALL with a lysate of TOM-1 cells resulted in a clear precipitation of P190bcr-abl (Fig 4, lane 1). In contrast, BP-ALL did not precipitate P210bcr-abl from either K562 or BV173 (Fig 4, lanes 2 and 3). These results imply that antiserum BP-ALL specifically recognizes the e1-a2 junction domain in P190bcr-abl.

We performed peptide blocking studies to confirm this notion. We first incubated BP-ALL with cognate peptide SP e1-a2 or, as a control, with a peptide corresponding to the b3-a2 bcr-abl junction. Next, TOM-1 lysate was added. As shown in Fig 5, preincubation of BP-ALL with cognate peptide SP e1-a2 prevented precipitation of P190bcr-abl completely (lane 2). However, precipitation of background bands was not inhibited. Preincubation of BP-ALL with SP b3-a2, absorbing antibodies directed against a2, had no effect on the immunoprecipitation (lane 4). This observation strengthens the notion that P190bcr-abl is precipitated by antibodies in
serum BP-ALL that are specifically directed against the e1-a2 junction.

Precipitation of P190

Finally, we investigated whether antiserum BP-ALL precipitated P190 from leukemic cells from a Ph'-positive ALL patient. Peripheral blood leukocytes of patient L were enriched by Ficoll-Hyapac centrifugation. Next, blast cells were lysed and incubated with BP-ALL. The immunoprecipitation was followed by the autophosphorylation assay. Figure 6 shows a clear precipitation of P190 from patient L (lane 2). Moreover, analogous to the chimeric proteins in the cell lines, the P210 protein from patient K was not recognized by BP-ALL (Fig 7, lane 3). This indicates that the e1-a2 joining region also in leukemic cells of a Ph'-positive patient antigenically is exposed on the P190 protein.

DISCUSSION

Chromosomal abnormalities in ALL are frequently observed and have prognostic significance. Particularly, the presence of the Philadelphia chromosome in ALL is of clinical importance. Ph'-positive ALL is associated with a lower remission rate and an overall worse prognosis than Ph'-negative ALL. Especially in childhood ALL the Ph' chromosome implicates a bad prognosis. It is obvious that for clinical diagnosis antibodies directed against the tumor-specific bcr-abl joining will be useful tools.

Recently we described the development of a polyclonal antiserum directed against the b2-a2 bcr-abl junction in P210. Now we report on the production of an antiserum, BP-ALL, specifically recognizing the e1-a2 bcr-abl joining region in P190. The specificity of the polyclonal antiserum, raised against a synthetic peptide corresponding to the e1-a2 junction, was tested in an immunoprecipitation assay, followed by an autophosphorylation. TOM-1 cells...
were used as a source of Ph'-positive cells. These cells are derived from a Ph'-positive patient by Okabe et al.19 Since the exact bcr-abl rearrangement in TOM-1 was not described, we performed the highly sensitive PCR technique to verify which bcr-abl junction occurred in TOM-1 cells. In these experiments we amplified cDNA from TOM-1 cells comprising the el-a2 bcr-abl junction, indicating a break-point in the m-bcr-1. Therefore, presence of P190' in these cells could be expected. We confirmed this notion by immunoprecipitation of P190' from TOM-1 cells using an antiserum directed against the amino-terminus of the bcr gene.

We subsequently showed that antiserum BP-ALL, raised against a peptide corresponding to the el-a2 junction, reacted in a highly specific way with the native protein P190' in TOM-1 cells. We argue that P190' is precipitated by antibodies in BP-ALL that specifically recognize the el-a2 junction. Antibodies reacting with el and/or a2 sequences only do not play a role, since no other bcr-abl chimeric proteins, b2-a2 and b3-a2 P210' , which also contain el and a2 sequences, were immunoprecipitated by BP-ALL. Moreover, the reaction of BP-ALL with P190' was specifically inhibited by blocking with cognate peptide. Thus, precipitation of P190' was completely abrogated after preincubation of BP-ALL with peptide el-a2. Preincubation with a related peptide SP b3-a2, which eliminates antibodies from BP-ALL directed against a2 sequences, had no effect on the immunoprecipitation of P190'. These observations provide strong evidence that the tumor-specific bcr-abl junction el-a2 is expressed in an antigenic way on the P190' molecule.

The el-a2 joining region in P190' in leukemic cells of a Ph'-positive ALL patient is similarly exposed as in the TOM-1 cell line. This observation confirms the idea that the el-a2 epitope can be used as an immunologic marker for Ph'-positive ALL.

The data presented in this report have implications for ALL diagnosis and classification. Here we have demonstrated the antigenic expression of the tumor-specific el-a2 bcr-abl junction in P190'. In general, antibodies directed against this determinant will be valuable tools in ALL diagnosis and detection of minimal residual disease after clinical treatment, such as chemotherapy and bone marrow transplantation. However, some Ph'-positive ALL patients express P210', just as in CML. It has been suggested that these patients are, de facto, CML patients in lymphoid blast crisis without or with a very short chronic phase. Ph'-positive ALL patients expressing P190' would then be de novo ALL patients. Without necessarily subscribing to this hypothesis, it is of interest to investigate whether Ph'-positive ALL with P190' differ from the cases of Ph'-positive ALL expressing P210' in terms of clinical features like prognosis, response to therapy, and survival. To this purpose a large group of ALL patients has to be studied. The development of well-defined reagents for immunocytochemistry, such as monoclonal antibodies directed against the tumor-specific bcr-abl junction in P190' and P210', is therefore invaluable for accurate diagnosis of ALL and CML.

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