TUMOR-SPECIFIC PROTEINS IN HUMAN CANCER

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van Denderen, Adriana Cornelia

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TUMOR-SPECIFIC PROTEINS IN HUMAN CANCER

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Promotor : Prof. dr. W. van Ewijk

Overige leden : Prof. dr. J.J.M. van Dongen
Dr. G. Grosveld
Prof. dr. C.J.M. Melief

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CHAPTER 1

General introduction

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Chapter 1

General introduction

1 Introduction

Normal cells grow, divide, communicate, and differentiate in a coordinated fashion. These highly complex processes are regulated by the programmed expression of different genes. It is generally assumed that tumor formation originates from alterations in genes involved in the control of cell proliferation. To get more insight in the process of tumorigenesis, many studies have focused on the detection of such altered genes followed by the elucidation of the function of the genes involved. Because chromosomal aberrations occur frequently in human cancer, they were suspected to form the basis of the alterations in the genes. Therefore, the earliest studies aimed at the identification of chromosomal breakpoints, were based on known cytogenetic aberrations, such as translocations. In this way, a considerable number of genes has been detected in which structural aberrations occur. The ultimate purpose of these studies was to elucidate the function of the proteins encoded by the altered genes, their role in tumorigenesis and the potentiality to be used as targets for specific tumor-therapy.

Aberrant genes and proteins are also highly important from a diagnostic point of view, since they are only expressed in tumor cells. Therefore, the malignant cells distinguish themselves from normal cells by the presence of these particular, *tumor-specific* genes. As a consequence, aberrant proteins encoded by these genes are also tumor-specific and as such excellent phenotypic tumor-markers, called *tumor-specific proteins* (*TSPs*).

Based on their type of structural alteration, TSPs can be classified in two groups:

- fusion-point TSPs, which are newly formed protein structures, generated by a fusion of amino acid chains, which are normally contiguous to each other;
- TSPs generated by point mutations within genes.

1.1 Fusion-point TSPs

Fusion-point TSPs are generated either by *chromosomal translocations*, *chromosomal insertions* or *inversions* or by *internal deletions*. In most instances, as a result of a *chromosomal translocation*, *insertion* or *inversion* two genes are disrupted and translocated into each other, resulting in a chimeric, tumor-specific gene, encoding hybrid, tumor-specific proteins. As shown in Figure 1A, a chimeric protein consists of parts of the normal counterparts involved in the translocation. As such, these particular parts of the protein are non-tumor-

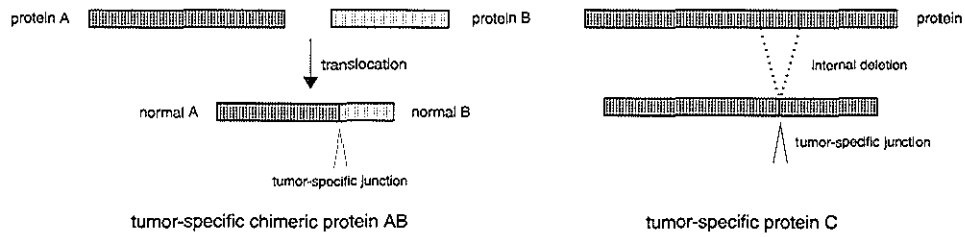

Figure 1A Schematic representation of a fusion-point TSP generated by a *translocation process*. The translocation process can be the result of a chromosomal translocation, insertion or inversion. The only tumor-specific part of the chimeric protein is formed by the junction of the non-tumor-specific proteins A and B.
Figure 1B Schematic representation of a fusion-point TSP generated by an *internal deletion*. The tumor-specific part of the protein is formed by the junction of the two parts of protein, which were not connected to each other in the normal protein.

specific. The only tumor-specific segment of this type of TSP is created by the junction at the fusion-point of two proteins.

Another mechanism of formation of a fusion-point TSP is by *internal deletion*. Here, due to deletion of an *internal* part of a gene, two non-contiguous segments of the same gene are fused (Figure 1B). As a result, the encoded TSP lacks a stretch of amino acids encoded by the deleted region. In this situation the newly formed tumor-specific site on the protein is generated by the fusion-point of the two parts of the protein which are normally separated from each other.

1.2 TSPs generated by point mutations.

A gene harboring a point mutation encodes a TSP which differs from the normally occurring protein in only one amino acid. Thus, the tumor-specific site on this particular TSP is formed only by the altered amino acid. As will be discussed in Chapter 2, in some genes the point mutations occur always at the same, highly distinct sites in all tumors involved. As a result the tumor-specific site on the TSP is identical in all tumors. However, other genes express point mutations which differ between individual tumors. As a consequence, also the tumor-specific sites on the TSP differ widely in the respective tumors.

1.3 Biological activity of TSPs

All TSPs, known thus far, share the property to be involved in growth regulation control, either as growth factor receptor, or as molecule involved in signal transduction or transcription regulation. However, the function of most TSPs is only provisionally described. In many cases the putative function of a TSP is deduced from sequence homologies and structural features which the

TSP in question shares with known proteins. For example, proteins involved in the signalling pathway may express specific GTP or ATP binding sites. Putative transcription factors may have very characteristic motifs similar to those implicated as DNA binding domains, or domains involved in protein-protein interactions, such as a helix-loop-helix motif, a leucine zipper or a Zn-finger motif (Jones, 1990; Nichols and Nimer, 1992).

As mentioned above, TSPs are supposed to be active in the regulation of cell growth. Therefore, it is commonly assumed that they play a role in *tumor-progression*. At the other hand, it is known that TSPs are also able to play a functional role in *tumor-rejection* by the immune system (reviewed by Urban and Schreiber, 1991). Since almost all TSPs are intracellular proteins, they cannot function in their native form as targets for the immune system. However, intracellular proteins are processed within the cell into peptides to be complexed to MHC molecules. Thus, peptides derived from a TSP will also associate with MHC molecules and will be presented to T cells at the cell membrane of the tumor cell. Both CD8-positive and CD4-positive T cells are described to recognize tumor-specific peptide sequences, in class I or class II MHC molecules on malignant cells, respectively (Jung *et al.*, 1991; Peace *et al.* 1992; Chen *et al.* 1992; Houbiers *et al.* 1993). As a result of the T-cell stimulation, the tumor may be eradicated either by the cytolytic activity of the CD8-positive T-cells, or by tumor infiltrating natural killer cells and macrophages, which are activated by cytokines produced by the CD4-positive T-cells.

When recognized by the immune system the respective tumor-specific proteins can be defined as **tumor-specific antigens** (TSAs).

2 Scope of the thesis

This thesis will give an overview of the TSPs which are presently known. Moreover, it will summarize our own experimental work on the immunologic characterization of the respective fusion-points within TSPs generated by the t(9:22)(q34;q11).

In *Chapter 2* the TSPs which are presently known, will be discussed. Special emphasis will be laid on the occurrence of the TSPs, their normal and altered function, and their putative role in tumor formation. From the diagnostic point of view, we will aim at the question whether the respective TSPs can be used as targets in tumor diagnosis. The TSPs generated by the t(9;22)(q34;q11) will be extensively discussed, since these particular TSPs form the basis of our experimental work as described in Chapter 3.

In *Chapter 3* our experimental work will be described on the immunologic characterization of three types of BCR-ABL fusion-points in TSPs generated by t(9;22)(q34;q11). We will focus on both the generation and on the application

of junction-specific antisera.

In the general discussion in *Chapter 4* the biological and clinical significance of the TSPs will be discussed, with special emphasis to their role in tumorigenesis, tumor rejection and tumor therapy. In relation to the results of our experimental work, we will address the relevance of applying tumor-specific antibodies in tumor diagnosis. Moreover, we will discuss techniques to improve the production of tumor-specific monoclonal antibodies.

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CHAPTER 2

Tumor-specific proteins

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Chapter 2

Tumor-specific proteins

In this chapter the tumor-specific proteins presently known will be discussed. These particular TSPs are summarized in Table 1, with reference to their underlying chromosomal aberration, their putative function and their occurrence.

In the text, first the fusion-point TSPs will be addressed. Subsequently, the TSPs generated by point mutations will be described.

2.1 Fusion-point TSPs

Fusion-point TSPs are generated by either chromosomal translocations, internal deletions, or chromosomal inversions and insertions. A common way by which TSPs have been discovered is as follows. First cytogenetic analysis of tumor cells revealed a chromosomal aberration, for example a translocation, which is strongly associated with a certain type of tumor. Next, the rearranged genes the genomic breakpoint are identified by molecular cloning, followed by the characterization of the complete genes involved, the mRNAs and the proteins. Based on sequence homologies and functional studies the identification of the TSP will be completed with respect to its biological function and its role in tumorigenesis. Since this type of work has been performed only over the last decade, the characterization of most TSPs is still at the level of DNA and mRNA analysis; hence protein studies are described for only a few TSPs.

In this section only those TSPs will be discussed which have been fully characterized at least at gene structure and mRNA composition.

2.1.1 Fusion-point TSPs generated by chromosomal translocations

The majority of the presently known TSPs is generated by chromosomal translocations. They will be discussed in a historical line based on their date of publication.

TSPs generated by t(9;22)(q34;q11): The Philadelphia translocation

The Philadelphia chromosome is the best known tumor-specific chromosomal aberration. In the next part of this thesis the Philadelphia chromosome 'from tumor-specific chromosome to tumor-specific protein' will be described.

The Philadelphia chromosome

In 1960 Nowell and Hungerford detected for the first time an abnormal, minute chromosome 22 in leukemic cells of patients with chronic myeloid leukemia (CML)(Nowell and Hungerford, 1960). Since this so called Philadelphia (Ph) chromosome was observed in more than 90% of all CML patients, it was consi-

chromosomal mechanism	genes involved	tumor-specific protein	(putative) function	observed in
reciprocal translocation				
t(9;22)(q34;q11)	*abl*(#9), *bcr*(#22)	BCR-ABL/ABL-BCR	tyr. kinase	CML, ALL, AML
t(1;19)(q23;p13.3)	*pbx1*(#1), *E2A*(#19)	E2A-PBX1	TF	pro-B-ALL
t(17;19)(q21-q22;p13)	*HLF*(#17), *E2A*(#19)	E2A-HLF	TF	pre-B-ALL
t(15;17)(q22;q12-21)	*pml*(#15), *RARα*(#17)	PML-RARα/ RARα-PML	TF	APL
t(11;17)(q23;q21)	*plzf*(11), *RARα*(#17)	PLZF-RARα	TF	APL
t(6;9)(p23;q34)	*dek*(#6), *can*(#9)	DEK-CAN	TF	AML
	dek(#6), *set*(#9)	SET-CAN	TF	AUL
t(4;11)(q21;q23)	*AF-4*(#4), *HRX*(#11)	HRX-AF-4/AF-4-HRX	TF	early pre-B-ALL
t(11;19)(q23;p13)	*HRX*(#11), *ENL*(#19)	HRX-ENL	TF	early pre-B-ALL
t(8;21)(q22;q22)	*ETO*(#8), *AML-1*(#21)	AML-1-ETO	TF	AML, M2
t(3;21)(q26;q22)	*EAP*(#3), *AML-1*(#21)	AML-1-EAP	TF	MDS, AML, CML
t(11;22)(q24;q12)	*Fli1*(#11), *EWS*(#22)	EWS-Fli-1/Fli-1-EWS	TF	Ewing sarcoma
t(12;22)(q13;q12)	*ATF-1*(#12), *EWS*(#22)	EWS-ATF-1	TF	malignant melanoma
t(12;16)(q13;p11)	*CHOP*(#12), *TLS*(#16)	TLS-CHOP	TF	myxoid liposarcoma
chromosomal inversion				
inv(16)(p13;q22)	*CBFß*(p13), *MYH11*(q22)	CBFß-SMMHC	?	AML, M4Eo
inv(10)(q11.2;q21)	*D10S170*(q21), *RET*(q11)	D10S170-RET	receptor	thyroid papillary carcinoma
chromosomal insertion				
ins(2;2)(p13;p11.2-14)	*rel*(#2)	REL-NRG	TF	B-cell lymphoma
unknown intrachromosomal rearrangement				
chr 1p32	*rlf*(#1), L-*myc*(#1)	RLF-L-MYC	TF	small cell lung cancer
chr 7p13-p12	*EGFR*	int. del EGFR	receptor	glioma
point mutation				
chr 1p13, 11p15.5, 12p12.1	*N-ras, H-ras, K-ras*	mutated P21	GTPase	various
chr 17q12-q13	*P53*	mutated P53	TF	various
chr 13q14	*Rb*	mutated P105-Rb	TF	prostate, bladder and small cell lung carcinoma
chr ?	*gsp*	α chain of G protein G_s	GTPase	pituitary tumors
chr ?	*gip2*	α chain of G protein G_{i2}	GTPase	endocrine tumors of ovary and adrenal cortex
chr 5q33-q35	*fms*	mutated CSF-1 receptor	receptor	AML and MDS

Table 1
Overview of tumor-specific proteins, with reference to the underlying chromosomal aberration, the genes involved, the (putative) function of the TSP and the disease in which the TSP can be expressed. All TSPs will be discussed in more detail in chapter 2. Abbreviations: TF: transcription factor; tyr. kinase: tyrosine kinase.

dered to be specific for this disease. However, later the Ph chromosome was also observed in acute leukemias. Two to five percent of all children with acute lymphoblastic leukemia (ALL) and 25-30% of all adult ALL patients harbor the Ph chromosome in their leukemic cells (Sandberg *et al.*, 1980; Priest *et al.*, 1980). Moreover, 2-3% of all patients with acute myeloid leukemia (AML) are Ph chromosome-positive (Whang-Peng *et al.*, 1970). Nevertheless, since the Ph chromosome only occurs in leukemic cells and never in normal cells, this chromosome is highly tumor-specific.

***Chronic myeloid leukemia** (CML) is a myeloproliferative disease arising from the pluripotent stem cell. CML is characterized by increased granulopoiesis, presence of immature myeloid progenitors in the peripheral blood, basophilia and hepato-and splenomegaly. Generally, the median age of the patients is 50-60 years. The disease shows a bi-phasic course, starting with a chronic phase which may last for 1-4 years. In the chronic phase leukocytosis combined with full maturation is observed. Chemotherapy or therapy with interferon α may control the chronic phase. Bone marrow transplantations are also performed in the chronic phase. The second phase or 'blast is characterized by a block in hematopoietic differentiation. Either immature lymphoid or myeloid blast cells are then present in the peripheral blood. Usually, patients in blast crisis poorly respond to therapy.*

***Acute leukemia** arises from a single transformed progenitor cell and is characterized by a high frequency of immature precursors in the bone marrow and the peripheral blood. Depending on the phenotype of the leukemic clone the leukemia will be classified as an acute lymphoblastic leukemia (ALL) or an acute nonlymphoblastic (ANLL) or myeloid leukemia (AML). This crude classification can be further specified following criteria based on either the morphology or the immunological phenotype of the malignant clone. Acute leukemia occurs at all ages. Except in myeloid leukemia, the acute leukemias have a peak of incidence in children around the age of two to three years. Due to the uncontrolled growth of the leukemic cells, growth and differentiation of the normal blood cells is disturbed. As a result patients suffer from anemia, hemorrhage and are highly sensitive for infections. In addition, leukemic cells may invade other tissues, such as the central nervous system, the testis, the skin and the eye. Therefore, without treatment patients die within weeks to month after diagnosis. Therapy of acute leukemias includes chemotherapy or bone marrow transplantation.*

Chromosome 9 and 22 breakpoints

The Philadelphia chromosome results from a reciprocal translocation between the chromosomes 9 and 22 (Rowley, 1973; De Klein *et al.*, 1982). Using chromosomal banding techniques, chromosomal breakpoints were found to occur within bands 9q34 and 22q11 as depicted in Figure 1 (Rowley, 1973). After establishment of a series of somatic cell hybrids, De Klein *et al.* (1982) demonstrated that the c-*abl* oncogene was translocated from chromosome 9q34 to chromosome 22q$^-$. Breakpoints in the c-*abl* gene were mostly found to occur scattered over a distance of approximately 200 kb 5' of exon 2 (termed a2), either within the first or the second intron (Figure 2) (Heisterkamp *et al.*, 1983; Leibowitz *et al.*, 1985; Grosveld *et al.*, 1986). In addition, an a small number of patients breakpoints were found to occur between exon 2 (a2) and 3 (a3) (Van der Plas

Figure 1 Schematic representation of the Philadelphia translocation, a reciprocal translocation between chromosome 9 and chromosome 22. As a result of the translocation a chimeric *abl-bcr* gene has been generated on the $9q^+$ chromosome and a chimeric *bcr-abl* gene has been generated on the $22q^-$ chromosome, the so called 'Philadelphia chromosome'.

et al., 1991; Chapter 3.4 of this thesis). The gene and the breakpoints on chromosome 22 were also identified (Groffen *et al*, 1984; Heisterkamp *et al*, 1985; De Klein *et al.*, 1986; Hariharan and Adams, 1987). This gene, termed *bcr* (breakpoint cluster region gene) comprises 18 exons. In contrast to breaks on chromosome 9, the breakpoints in *bcr* are clustered in two regions defined as the 'minor' breakpoint cluster region (m-bcr) and the 'major' cluster breakpoint region (M-bcr) (Hermans *et al.*, 1987; Hermans *et al.*, 1988; Kurzrock *et al.*, 1988; Gale and Goldman, 1988; Berger et al. 1990). In CML, almost all breakpoints occur in the M-bcr that spans 4 exons, b1-b4, corresponding to exons 12-15 of the gene (Figure 2). The respective breakpoints were detected either in either the intron between b2 and b3 or between b3 and b4. Breakpoints outside the M-bcr are extremely rare in CML (Selleri *et al.*, 1987; Selleri *et al.*, 1990; Van der Plas, 1991).

In ALL the majority of *bcr* breakpoints were found within the m-bcr region, which is localized between the first (e1) and the second (e2) exon of the gene (Figure 2). Within the population of ALL patients carrying a *bcr-abl* translocation 85% of all children and 68% of all adults show m-bcr breakpoints (Maurer et al., 1991). The remaining group of patients has CML-like breakpoints in the M-bcr, with equal chance of a b2/b3 or a b3/b4 breakpoint (Kurzrock et al., 1988; Berger et al., 1990; Maurer *et al.*, 1991).

As a result of the reciprocal translocation, parts of the *bcr* and *abl* genes are fused. Depending on the localization of the *bcr* breakpoints various chimeric genes can be generated. The hybrid gene on chromosome 22 consists of a 5' *bcr* head and a 3' *abl* tail. Besides the above mentioned exceptions formed by

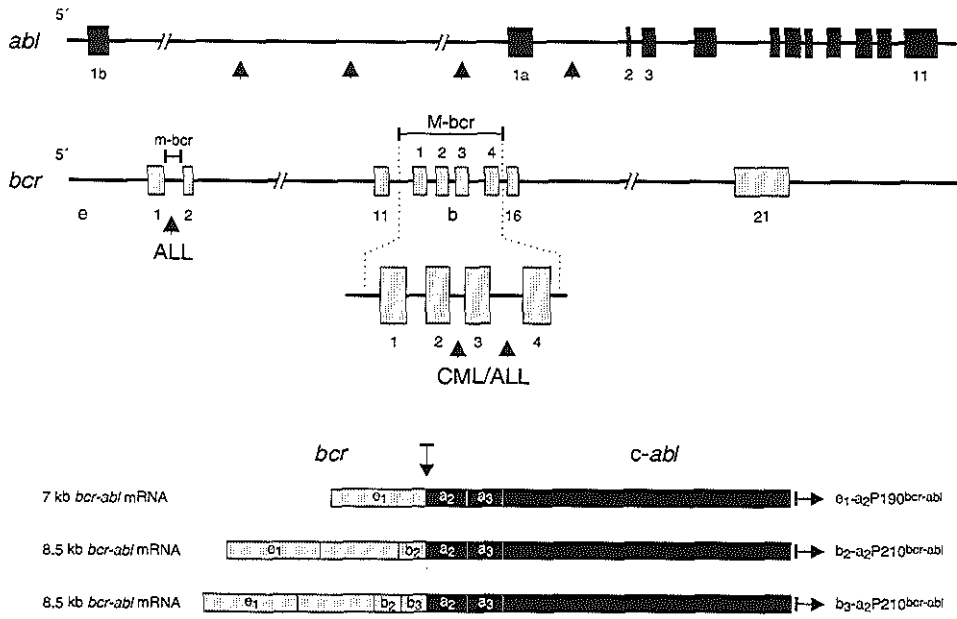

Figure 2 Schematic representation of the normal *abl* and *bcr* genes and of the chimeric *bcr-abl* mRNAs and proteins. Black boxes and black bars represent *abl* exons and *abl* derived mRNA and protein sequences, respectively. Dotted boxes and bars symbolize *bcr* exons and *bcr* derived mRNA and protein sequences, respectively.

a2/a3 breakpoints, the *abl* sequences are generally spliced to the *bcr* sequences through exon a2 after transcription, due to alternative splicing (Figure 2) (Shtivelman *et al.*, 1986; Grosveld *et al.*, 1986). This results in three chimeric mRNAs each comprising a different *bcr-abl* junction: a 7-kb mRNA comprising the e1-a2 junction (Hermans *et al.*, 1987), or 8.5-kb mRNA either with the b2-a2 or the b3-a2 junction (Figure 2) (Shtivelman *et al.*, 1986; Grosveld *et al.* 1986).

The *bcr-abl* fusion results in an in-frame fusion of the two open reading frames, which implies that the mRNAs are translated in functional hybrid proteins (Shtivelman *et al.*, 1985; Hermans *et al.*, 1988). Thus, breakpoints in the m-bcr give rise to a 190-kD protein, designated as e1-a2 $P190^{bcr\text{-}abl}$, whereas M-bcr breakpoints encode 210-kD proteins, either with the b2-a2 or the b3-a2 junction. Both these proteins are termed b2-a2 $P210^{bcr\text{-}abl}$ and b3-a2 $P210^{bcr\text{-}abl}$, respectively (Figure 2) (Ben-Neriah *et al.*, 1986b; Clark *et al.*, 1987; Chan *et al.*, 1987; Hermans *et al.* 1987; Clark *et al*, 1988). Both P210 molecules differ only 25 amino acids, encoded by the b3 exon, a difference too small to be detected on polyacrylamide gels.

In most CML and ALL patients usually one *bcr-abl* junction is found. However, simultaneous expression of two chimeric mRNAs has also been reported (Dobrovic *et al.*, 1988; Marcelle *et al.*, 1989; Lee *et al.*, 1989; Van der Plas, 1991; Maurer *et al.*, 1991; Chapter 3.4). This phenomenon may be explained either by bi-clonality of the leukemia, where each clone comprises its own junction or by alternative splicing, resulting in more than one mRNA. In Chapter 3.4 it will be described that, although two mRNAs are found only one chimeric protein is ultimately detected. It is not known whether this discrepancy reflects the actual situation, or whether it is caused by the difference in sensitivity of the used detection methods.

Several investigators have pointed at the question whether there is any correlation between type of junction and prognosis or response on therapy (reviewed by Secker-Walker and Craig, 1993; Kantarjian *et al.*, 1993; Mills, 1993). However, with respect to correlation the reports reviewed are not univocal, suggesting that there is no direct relationship.

Presence of the reciprocal *abl-bcr* transcript, encoded by the chimeric *abl-bcr* gene on chromosome $9q^+$ has also been reported (Stam *et al.*, 1985; Melo *et al.*, 1993a and b; Mackenzie *et al.*, 1993). Both in CML, chronic and acute phase, and in ALL the *abl-bcr* mRNA are described to occur. Depending on the localization of the breakpoint in the *abl* and *bcr* genes, all expected reverse recombinations between *abl* and *bcr* are detected: 1b-e2, 1a-e2, 1b-b3, 1a-b3, 1b-b4 and 1a-b4 (Figure 3) (Melo *et al.*, 1993a and b). However, in one third of the CML patients with rearranged *bcr-abl* transcript, the reciprocal *abl-bcr* mRNA expression was not detected, probably due to a deletion of the fragment (De Klein *et al.* 1986; Melo *et al.* 1993a). Therefore, it may be argued that

Figure 3 Schematic representation of the chimeric *abl-bcr* transcripts. Black bars represent *abl* derived sequences, whereas the dotted bars symbolize *bcr* derived sequences.

presence of the ABL-BCR protein, although not yet identified, is not essential for the generation of the leukemia.

Function of the normal BCR protein

Several different BCR proteins have been reported: 160-kD and 180-kD proteins by Stam *et al.* (1987), a 190-kD protein by Ben-Neriah *et al.* (1986a), and a 130-kD protein by Dhut *et al.* (1988). In addition to these proteins Li *et al.* (1989) showed evidence for the presence of 125-kD, 108-kD and 83-kD BCR proteins (Li *et al.* 1989). Antibody analysis showed that all BCR proteins share epitopes with the BCR aminoterminus of chimeric BCR-ABL proteins.

The origin of the multiple BCR proteins is not yet known, because in addition of the *bcr* gene involved in the Philadelphia translocation, 3 *bcr*-related genes were found (reviewed by Campbell and Arlinghaus, 1991). However, it is still under investigation whether the various BCR proteins are products of different genes, or whether they are generated by one gene which is transcribed to multiple mRNAs by alternative splicing. A combination of these two possibilities is also feasible (Campbell and Arlinghaus, 1991). Although the function of all the BCR proteins is not yet known, sequence homology with known protein kinases suggests that the BCR proteins are contain a serine/threonine kinase domain in their N-terminal moiety (Maru and Witte, 1991). In addition, in its C-terminal part BCR shows homology to the catalytic domain of the GTPase activating protein (GAP) for $P21^{rho}$, a RAS related protein (Diekmann *et al.*, 1991). Diekmann *et al.* (1991) found that BCR itself is a GAP protein for another RAS related protein, termed $P21^{rac}$. GAP proteins catalyze the transition of RAS proteins from their active, GTP bound state, to their inactive GDP bound form. GAP proteins control the rate of GTP hydrolysis and are negative regulators of the RAS signalling pathway. Thus, both the kinase activity and the homology of BCR with GAP proteins suggests that BCR proteins are involved in signal transduction.

Protein studies concerning the tissue distribution of BCR *proteins* are very limited in number. The characterization of the BCR proteins is performed in human cell lines of multiple origin. Dhut *et al.* (1988) showed presence of BCR P130 and P160 in various hematopoietic cell lines as well as in fibroblastic and neuroblastoma cell lines. The BCR proteins were mainly expressed in the cytoplasm.

Additional studies were aimed at the expression of BCR encoding *mRNA*. BCR mRNA is reported to be present in all so far tested human cells, such as cultured human cells of hematopoietic lymphoid, myeloid and erythroid origin, as well cultures of skin and bone marrow fibroblasts (Collins *et al.*, 1987). Thus, the expression of BCR is not limited to one cell type, but is expressed in a broad variety of cell types. This notion lead to the suggestion that BCR plays a general,

non-tissue-specific role in cell metabolism.

It is likely that the expression of BCR is inversely related with the maturation of normal myeloid cells: Wetzler *et al.* (1993) reported a high expression of BCR proteins in immature myeloblasts and promyelocytes, whereas the expression in mature granulocytes was weakly positive.

Function of the normal ABL protein

The 145-kD ABL protein ($P145^{abl}$) is encoded by the *abl* gene which is expressed as two distinctively sized mRNAs of 6.0 and 7.0 kb occurring in normal cells and tissues of both hematopoietic and non-hematopoietic origin (Ben-Neriah *et al.*, 1986b; Wang and Baltimore, 1983). It belongs to the family of non-receptor tyrosine kinases (Hunter and Cooper, 1985). The ABL protein occurs in two forms, differing in their N-terminal sequences which are encoded either by exon 1b or 1a (Figure 2) (Ben-Neriah *et al.*, 1986b). The type 1b protein is myristoylated on the N-terminal glycine providing a site for membrane association (Jackson and Baltimore, 1989). Most likely, the type Ia protein is not myristoylated. Van Etten *et al.* (1989) showed that the mouse homologue of the human type 1b-ABL localizes in two different cell compartments: *i.* in the cytoplasm associated with actin of the cytoskeleton and *ii.* to a much higher degree in the nucleus. Amino acid analysis revealed that in its C-terminal part the ABL protein harbors a nuclear localization signal, consisting of a pentalysine motif, which may account for the nuclear expression (Van Etten *et al.* 1989). The distribution of the alternate type ABL protein, i.e. the mouse homologue of the human 1a-ABL protein, is not yet known. However, presence of the pentalysine motif also in this particular ABL-type suggests localization in the nucleus.

The nuclear localization of ABL points to involvement of ABL in transcription regulation. Presence of a DNA binding domain in the C-terminal part of the protein supports this hypothesis (Kipreos and Wang, 1992). Both the localization of c-ABL in association with the cytoskeleton and in the nucleus might allow c-ABL mediated transduction of signals from cell-surface molecules directly into the nucleus.

The kinase function of the ABL protein is extensively studied. $P145^{abl}$ is a tyrosine kinase harboring so called 'SH2-SH3 domains' N-terminal of the kinase domain (Pawson, 1988). The SH2 (src-homology 2) domain has a positive regulatory influence on the kinase domain, enhancing its activity, whereas the SH3 (src-homology 3) region regulates the kinase domain negatively. Due to a balance between the SH2, SH3 and the kinase domain, $P145^{abl}$ has low tyrosine kinase activity (Jackson and Baltimore, 1989). Mutation or deletion of the SH3 domain triggers the activation of the tyrosine kinase. At the same time, the ABL protein has been shown to acquire transforming activity, suggesting that high

tyrosine kinase activity is necessary for transformation (Jackson and Baltimore, 1989). It has been hypothesized that the SH3 inhibiting function is mediated by other cellular proteins which bind to the SH3 sequences (Jackson and Baltimore, 1989). Actually, Ren *et al.* (1993) showed that proteins containing a proline rich stretch of 10 amino acids are able to bind to the c-ABL SH3 domain. Deletions in SH3, might prevent binding such proteins and result in loss of the kinase inhibiting function of the SH3 domain.

Function of the chimeric BCR-ABL proteins

The chimeric BCR-ABL proteins, e1-a2 $P190^{bcr\text{-}abl}$, b2-a2 $P210^{bcr\text{-}abl}$ and b3-a2 $P210^{bcr\text{-}abl}$, localize predominantly in the cytoplasm associated with actin filaments in the cytoskeleton (McWhirter and Wang, 1991; Wetzler *et al.*, 1993). This observation means that under the influence of the N-terminal BCR sequences the subcellular localization of c-ABL has changed from both a cytoplasmic and nuclear into an exclusively cytoplasmic localization.

More recently, McWhirter and Wang (1993) showed that there is a direct relationship between the association of the chimeric BCR-ABL proteins with the cytoskeleton and their transforming capability: mutations in the actin binding domain, as present the C-terminal ABL part of $P210^{bcr\text{-}abl}$, abolished binding of $P210^{bcr\text{-}abl}$ to the actin filaments. At the same time, the mutant $P210^{bcr\text{-}abl}$ proteins showed a reduced potential to transform Rat-1 fibroblasts in vitro. Thus, association of chimeric BCR-ABL proteins with the cytoskeleton is required for their transforming activity.

All three types of BCR-ABL chimeric proteins have an enhanced tyrosine kinase activity in comparison to the normal ABL protein (Konopka *et al.*, 1984; Clark *et al.*, 1987; Chan *et al.*, 1987). However, the complete ABL derived regulatory SH2-SH3 domains are present in the chimeric proteins, predicting low tyrosine kinase activity. Therefore, it has to be concluded that the SH2/SH3 mediated regulation of the tyrosine kinase activity is disturbed in the chimeric proteins by the replacement of 5' ABL sequences by BCR sequences. Data from Pendergast *et al.* (1993) show that BCR sequences encoded by the first exon of the *bcr* gene can bind to the ABL-SH2 domain. It has been suggested, that as a result of this binding, conformational changes in the chimeric protein are induced, through which the negative regulatory function of the SH3 domain might be switched off (Figure 4) (Sawyers, 1992). This hypothesis is supported by the finding that deletion of regulatory sequences in the SH3 domain of chimeric proteins in BCR-ABL-positive patients, does not influence the tyrosine kinase activity (Chapter 3.3).

Recently, Pendergast *et al.* (1993) showed BCR sequences, encoded by the first *bcr* exon does not exclusively bind to the ABL-SH2 domain but also to the SH2 domain of another SH2-SH3 containing protein, termed GRB-2. The GRB-2

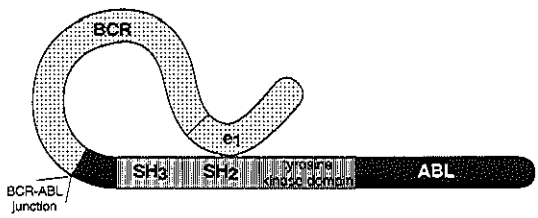

Figure 4 Model for disruption of the SH2-SH3 mediated regulation of the tyrosine kinase activity in chimeric BCR-ABL proteins. BCR derived e1 sequences bind to the ABL derived SH2 domain, inducing such conformational changes that the kinase inhibitory function of SH3 is switched off (according to Sawyers,1992).

protein has been described to link tyrosine kinase activity to activation of the RAS signalling pathway. Pendergast *et al.* (1993) showed that BCR-ABL chimeric proteins induce RAS activation through direct interaction with GRB-2. After mutation in the GRB-2 binding site of the BCR-ABL protein the RAS activation is abolished. Moreover, the mutant chimeric proteins have impaired capacity in in vitro transformation assays. This observation indicates that RAS activation is an essential component in the BCR-ABL induced transformation process.

Identical to the normal BCR and ABL proteins the expression of chimeric $P210^{bcr\text{-}abl}$ molecules in myeloid hematopoietic cells is inversely related to the degree of maturation. Bedi *et al.* (1993) described that the expression of $P210^{bcr\text{-}abl}$ mRNA and protein is absent or very low in primitive CML progenitors ($CD34^+$, $CD33^-$, $CD19^-$, $CD5^-$), although there is *bcr-abl* gene rearrangement. This may suggest that the expression is regulated at the transcriptional level. Based on these results it is likely that $P210^{bcr\text{-}abl}$ is expressed as a wave: absent or low in stem cell like CML progenitors, high in myeloblasts and promyelocytes and low in mature granulocytes.

In summary, replacement of aminoterminal ABL sequences by BCR sequences has the following consequences:
- BCR sequences are responsible for localization of BCR-ABL chimeric proteins exclusively in the cytoplasm associated with the cytoskeleton;
- presence of aminoterminal BCR sequences enhances the ABL tyrosine kinase and activates its transforming activity;
- aminoterminal BCR sequences are required for linking BCR-ABL activity to the RAS signalling pathway.

The role of chimeric proteins in leukemogenesis in vivo was tested in two systems. Heisterkamp *et al.* (1990) generated transgenic mice containing the $P190^{bcr\text{-}abl}$ encoding gene. Such transgenic mice developed myeloid or B-lymphoid

leukemia and died within 10-58 days after birth. At the same time, several other groups transplanted bone marrow cells in vitro infected with retrovirus comprising $P210^{bcr\text{-}abl}$ encoding DNA (Daley *et al.*, 1990; Elefanty *et al.*, 1990; Kelliher *et al.*, 1990). In these mice myelomonocytic, granulocytic and B-ALL leukemias were observed. These studies show that BCR-ABL chimeric proteins may directly induce leukemia and thus play a primary role in the generation of leukemia in vivo.

Chimeric DNA, mRNA and proteins as diagnostic, tumor-specific markers for ALL and CML

Chimeric *bcr-abl* DNA, mRNA and proteins are 'per definition' tumor-specific markers for CML and ALL, since they only occur in leukemic cells. Several techniques are now applied to detect these chimeric sequences at the DNA and mRNA level, such as Southern blot analysis, pulsed field gel electrophoresis and Northern blotting (Hooberman *et al.*, 1989).

The detection of tumor-specific, chimeric proteins in single leukemic cells using antibodies is another approach to diagnose Ph-positive CML and ALL. However, although the proteins as such are tumor-specific, they are composed by parts of the normal, non-tumor-specific BCR and ABL proteins. The only tumor-specific epitopes of the chimeric proteins are formed by the three respective BCR-ABL junctions, e1-a2, b2-a2 and b3-a2. The amino-acids on these particular sites are called *fusion-point epitopes*.

The experimental work as described in Chapter 3 of this thesis was focused on the three particular fusion-point epitopes in the chimeric BCR-ABL proteins: e1-a2, b2-a2 and b3-a2. The work aimed at the questions (*i*) whether it was possible to generate specific antibodies against the fusion-point epitopes and (*ii*) whether these particular antibodies could be used in diagnosis of Ph-positive CML and ALL.

TSPs generated by t(1;19)(q23;p13) and t(17;19)(q21;p13)

The reciprocal translocation t(1;19)(q23;p13.3) is the most frequently occurring chromosomal aberration in childhood pre-B-ALL: in 25% of all cases this particular translocation can be observed (Crist *et al.*, 1990). Two genes are involved in the translocation; on chromosome 1, a gene termed *pbx1*, (also called *PRL* (pre-B cell leukemia), and on chromosome 19 a gene called *E2A* (E2-box binding). Both genes encode transcription factors (Nourse *et al.*, 1990; Kamps *et al.*, 1990). As a result of the translocation, a chimeric *E2A-pbx1* gene is generated on chromosome 19, encoding chimeric E2A-PBX1 proteins. The hybrid protein shares functional domains of both parental proteins. The *E2A* gene, spanning at least 17 exons, encodes two transcription factors, E12 and E47, which are generated by alternative splicing of the *E2A*. The molecular mass of

these particular factors is probably 75 kD (Bain *et al.*, 1992). Both proteins contain a proline and glycine rich N-terminal transactivation domain, a leucine zipper and a basic helix loop helix (bHLH) domain, of which the latter is needed for sequence specific binding of DNA (Quong *et al.*, 1993). E12 and E47 differ from each other in their bHLH domain only (Kamps *et al.*, 1990; Murre *et al.*, 1989). Breakpoints within the *E2A* gene are clustered in a 3-kb region between exon 13 and 14 (Mellentin *et al.*, 1990). This implies that the transactivation domain and the leucine zipper, encoded by 5′ exon 13 sequences are still present in the chimeric E2A-PBX1 protein. In contrast, the bHLH encoded by sequences 3′ of exon 14 is lost and replaced by PBX1 sequences. The normal *pbx1* gene encodes 46.5-kD and 38.4-kD homeodomain proteins which are expressed in almost all tissues except in cells of the B and T cell lineage (Monica *et al.*, 1991). Breakpoints within the *pbx1* gene are scattered over a region of at least 50 kb, localized 5′ of the homeodomain (Mellentin *et al.*, 1990). Thus, in the t(1;19) positive leukemias the DNA binding homeodomain of PBX1, which is normally not present in B-cells, is transcriptionally activated by fusion to E2A sequences of E2A. In contrast, the reversed PBX1-E2A molecule, is never detected because in most patients the complete 1q⁻ chromosome is lost, or if present, the gene is not transcribed as it is under the control of the silent *pbx1* promoter.

Kamps *et al.* (1991) have identified five E2A-PBX fusion proteins: $P87^{E2A\text{-}Pbx1}$, $P85^{E2A\text{-}Pbx1}$, $P83^{E2A\text{-}Pbx1}$, $P77^{E2A\text{-}Pbx1}$ and $P72^{E2A\text{-}Pbx1}$, respectively. Two of them, $P85^{E2A\text{-}Pbx1}$ and $P77^{E2A\text{-}Pbx1}$, are most abundantly present in t(1;19) positive cells. The DNA's encoding these two proteins have been cloned (Kamps *et al.* 1990). Sequence analysis showed that both proteins have identical E2A-PBX1 fusion-points. It is yet unknown whether the other three E2A-PBX1 proteins have also the same fusion-point. It has been suggested that all five proteins are primary translation products of different, alternatively spliced transcripts which vary in their *pbx1* moiety (Kamps *et al.* 1991).

The role of $P85^{E2A\text{-}Pbx1}$ and $P77^{E2A\text{-}Pbx1}$ in oncogenesis has been studied in vitro and in vivo. In vitro both E2A-PBX1 proteins were found to be able to transform NIH3T3 cells (Kamps *et al.*, 1991). However, $P77^{E2A\text{-}Pbx1}$ was more potent in transformation than $P85^{E2A\text{-}Pbx1}$, suggesting the presence of regulatory elements in the 97-amino acid carboxyl terminus of $P85^{E2A\text{-}Pbx1}$, which is not present in $P77^{E2A\text{-}Pbx1}$. In vivo, the leukemogenic potential was tested in two ways. (*i*) In mice, tumors were obtained by transplantation of murine bone marrow cells infected in vitro with retrovirus harboring a *E2A-pbx1* gene encoding $P85^{E2A\text{-}Pbx1}$ (Kamps and Baltimore, 1993). The transplanted mice developed an acute myeloid leukemia. (*ii*) Transgenic mice were generated expressing the *E2A-pbx1* gene under the control of the immunoglobulin heavy chain enhancer (Dedera *et al.*, 1993). Although the *E2A-pbx1* gene is associated with B-cell lymphomas, these mice developed fatal T-cell lymphomas. Interestingly, in premalignant animals

a high number of apoptotic thymocytes was observed. Therefore, it has been suggested that the abnormal transcription factor E2A-PBX1 interacts both with genes involved in the control of cell proliferation and with genes regulating cell death. However, this hypothesis has to be confirmed. Nevertheless, in vitro suggest that *E2A-pbx1* is a transforming gene and the in vivo studies show that it is able to induce leukemia.

Since E2A-PBX1 proteins are only expressed in t(1;19) positive leukemic cells, they are 'per definition' tumor-specific proteins. However, also here, the only tumor-specific epitopes are exclusively formed by the amino acids at the E2A-PBX1 junction. In t(1;19) positive pre-B-ALL the majority of all patients express identical fusion-points at the mRNA level (Hunger *et al.* 1991). Recently, three cases have been reported to co-express an additional E2A-PBX1 mRNA, with an in frame insertion of 9 amino acids at the E2A-PBX1 fusion point (Izraeli *et al.*, 1993.) All these patients showed the same insertion. Nonetheless, because of the bad prognosis of t(1;19) positive pre-B-ALL (Crist *et al.*, 1990), immunodetection of the E2A-PBX1 proteins by using antibodies, specific for the regular E2A-PBX1 junction, will be of great diagnostic importance.

Interestingly, involvement of E2A has also been shown in t(17;19)(q21;p13)-positive-leukemias (Inaba *et al.*, 1992; Hunger *et al.*, 1992). Here, the chromosome 17 encoded protein, HLF (hepatic leukemia factor), has been characterized as a 295 amino acid protein with a putative molecular mass of 43 kD, abundantly transcribed in liver, but, like PBX1, silent in hematolymphoid cells. HLF shows homology with leucine zipper proteins and is probably involved in transcription regulation. In the three cases thus far reported the *E2A* breakpoints coincide exactly with the *E2A* breakpoints in t(1;19) (Inaba *et al.* 1992; Hunger *et al.* 1992). The *HLF* breakpoints are found to occur 5' of the leucine zipper domain. As a result, the predicted E2A-HLF chimeric protein comprises an aminoterminal E2A part and a HLF derived carboxyl terminus. However, depending on the patient, a 20-30 stretch of amino acids is inserted between the E2A and HLF sequences, thus causing heterogeneity in E2A-HLF junctions. Interestingly, the nucleotides encoding these amino acids originate from the flanking breakpoint introns of *E2A* and *HLF*. It has been suggested that these nucleotides are added as a result of terminal deoxynucleotidyl transferase activity since sequences in the E2A-breakpoint cluster region show homology to heptamer signal sequences (Hunger *et al.* 1992). It is also possible that the heterogeneity is caused by cryptic splice acceptor and donor sites in the respective introns (Hunger *et al.*,1992; Inaba *et al.* 1992).

The above mentioned 9 amino acid insertion in the chimeric E2A-PBX1 protein may be generated by the same process, suggesting that the shared E2A part may be responsible for the adoption of the additional amino acids (Izraeli *et al.*, 1993).

So far, the role of E2A-HLF in leukemogenesis has not been studied extensively. However, Hunger *et al.* (1992) show that the chimeric E2A-HLF protein has impaired DNA binding capacity in comparison with the wild type HLF, suggesting that the transcription of, yet unknown, target genes will be deregulated. This hypothesis is further supported by the fact that HLF is expressed ectopically in B lymphocytes, which may cause deregulation of normal regulatory systems in growth and differentiation of early B cells.

TSPs generated by t(15;17)(q22;q21) and t(11;17)(q23;q21.1)

The reciprocal translocation t(15;17)(q22;q21) is present in almost all cases of acute promyelocytic leukemia (APL) (Larson *et al.*, 1984). As a result of the translocation, the gene encoding the retinoic acid receptor α (RARα) on chromosome 17, and the gene called *pml* (promyelocytic leukemia) or *myl* (myelocytic leukemia) on chromosome 15 are disrupted (de Thé *et al.*, 1990; Kakizuka *et al.*, 1991; De Thé *et al.*, 1991).

Chimeric genes, encoding functional chimeric mRNA, are reported to be generated on both chromosome $15q^+$ and chromosome $17q^-$ (De Thé *et al.*, 1990; Kakizuka *et al.*, 1991; De Thé *et al.*, 1991; Chang *et al.*, 1992; Alcalay *et al.*, 1992). Molecular studies show that the chromosome 17q breakpoints are consistently localized within the second intron of the *RARα* gene (Biondi *et al.*, 1992). In contrast, within the *pml* gene on chromosome 15, three breakpoint clusters (bcr1-3) have been identified. Breakpoints in bcr1 are localized in *pml* intron 6; bcr2 breakpoints are found within *pml* exon 6, bcr3 breakpoints occur in *pml* intron 3. As a consequence, different *pml-RARα* junctions are generated on chromosome $15q^+$ (Biondi *et al.*, 1992; Pandolfi *et al.*, 1992). These types of *pml-RARα* junctions are consistent within the groups containing the bcr1 and bcr3 breakpoints. However, the *pml-RARα* junctions in bcr2 positive patients differ from case to case (Pandolfi *et al.* 1992).

Next to the breakpoints in these clusters there is additional heterogeneity due to alternative splicing of the *pml* moiety: Pandolfi *et al.* (1992) showed that in individual APL cases differently spliced *pml-RARα* transcripts coexist with one of the above mentioned fusion-points. This means that there are on the one hand three 'consistent' types of *pml-RARα* transcripts. On the other hand, there are multiple additional types of *pml-RARα* transcripts caused by alternative splicing of the 5' *pml* exons.

As a result of the above mentioned diversity, several PML-RARα chimeric proteins have been described. Pandolfi *et al.* (1992) detected in COS cells transfected with either the bcr1- or bcr2-type of transcript a chimeric PML-RARα of approximately 110 kD. A 90-kD chimeric protein was observed in bcr3-type transfected COS cells. The predicted molecular masses of the proteins encoded

by the transcripts, alternatively spliced in the 5' *pml* exons, vary from 83 kD to 100 kD.

Alcalay *et al.* (1992) investigated the heterogeneity of the reverse *RARα-pml* fusion on chromosome 17q⁻. Here, two types of *RARα-pml* junctions were found to exist. In bcr1 and bcr2 patients a junction between *RARα* exon 2 and *pml* exon 7 was demonstrated, whereas in bcr3 patients a fusion of *RARα* exon 2 and *pml* exon 4 was found to exist. In addition, also other chimeric transcripts were detected, which were found to be caused by alternative splicing of the 3' *pml* exons.

Thus, in t(15;17)-positive APL cells, a variety of chimeric RARα-PML and PML-RARα proteins can be expressed simultaneously. Their function in the generation of APL is still undefined. However, it has been suggested that the PML-RARα protein plays the most important role in leukemogenesis, since the *pml-RARα* rearrangement is always observed in t(15;17) positive APL, whereas the reversed form, *RARα-PML* is not always detected (Biondi *et al.*1991; Alcalay *et al.* 1992).

The normal RARα protein is a nuclear receptor (mol. mass 48 kD) which regulates gene expression after binding of retinoic acid. RARα is described to be involved in the control of terminal myeloid differentiation (Collins *et al.*, 1990). Although the definite function of normal PML is unknown, sequence analysis indicates that it is a putative transcription factor (mol. mass ~ 70 kD) (Kakizuka *et al.*, 1991;, De Thé *et al.*, 1991).

The function of the chimeric PML-RARα is still uncertain. However, the trans-activating activity of RARα is impaired by the substitution of aminoterminal PML sequences, which means that in the leukemic cells the normal signal transduction pathway of RARα is disturbed (De Thé *et al.*, 1991; Kakizuka *et al.*, 1991).

Recently, another RARα fusion product has been described, which is observed in a single APL patient with a t(11;17)(q23;q21.1) (Chen *et al.*, 1993). In this case, the RARα moiety is fused to a transcription factor, termed PLZF (promyelocytic leukemia zinc finger). Thus, the function of the normal RARα protein can be disturbed by fusion with various other transcription factors.

It is possible that the differentiation of promyelocytes, which may be normally induced by genes activated via RARα, is blocked in APL due to the impaired function of the RARα protein after fusion to another transcription factor. This hypothesis is supported by the observation that high dose retinoic acid, strongly stimulating the PML-RARα, given to APL patients, can overcome the leukemic state through induction of differentiation in the leukemic cells (Castaigne *et al.*, 1990). In addition, Grignani *et al.* (1993) showed that the myeloid precursor cell

line U937 lost the capacity to differentiate after transfection with *PML-RARα* cDNA. Moreover, the PML-RARα expressing U937 cells became highly sensitive to retinoic acid and showed a higher growth rate, reflecting the situation in leukemic APL cells.

Next to the deregulation of RARα in APL cells, most likely, the normal PML function is also disturbed as a result of the fusion with RARα. However, little is known about this issue. PML is localized in normal cells in subnuclear compartments corresponding to nuclear bodies, also termed 'PML oncogenic domains' (Kastner *et al.*, 1992; Weis *et al.*, 1994; Dyck *et al.* 1994). However, APL cells expressing both PML and PML-RARα showed a fine granular staining pattern using an anti-PML antiserum (Weis *et al.* 1994; Dyck *et al.* 1994). Interestingly, in the presence of retinoic acid a complete redistribution of the anti-PML staining was observed, resembling the situation in normal cells. These data suggest that the aberrant nuclear localization of PML and PML-RARα may play a role in the generation of APL.

Since the chimeric proteins are as such tumor-specific, they are in principle useful targets for antibody diagnosis using junction specific antibodies. Although a broad heterogeneity exists in PML-RARα junctions, always one of the bcr1, bcr2 or bcr3 comprising PML-RARα proteins is expressed in the individual APL cells. However, antibody diagnosis should be aimed at the bcr1 and bcr3 comprising proteins, because bcr2 breakpoints are heterogeneous within the group of patients.

TSPs generated by t(11;22)(q24;q12) and t(12;22)(q13;q12)

In more than 90% of all patients with Ewing sarcoma or related primitive neuroectodermal tumors, the reciprocal translocation t(11;22)(q24;q12) has been observed (Turc-Carel *et al.* 1984; Whang-Peng *et al.*, 1986). Recently performed molecular studies have shown that two genes are involved in this particular translocation: the *Fli-1* (Friend leukemia integration-site 1) gene on chromosome 11 and the *EWS* (Ewing Sarcoma) gene on chromosome 22 (Delattre *et al.*, 1992). *Fli-1* is a member of the *ETS* family of transcription factors (predicted mol. mass ~ 42 kD), whereas *EWS* encodes a putative RNA binding protein (predicted mol. mass ~ 65 kD). Both reciprocal transcripts were detected, although the derivative 11 transcript was expressed at a very low level (Delattre *et al.*, 1992). Moreover, since in some Ewing sarcomas the derivative 11 chromosome can be lost it is suggested that the derivative 22 fusion gene will be the most essential one for tumorigenesis of Ewing sarcoma.

In the EWS-Fli-1 protein (predicted mol. mass ~ 47 kD) encoded by the derivative 22 chimeric gene the RNA binding domain of the EWS protein, is

replaced by FLi-1 DNA binding domain. After PCR amplification four types of chimeric transcripts were detected. Heterogeneity may be generated either by variation in localization of the breakpoints or by alternative splicing (Delattre *et al.*, 1992; May *et al.*, 1993). Little is known about the function of these chimeric proteins. Using deletion mutants of EWS-Fli-1 in NIH3T3 transforming assays, May *et al.* (1993) demonstrated that the chimeric protein has transforming capacity and is probably an aberrant transcription factor, which activates ETS-responsive genes in an abnormal way.

Recently, other chimeric transcripts have been detected involving the *EWS* gene (Zucman *et al.*, 1993). From a cell line and from tumor material obtained from a patient with malignant melanoma of the soft parts carrying a translocation t(12;22)(q13;q12), chimeric transcripts were isolated comprising 5′ *EWS* sequences and 3′ sequences of a gene termed *ATF-1* (c-AMP-dependent transcription factor). The localization of the breakpoint within the *EWS* gene was found to be identical in both samples, but different from the breakpoint in Ewing sarcomas (Zucman *et al.*, 1993). Similar to the *Fli-1* gene, *ATF-1* encodes also a transcription factor of the ETS-family. As a result of the t(12;22) translocation, the RNA binding domain of the EWS protein is in this particular translocation replaced by the DNA binding sequences of ATF-1 (Zucman *et al.* 1993).

Therefore, it seems that the EWS protein can be activated after exchanging its RNA binding domain for various DNA binding domains, thereby losing its normal RNA binding function. As a consequence, it is possible that the regulation of protein translation is disturbed, through which tumor formation may occur. It is also likely, that as a result of the fusion of EWS to various ETS related genes, ETS target genes are abnormally activated, which may cause a tumor.

TSPs generated by t(6;9)(p23;q34)

The reciprocal translocation t(6;9)(p23;q34) is observed in a specific subtype of acute myeloid leukemia, termed M2/M4. This particular translocation is correlated with a poor prognosis. The incidence is very low. Since Rowley for the first time reported on this particular translocation in 1976, until now 51 patients have been described carrying the t(6;9) (Rowley and Potter, 1979; Von Lindern, 1992). Two genes are involved in the t(6;9) translocation: the *dek* gene on chromosome 6, and the *can* gene on chromosome 9. The translocation results in a *dek-can* chimeric gene encoding chimeric mRNA and chimeric protein (Von Lindern, 1992a) In both genes the breakpoints are clustered within one specific intron termed icb-6 and icb-9 (intron containing the breakpoints on chromosome 6 and 9, respectively). However, in one case of acute undifferentiated leukemia (AUL) *can* was coupled to another gene on chromosome 9, named *set* (Von Lindern *et al.*, 1992b; Von Lindern *et al.*, 1992c). In this case, the chromosome 9 breakpoint occurred also in icb-9, resulting in a chimeric *set-can* mRNA

comprising 3' *can* sequences fully corresponding to the chimeric *dek-can* message. This observation suggests that also CAN can be activated by at least two other genes.

The functions of the DEK, CAN and SET proteins are still under investigation. Their molecular masses are 43 kD, 220 kD and 32 kD respectively (Von Lindern *et al.* 1992a; Von Lindern *et al.*, 1992c). Sequence homology studies suggest that they could play a role in transcription activation. Immunofluorescence studies, using polyclonal antibodies showed that DEK is expressed in the nucleus, whereas CAN is mainly expressed in the cytoplasm (Von Lindern, 1992). Recently, Kraemer *et al.* (1994) have identified CAN as a nucleoporin, localized on the cytoplasmic side of the nucleopor complex. The chimeric DEK-CAN protein (mol. mass 165 kD) is localized in the nucleus. This localization corresponds with the putative transcription activation function of the chimeric protein.

Although the incidence of t(6;9) is very low, the poor prognosis, combined with the relatively young age (28) of the patients acquiring the disease, justify accurate diagnosis (Soekarman *et al.*, 1992). In this respect, Soekarman *et al.* (1992) showed that junction-specific PCR at the mRNA level is a reliable method to diagnose this group of patients. In principle, the immunological detection of the tumor-specific proteins using junction specific antibodies could be another approach. However, the frequency of patients carrying a t(6;9) translocation is too low to justify the production of junction-specific monoclonal antibodies.

TSPs generated by t(4;11)(q21;q23) and t(11;19)(q23;p13)

Cytogenetic studies showed that band 11q23 is frequently involved in a large group of leukemias, such as Hodgkin's and non-Hodgkin's lymphomas as well as childhood ANLL and ALL. (Abe and Sandberg, 1984; Raimondi *et al.*, 1989; Raimondi, 1993). Several different chromosomal loci may participate in translocation-mediated exchanges with band 11q23, for example in t(9;11)-(p22;q23), t(11;17)(q23;q25), t(11;19)(q23;p13), t(10;11)(p15;q23) t(6;11)(q27;q23) and in t(4;11)(q21;23). The latter translocation is the most common within this group. These observations suggested that a gene important in lymphoid-myeloid differentiation was localized in 11q23. Tkachuck *et al.* (1992) and Gu *et al.* (1992) reported contemporarily on the identification of the affected gene. This particular gene is a human homologue of the *Drosophila* thritorax gene, termed *HRX*, or *HTRX* (for human thritorax), *ALL-1* (for acute lymphoblastic leukemia), or *MLL* (for myeloid /lymphoid leukemia, or mixed lineage leukemia) (Tkachuk *et al.*, 1992; Cimino *et al.*, 1991; Ziemin *et al.*, 1991; Djabali *et al.*, 1992). The *HRX* gene spans approximately 100 kb, it contains at least 21 exons and encodes a protein with a predicted molecular mass of 431 kD. The protein is a putative transcription factor and contains two DNA binding domains: an aminoterminal "AT hook" and a zinc finger motif. Breakpoints occur

between these two domains.

Thus far, only the rearrangements in t(4;11)(q21;q23) and t(11;19)(q23;p13) have been extensively studied. In these particular translocations the chromosome 4 and 19 "acceptor" genes are known (Morissey *et al.*, 1993; Tkachuk *et al.*, 1992).

In the (4;11) translocation *HRX* is fused to the chromosome 4 derived gene called *AF-4* (for ALL-1 fused gene from chromosome 4) or *FEL* (Morissey *et al.* 1993). The normal function of AF-4, which is detected in both B- and T-lymphoid cell lines, has not yet been defined. The predicted molecular mass of AF-4 is 140 kD (Morissey, 1993). The chimeric HRX-AF-4 proteins (predicted molecular mass 240 kD) are probably heterogeneous due to the variety of breakpoints in both the *HRX* and the *AF-4* gene (Gu *et al.* 1992; Hilden *et al.*, 1993). Reciprocal *AF-4-HRX* fusion transcripts have also been detected (Gu *et al.*, 1992) However, at present it is not known which of the two types of chimeric proteins is the most important in tumorigenesis.

Chromosome 19 breakpoints have been reported to occur within a gene called *ENL*, encoding a predicted 62-kD protein (Tkachuk *et al.*, 1992). Since the chromosome 19 breakpoint is found to occur between the fourth and the fifth amino acids, almost the entire ENL is fused to HRX, resulting in a chimeric HRX-ENL protein with a predicted molecular mass of 217 kD. The normal function of ENL is not known and sequence homology studies so far have not shown any similarity with any other proteins (Tkachuk *et al.*, 1992).

Interestingly, both ENL and AF-4 comprise extremely serine/proline rich stretches which are entirely coupled to the HRX residues. Proline/serine rich domains have been associated with transcriptional activator proteins. Therefore, theoretically due to the fusion of the HRX derived DNA binding sequences to different proline/serine rich partners, the chimeric proteins interact inappropriately with DNA and act as abnormal transcription factors.

Hunger *et al.* (1993) analyzed several other translocations with respect to *HRX* rearrangements. They observed that, whatever translocation, all breakpoints are localized in a 8-kb region, spanning exons 5-11 of the *HRX* gene (Hunger *et al.*, 1993). This suggests that all chimeric proteins contain the same aminoterminal HRX moiety and that, identical to the above described fusion proteins, the HRX protein may be activated after fusion with multiple fusion partners. If this is true all fusion partners in the respective translocations are expected to share the serine/proline rich parts (as found in AF-4 and ENL) and to act as aberrant transcription factors.

Diagnosis of t(4;11)-positive acute leukemia based on tumor-specific HRX-AF-4

junctions has been recently performed in a small group of 11 patients using the polymerase chain reaction (Hilden *et al.*, 1993). Within this group six unique fusion sequences were detected, indicating heterogeneity of breakpoints. Therefore, junction-specific antibody diagnosis will not the be most obvious approach for the detection of t(4;11)-positive acute leukemia.

TSPs generated by t(8;21)(q22;q22) and t(3;21)(q26;q22)

In 40% of all children with acute myeloid leukemia (AML) and in 10% of all adult AML patients the translocation t(8;21)(q22;q22) has been observed (Mitelman and Heim, 1992). This translocation is highly predictive for the AML subtype M2, which is associated with granolycytic differentiation. Recently, two genes have been identified, which are involved in the translocation (Miyoshi *et al.*, 1991; Gao *et al.*, 1991): the *AML1* (acute myeloid leukemia-1) gene on chromosome 21 and the *MTG8* (for myeloid translocation gene on chromosome 8) or *ETO* gene (for eight twenty one) on chromosome 8. As a result of the translocation both genes are interrupted and a chimeric *AML1-ETO* gene is generated (Erickson *et al.*, 1992).

Homology studies indicate sequence homology between the *AML1* gene and the *Drosophila melanogaster* segmentation gene *runt*, including the DNA binding motif in *runt* (Daga *et al.* 1992; Erickson *et al.*, 1992). This DNA binding motif is retained in the fused gene, suggesting a putative role of the AML1-ETO protein in transcription regulation. More interestingly, recently the AML1 gene has been shown to be identical to the transcription factor CBFα (core binding protein α), also described as PEBP2α (Wang *et al.*, 1993). CBFα is part of the CBF complex, consisting of CBFα and CBFβ. As will be discussed later, CBFβ is structurally altered as a result of an inversion of chromosome 16 in another type of AML, termed M4Eo (Liu *et al.*, 1993). The AML type M2 is characterized by granolocytic maturation, whereas the tumor cells in M4Eo patients express myelomonocytic differentiation; moreover AML/M4Eo patients are distinguished by eosinophilia in bone marrow and peripheral blood (Adriaansen *et al.*, 1993).

These observations indicate that the role of the CBF complex is highly important in the control of proliferation and differentiation of myeloid cells. Disruption of the α chain after fusion with ETO may block the ability for myelocytic cells to differentiate into mature myeloid cells. In contrast, after deregulation of the β chain both differentiation and proliferation are stimulated.

The ETO gene has not been sequenced entirely. Thus far, no sequence homology has been found between this gene and any known reported sequences (Erickson *et al.*, 1992). However, the *ETO* gene is not expressed in normal myeloid cells (Chang *et al.*, 1993). Thus, as a result of the t(8;21) translocation *ETO* is activated by *AML1* and shows an aberrant expression pattern.

Molecular studies have shown that the AML1-ETO fusion-points are highly

consistent within the t(8;21) positive group of patients (Nucifora *et al.*, 1993a; Downing *et al.*, 1993). This indicates that the *AML1-ETO* junction is appropriate to use for diagnostic purposes, either by applying PCR amplification techniques or by using junction specific antibodies

Interestingly, very recently Nucifora *et al.* (1993b) reported on another translocation in which the *AML1* gene is fused to the *EAP* (EBER associated protein) gene. EAP is a highly conserved protein which is able to associate with EBER1, a loop in the Epstein-Barr virus small RNA, and which has also been identified as a ribosomal protein, termed L22 (Nucifora *et al.*, 1993b). This particular translocation, t(3;21)(q26;q22), is found in a small number of patients with therapy related myelodysplastic syndrome or acute myeloid leukemia and in some patients with chronic myeloid leukemia in blast crisis (Rubin *et al.*, 1990). The fused *AML1-EAP* transcript comprises also the *runt* homologous 5′ part of *AML1* and at least one additional exon of *AML1* compared to the *AML1-ETO* fusion transcript. However, since the *AML1-EAP* fusion does not maintain the correct reading frame of *EAP*, the existence of a chimeric AML1-EAP protein is unlikely. Nevertheless, Nucifora *et al.* (1993b) formulated three hypotheses which could explain existence of a functional abnormal AML1 protein: *i.* a stable *AML1-EAP* transcript encodes only the DNA-binding region of AML1, resulting in a truncated AML1 protein; *ii.* the chimeric cDNA clone isolated represents the most abundant transcript but not the active one; and *iii.*ribosome frame shifting could result in the translation of the chimeric *AML1-EAP* message.

However, it is highly suggestive that structural aberrations in the AML protein, either by fusion to other proteins or by truncation of the protein may alter its normal function. Although not defined as yet, this event could be an important step in the generation of AML.

TSPs generated by t(12;16)(q13;p11)

In myxoid liposarcoma (MLPS), a sarcoma of adipose tissue origin, a characteristic reciprocal translocation t(12;16)(q13;p11) has been observed (Turc-Carel *et al.*, 1986). Recently, the genes involved in this particular translocation have been identified (Rabbitts *et al.*, 1993; Crozat *et al.*, 1993). The breakpoint on chromosome 12 occurs within a gene termed *CHOP* or *GADD153*. The chromosome 16 breakpoint was found to occur within a gene called *FUS* or *TLS* (translocated in liposarcoma).

The normal *CHOP* gene encodes a 29-kD nuclear protein. The CHOP protein contains a leucine zipper motif and is involved in transcription regulation (Ron and Habener, 1992). CHOP is silent in dividing cells, but inducible in response to DNA damage (Fornace *et al.*, 1989). Moreover, CHOP is induced in the differentiation of adipocytes (Ron and Habener, 1992).

The normal TLS is a 68-kD nuclear protein with RNA binding activity (Crozat

et al., 1993). There is a large homology between TLS and the EWS protein, involved in Ewing sarcoma (as mentioned above). As a result of the t(12;16) a 75-kD chimeric TLS-CHOP protein is generated, which is also expressed in the nucleus. In the chimeric protein the C-terminal *RNA binding* sequences of TLS are replaced by the full length CHOP protein, including the *DNA binding* CHOP sequences.

The role of TLS-CHOP in the generation of liposarcomas is only speculative. It could be that under the control of the TLS promoter, CHOP is expressed in phases of the cell cycle where it is normally silent. This aberrant expression could probably lead to abnormal activation of yet unknown target genes, resulting in abnormal cell proliferation.

At present, the TLS-CHOP rearrangement is only detected in two liposarcoma cell lines (Rabbitts *et al.*, 1993;Crozat *et al.*, 1993). Interestingly, in both cell lines identical TLS-CHOP fusion-points were observed. To investigate whether the tumor-specific TLS-CHOP junction is a useful, uniform target in immunodiagnosis of MLPS, fresh tumor material has to be analyzed. Åman *et al.* (1992) already reported on Southern blot analyses of fresh tumor material with respect to *CHOP* rearrangement. This study indicates that, analogous the MLPS cell lines, also in patients the CHOP coding region is not interrupted by the translocation. This finding suggests that at least the CHOP part of the TLS-CHOP junction will be identical in all patients.

2.1.2 Fusion-point TSPs generated by 'internal deletion'

The only thus far known TSPs generated by internal deletion are structurally altered forms of the Epidermal Growth Factor Receptor (EGFR). In human glioma's expression of the EGFR is frequently disturbed, due to *amplification* or *overexpression* of the EGFR gene (Humphrey *et al.*, 1988). Moreover, in combination with overexpression or amplification, *structural rearrangements* of the EGFR gene are observed. In this context, three types of deletion mutant EGFR have been observed (Humphrey *et al.*, 1988; 1990 and 1991).

The normal EGFR gene encodes a 170-kD receptor with tyrosine kinase activity (Humphrey *et al.* 1991). This protein consists of an extracellular, an intracellular and a transmembrane domain. Deletions have been reported to occur in the extracellular domain (Humphrey *et al.* 1991). In mutant EGFR type I almost the complete aminoterminal extracellular domain is deleted, including the binding domain for Epidermal Growth Factor (EGF). This particular deletion, which is found in only one single case, results in a truncated EGFR. Cells carrying this type of mutant EGFR are not able to respond to EGF stimuli (Humphrey *et al.* 1988).

Both other types show internal (in frame) deletions: in type II a 83 amino acid segment is deleted (amino acid 520-603), whereas in type III a 267 amino acid

segment is deleted (amino acid 6-273). In these particular mutated proteins the EGF binding domain is not affected (Humphrey *et al.* 1991). Therefore, it has been suggested that in human gliomas the deletion in the EGFR is a minor molecular abnormality. Overexpression of the mutant protein is probably more important in the generation of the tumor (Humphrey *et al.*, 1991). However, it could be possible that the internally deleted protein may be more stable than the normal EGFR, which may favor growth of tumor cells.

As a result of the deletions new, tumor-specific amino acid sequences are generated at the point of the respective fusions. Specific polyclonal antibodies have been raised against the internal fusion-point in the type III mutation, which is the most frequently occurring mutation, i.e. in 17% of all glioblastomas (Humphrey *et al.*, 1990). The antibodies recognize deletion-mutant proteins highly specifically in immunoprecipitation experiments and in immunohistological analysis of frozen sections of tumor tissue. Internalization studies demonstrate also uptake of the antibodies by living glioma cells (Humphrey *et al.* 1990). Thus, the antibodies have diagnostic and most likely, when coupled to cytotoxic drugs, therapeutic potential.

2.1.3 Fusion-point TSPs generated by intrachromosomal rearrangements

Due to *intrachromosomal* rearrangements, parts of the *same* chromosome are translocated and fused to each other. When the rearrangement is in frame, a new chimeric gene is generated analogous to the above mentioned fusion process occurring in reciprocal translocations.

Four TSPs of this type will be discussed. Two types are generated by a chromosomal inversion, inv 10 and inv 16, respectively. The third TSP is the result of an unidentified intrachromosomal rearrangement on chromosome 1. The last type of TSPs which will be discussed in this paragraph is generated by a chromosomal insertion on chromosome 2.

TSPs generated by inv (10)(q11.2;q21)

In five out of 20 patients with thyroid papillary carcinoma, Grieco *et al.* (1990) identified an activated form of the *ret* gene, which was called *ret*/PTC (for *ret* in papillary thyroid carcinoma).

The normal *ret* gene encodes a putative tyrosine kinase receptor (Takahashi *et al.*, 1985). Molecular cloning studies have shown that the *ret*/PTC gene consisted of the 3′ tyrosine kinase domain of the *ret* gene (Grieco *et al.*, 1990; Pierotti *et al.*, 1992). The 5′ sequences of *ret*, encoding the putative transmembrane and extracellular receptor domains, were replaced by sequences of a so far unknown gene called *H4* or *D10S170*. Therefore, this observation suggests that the the activated RET protein (RET/PTC or H4/D10S170-RET) has changed the putative normal transmembrane position for localization in another cell

compartment, where it functions in an abnormal way under the regulation of the D10S170 promoter. Moreover, cells carrying the activated RET are probably unable to react to signals from the, yet unknown, ligand of the normal RET protein. Both these mechanisms may contribute to the malignant state of the tumor cells.

In principle, the fusion-point of D10S170 and RET might be a tumor-specific determinant on the chimeric protein. So far, the nucleotide sequence of the junctional region has not been described.

TSPs generated by inv (16)(p13;q22)

In about 8% of all patients with acute myeloid leukemia (AML) a specific chromosome 16 abnormality is observed, inv (16)(p13;q22) (Mitelman and Heim, 1992). In all these patients the AML is of the so called 'AML-M4Eo' type. Recently, Liu *et al.* (1993) reported that due to the inversion, the gene encoding the β chain of the transcription factor CBF (core binding factor), termed *CBFβ* or PEBP2β, is fused to the *MYH11* gene encoding the human smooth muscle myosin heavy chain (SMMHC). As a result of the fusion a chimeric *CBFβ-MYH11* gene is generated.

The CBFβ protein itself, comprising 182 amino acids, does not show any sequence homology with other proteins. However, CBFβ together with another chain, CBFα, forms a heterodimer complex, CBF, which is reported to bind to the enhancers of the T cell receptor (Redondo *et al.*, 1992). Since the CBFβ chain is not able to bind to DNA sequences directly, it has been suggested that the CBFβ chain stabilizes the interaction of the CBFα chain with DNA (Wang *et al.*, 1993). As mentioned above (page 34), the CBFα chain is identical to AML1, the transcription factor involved in reciprocal translocation t(8;21) which occurs in AML, subtype M2 (Miyoshi *et al.* 1991; Gao *et al.* 1991; Wang *et al.* 1993). Thus, the role of an abnormal CBF-αβ complex seems to be important in myeloid leukemogenesis: deregulation of the α chain results in AML-M2, which is characterized by granulocyte maturation; disruption of the β chain causes AML-M4Eo, which is accompanied by granulocytic and monocytic differentiation and abnormal eosinophilia (Adriaansen *et al.* 1993).

The role of the SMMHC part in the chimeric protein is only speculative. It is possible that, due to the repeated α helical structures of SMMHC, as present in the chimeric protein, the dimerization of CBFβ moiety with the α chain is reinforced (Liu *et al.*, 1993). This might result in a more stable CBF complex, which can influence transcription of target genes, either positively or negatively as compared to wild type CBF. However, target genes of CBF are not identified in the myeloid lineage. It has been shown that CBF binds to a specific nucleotide sequence, termed the δE3 site, in the enhancers of the T-cell receptor δ (TCRδ)

gene (Redondo *et al.*, 1992). In T-cells, binding of CBF to the δE3 site is necessary, but not sufficient for transcription activation of the TCRδ gene. Abnormalities in the T-cell compartment as a result of alterations in the CBFα and CBFβ chain have not been described, but it could be possible that CBF binds to δE3 homologous sequences in myeloid cells.

At present, it is not obvious whether antibody diagnosis of AML using CBFβ-SMMHC junction-specific antibodies will be useful. Liu *et al.* (1993) reported on three types of CBFβ-SMMHC mRNA rearrangements within a group of six patients, suggesting heterogeneity of junctions. Sequencing of PCR products resulted in the observation that *CBFβ* breakpoints were identical in all patients. The heterogeneity was found to be caused by the diversity of breakpoints within the SMMHC encoding mRNA (Liu *et al.* 1993).

Fusion-point TSPs generated by a chr 1 rearrangement

In two small cell lung cancer cell lines and in one patient with lung cancer an abnormal L-MYC protein is detected (Mäkelä *et al.*, 1991; 1992a and b). Immunoprecipitation studies using anti-L-MYC antibodies resulted in the precipitation of a 72-77-kD protein triplet instead of the normal 62-67-kD protein triplet. After molecular cloning, an *L-myc* fusion DNA was isolated in which exon I sequences were replaced by nucleotides from an unknown gene, which was then called *rlf* (rearranged L-*myc* fusion).

The role of the newly formed chimeric protein in the generation of lung cancer is not known, since the normal function of RLF is still unidentified. Nevertheless, most likely the normal function of L-MYC, (a transcription factor) could be disturbed, because the expression of the chimeric gene is under the control of the *rlf* promoter.

With respect to tumor diagnosis, in all cell lines the *rlf-L-myc* junction at the mRNA level was identical, but differed at the DNA level. Mäkelä *et al.* (1992a) have detected in a sample of a primary lung cancer another rearranged *rlf-L-myc* DNA. Whether this particular DNA gives rise to the same mRNA as occurs in the cell lines, is not known. Thus, more patient studies have to be performed before one can evaluate prospects of immunodiagnosis of tumor-specific RLF-L-MYC junctions.

Fusion-point TSPs generated by insertion (2;2)(p13;p11.2-14)

An abnormal *rel* gene was detected in a human diffuse large cell lymphoma cell line carrying an insertion (2;2)(p13;p11.2-14) (Lu *et al.*, 1991). After isolation and sequencing of the abnormal gene, it became evident that the 3' part of the *rel* gene was replaced by sequences of an unknown gene, which was called *nrg* (non-*rel*-gene).

REL is a transcription factor which belongs to the nuclear factor κB family of

transcription factors (Gilmore, 1990) The hybrid *rel-nrg* gene may encode a chimeric protein containing the N-terminal 284 amino acids of the REL protein, comprising the DNA binding domain, and 156 amino acids encoded by the *nrg* gene. Thus far, the chimeric *rel-nrg* sequences are only detected in cell lines and are not observed in patients with a chromosomal aberration in the *rel* comprising band region p11.2-14 of chromosome 2.

2.2 TSPs generated by point mutations

In this part of the chapter TSPs generated by point mutations will be discussed. Major attention will be given to the incidence of point mutations in malignancy, the randomness or nonrandomness of the point mutations and the relevance of the point mutations to tumor diagnosis and / or tumor therapy. The effect of the point mutations on the biological function of the affected proteins will be briefly discussed.

TSPs encoded by mutations within ras genes

In various types of tumors mutant RAS proteins are reported to occur (reviewed by Bos, 1989). The highest incidence is found in pancreatic adenocarcinoma: approximately 80% of these tumors harbors a mutated RAS encoding gene. Also half of the carcinomas of the colon and follicular and undifferentiated carcinomas of the thyroid present a mutated *ras* gene. In a considerable part of all seminomas (40%), acute myeloid leukemias (35%), myelodysplastic syndromes (27%) and adenocarcinomas of the lung (27%) *ras* mutations are manifest. In other types of tumors *ras* mutations are reported to occur in a minority of all cases, such as chronic myeloid leukemia (13%), melanoma (14%), bladder carcinoma (13%) and liver and kidney carcinoma (10%).

The normal RAS proteins are 21-kD proteins ($P21^{ras}$) which are associated with the inner cell membrane (Barbacid, 1987). The $P21^{ras}$ proteins are involved in signal transduction and belong to the GTPase superfamily (Barbacid, 1990). They are able to bind GDP and GTP and are also capable to hydrolyze GTP.

GDP is bound to the *inactive* form of $P21^{ras}$. After stimulation, probably through a receptor mediated event, GDP is exchanged by GTP. At that moment $P21^{ras}$ will be in an *active* state. Activated $P21^{ras}$ interacts with intracellular effector molecules.

$P21^{ras}$ will be inactivated by its own GAP-activated GTPase activity, which hydrolyses GTP to GDP. Mutation activated $P21^{ras}$ has a decreased GTPase activity (Barbacid, 1987 and 1990). As a result, GTP will not be hydrolyzed and the activation of $P21^{ras}$ will be maintained, mimicking continuous signal transduction. This process may cause deregulated cell growth and tumor formation (Barbacid, 1987 and 1990). However, the definite role of the RAS proteins in tumorigenesis is still unclear. Mutations in *ras* are also found in adenoma of the colon, a benign tumor-type which can progress into a malignant carcinoma (Bos *et al.*, 1987). Therefore the *ras* mutation can be in principle an early event in the transition from adenoma to carcinoma (Bos *et al.*, 1987). Strikingly, in many types of tumors *ras*-mutations are found to occur only in a minority of all cases. Thus, activation of $P21^{ras}$ is not a tumorigenic event in all tumors.

There are three types of highly homologous RAS proteins, termed N-RAS, K-

RAS and H-RAS respectively (reviewed by Bos, 1989). Point mutations are found to occur in all three types of RAS proteins always at the same distinct sites: in amino acid residue 12, 13 or 61. Due to a replacement of only one nucleotide in the coding triplet the original amino acid residue can be replaced by various others. For example, glycine at position 12 can be substituted by either aspartic acid, valine, serine, arginine, alanine or cysteine. All mutations have in common that they impair the $P21^{ras}$-GTPase activity. At present, there seems to be no correlation between tumor-type, localization of the mutation, and amino acid substitution. The only relationship which has been reported is the preference of **N**-*ras* mutations for myeloid disorders, and **K**-*ras* mutations for adenocarcinomas of lung, colon and pancreas (Bos, 1989).

It is obvious that point mutations in *ras* are not specific for one type of tumor. However presence of a mutated *ras* gene in tumor-derived cells, is an indication for a malignant or pre-malignant state of the tumor. Therefore, identification of *ras* mutations will be of clinical importance. Currently, to detect point mutations PCR techniques are applied, which amplify cDNA comprising the mutated codons followed by hybridization with mutation specific probes (Diamandis, 1992). At the other hand, the mutated amino acids create per definition tumor-specific epitopes on the proteins. Thus, in principle they are excellent targets for antibody diagnosis. Antibodies recognizing amino acid substitutions at position 12, arginine, valine, serine and aspartate respectively, have been reported (Wong *et al.*, 1986; Pullano *et al.*, 1989). Unfortunately, these antibodies function only in Western blot systems. Detection of mutated RAS proteins at the single cell level using immunohistochemical techniques has not yet been described.

From the therapeutic point of view, $P21^{ras}$ cannot act as target for humoral, antibody mediated tumor therapy, due to its intracellular localization. However, the intracellular $P21^{ras}$ might be processed into peptides to be presented at the cell surface in MHC molecules. In this way, a cellular peptide specific immune response might be elicited. Thus far, Jung and Schluesener (1991) reported on the establishment of human $CD4^+$ positive T cell lines, which specifically recognize a mutated $P21^{ras}$ with a glycine --> valine substitution at position 12. Also in the mouse the generation of T-cell lines has been published (Peace *et al.*, 1991). These findings offer new perspectives for specific T-cell mediated immunotherapy of $P21^{ras}$-mutation-positive tumors.

TSPs generated by mutations within the RB gene

The retinoblastoma susceptibility gene *RB* encodes a 105-kD protein, the so called P105-RB (Lee *et al.*, 1987). The protein is *absent* in retinoblastomas, in a large fraction of small cell lung carcinomas, in some osteosarcomas, in soft tissue sarcomas and in human breast cancers (Lee *et al.*,1988; Harbour *et al.*, 1988; Varley *et al.*,1989; Mori *et al.*, 1990; Marshall, 1991). *Mutant* P105-RB

proteins are detected in cell lines derived from a prostate carcinoma (Bookstein *et al.*, 1990), a bladder carcinoma (Kaye *et al.*, 1990) and several small cell lung cancer samples (Horowitz, *et al.* 1990). Here, point mutations are described to occur at various sites, but always within or around exon 21. In the bladder carcinoma derived cell line, the point mutation is found to occur just within a splice acceptor site, resulting in the deletion of the complete exon 21 (Kaye *et al.*, 1990). As a consequence the exon 20 and 22 sequences are fused. Thus, in principle this type of mutation belongs to the category of 'fusion-point' TSPs.

P105-RB is a nuclear phosphoprotein, which plays a critical role in cell proliferation control (reviewed by Levine, 1993). In the G_0/G_1 phase of the cell-cycle, the hypophosphorylated form of P105-RB is complexed to the transcription factor E2F-1, which regulates the transcription of cell proliferation genes (Johnson *et al.*, 1993). P105-RB-bound E2F-1 is inactive and cells remain in G_0/G_1. Under the influence of the kinase activity of cyclin D-dependent kinase CDK4, which is also complexed to P105-RB, P105-RB is phosphorylated and binding of E2F-1 is prevented (Kato *et al.*, 1993, Ewen *et al.* 1993). As a result, E2F-1 responsive genes are activated and the cell enters into the S-phase.

Thus, in hypophosphorylated form P105-RB has the potential to *suppress* cell proliferation. Kaye *et al.* (1990) have reported that mutated P105-RB cannot undergo dephosphorylation. As a consequence, the negative control of cell proliferation by hypophosphorylated P105-RB has been eliminated, resulting in abnormal cell proliferation. Therefore, the *RB* gene is a so called 'tumor-suppressor gene': due to absence of the normal function of the protein, either by absence of the protein itself or by loss of function due to mutations, inadequately controlled cell proliferation may lead to tumor formation.

Thus far, the point mutations in P105-RB are only reported to occur in cell lines. Further studies are required to look for the same mutations in freshly isolated tumor material. Immunodiagnosis based on point mutations in the P105-RB protein will not be obvious, because the location of the point mutations is not identical in all tumors.

TSPs generated by point mutations within the P53 gene

Point mutations in the tumor suppressor gene P53 are common in various types of human cancer, such as breast, colon, bladder, and testicular tumors and sarcomas and melanomas (Hollstein *et al.*, 1991). Also in leukemias and lymphomas P53 point mutations have been reported (Hollstein *et al.*, 1991). The point mutations are clustered in so called 'hot-spots' in the exons 5-9, between codon 110-307. Hollstein *et al.* (1991) have summarized 280 base substitutions, which are distributed over 90 codons. Due to the diversity of the point mutations, at some positions the original amino acid can be replaced by as many as 5 different residues.

As a result of the point-mutations the normal DNA-binding function of P53 is disturbed. Mutant P53 has a dominant negative effect, since it is able to dimerize with the wild-type P53 in the cytoplasm, preventing entrance of wild-type P53 into the nucleus. The role of wild type P53 in cell metabolism is complex and still under investigation. Raycroft *et al.* (1990) reported that normal P53 can act as a transcriptional activator, probably directly by binding to DNA or indirectly by interacting with other DNA binding proteins. Point mutations might result in conformational changes in P53 which could prevent P53 to complex with DNA or with other proteins, leading to uncontrolled cell proliferation. In addition, it has been suggested that P53 acts as a so called 'checkpoint control' of the cell cycle in the transition from the G1- into the S-phase (reviewed by Levine, 1993): in response to DNA damage wild type P53 protein is stabilized which results in an increased intracellular level of P53. Cells with these high levels of P53 are inhibited to enter into the S-phase. As a result, the G1 phase is prolonged to allow DNA repair. In contrast, cells with mutant P53 do not pause in G1 and proceed into the S phase with unrepaired DNA, which may cause deregulated cell proliferation. P53 controls the entry of cells from G1 into S phase indirectly, by activation the transcription of other genes, which regulate the cyclin D-dependent kinase CDK4, involved in activation of P105-RB (Xiong *et al.* 1993; Serrano *et al.* 1993)

Mutant P53 is expressed in many types of tumors. Therefore, P53 is a reliable marker for 'malignancy'. Poly- and monoclonal antibodies (Gannon *et al.*, 1990; Yewdell *et al.*, 1986; Bartek *et al.*, 1991) have been applied in tumor diagnosis. The respective antibodies react with mutated P53, expressing conformational epitopes generated by various point mutations. These epitopes are per definition tumor-specific antigens. In principle, all mutated amino acids are tumor-specific antigens and could function as target for antibody diagnose. However, due to the large scale heterogeneity of the point mutations, it will be difficult to prepare as many antibodies as there are tumor-specific epitopes induced by the point mutations.

With respect to tumor therapy, Houbiers *et al.* (1993) have cloned human cytotoxic T cells which recognize peptides derived from either wild type or mutant P53 (1993). These particular T cells were able to lyse peptide-loaded target cells. Although processing of native P53 has not yet been shown, a first step has been taken into the way of specific P53-focused cellular immunotherapy.

TSPs generated by point mutations within genes encoding G proteins

G proteins are associated with the cytoplasmic surface of the plasma membrane and are involved in signal transduction (Kaziro *et al.*, 1991). They are functionally and structurally related to RAS proteins. Alike RAS, G proteins

belong to the GTPase superfamily.

Point mutations have been observed in the α chain of two types of G proteins, termed G_s and G_{i2} (Landis *et al.*, 1989; Lyons *et al.*, 1990). Such mutations are frequently found in endocrine tumors: in 43% of all patients with growth hormone producing pituitary tumors the α chain of G_s ($G_{s\alpha}$) is mutated, whereas 30% of the so far tested endocrine tumors of the ovary and 27% tumors of the adrenal cortex show a mutation in the α chain of G_{i2} ($G_{i2\alpha}$) (Landis *et al.*, 1989; Lyons *et al.*, 1990).. The genes encoding the mutated α genes are called *gsp* (encoding mutated $G_{s\alpha}$ protein) and *gip2* (encoding mutated $G_{i2\alpha}$ protein).

G_s, adenylate cyclase stimulating G protein, is a heterotrimer, composed of a α, β and γ chain. Due to its GTPase activity, the α chain plays a critical role in signal transduction. Point mutations are detected within the α chain either at position 227 (gln --> arg) or 201 (arg-->cys or his) (Lyons *et al.*, 1990). The mutated sequences, highly conserved within all known G-proteins, are functionally important in the GTPase activity of the α chain. The point mutations inhibit the GTPase activity of the α chain. As a result the α chain remains active and cell division is stimulated continuously.

The G_{i2}, inhibitory G protein-2, is constructed by the same β and γ chains as Gs, but by another α chain. In contrast to $G_{s\alpha}$, activated $G_{i2\alpha}$ inhibits adenylate cyclase.

Point mutations in $G_{i2\alpha}$ are found in an arginine encoding codon at position 179. This particular Arg^{179} is homologous to the Arg^{201} in $G_{s\alpha}$ and is also substituted either by a cysteine or a histidine residue. Therefore, with respect to tumor diagnosis, this particular amino acid in combination with the surrounding, highly conserved region could be a target for antibody diagnosis.

TSPs generated by point mutations in c-FMS

The *fms* gene encodes the receptor of the macrophage- and monocyte-specific growth factor, colony-stimulating factor 1 (CSF-1) (Sherr *et al.*, 1985). The receptor is a cell surface glycoprotein, expressed by cells of the monocyte / macrophage lineages. Functionally, the receptor possesses ligand dependant tyrosine kinase activity (Sherr, 1990). Point mutations in the FMS encoding gene have been reported in acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS). Here, codons 301 and 969 are involved exclusively. Most likely, the respective codons are important for activation of the receptor (Roussel *et al.*, 1988). Therefore, it is has been suggested that the point mutations in the receptor mimic permanent ligand binding.

Although it seems plausible that point mutations in c-FMS could play an important role in the pathogenesis of AML, studies on this subject are not univocal. Ridge *et al.* (1990) and Tobal *et al.* (1990) report on point mutations

in 18% of all AML and in 15% of all MDS patients. On the other hand, in comparable groups of patients, Shepherd *et al.* (1990) and Springall *et al.* (1993) fail to detect any point mutations. Therefore, focusing on substituted amino acid residues as targets for either tumor diagnosis or tumor therapy is not relevant before this controversy has been elucidated.

TSPs generated by point mutations in immunoglobulin genes

B-cell lymphomas are characterized by the unrestricted outgrowth of one particular B-cell clone. In B-cell malignancies which express immunoglobulin molecules on the cell surface, the malignant clone differs from the other non-malignant B-cells by its own antigen-specific idiotype. Consequently, the idiotype complies with the definition of a tumor-specific protein. However, there is one restriction: the idiotypic TSP is not a *common* TSP, but a *patient-specific* TSP. A particular idiotype might be expressed as a TSP on a malignant clone in the one patient, whereas in another patient the same idiotype cannot be classified as a TSP, since it is expressed on a normal, non-malignant cell.

Since fine diversity of idiotypes is a.o. generated by somatic point mutations in the hypervariable regions of the heavy and light chains of the immunoglobulin molecule, idiotypic antigens in B-cell lymphomas can in principle be classified as TSPs encoded by point mutations. However, most likely, each B-cell may develop from normal into malignant, independently on the specificity of its idiotype. Consequently, the number of tumor-specific idiotypic antigens is theoretically 10^{10}, corresponding to the number of B-cells with different antigenic specificity. Thus, due to the very broad variety, antibody diagnosis using anti-idiotypic antibodies will not be practicable.

From the therapeutical point of view, treatment of patients with monoclonal anti-idiotypic antibodies seemed to be successful. Meeker *et al.* (1985a) described the effective treatment of a patient with a follicular B-cell lymphoma with anti-idiotypic antibodies. However, there are still certain shortcomings attached to this approach. (*i*) The anti-idiotypic antibodies are exclusively applicable for only one patient. Thus it will be necessary to prepare new, specific monoclonal antibodies for each particular patient. This makes the therapy rather laborious and time consuming. (*ii*) The idiotypes are encoded by the hypervariable regions of the H and L chains. As a result of new mutations within the idiotype, the malignant clone may escape from the therapy during the anti-idiotypic therapy (Meeker *et al.*, 1985b). In this particular case, the therapy must be targeted to the newly created idiotype. Thus, although idiotypes are by definition tumor-specific proteins, their practical applicabilities are presently insufficient.

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CHAPTER 3

Antibody recognition and immunologic characterization of tumor-specific BCR-ABL fusion points in Philadelphia chromosome-positive leukemias

Contents

CHAPTER 3.1

Antibody recognition of the tumor-specific BCR-ABL joining region in chronic myeloid leukemia

Janneke van Denderen[1], André Hermans[1], Toon Meeuwsen[1], Christine Troelstra[1], Netty Zegers[2], Wim Boersma[2], Gerard Grosveld[1] and Willem van Ewijk[1]

[1]Departments of Cell Biology, Immunology and Genetics, Erasmus University, Rotterdam, and [2]Medical Biological Laboratory TNO, Rijswijk, The Netherlands

Summary

Chronic myeloid leukemia (CML) is characterized by the presence of a 210-kD protein ($P210^{bcr\text{-}abl}$) in the cytoplasm of leukemic cells, generated by the reciprocal translocation between chromosome 9 and chromosome 22. Due to this translocation, the *abl* oncogene is coupled to the *bcr* gene, forming a new determinant in this protein encoded by the *bcr-abl* joining region. In the joining region itself, either the bcr exon 2 is coupled to the *abl* exon 2 (b2-a2), or the bcr exon 3 is coupled to the *abl* exon 2 (b3-a2). Thus, these joining regions form by definition new tumor-specific determinants in the respective chimeric $P210^{bcr\text{-}abl}$ molecules.

This paper addresses the question as to whether these tumor-specific joining regions are exposed on the $P210^{bcr\text{-}abl}$ molecule in such a way that antibodies can be generated to detect these sites. To test this possibility a polyclonal antiserum, termed BP-1, was raised against a synthetic peptide representative for the b2-a2 joining region. The reactivity of BP-1 was analyzed in an ELISA system on various synthetic peptides. Peptide inhibition studies showed the presence of antibodies to different parts of the b2-a2 peptide in the polyvalent antiserum.

The reactivity of BP-1 was then tested with native $P210^{bcr\text{-}abl}$ molecules in various CML cell lines (K562, LAMA-84, and BV173) using a protein kinase assay. In this context, the *bcr-abl* junctions were first analyzed at the DNA and RNA level. The present study indicates that BP-1 specifically recognizes the b2-a2 junction in native $P210^{bcr\text{-}abl}$. Furthermore, BP-1 clearly discriminates between b2-a2 $P210^{bcr\text{-}abl}$ and b3-a2 $P210^{bcr\text{-}abl}$. We conclude that the tumor-specific b2-a2 joining region is antigenically exposed on the native $P210^{bcr\text{-}abl}$ molecule.

Introduction

Chronic myeloid leukemia (CML) is a pluripotent stem cell disorder characterized by the presence of the Philadelphia (Ph) chromosome in the leukemic cells of 96% of all CML patients (1). The Ph chromosome is formed by a reciprocal translocation between chromosomes 22 and 9 (2,3). In this translocation, the c-*abl* oncogene has moved from chromosome 9 into the breakpoint cluster region (bcr), within the *bcr* gene on chromosome 22, resulting in a chimeric *bcr*-c-*abl* gene (3,4). The fused gene encodes an 8.5-kb chimeric mRNA (5,6), which is translated into a 210-kD protein (7). This $P210^{bcr\text{-}abl}$ protein shows tyrosine kinase activity and is uniquely present in the leukemic cells of CML (and a number of Ph-positive ALL) patients (8-12).

The breakpoint in the *bcr* gene occurs either between bcr exon 2 (b2) and 3 (b3), or alternatively between bcr exon 3 (b3) and 4 (b4). Therefore, in the mature *bcr-abl* mRNA, either b2 or b3 is spliced to *abl* exon a2, which results in two alternative chimeric $P210^{bcr\text{-}abl}$ proteins, comprising either the b2-a2 or b3-a2 junction (13). As such, the two different amino acid sequences at the point of the junction represent unique tumor-specific determinants.

In this study we investigate whether these joining determinants are exposed on the $P210^{bcr\text{-}abl}$ molecule in an immunogenic fashion. Our data indicate that the joining determinants b2-a2 can indeed be recognized by antibodies. The strategy we used to generate and characterize the anti-b2-a2 antiserum has potential for the further development of antibodies detecting tumor-specific proteins resulting from chromosomal rearrangements.

Materials and methods

Cell lines

K562, LAMA-84, and BV173 are Ph-positive cell lines derived from patients during the blast crisis phase of CML. Cells were cultured in RPMI medium supplemented with 7.5% FCS, 100 µg/ml penicillin, and 60 µg/ml streptomycin.

Peptide synthesis, purification and conjugation

Peptides were synthesized according to the solid phase method developed by Merrifield (14) on polystyrene resin (1% crosslinking), using an automated peptide synthesizer (SAM-2; Biosearch, San Rafael, CA). The reaction sequence was performed according to the standard protocol using tertiary-butyl-oxycarbonyl amino acids with the following sidechain protection: lys-2-chlorocarbobenzoxy, glu-benzyl, gly-benzyl, cys-tertiary-butyl-mercapto, all commercially available (Fluka AG, Buchs, Switzerland and Bachem AG Bubendorf, Switzerland). Boc-arg-4-methoxybenzenesulfonyl was synthesized from Boc-Arg (Fluka AG, Buchs) and MBS-Cl (Aldrich Chemical Co., Milwaukee, WI) according to Nishimura and Fujino (15). Final deblocking and cleavage from the resin was performed by treatment with trifluoromethanesulfonic acid/Thioanisol/*m*-Cresol in

trifluoro acetic acid (TFA) for 1 h at 0°C (16), followed by filtration and precipitation from ether/*n*-pentane. Cys-containing peptides were treated with 10 equivalent threo-1,4-dimercapto-2,3-butanechol, pH 8, for 1 h and lyophilized. Gelfiltration of the crude peptide was performed on Sephadex-G15 (Pharmacia Fine Chemicals, Piscataway, NJ) using 5% (vol/vol) acetic acid as the eluent. For purification by HPLC (Pharmacia Fine Chemicals) a reverse phase ultrasphere C18 column, 10 x 250 mm (Beckman Instruments, Inc., Palo Alto, CA), was used applying a linear gradient from 15% to 40% solution B (0.1% TFA in acetonitrile) into solution A (0.1% TFA in water), in 15 min at 2.5 ml/min. Amino acid analysis was performed on the hydrolyzed peptide using precolumn derivatisation of the amino acids according to Janssen et al. (17), confirming the expected composition. Peptides were coupled to a carrier protein (chicken gamma globulin, and BSA) via the terminal cystein residue using *m*-maleimidobenzoyl-sulfosuccinimide-ester (18) (MBS) as a bi-functional coupling agent.

Immunizations and Ig purification

Flemish rabbits were primed intracutaneously with 250 μg protein complex consisting of SP b2-a2 coupled via MBS to CGG, emulsified in an equal volume of Complete Freund's Adjuvans. Rabbits were boosted twice after intervals of 4 weeks; the first time with 250 μg of protein in Complete Freund's Adjuvans, the second time with the same dose in Incomplete Freund's Adjuvans. 14 days after the last boost rabbits were bled and sera were collected. The Igs were purified from the serum by precipitation with 16% Na_2SO_4.

Preparation of anti-BCR antiserum

A polyclonal antiserum directed against the NH_2 terminus of the BCR protein was generated using the bacterial expression vector pEX (19). A 513bp Bam HI-Pst I cDNA fragment, derived from the first exon of the *bcr* gene, was cloned in the Bam HI and Pst I site of pEX2, thus maintaining the translational reading frame of the normal BCR protein. The resulting hybrid β-galactosidase-BCR protein was expressed according to standard procedures (7) and isolated from the bacteria as follows. The bacteria were collected and sonicated for 6 min in PBS on ice. After a 5-min centrifugation in an Eppendorf centrifuge, the pellet was resuspended in PBS and emulsified in an equal volume of Freund's Adjuvans. Immunization procedures and Ig purification were as described above.

ELISA

Binding of antiserum to the synthetic peptides was investigated in a micro ELISA system as described previously (20). Terasaki trays were coated with 0.1 μg antigen per well. Sera were diluted in PBS supplemented with 0.05% Tween-20. An optimal dilution of donkey anti-rabbit $F(ab)_2$ fragments conjugated to β-galactosidase (Amersham Corp., Arlington Heights, IL) was used as detecting reagent. Binding of antibodies was visualized by incubation with the fluorogeneic substrate 4-methyl-umbelliferyl-β-galacto-pyranoside. Binding is expressed as arbitrary fluorescence units.

Immunoprecipitation and protein kinase reaction

Immunoprecipitation and kinase reaction were carried out according to Chan et al. (21) with some minor modifications. Cells (5×10^6) were washed once in PBS, then lysed for 5 min in 0.5 ml ice-cold lysis buffer (1% Triton X-100, 0.05% SDS, 150 mM NaCl, 5 mM EDTA in 10 mM sodium phosphate, pH 7.0), supplemented with 0.6 mg/ml gelatine, 4 mM PMSF, and 0.3 mg/ml of each of the following protease inhibitors: aprotinin, trypsin inhibitor, leupeptin, and bestatin (Sigma Chemical Co., St. Louis, MO). After centrifugation of the lysate in an Eppendorf centrifuge, 5-50 µl of Na_2SO_4-precipitated Igs were added. Antigen-antibody interaction was allowed for 2 h at 4°C. Next, 40 µl of a 1:3 dilution of packed protein A Sepharose beads (Pharmacia Fine Chemicals) was added. Beads were collected after a 30-min incubation at 4°C, and washed twice in lysis buffer without SDS and subsequently washed with 50 mM Tris-HCl, pH 7.0. The beads were resuspended in 20 1µl 20 mM Pipes buffer, pH 7.0 (Sigma Chemical Co.), supplemented with 20 mM $MnCl_2$ and 20 µCi (γ-[^{32}P]) ATP was added (Amersham Corp.). Incubation was allowed for 10 min at 37°C. Beads were then washed twice with lysis buffer without SDS and boiled in 100 µl sample buffer. Samples were run at 6% polyacrylamide gels. Gels were washed in distilled water, dried, and autoradiographed for 30 min, using Fuji (RX-NIF) films.

RNA analysis

RNA was extracted according to the Li/Cl method (22). Samples were subjected to electrophoresis on formaldehyde 1% agarose gels, transferred to nitrocellulose, and after hybridization, exposed for 3 days to Kodak XAR 5 films using intensifying screens. The oligonucleotides covering the *bcr-abl* junctions were composed as follows: (b3-a2): 5'TGGATTTAAGCAGAGTTCAAAAGCCCTTCAGCGGCCAGTA3'; (b2-a2): 5'GCTGAC-CATCAATAAGGAAG AAGCCCTTCAGCGGCCAGTA3'; (primer): 5'TACTGGCC3'. ^{32}P-labeled probes were synthesized by extension of the 8-mer primer hybridized to one of the 40-mers. The primer extension mixture contained 6 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 6 mM β-mercaptoethanol, 50 mM NaCl, 30 µCi (α-[^{32}P]) dATP, 30 µCi (α-[^{32}P]) dCTP, 100 mM dGTP, 100 mM dTTP, 8 ng of the 40-mer, 4 ng of the primer, and 5 U Klenow polymerase in a total volume of 10 µl. The reaction was performed at room temperature for at least 1 h and stopped by the addition of 90 µl TES (10 mM Tris-HCl, pH 7.5; 5 mM EDTA; 0.1 % SDS) followed by removal of free nucleotides on a Sephadex-G50 column.

Amplification of cDNA

The cDNA amplification was performed using the polymerase chain reaction. 10 µl of total RNA was ethanol precipitated; washed with 70% ethanol, and dried in an desiccator. The pellet was dissolved in 9 µl of annealing buffer (250 mM KCl, 10 mM Tris-HCl, pH 8.3 at 42°C, 1 mM EDTA) and 1 µg of the c-*abl* primer 5'GAGCTCGGATC-CACTGGCCACAAAATCATACAGT3' was added. The sample was heated for 3 min at 80°C and transferred to a 65°C waterbath to allow annealing of the primer for 1 h. 15µl of cDNA buffer (24 mM Tris-HCl, pH 8.3 at 42°C, 16 mM $MgCl_2$, 8 mM DDT, 0.4 mM dNTP) and 5 U of avian myeloblastosis virus reverse transcriptase were added. This sample was incubated for 1 h at 42°C. After this step, 1 µg of a bcr primer 5'GAAGAA-

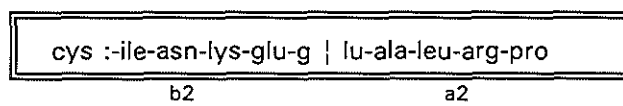
GTGTTTCAGAAGCTTCTCCC3' (from exon b2) was added and 26 cycles of the polymerase chain reactions were performed directly in a volume of 100 µl, using Taq polymerase.

Results

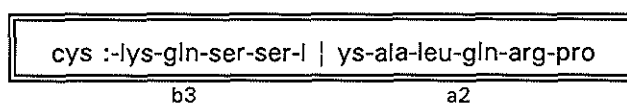
Synthesis of peptides corresponding the BCR-ABL junction

Two peptides were produced by solid phase synthesis according to Merrifield (14) based on the previously published (6) nucleotide sequence of the chimeric *bcr-abl* gene. The peptides were prolonged with an NH_2-terminal cysteine providing a specific coupling site to carrier molecules .

(*a*) Synthetic peptide b2-a2 (SP b2-a2) represents the fusion part of b2-a2 $P210^{bcr\text{-}abl}$ and consists of the amino acids:



(*b*) Synthetic peptide b3-a2 (SP b3-a2) represents the b3-a2 fusion part of b3-a2 $P210^{bcr\text{-}abl}$ and consists of the amino acids:



Since the fusion of the *bcr* and *abl* genes occurs within a coding triplet (6), the second glu in SP b2-a2 and the second lys in SP b3-a2 are newly generated by the translocation process. Thus, both peptides contain newly formed amino acids, share the c-ABL COOH-terminal amino acids and differ at the NH_2-BCR terminus.

Antibody binding to synthetic peptides

Antiserum BP-1 was raised against SP b2-a2. Here, we show the binding of BP-1 to the peptide in a micro ELISA system. Terasaki trays were coated with SP b2-a2 conjugated to BSA. As a control, Terasaki plates were coated with BSA-MBS or BSA-MBS-SP b3-a2. As shown in Figure 1a, BP-1 bound to BSA-MBS-SP b2-a2 and, to an equal extent, to BSA-MBS-SP b3-a2 (Figure 1b). However, binding to BSA-MBS occurred to a much lower extent (Figure 1c). This indicates that antiserum BP-1 contains antibody molecules directed against MBS and against determinants on both synthetic peptides, most probably on the

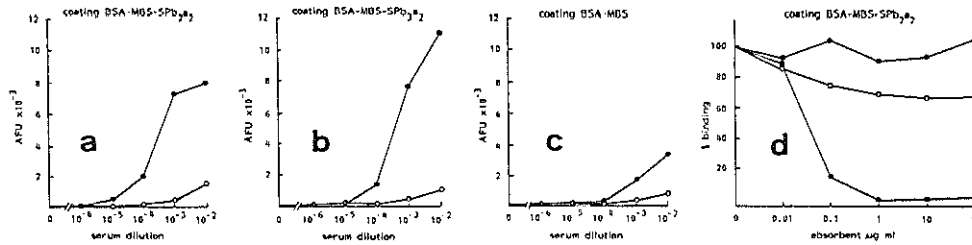


Figure 1 Binding characteristics of antiserum BP-1 to BSA-MBS +/- SPs (a-c) and to BSA-MBS-SP b2-a2 after preabsorption with BSA-MBS + /- SPs (d). (a-c) Binding of different concentrations of antiserum BP-1 (●) and normal rabbit serum (○) to BSA-MBS-SPb2-a2 (a), BSAMBS-SP b3-a2 (b), and BSA-MBS (c) in a micro ELISA system. Methods were as described by van Soest et al. (20). Binding is expressed as arbitrary fluorescence units (AFUs). (d) Binding of antiserum BP-1 to BSA-MBS-SP b2-a2 after preabsorption with BSA-MBS-SP b2-a2 (■), BSA-MBS-SP b3-a2 (□), and BSA-MBS (●). Binding is expressed as percentage of the binding of a 1:1,000 dilution of the unabsorbed serum.

shared a2 part of the peptides.

We then investigated the presence of antibodies in the serum directed against the b2 side of SP b2-a2 by absorption studies (Figure 1d). As expected, preincubation of antiserum BP-1 with BSA-MBS-SP b2-a2 completely abrogated binding to BSA-MBS-SP b2-a2. However, absorption with BSA-MBS maintained binding of BP-1 to BSA-MBS-SP b2-a2, suggesting a predominance of anti-SP b2-a2 antibodies in the serum. Absorption of BP-1 to BSA-MBS-SP b3-a2 showed only partial inhibition of antibody binding to BSA-MBS-SP b2-a2. This indicates that the majority of the anti-SP b2-a2 antibodies in serum BP-1 is directed against the b2 side and/or the b2-a2 joining region of the peptide.

DNA and RNA analysis of bcr-abl joining regions in CML cell lines

As a source for native $P210^{bcr\text{-}abl}$ we used three cell lines derived from CML patients in blast crisis: K562 (23), BV173 (24), and LAMA-84 (25). To determine whether the cell lines contain b3-a2 $P210^{bcr\text{-}abl}$ or b2-a2 $P210^{bcr\text{-}abl}$ we first localized at the DNA and RNA level the bcr breakpoints and *bcr-abl* junctions of the BV173 and LAMA-84 cells. As previously published, K562 cells contain a breakpoint 3′ of exon b3, and express a hybrid mRNA in which exon b3 is spliced to exon a2 (6). Southern blot analysis was performed using Bam HI and Bgl II restriction enzyme digests to pinpoint to genomic breakpoints on chromosome 22 in BV173 and LAMA-84 (Figure 2). One extra band was visible using 5′ *bcr* probes (probes a and b), while two rearranged bands were visible in both digests of LAMA-84 DNA, using a 3′ *bcr* probe. This indicates that the 3′ *bcr* probe (probe c) detects both the $22q^-$ and the $9q^+$ hybrid fragments in LAMA-84 DNA, which maps the breakpoint within this 1.2-kb 3′ Hind III-Bgl II

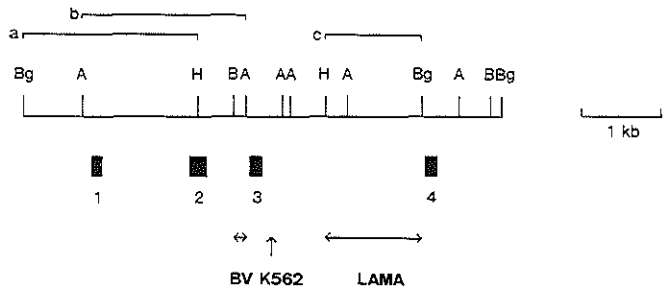


Figure 2 Genomic breakpoints in the bcr of three CML cell lines, in relation to the bcr exons and to the *bcr-abl* fusion mRNA. A restriction enzyme map is depicted of the bcr involved in CML. Bcr exons (b1-b4) are indicated by black boxes below the restriction enzyme map, and the probes used are shown above it ((a): 5′ *bcr*, a 2.2-kb Bgl II-Hind III fragment; (b): 5′ *bcr* Ava I a 2-kb AvaI fragment; (c) 3′ *bcr*, a 1.2-kb Hind III-Bgl II fragment).

fragment. Thus, LAMA-84 contains a breakpoint in the intron between exon b3 and b4. In BV173 DNA, only in the Bgl II digest was an extra band visible, using the 5′ *bcr* probe. However, using another 5′ *bcr* Ava I probe (probe b), two extra bands were seen in the Bam HI digest, apart from the two normal fragments. Thus, the breakpoint in BV173 is located just 5′ of exon b3 within the Bam HI-Ava I fragment.

To confirm the expected RNA structure surrounding the *bcr-abl* junction, we hybridized specific oligonucleotides (40-mers) comprising the b3-a2 and b2-a2 junction sequences to RNA preparations of the CML cell lines on Northern blots (Figure 3). After hybridization, stringent washings were performed, i.e., above the Tm of the three different 20-mers (b2, b3, and a2). Thus, a signal could only be obtained when a hybrid *bcr-abl* mRNA was present. As shown in Figure 3, the b3-a2 oligomer hybridizes exclusively to the 8.5-kb hybrid *bcr-abl* mRNA of the K562 and LAMA-84 cell lines. The b2-a2 oligomer however, only hybridizes to the *bcr-abl* mRNA of BV173. These results are in concordance with the DNA breakpoints in the *bcr* gene of these cell lines, and strongly indicate that BV173 cells contain b2-a2 $P210^{bcr\text{-}abl}$, whereas K562 and LAMA-84 cells both contain b3-a2 $P210^{bcr\text{-}abl}$. Surprisingly, we do not detect a b2-a2 alternative splice product in K562 RNA, as was previously found by others (13).

To confirm these observations we decided to use a highly sensitive assay based on the polymerase chain reaction (PCR) (26). A cDNA synthesis of K562 and BV173 RNA was performed, using a c-*abl* primer from exon a2. This cDNA was amplified using the PCR with *bcr* and c-*abl* primers mapping 345 and 270 bp apart in the b3-a2 and b2-a2 cDNAs, respectively. As is shown in Figure 4, the amplified fragment from K562 RNA is 345 bp long and hybridizes to the b3-a2 oligonucleotide, while no 270-bp fragment is detected after hybridization

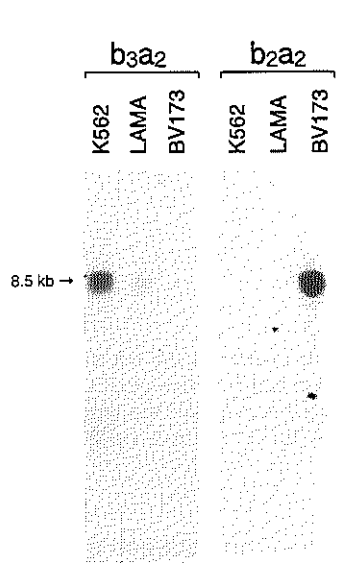


Figure 3 Northern blots of K562, LAMA - 84,and BV173 total RNA preparations. Northern blots were hybridized to ^{32}P-labeled 40-mers spanning the b3-a2 or b2-a2 *bcr-abl* junctions.
The filters were washed in 0.2 x SSC at 60°C. Size of the mRNA is indicated in kb.

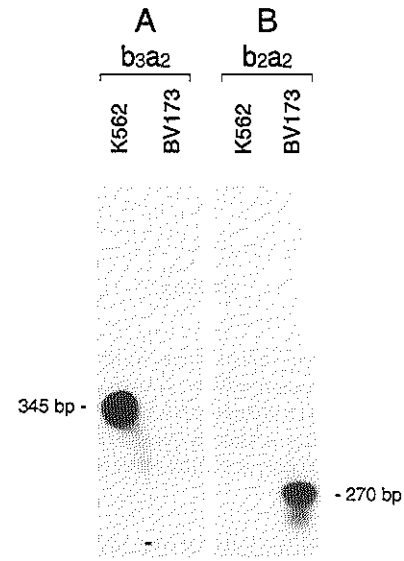


Figure 4 Amplification of the *bcr-abl* junction from K562 and BV173 cDNA. 2% agarose gels were run with 25% of the yield of the final PCR reactions. *A* was hybridization to the b3-a2 40-mer and *B* to the b2-a2 40-mer. Hybridization and washing conditions were as in Figure 3.

with the b2-a2 oligonucleotide. In contrast, in BV173 the 270-bp fragment is amplified and hybridizes to the b2-a2 oligonucleotide. These findings strongly argue against a b2-a2 alternative splice in the K562 cell line we used for the present experiments.

Immunoprecipitation of P210$^{bcr-abl}$ from CML cell lines

Binding of antiserum BP-1 to both b2-a2 P210$^{bcr-abl}$ and b3-a2P210$^{bcr-abl}$ was tested by immunoprecipitation and autophosphorylation of the proteins. Figure 5 shows a clear precipitation of b2-a2 P210$^{bcr-abl}$ from BV173 cells (lane 2). Strikingly, b3-a2 P210$^{bcr-abl}$ from LAMA-84 and K562 cells was not precipitated by BP-1 (Figure 5, lanes 4 and 6). To show that b3-a2 P210$^{bcr-abl}$ indeed is present in our K562 and LAMA-84 cells, we precipitated these molecules with a polyclonal antiserum directed against the NH_2 terminus of the BCR protein (Figure 6, lanes 2 and 4). Since P210$^{bcr-abl}$ is clearly precipitated, we conclude that antiserum BP-1 recognizes an antigenic determinant on b2-a2 P210$^{bcr-abl}$ which is not expressed on b3-a2 P210$^{bcr-abl}$.

To analyze the specificity of the binding of BP-1 to P210$^{bcr-abl}$ in more detail,

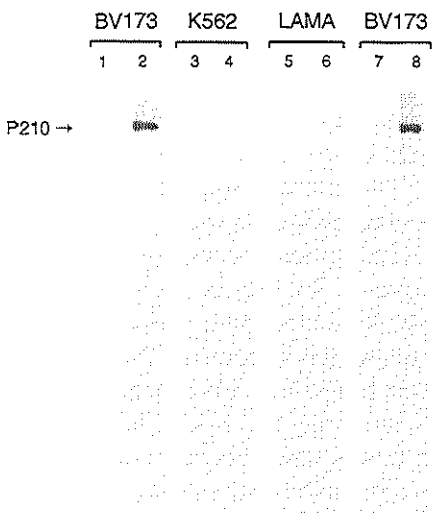


Figure 5 Immunuprecipitation analysis of the specificity of antiserum BP-1. BV173, K562, and LAMA-84 cells were lysed and immunoprecipitated with 10 µl Na_2SO_4 precipitated normal rabbit serum (lanes 1, 3, and 5) and with 10 µl Na_2SO_4 precipitated serum BP-1 (lanes 2, 4, and 6). (Lane 7 and 8) Immunoprecipitation analysis of BV173 cells with serum BP-1 after addition of 0.25 mM SP b2-a2 (lane 7) and 0.25 mM SP b3-a2 (lane 8). Immunoprecipitation and kinase reaction were carried out according to Chan et al. (21).

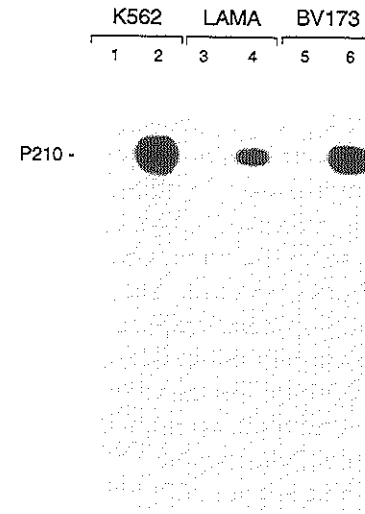


Figure 6 Immunoprecipitation analysis of K562, LAMA-84, and BV173 cells with a polyclonal serum directed against the NH_2 terminus of the BCR protein. The cells were lysed and precipitated with 25 µl Na_2SO_4 precipitated normal rabbit serum (lanes 1, 3, and 5) and with 25 µl Na_2SO_4 precipitated anti-BCR antiserum (lanes 2, 4, and 6).

peptide blocking studies were performed, either with unconjugated SP b2-a2 or with SP b3-a2. Figure 5 shows that immunoprecipitation of b2-a2 $P210^{bcr\text{-}abl}$ was prevented after incubation of BP-1 with the cognate peptide b2-a2 (Figure 5, lane 7). In contrast, incubation with SP b3-a2 (Figure 5, lane 8) had no effect on immunoprecipitation. This indicates that precipitation of b2-a2 $P210^{bcr\text{-}abl}$ could not be caused by antibodies directed against the a2 part of $P210^{bcr\text{-}abl}$ since those antibodies were removed by absorption with the b3-a2 peptide.

Discussion

$P210^{bcr\text{-}abl}$ can be considered as a highly tumor-specific protein for CML (8-11). In addition, the molecule is also detected in 10-25% of Ph-positive ALL patients (12). It has been suggested that these patients have a CML blast crisis without or with a very short, preceding chronic phase (27).

The tumor-specific character of $P210^{bcr\text{-}abl}$ is different from that of other previously described tumor-specific or tumor-associated antigens. A problem occurring for many tumor-associated antigens is that they are not only produced by tumor cells, but also, although in a lower degree, by normal cells (28). The P97 antigen, for example, is highly associated with malignant melanoma, but is also expressed on normal tissue (29). Other antigens, such as the TAG-72 antigen (30), are not specific for one particular tumor but are expressed on a variety of tumors. In contrast, $P210^{bcr\text{-}abl}$ can only be produced by cells carrying the CML-specific $^{bcr\text{-}abl}$ translocation and is therefore, by definition, a tumorspecific antigen. $P210^{bcr\text{-}abl}$ however, is composed of the normal BCR and ABL proteins, which are, as such, non-tumor-specific determinants on the fusion protein. Theoretically, the only tumor-specific determinant on the $P210^{bcr\text{-}abl}$ molecule is formed just by the joining between BCR and ABL.

In this report we show the possibility to produce a polyclonal antiserum recognizing the BCR-ABL joining region by using a synthetic peptide corresponding the b2-a2 junction as an immunogen. Molecular studies revealing the nucleotide sequence at this junction (6) made this direct approach possible. As such, our approach differs principally from other strategies meant for the production of tumor-specific antibodies, where tumor cells, tumor cell membranes, or purified proteins were used as immunogens (30-32).

The peptide we synthesized consisted of four amino acids derived from b2 and five amino acids derived from a2. Between these amino acids, one amino acid is located, which is newly generated by the Philadelphia translocation. The polyclonal antiserum we obtained after immunization with the peptide reacted in a very specific way with the native b2-a2 $P210^{bcr\text{-}abl}$ molecule using the protein kinase assay. In contrast, b3-a2 $P210^{bcr\text{-}abl}$ was not recognized by BP-1 in this assay. Our ELISA data indicated, however, that BP-1 reacted with different parts of the peptide; the a2 part as well as the b2 and/or b2-a2 junction were recognized by BP-1. Since no precipitation of b3-a2 $P210^{bcr\text{-}abl}$ was detected, the b2 and a2 amino acids were not recognized in the native b3-a2 $P210^{bcr\text{-}abl}$ molecules. We conclude, therefore, that the individual b2 and a2 determinants, as exposed on the peptide, are hidden within the tertiary structure of the native b3-a2 $P210^{bcr\text{-}abl}$ and are inaccessible for anti-b2 and anti-a2 antibodies.

We also showed that b2-a2 $P210^{bcr\text{-}abl}$ was not precipitated by antibody molecules directed against a2 sequences, because after removing anti-a2

reactivity from BP-1 by absorption, b2-a2 was still precipitated. This leaves us with the conclusion that under the present experimental conditions, b2-a2 $P210^{bcr\text{-}abl}$ from BV173 cells is precipitated by antibodies recognizing the b2-a2 joining region itself or by antibodies that recognize newly created tertiary b2 or a2 determinants introduced by the BCR-ABL joining region.

Furthermore, the observation that no b2-a2 $P210^{bcr\text{-}abl}$ is precipitated by BP-1 from the K562 cells was an extra confirmation of our molecular data, i.e., the absence of a b2-a2 alternative splice in the K562 cell line we used.

In summary, we have shown that breakpoint-specific DNA-encoded sequences are exposed on $P210^{bcr\text{-}abl}$ molecules and that, under the present experimental conditions, such sequences can be visualized at the protein level by antibodies generated through the use of synthetic peptides encoded by these sequences as immunogens. Obviously, antibodies obtained in this way may further aid in the clinical diagnosis of CML and in the distinction of the various malignant disorders where chromosome rearrangements are involved. Studies to detect individual tumor cells using immunohistochemical techniques are now in progress.

Acknowledgements

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CHAPTER 3.2

Antibody recognition of the tumor-specific b3-a2 junction of BCR-ABL chimeric proteins in Philadelphia chromosome-positive leukemias

Janneke van Denderen[1], Paulien ten Hacken[1], Paul Berendes[1], Netty Zegers[2], Wim Boersma[2], Gerard Grosveld[3] and Willem van Ewijk[1]

[1]Department of Immunology, Erasmus University, Rotterdam, [2]Medical Biological Laboratory, TNO, Rijswijk, [3]Department of Cell Biology and Genetics, Erasmus University, Rotterdam

Summary

The reciprocal translocation between chromosome 9 and chromosome 22, as observed in chronic myeloid leukemia (CML) as well as in acute lymphoblastic leukemia (ALL), results in a 22q$^-$ chromosome, the so called Philadelphia chromosome. The translocation event creates on the Philadelphia chromosome a fusion between two genes: *bcr* and *abl*. Depending on the localization of the breakpoint in the *bcr* gene different chimeric *bcr-abl* genes are generated, each encoding their own tumor-specific protein: e1-a2 P190$^{bcr-abl}$, b2-a2 P210$^{bcr-abl}$ or b3-a2 P210$^{bcr-abl}$. Especially in ALL, the presence of such a tumor-specific protein is highly associated with a poor prognosis. Detection of these proteins therefore has a strong clinical significance. In this study a polyclonal antiserum, termed BP-2 was raised against a synthetic peptide, corresponding to the tumor-specific 'fusion-point' epitope of the b3-a2 P210$^{bcr-abl}$ protein. The specificity of BP-2 for the BCR-ABL joining region in b3-a2 P210$^{bcr-abl}$ is demonstrated by means of peptide inhibition studies in combination with immunoprecipitation. In addition we show the reactivity of BP-2 with BCR-ABL proteins in leukemic cells of a Philadelphia chromosome-positive ALL patient.

Introduction

The Philadelphia (Ph) chromosome, a minute chromosome 22, is the best known chromosomal aberration in cancer cytogenetics. Since the Ph chromosome is detected in more than 90% of all patients with chronic myeloid leukemia (CML), it can be considered as a highly reliable tumor-marker for CML (1). However, the Ph chromosome is also demonstrated in acute leukemias (2). A minority (2-5%) of all patients with acute myeloid leukemia (AML) and childhood acute lymphoblastic leukemia (ALL) shows expression of the Ph chromosome (3,4). In contrast, in adult ALL the Ph chromosome is the most

common chromosomal abnormality with an incidence of 30% (5). Moreover, with respect to both childhood and adult ALL, various studies have demonstrated a strong association between the presence of a Ph chromosome and a poor prognosis of the disease (6). In view of the most appropriate therapy to be applied, it is of great importance to develop sensitive techniques using antibodies for the accurate detection of Ph-positive ALL.

The Philadelphia chromosome results from a reciprocal translocation between chromosomes 9 and 22 (1,7). Molecular studies have shown that two genes are involved in this process: the *abl* gene on chromosome 9 and the *bcr* gene on chromosome 22 (8). As the result of the translocation both genes are interrupted and translocated resulting in a chimeric *bcr-abl* gene on chromosome 22.

Breakpoints on chromosome 9 are scattered over a distance of more than 175 kb upstream of the *abl* exon 2 (a2). On the other hand, breakpoints in the *bcr* gene occur within two well-defined areas, the so called 'minor' (m-bcr) and 'major' breakpoint cluster region (M-bcr) (9-11). The m-bcr is localized between the first (e1) and the second exon (e2) of the *bcr* gene. The M-bcr spans the exons 12 to 15 in the middle of the *bcr* gene, termed b1 to b4. Here, breakpoints occur either between exon b2 and b3, or between b3 and b4 (12).

Depending on the localization of the respective breakpoints within the *bcr* gene three different chimeric *bcr-abl* genes are expressed, either with a junction between the exons e1 and a2, b2 and a2, or b3 and a2. As a consequence, three different proteins are encoded by the respective chimeric genes. A breakpoint in the m-bcr results in translation of a 190-kD protein, $P190^{bcr\text{-}abl}$, comprising the e1-a2 BCR-ABL junction (13-16). Chimeric genes with M-bcr breakpoints encode 210-kD proteins, $P210^{bcr\text{-}abl}$, either comprising the b2-a2 or the b3-a2 junction (17,18). All BCR-ABL proteins have an elevated tyrosine kinase activity (13-15, 19).

In CML, breakpoints occur almost exclusively in the M-bcr, resulting in expression of either b2-a2 or b3-a2 $P210^{bcr\text{-}abl}$. In around 30% of all Ph-positive ALL patients breakpoints are found in the M-bcr, while almost 70% of all Ph-positive ALL patients express breakpoints in the m-bcr, resulting in e1-a2 $P190^{bcr\text{-}abl}$ protein (21). Therefore, both expression of $P210^{bcr\text{-}abl}$ and $P190^{bcr\text{-}abl}$ in ALL indicates presence of the Ph chromosome with the implication of a poor prognosis. In this respect, $P210^{bcr\text{-}abl}$ and $P190^{bcr\text{-}abl}$ can be considered as highly valuable tumor-specific markers. Accurate detection of $P210^{bcr\text{-}abl}$ and $P190^{bcr\text{-}abl}$ is therefore of great importance.

Although $P210^{bcr\text{-}abl}$ and $P190^{bcr\text{-}abl}$ are tumor-specific proteins, they are composed of amino acid sequences present in the normally occurring cellular proteins BCR and ABL, the only tumor-specific part of the proteins are the respective junctions between BCR and ABL.

In this study, we report on the generation of an antiserum, termed BP-2, specifically recognizing the b3-a2 junction on $P210^{bcr\text{-}abl}$. This finding indicates that the b3-a2 junction is antigenically exposed on the chimeric protein, enabling immunodetection, as we described previously for both the b2-a2 junction in $P210^{bcr\text{-}abl}$ and the e1-a2 junction in $P190^{bcr\text{-}abl}$(16, 18).

Analysis of a blood sample of a Philadelphia-positive ALL patient shows that antiserum BP-2 can be used in the diagnosis of ALL.

Materials and methods

Cell lines

K562 and BV173 are cell lines derived from patients during blast crisis of CML (21, 22). TOM-1 is a cell line derived from a Ph-positive ALL patient (23). Cells were cultured in RPMI medium supplemented with 10% fetal calf serum, 100µg/ml penicillin and 60 µg/ml streptomycin.

ALL patient

Patient S. is a 51 year old female patient exhibiting a standard Philadelphia translocation, t(9;22)(q34;q11). She was diagnosed as common ALL based on immunologic criteria. Blast cells were isolated from peripheral blood by Ficoll-Hypaque centrifugation. After cryo-preservation using a controlled freezing apparatus (Planer Biomed, Sunbury-on-Times, UK), the cells were stored under liquid nitrogen.

Peptide synthesis

Peptides were synthesized using solid-phase synthesis as described previously (18). Purification of the peptides was performed by gel filtration and reverse phase high liquid chromatography.

Peptides corresponding to the b3-a2 junction in $P210^{bcr\text{-}abl}$ were coupled through 1-ethyl-3 (3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) to the carrier molecule keyhole limpet hemocyanin (KLH).

During synthesis the peptide corresponding to the ABL protein was elongated with a cysteine residue at the amino-terminal part. This peptide was coupled to carrier protein chicken gamma globulin (CGG) via the terminal cystein residue using sulfo-m-maleimido-benzoyl-sulfosuccinimide-ester (S-MBS).

Immunizations, antisera and purification of immunoglobulin

Antiserum BP-2 Flemish giant rabbits (MBL-TNO, Rijswijk, the Netherlands) were primed intracutaneously with 250 µg antigen, consisting of peptide b3-a2 coupled through EDC to KLH, emulsified in an equal volume of Complete Freund's Adjuvanst. Rabbits were boosted twice after intervals of four weeks. The first time with 250µg antigen in CFA, the second time with the same dose in Incomplete Freund's Adjuvans. Fourteen days after the last boost rabbits were bled and sera were collected.

Anti-ABL antiserum Anti-ABL antiserum is a polyclonal rabbit antiserum raised against

a synthetic peptide with proven antigenicity, comprising the ABL sequence SISDEVEKEL-GK (24). Flemish giant rabbits were immunized with 250 µg antigen consisting of ABL peptide coupled via MBS to the carrier protein chicken gamma globulin. The immunization procedure was as described above.

Anti-BCR antiserum Anti-BCR antiserum is a polyclonal rabbit antiserum directed against amino acid sequences encoded by the first exon of the *bcr* gene. The antiserum was prepared as described previously (18).

Antiserum BP-1 Antiserum BP-1 is a polyclonal rabbit antiserum detecting specifically the b2-a2 junction $P210^{bcr\text{-}abl}$. This antiserum was prepared as described previously (18).

Antiserum BP-ALL Antiserum BP-ALL is a polyclonal antiserum specific for the sequence overlapping the e1-a2 junction in $P190^{bcr\text{-}abl}$. This antiserum was prepared as described previously (16).

Immunoglobulin purification

The Ig fraction of all sera was purified by precipitation with 16% Na_2SO_4 (final concentration). The precipitates were dissolved in phosphate-buffered saline and desalted using Sephadex G-25M columns (Pharmacia, Uppsala, Sweden).

Protein tyrosine kinase assay

Immunoprecipitations and tyrosine kinase assays were performed as described previously (16).

Results

Peptides corresponding to bcr-abl junctions in $P210^{bcr\text{-}abl}$

Based on the previously published nucleotide sequence (25) a peptide was synthesized corresponding to the b3-a2 junction in $P210^{bcr\text{-}abl}$. The localization of the chosen peptide within the breakpoint region was based on calculations, in such a way that a peptide with optimal hydrophilicity was selected. This peptide, termed SP b3-a2, consists of the following amino acids:

G F K Q S S K^* A L Q

In the b3-a2 $P210^{bcr\text{-}abl}$ encoding gene the *bcr* and *abl* sequences are fused within a coding triplet (25). As a result, the amino acid encoded by this triplet, i.e. K^* (lysine), is newly generated during the translocation process. In the synthetic peptide the amino acids GFKQSS are BCR derived, whereas ALQ are ABL derived residues.

As controls for peptide inhibition studies the following peptides were constructed:

'control' SP b3-a2 : CKQSS K^+ **ALQ**, sharing all the amino acids with the above mentioned SP b3-a2 around the BCR-ABL fusion-point, except the amino acids G and F

SP e1-a2 : CAFHGDAE**ALQ**, sharing the a2-derived amino acids **ALQ** with SP b3-a2

SP b2-b3 : CINKEKQSS, sharing the b3-derived amino acids KQSS with SP b3-a2. SP b2-b3 does not correspond with an amino acid sequence occurring naturally in the native chimeric protein. The residues INKE are the hind amino acids encoded by exon b2, whereas KQSS are encoded by the last part of exon b3.

Binding of antiserum BP-2 to BCR-ABL chimeric proteins

The polyclonal antiserum BP-2 was raised in rabbits against SP b3-a2. Reactivity of BP-2 with native BCR-ABL chimeric proteins in the CML cell lines K562 and BV173 was tested with an immunoprecipitation assay followed by an autophosphorylation reaction. As described previously, in K562 cells b3-a2 $P210^{bcr\text{-}abl}$ and in BV173 cells b2-a2 $P210^{bcr\text{-}abl}$ is expressed (18). As shown in Figure 1, b3-a2 $P210^{bcr\text{-}abl}$ is clearly precipitated from a K562 lysate by antiserum BP-2 (K562, lane 2). In contrast, b2-a2 $P210^{bcr\text{-}abl}$ in a lysate of BV173 cells is not recognized by BP-2 (BV173, lane 2). $P210^{bcr\text{-}abl}$ is present in BV173 cells, because the protein is precipitated by a control antiserum directed

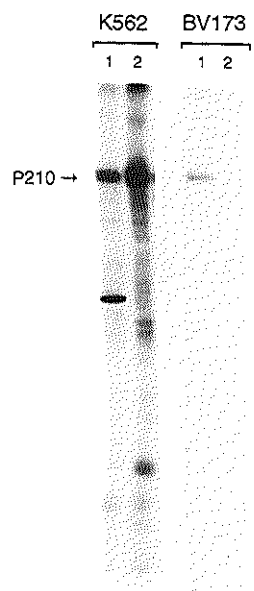

Figure 1 Immunoprecipitation analysis of antiserum BP-2. K562 cells (harboring b3-a2$P210b^{bcr\text{-}abl}$) and BV173 cells (harboring b2-a2 $P210^{bcr\text{-}abl}$) were lysed and proteins were precipitated with 25µl Na_2SO_4 precipitated antiserum BP-2 (lanes 2). As a control, proteins were precipitated with 25µl Na_2SO_4 anti-BCR antiserum (lanes 1).
Immunoprecipitation was followed by autophosphorylation of the proteins (18).

against the amino-terminus of the BCR protein (BV173, lane 1). These results imply that antiserum BP-2 is directed to the b3-a2 junction in b3-a2 P210b$^{cr\text{-}abl}$.

To further confirm the specificity of BP-2 for b3-a2 P210$^{bcr\text{-}abl}$ junctions only, we performed peptide inhibition studies. Antiserum BP-2 was first incubated with its cognate peptide SP b3-a2. Next the peptide-incubated BP-2 was added to a lysate of K562 cells for the immunoprecipitation analysis. As shown in Figure 2, lane 2, precipitation of b3-a2 P210$^{bcr\text{-}abl}$ was completely abrogated after incubation of BP-2 with 6 mM SP b3-a2. This finding indicates that the antibodies directed against the SP b3-a2 also precipitate the native protein P210$^{bcr\text{-}abl}$.

However, due to the polyclonal character of antiserum BP-2 three different types of antibody molecules could be responsible for the precipitation of b3-a2 P210$^{bcr\text{-}abl}$:

- antibodies directed against b3-derived amino acids (GFKQSS) or
- antibodies directed against a2-derived amino acids (ALQ) or
- antibodies directed against amino-acids forming a 'fusion-point epitope'.

We therefore performed several control experiments.

First, to show that the inhibition of the immunoprecipitation with BP-2 of b3-a2 P210$^{bcr\text{-}abl}$ is not restricted to the cognate peptide, but can also be performed with a slightly different peptide, we used another b3-a2 peptide, the so-called 'control' SP b3-a2. The 'control' peptide differs from the cognate peptide at the amino-terminus: in 'control' SP b3-a2 two amino-acids (G and F) are lacking compared to the cognate peptide. So, in this experiment we tested whether there was anti-G and/or anti-F reactivity present in BP-2. As shown in Figure 2, lane 3, the immunoprecipitation of b3-a2 P210$^{bcr\text{-}abl}$ is inhibited by pre-incubating BP-2 with the 'control' SP b3-a2 to the same extent as by pre-incubation of BP-2 with cognate SP b3-a2 (Figure 2, lane 2). This result indicates that immuno-

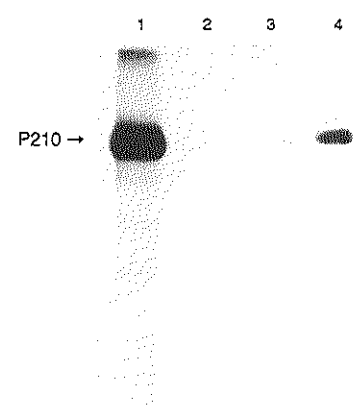

Figure 2 Immunoprecipitation analysis of the specificity of antiserum BP-2. K562 cells were lysed and immunoprecipitated with 40 µl Na_2SO_4 precipitated antiserum BP-2 after preincubation of BP-2 with: lane 1: 10 µl PBS; lane 2: 5 µl 6 mM SP b3-a2 + 5 µl PBS; lane 3: 5 µl 6 mM 'control' SP b3-a2 + 5 µl PBS; lane 4: 5 µl 6 mM SP b2-b3 + 5 µl 6 mM SP e1-a2.

globulins recognizing an epitope that comprising the G and F amino-acids at the b3-part of the molecule are not responsible for the immunoprecipitation of b3-a2 $P210^{bcr\text{-}abl}$. Moreover, we show that the inhibition is not restricted to the configuration of the peptide.

Next, to elucidate further anti-b3 and anti-a2 reactivity in the antiserum, BP-2 was pre-incubated with a mixture of the peptides SP b2-b3 and SP e1-a2. Here, SP b2-b3 represents the b3-derived amino acids, while SP e1-a2 represents the a2-derived amino-acids, as present in the 'control' SP b3-a2. After incubation with these peptides, antibodies recognizing epitopes on the b3-part only, as well as antibodies recognizing epitopes on the a2-part only, can be considered inactive. As a consequence these immunoglobulins can not be held responsible for the immunoprecipitation of b3-a2 $P210^{bcr\text{-}abl}$. As shown in Figure 2, lane 4, b3-a2 $P210^{bcr\text{-}abl}$ is still precipitated by BP-2 pre-incubated with the peptide mixture. Since the signal is less intense compared to the control band in lane 1 (Figure 2), we conclude that, there was anti-b3 and/or anti-a2 reactivity present in antiserum BP-2. However, the most important conclusion is that the precipitation of b3-a2 $P210^{bcr\text{-}abl}$ as observed in lane 4 can only be caused by antibodies recognizing a b3-a2 'fusion-point epitope' present in the chimeric b3-a2 $P210^{bcr\text{-}abl}$ protein.

Sensitivity of the tyrosine kinase assay by using antiserum BP-2

To determine the detection limit of $P210^{bcr\text{-}abl}$ by using antiserum BP-2 in the tyrosine kinase assay we performed dilution experiments. To this purpose, b3-a2 $P210^{bcr\text{-}abl}$ containing K562 and e1-a2 $P190^{bcr\text{-}abl}$ expressing TOM-1 cells were mixed in various ratios. Next, the cells were lysed and b3-a2 $P210^{bcr\text{-}abl}$ from the K562 cells was precipitated with antiserum BP-2. As shown in Figure 3 (lane 4), a clear $P210^{bcr\text{-}abl}$ band is still detectable after precipitation of a mixture of 0.5% K562 cells and 99.5% TOM-1 cells. Here, 2.5×10^3 K562 cells were present in the sample. These results indicate that, using antiserum BP-2 in the tyrosine kinase assay, we can detect in principle 1 aberrant cell in 200 normal cells.

Precipitation of BCR-ABL proteins from leukemic cells of a Ph-positive ALL patient

Next, we investigated whether BP-2 precipitated $P210^{bcr\text{-}abl}$ not only from cell lines but also from leukemic cells derived from a Ph-positive ALL patient. Blast cells from peripheral blood of patient S. were enriched by Ficoll-Hypaque centrifugation and, the cells were then lysed and incubated with BP-2. The immunoprecipitation was followed by an autophosphorylation reaction. Figure 4 clearly shows precipitation of b3-a2 $P210^{bcr\text{-}abl}$ from the leukemic cells of patient S (lane 2). In contrast, the chimeric protein was neither precipitated by

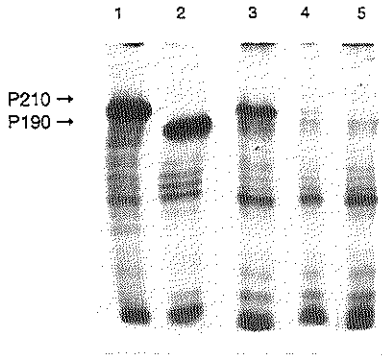

Figure 3 Analysis of the detection limit of the tyrosine kinase assay using antiserum BP-2.
As controls, $5x10^6$ K526 cells (lane 1) and $5x10^6$ TOM-1 cells (lane 2) were lysed and precipitated with 50 µl anti-ABL antiserum. K562 (b3-a2P210$^{bcr-abl}$) cells and TOM-1 (e1-a2-P190$^{bcr-abl}$) cells were mixed at ratio: 1:20 (lane 3) or 1:200 (lane 4). Lane 5 represents TOM-1 cells alone. The total amount of cells (5×10^6) was lysed and b3-a2P210$^{bcr-abl}$ was precipitated using 50 µl Na_2SO_4 precipitated BP-2.

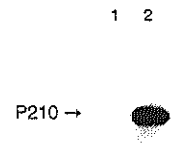

Figure 4 Immunoprecipitation analysis of Ficoll-Hypaque-enriched peripheral blood cells of ALL patient S.
Cells were lysed and proteins were precipitated with 50 µl Na_2SO_4 precipitated anti-b2-a2 P210$^{bcr-abl}$ (BP-1) antiserum (lane 1), or with 50 µl Na_2SO_4 precipitated anti-b3-a2P210$^{bcr-abl}$ (BP-2) antiserum (lane 2), or precipitated with 50 µl Na_2SO_4 precipitated anti-e1-a2P190$^{bcr-abl}$ (BP-ALL) antiserum (lane 3).

antiserum BP-1 (lane 1) nor by BP-ALL (lane 3), recognizing the BCR-ABL junction in b2-a2 P210$^{bcr-abl}$ and in e1-a2 P190$^{bcr-abl}$ respectively.

This observation demonstrates that the b3-a2 fusion-point is also antigenically exposed in b3-a2 P210$^{bcr-abl}$ in leukemic cells of a Ph-positive patient.

Discussion

Presence of the Philadelphia chromosome both in children and in adults with ALL is correlated with a very poor prognosis (6). Therefore, sensitive and reliable methods for the detection of the Ph-positive ALL are extremely important in the diagnosis of ALL. In this respect various techniques have been described, all based on the unique molecular *bcr-abl* rearrangements caused by the Philadelphia translocation.

A conventional technique is the detection of the Ph chromosome by cytogenetic analysis. Other methods include the detection of the specific *bcr-abl* rearrangements by Southern blot analysis, pulsed-field gel electrophoresis and Northern blot analysis. With these techniques the detection limit is 1-5% malignant cells (26, 27). More sensitive is the polymerase chain reaction (PCR), by which at the RNA level the *bcr-abl* junctions are amplified in a highly specific way. The detection limit of the latter technique is approximately 1 leukemic cell

in 100,000 (27). However for routine application in a diagnostic laboratory this technique is not the most obvious, due to a high risk for contamination of RNA from positive to negative samples.

In this paper we have reported on a highly specific detection of BCR-ABL junctions at the protein level, using polyclonal antibodies. With the applied method, i.e. immunoprecipitation followed by autophosphorylation, we detect malignant cells in a frequency of approximately 1:200. However, this is probably an overestimate, because the chimeric *bcr-abl* gene is over-expressed in K562 cells (8).

Previously we described specific antibody recognition of the b2-a2 and the e1-a2 junction in the respective BCR-ABL chimeric proteins in CML and ALL (16,18). In the present study we show that also the b3-a2 junction in $P210^{bcr\text{-}abl}$ can be recognized by a specific polyclonal antiserum BP-2. By using the tyrosine kinase assay serum BP-2 reacted in a highly specific way with $P210^{bcr\text{-}abl}$ comprising the b3-a2 junction. No reactivity was found with the other BCR-ABL chimeric proteins, b2-a2 $P210^{bcr\text{-}abl}$ and e1-a2 $P190^{bcr\text{-}abl}$. From these results we conclude that the amino-acid sequence at the b3-a2 BCR-ABL junction is recognized by antibody molecules in antiserum BP-2. In addition these results indicate that similar to the b2-a2 and the e1-a2 junction, the b3-a2 BCR-ABL junction is also antigenically expressed on the chimeric protein.

However, by performing peptide inhibition studies we demonstrated that antiserum BP-2, in addition to antibody molecules directed against the specific 'fusion-point epitope', comprises also antibody molecules recognizing b3-derived amino acids. Therefore, due to this polyclonal character of the serum, BP-2 is not optimal for cell staining experiments, because next to the chimeric BCR-ABL proteins also the normal BCR proteins will be stained. This problem will be solved when 'fusion-point epitope' monoclonal antibodies are available. We have demonstrated in this study that, in principle, such antibodies can be raised.

Acknowledgements

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CHAPTER 3.3

A novel variant of the BCR-ABL fusion product in Philadelphia chromosome-positive acute lymphoblastic leukemia

D. Soekarman[1], J. van Denderen[2], L. Hoefsloot[1], M. Moret[1], T. Meeuwsen[2], J. van Baal[1], A. Hagemeijer[1] and G. Grosveld[1].

[1]Department of Cell Biology and Genetics, [2]Department of Immunology, Erasmus University, Rotterdam, The Netherlands

Summary.

Two patients with Philadelphia chromosome-positive acute lymphoblastic leukemia showed novel variants of the chimeric *bcr-abl* mRNA. The *bcr-abl* breakpoint region on cDNA derived from the chimeric mRNA was amplified, using the polymerase chain reaction (PCR). Sequence analysis of the breakpoint containing fragment showed that in both patients exon a2 of the *abl* gene was deleted, giving rise to an in frame joining at the mRNA level of 5' *bcr*-sequences to the *abl* exon a3. These findings were confirmed by Southern blot analysis and cloning of chromosomal DNA. Protein studies showed a BCR-ABL protein with heightened tyrosine kinase activity in blast cells of both patients: one of the P190 type, the other of the P210 type. The significance of these findings and the role of this new type of translocation in the deregulation of the *abl* gene are discussed.

Introduction

In 95% of the patients with chronic myeloid leukemia (CML) the Philadelphia (Ph) chromosome is found, which is the result of a reciprocal translocation between chromosomes 9 (q34) and 22 (q11) (1). Due to this event the *abl* oncogene is translocated to the major breakpoint cluster region (M-bcr-1) of the *bcr* gene on chromosome 22 (2,3). So far, the breakpoint in the *abl* gene on chromosome 9 seems to occur in a 200-kilobase (kb) intron, always 5' of *abl* exon a2 (4). Most breakpoints in the M-bcr-1 region on chromosome 22 are located between exons b2 and b3 or b3 and b4. The *bcr-abl* fusion gene on the Ph chromosome is transcribed into an 8.5-kb chimeric *bcr-abl* mRNA which shows either a b2-a2 or a b3-a2 joining at the mRNA level (5-8). The 8.5-kb *bcr-abl* mRNA is translated into a 210-kilodalton (kD) BCR-ABL fusion protein showing enhanced in vitro tyrosine kinase activity when compared to the

normal, 145-kD ABL protein (9).

The Ph chromosome does not solely occur in CML but can also be found in approximately twenty percent of adult patients with acute lymphoblastic leukemia (ALL) (10). Roughly fifty percent of these cases show a *bcr-abl* joining at the mRNA level similar to CML (11). The remaining fifty percent has a breakpoint 5' upstream from the M-bcr-1 region, i.e. in the first intron of the *bcr* gene (minor breakpoint cluster region, m-bcr-1) (12). In the latter case the *bcr-abl* fusion gene on chromosome 22 gives rise to a 7.0-kb mRNA. Here the first exon of the *bcr* gene (e1) is spliced to the second exon of the c-*abl* gene (a2), resulting in an e1-a2 joining (13). The corresponding hybrid protein has a molecular weight of 190 kD, and also shows enhanced tyrosine kinase activity (14-17, for review see 18).

The elucidation of the molecular structure of the *bcr-abl* chimeric product and the development of the highly specific and sensitive polymerase chain reaction (PCR), allows us to diagnose Ph positivity in patients with leukemia. Therefore, patients with a Ph chromosome are routinely screened in our laboratory by PCR analysis to determine breakpoint junctions at the mRNA-level, i.e. e1-a2, b2-a2 and b3-a2 (19). Surprisingly two patients with ALL were found who showed an amplified *bcr-abl* fragment which did not hybridize to any of the available breakpoint probes. Sequence analysis revealed a deletion of *abl* exon a2 in the *bcr-abl* mRNA, which was confirmed by DNA-studies. Both patients expressed a BCR-ABL protein, one of the P190 type, the other of the P210 type. To our knowledge, these two patients are the first reported to have such a deletion in association with a Ph-positive leukemia.

Materials and methods

Patients

Both patients were diagnosed and treated in Rotterdam, the Netherlands: patient 1 at the University Hospital Dijkzigt, patient 2 at the Dr. Daniel den Hoed Cancer Center. The diagnosis ALL was based on the clinical and hematologic data, bone marrow morphology and immunophenotyping of the blast cells (Table 1).

At the time of diagnosis patient 1, a 39-years-old female presented with a history of bleeding and infections. On clinical examination there were no signs of lymphadenopathy or hepatosplenomegaly. The white blood cell count (WBC) was $8.4 \times 10.^{9}$/l showing 60% lymphoblasts. A bone marrow aspirate showed hypercellularity with 75% lymphoblasts. Cytochemically, 70% of the blasts were characterized by positivity for ANA-esterase with resistance to NaF and 30% of the blasts were PAS-positive. Peroxidase and acid phosphatase staining were negative. Immunophenotypical analysis of the lymphoblasts showed reactivity with antibodies for CD10, CD19, CD24, HLA-DR and TdT. Partial positivity for CD20 and CD34 existed. From this pattern it was concluded that the patient had pre-pre-B acute lymphoblastic leukemia. Remission-induction treatment

consisted of daunorubicin, vincristine, L-asparaginase and prednisone. In addition CNS prophylaxis was given (methotrexate and dexamethasone). Complete remission was attained 2.5 months after diagnosis. Despite consolidation-maintenance therapy, three months later a florid relapse occurred with more than 80% blast cells in bone marrow and peripheral blood. The patient died 9 months after diagnosis.

Table 1 Hematologic and cytogenetic data of cells used for the molecular investigation of patient 1 and 2

patient	age sex years	clinical phase	WBC $x10^9/l$	%blast PB	%blast BM	immuno-phenotype	karyotype
1	39 , F	relapse	40.0	89	82	pre-pre-B	46,XX(70%)/46,XX, t(9;22) + other aberrations (30%)
2	61,F	diagn.	209.0	79	53	pre-B	46,XX(44%)/46,XX t(9;11;22)(56%)

Data of patient 2 have been published previously (ref.11: patient R6, 20). Briefly, at the time of diagnosis this 61-years-old female presented with a 3 months history of fatigue and fever. Clinical examination showed a hepatosplenomegaly and a leucocytosis (WBC 209x10.^{9}/l).The differential count showed 53% blast cells, 1% myelocytes, 5% metamyelocytes, 20% neutrophils, 9% bands, 4% monocytes, 1% eosinophils, 6% lymphocytes. The bone marrow was hypercellular with 79% blast cells, which were Sudan Black negative and PAS positive. The blasts were of the immunologic pre-B phenotype (Tdt+, CD10+, Clg+). The diagnosis ALL was made although a CML in blast crisis at presentation could not be ruled out. Remission-induction treatment (daunorubicin, vincristine and prednisolone) and CNS prophylaxis was given and subsequently a complete remission was achieved. Notably, although cytogenetic study of the bone marrow showed persistence of the Ph chromosome in 70% of the metaphases, there were no hematologic features during remission to suggest a chronic phase of CML. 10.5 months later this patient suffered from a relapse of the ALL and died after a total survival time of 14 months.

Samples

Bone marrow aspirates and blood samples were part of diagnostic and clinical follow-up studies. Data of specimen used for the molecular investigations are presented in Table 1. Sterile samples were collected in heparinized tubes. After isolation of blast cells by Dextran or a Ficoll-Hypaque gradient, cells were frozen and stored in liquid Nitrogen until used.

Karyotyping

Cytogenetic studies were performed using standard procedures. Chromosomes were identified by G,Q and R banding techniques and classified according to the ISCN (1985) (21). In both patients PHA stimulated blood cultures were used to determine the constitutional karyotype.

Polymerase chain reaction (PCR)

The RNA-cDNA preparation was performed as described by Hermans et al. (19) with the following modifications for the PCR. Half of the cDNA preparation was used for amplification of a control *abl* fragment, the other half was used for amplification of the *bcr-abl* fragment. The PCR was performed in a volume of 55 µl. Changes in the composition of the Taq polymerase buffer were as follows: the molarity of MgCl2 was raised from 6 mM to 9 mM and 40 mM KCl was added. The molarity of the dNTPs was raised from 2.5 mM to 5 mM each. Amplification of the *bcr-abl* fragment was performed by adding 0.25 µg of primer 2 and 3 per PCR-reaction (Figure 1). As a positive control for the PCR experiment a 450-bp *abl* fragment was amplified, which is present both in healthy individuals and in patients with leukemia. This was done by combining primer 1 with a 25-mer sense oligonucleotide located in exon IB of the human *abl* gene (ATGCAGCGAATGTGAAATCCCACGT, primer 4 in Figure 1).

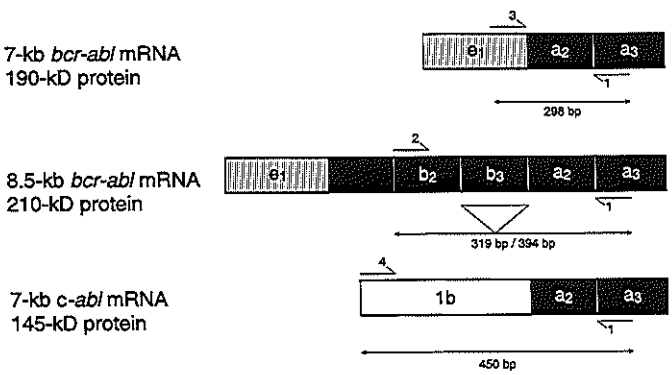


Figure 1 The various mRNA-molecules studied by cDNA preparation and the PCR are represented by bars. Exons derived from the *bcr* gene are hatched and exons from the *abl* gene are open. Primers for the PCR are depicted as arrows and numbered according to the text. Our primers 1, 2 and 3 are the same as c-*abl* a3, mcr b2 and bcr gene 1st exon e1 respectively in Hermans et al (19). The size of the fragments generated by the PCR is given underneath the corresponding fragments. The triangle indicates that exon b3 may be present or absent in the 8.5 kb-*bcr-abl* mRNA.

To exclude contamination during the PCR by *bcr-abl* containing fragments, RNA from a healthy individual was reverse transcribed and amplified as a control in all the experiments. One Unit of Taq polymerase (Cetus) was added per PCR-reaction, and the reaction mixture was covered by a layer of paraffin oil. Denaturation of the sample was allowed for 3 min. at 93°C and annealing of the primers was allowed for 2 min. at 55°C. This was followed by 24 cycles of extension (5 min., 70°C), denaturation (1 min., 93°C) and annealing (1 min., 55°C). Ten percent of the reaction was used for another 24 cycles after adding new reagents. Ethanol precipitation was followed by electrophoresis of thirty percent of the reaction mixture after 48 cycles of amplification through a 2% agarose gel. Four identical Southern blots on nylon filter (Zeta probe) were prepared from this gel. Each filter was hybridized separately to 32P end-labeled breakpoint oligonucleotides specific for the b2/b3-a2 and e1-a2 junctions at 65°C (probes described in ref. 19). A 25-mer oligonucleotide (TTGAACCCTCTTCTGGAAAGGGGTA), situated 43 nucleotides

3′ of primer 4 in the IB exon of the human *abl* gene, was hybridized at 42°C to detect the normal *abl* fragment.

Construction and screening of a λ EMBL-3 library of patient 2

High molecular weight DNA of patient 2 was partially digested with MboI and cloned into the BamHI site of λ EMBL-3 as described by Frischauf et al (22). 10^6 independent plaques were screened according to the method of Benton and Davis (23), using a genomic 2-kb 5′ M-bcr BglII-HindIII fragment. Four hybridizing plaques were rescreened with the 0.9-kb SaulI-KpnI genomic *abl* fragment. One out of the four plaques appeared to hybridize to the *abl* probe and contained a 13.5 kb insert. The 3.5-kb *bcr-abl* chimeric BglII fragment was subcloned into the BamHI site of PUC 19.

Sequence analysis

After amplification of the *bcr-abl* fragment of patient 1 by the PCR, the reaction-mixture was loaded on a 2% low-melting point agarose gel (BRL) and the band of interest was cut out from the gel. DNA was isolated using phenol/chloroform extraction and the amplified fragment was cloned into a Bluescript vector (Stratagene). Nucleotide sequences were obtained by the dideoxy chain termination method (24) on double stranded DNA (25). In the case of patient 2 separation of the amplified *bcr-abl* fragment from the PCR amplification primers was obtained by filtration over a Centricon S-100 device (Amicon) (26). Direct sequencing of the purified fragment was performed according to Winship (27).

For sequence analysis of chromosomal DNA fragments of patient 2, a 0.5-kb M-bcr PstI-SmaI genomic fragment which contains the M-bcr 5′ Bam HI site was subcloned into PUC 19. The DNA sequence of both strands was determined according to the method of Maxam and Gilbert (28), using PstI, BamHI and SmaI as labelling sites. The sequence strategy for the genomic SaulI-KpnI *abl* fragment containing parts of *abl* exons a2 and a3 are published by Grosveld et al, 1986 (6). The sequence of the chimeric portion of the 3.5-kb BglII *bcr-abl* fragment of patient 2 was determined by the method of Maxam and Gilbert (28), using the BamHI site in M-bcr and the SalI site in *abl* as labelling sites.

Southern blot analysis of patient 1

High molecular weight DNA was extracted by the usual techniques and blotted, after digestion with the restriction enzymes BglII, BamHI or KpnI (29). As a probe for the detection of a breakpoint between *abl* exon a2 and a3 a 0.9-kb SaulI-KpnI genomic fragment was used which covers the intron between both exons (6).

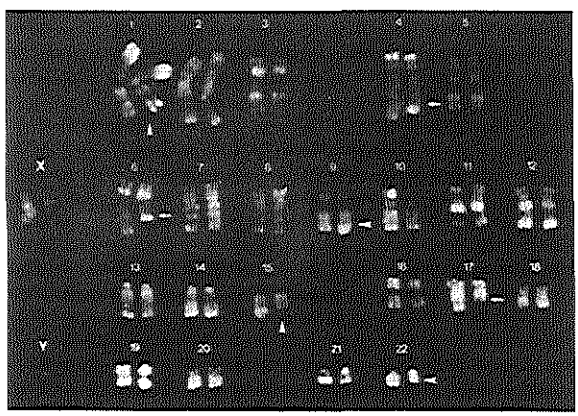
Protein studies

Chimeric proteins from blast cells of both patients were analyzed by immunoprecipitation, followed by autophosphorylation as described previously (30). BCR-ABL proteins were precipitated with anti-ABL and anti-BCR polyclonal antisera. The anti-ABL antiserum was raised a synthetic peptide with proven antigenicity, comprising the amino acids: CSISDEVEKELGK (31). The polyclonal anti-BCR antiserum directed against the amino terminal side of the BCR protein was raised in a rabbit as described previously (30). Blast cells of an ALL patient with e1-a2 $P190^{bcr\text{-}abl}$ were precipitated as a control for patient

1. In the case of patient 2, blast cells of a CML patient and BV173 cells, both containing b2-a2 $P210^{bcr\text{-}abl}$, were precipitated as controls.

Results

At the time of diagnosis only five metaphases from peripheral blood of patient 1 could be analyzed. They showed a 46,XX normal karyotype. At the time of relapse, cytogenetic analyses were performed twice on bone marrow and blood, mounting to a total of 110 cells karyotyped. These showed a mosaicism of normal and Ph-positive cells with additional changes of the following type: 46,XX(70%) / 46,XX,t(9;22)(q34;q11)(2%) / 46,XX,t(9;22),inv(1)(p36;q42),



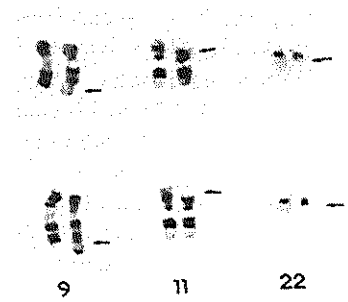


Figure 2A Karyogram of patient 1 showing t(9;22), inv(1), del(15), t(4;6;17). R-banding with acridine orange.
Figure 2B Partial karyotype of patient 2 showing t(9;11;22). G-banding with Trypsine-Giemsa staining.

del(15)(q22)(10%)/46,XX,t(9;22),inv(1),del(15),t(4;6;17)(q26;q16;q24)(18%) (Figure 2A).

Patient 2 was previously reported (11) and showed a variant of the Ph translocation at diagnosis i.e t(9;11;22) in 56% of the metaphases (Figure 2B). Seventy percent of the karyotypes remained Ph-positive during clinical and hematologic remission and additional chromosome abnormalities heralded progression and relapse of the disease.

Since patient 1 showed a Ph chromosome in metaphases of blood and bone marrow cells, PCR analysis was performed on cDNA preparations of RNA of this patient in order to determine the type of *bcr-abl* joining at the mRNA level. None of the available oligonucleotide probes, complementary to the already known

breakpoint joining regions which occur in Ph positive-leukemia (i.e. e1-a2, b2-a2 or b3-a2) hybridized to any *bcr-abl* fragment in the PCR mixture of this patient (Figure 3, sensitivity of the PCR $1:10^4$-10^5, D. Soekarman, unpublished results). However, the PCR generated a fragment of approximately 120 bp, which was clearly visible on the agarose gel stained with ethidium bromide (Figure 3, patient 1). We hypothesized that a fragment of such size could be produced if *abl* exon a2, spanning 174 bp, missed from the 298-bp fragment normally detected after amplification of the e1-a2 *bcr-abl* joining in ALL patients (19).

We described one other patient with Ph positive-leukemia previously, analyzed by Northern blotting in our laboratory, whose *bcr-abl* mRNA apparently lacked *abl* exon a2 (20). We proposed that in this chimeric RNA, joining had occurred of *bcr* exon b2 to *abl* exon a3. To prove this hypothesis PCR was performed on cDNA derived from this patient using primers 1,2 and 3. A fragment of approximately 150 bp length was generated (Figure 3, patient 2) which matched the size of a fragment covering the b2-a2 junction missing the 174 bp of *abl* exon a2 (319-174 = 145 bp).

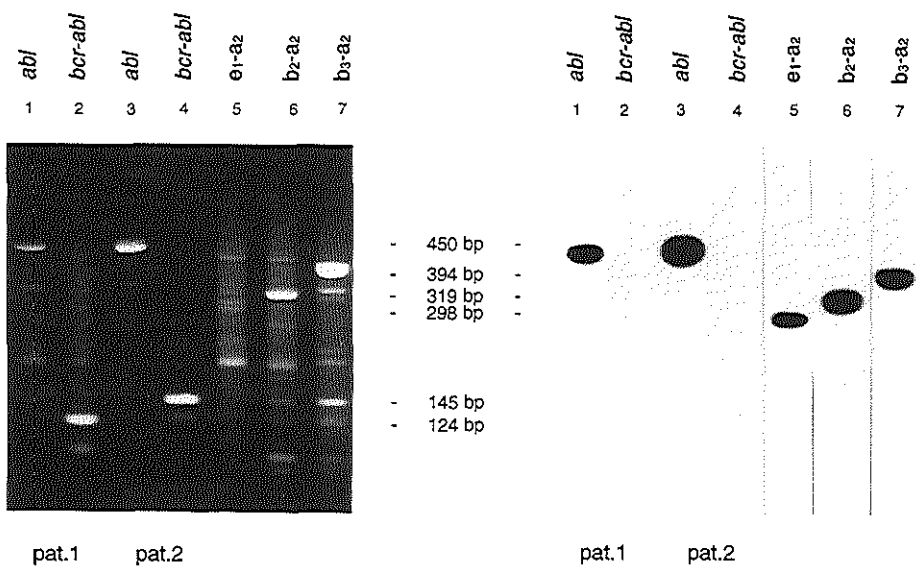


Figure 3 On the left side of the figure the results of the PCR-experiment are shown on gel; on the right side the corresponding autoradiogram is depicted. Lanes 1 and 3 contain the PCR-mixture of patient 1 and 2 after amplification of the 450-bp *abl* fragment. Lanes 2 and 4 contain the PCR-mixture of patient 1 and 2 after amplification of the *bcr-abl* fragment. Lanes 5 to 7 contain the result of amplification of the *bcr-abl* fragment of 3 patients with Ph positive leukemia who show respectively an e1-a2, b2-a2 and b3-a2 joining. Sizes of the PCR-fragments usually found in our experiments are given in the middle of the figure. Arrowheads indicate the aberrant *bcr-abl* fragments of patient 1 and 2 on gel.

Sequence analysis of the amplified *bcr-abl* fragments of both patients confirmed our suppositions: the joining in patient 1 consisted of the first exon of the *bcr* gene to *abl* exon a3, giving rise to an e1-a3 *bcr-abl* mRNA, while in patient 2 a b2-a3 joining was found (data not shown).

These data were confirmed by Southern blot analysis. DNA of patient 1, digested with restriction enzymes BamHI, BglII and KpnI, shows two extra bands in all digests after hybridization with a SauI-KpnI fragment which covers the intron between *abl* exon a2 and a3 (Figure 4). Since no aberrant bands are detected in corresponding digests of normal human thymus DNA we conclude that the breakpoint of patient 1 in the *abl* gene is located between *abl* exon a2 and a3.

Southern blot analysis of patient 2 (R6 in ref. 11) showed that 5'M-bcr probes hybridized to a 3.5-kb rearranged BglII M-bcr fragment on the Ph chromosome.

To investigate the molecular structure this fragment was cloned in E. Coli in order to sequence the *bcr-abl* junction. From the DNA of the leukemic cells of

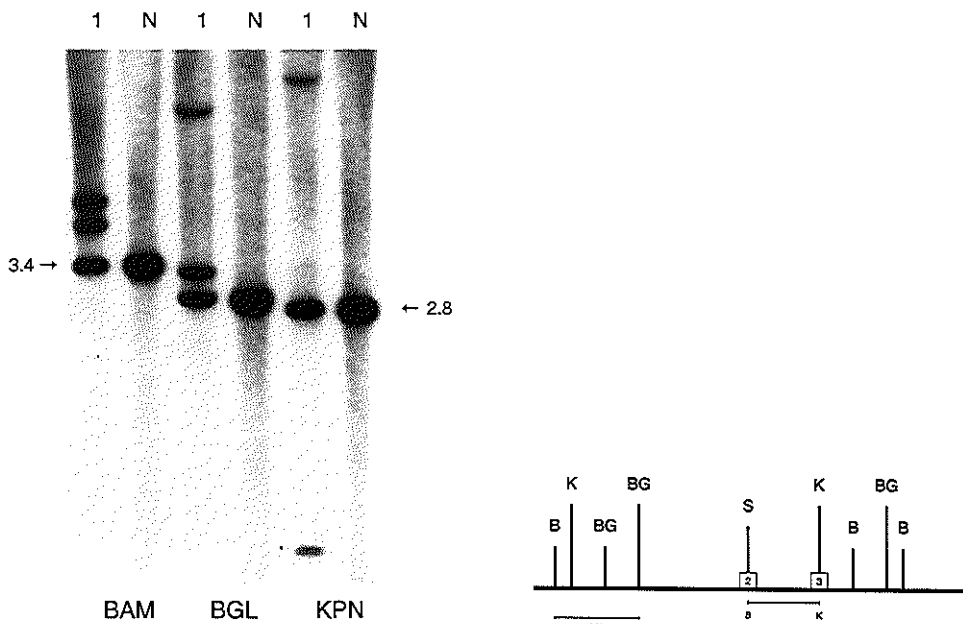
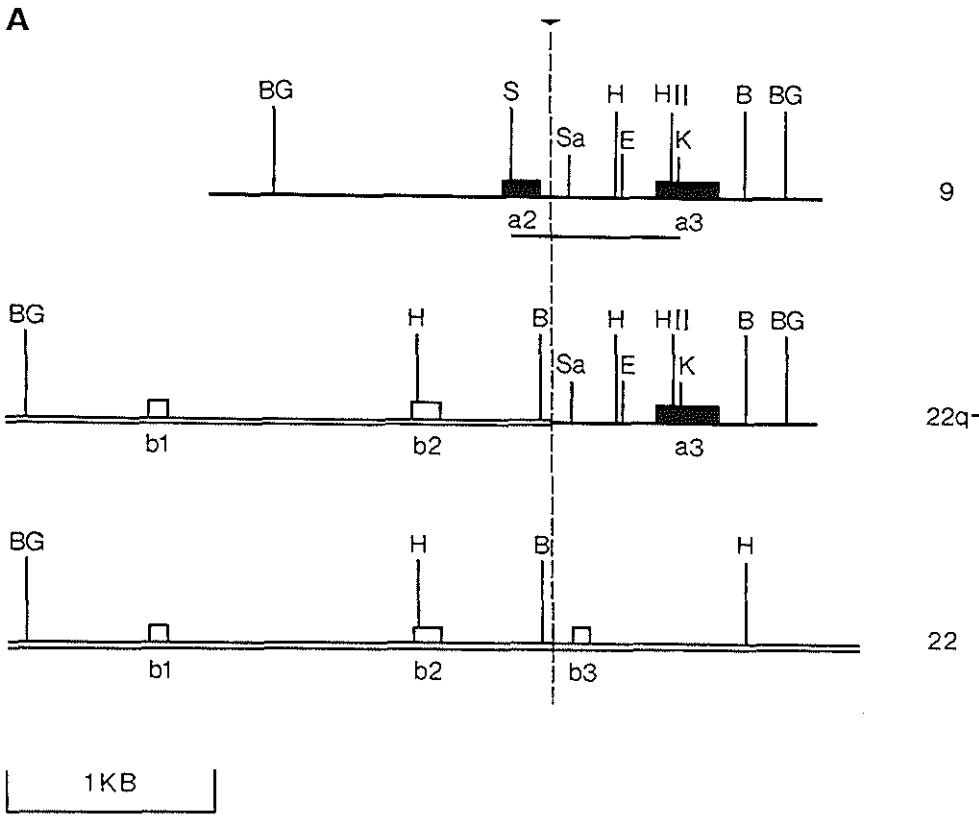


Figure 4 Southern blot analysis of patient 1. DNA of patient 1 was digested with restriction enzymes BamHI, BglII and KpnI. Normal human thymus DNA (N) was used as a control. A 0.9 kb-SauI-KpnI fragment was used as a probe. Germline bands are indicated by arrowheads and the size of the fragments is given on the left for the BamHI digest and on the right for the BglII and KpnI digests. Next to the Southern blot a simplified restriction map of the region of interest is given. B = BamHI, BG = BglII, K = KpnI, S = SauI. Abl exon a2 and a3 are depicted as boxes and numbered accordingly 2 and 3. The localization of the SauI-KpnI fragment used as a probe is given.



B

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                                        ←                       ↓
TTTGT GGCCA GTGGA GATAA CACTC TAAGC ATAAC TAAAG GTAAA AGGGT TGTGG GCAGC TAGTG    9
ATTAC ACTTC GAGTC ACTGG TTTGC CTGTA TTGCT AAACC AACTG GATCC TGAGA TCCCC AAGTG   22q-
ATTAC ACTTC GAGTC ACTGG TTTGC CTGTA TTGTG AAACC AGCTG GATCC TGAGA TCCCC AAGAC   22

GTGGT TGCAG GAGAT AGAAA TCTGG GAATT GCGGT TTGAC CTACC ACCCT TTGCT CGTTA AAGGA    9
GTGGT TGCAG GAGAT AGAAA TCTGG GAATT GCGGT TTGAC CTACC ACCCT TTGCT CGTTA AAGGA   22q-
AGAAA TCATG ATGAG TATGT TTTTG GCCCA TGACA CTGGC TTACC TTGTG CCAGG CAGAT GGCAG   22
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Figure 5

A Restriction map of part of the *abl* gene on chromosome 9 (upper line), the 3.5 kb-BgI fragment containing the *bcr-abl* joining of patient 2 (middle line) and part of the *bcr* gene on chromosome 22 (lower line). Chromosome 9 sequences are depicted as black lines, chromosome 22 as open lines. Exons of the *abl* gene are depicted as black boxes and of the *bcr* gene as open boxes. Exons are numbered according to the text. The arrowhead and the dashed line indicate the breakpoint localization of patient 2. The 0.9 kb-SauI-KpnI probe is depicted as a black bar underneath the restriction map of part of the *abl* gene. BG = BgI II, S = SauI, HII = HincII, K = KpnI, H = HindIII, B = BamHI, Sa = SalI, E = EcoRI.

B Corresponding sequence analysis of part of the fragments depicted in figure 5A. The ← indicates the end of exon a2 of *abl*. The ↓ points at the AG dinucleotide at the transition of the *bcr-* to the *abl* sequences. The Bam HI site in the *bcr* sequence is underlined.

this patient a MboI-partial library was constructed in λ EMBL-3. Screening of the library with a 5'M-bcr probe (2-kb BglII-HindIII fragment) yielded fourindependent hybridizing phage-plaques. Since Northern blot analysis indicated that the breakpoint in the *abl* gene would map between *abl* exons a2 and a3 (20), the phage-plaques were rescreened with a probe covering the intron between a2 and a3 (0.9-kb SauI-KpnI, Figures 4 and 5A). One out of the four plaques appeared to hybridize to the *abl*-probe. This phage clone contained an insert of 13.5 kb, which was mapped with the enzymes BglII, HindIII, BamHI, SalI and EcoRI (data not shown). Comparison of the map with the known *bcr* and *abl* maps indicated that this phage insert consisted of 11.5 kb of 5' *bcr* sequences linked to 2 kb of *abl* sequences. Furthermore, the clone contained the 3.5-kb aberrant BglII M-bcr fragment that was also detected on the Southern blots of patient 2. This fragment was subcloned and analyzed in more detail. Its 5' side was identical to 5' M-bcr sequences, but the similarity stopped just 3' of the BamHI side. The 3' part of the 3.5-kb BglII fragment was identical to the c-*abl*-map, starting in or just 3' of exon a2 of *abl* (Figure 5A). To exactly localize the breakpoint, the area of the breakpoint of the 3.5-kb BglII fragment was sequenced on both strands. Comparison with the corresponding *bcr* and *abl* sequences showed that chromosome 22 sequences stop 11 to 14 bp 3' of the M-bcr BamHI site (i.e. 175 bp 5' of exon b3) and the 9 sequences start 21 to 24 bp 3' of *abl* exon a2. At the site of the breakpoint the redundant dinucleotide AG is present which could be derived from either chromosome (Figure 5B). No nucleotides are inserted or deleted during the translocation event.

Finally, blast cells of patient 1 and 2 were analyzed in an immunoprecipitation assay followed by autophosphorylation. Using antisera directed against ABL sequences and BCR sequences, a protein of approximately 190 kD was precipitated from blast cells of patient 1. This 'P190$^{bcr\text{-}abl}$-like' protein exhibits similar tyrosine kinase activity as e1-a2 P190$^{bcr\text{-}abl}$ in blast cells of a Ph-positive ALL patient, as equivalent amounts of cells were taken (Figure 6A). From blast cells isolated from patient 2, a protein was precipitated both by anti-ABL and anti-BCR antisera with a molecular weight of 210 kD. The tyrosine kinase activity of this 'P210$^{bcr\text{-}abl}$-like' protein is comparable with the activity of the b2-a2 P210$^{bcr\text{-}abl}$ molecule, derived from a comparable number of blast cells from a Ph-positive CML patient and BV173 cells (Figure 6B).

Discussion

In this article we present the clinical, cytogenetic and molecular data of two patients with Ph-positive ALL. We investigated the *bcr-abl* rearrangements in both patients using the polymerase chain reaction (PCR) on cDNA. Sequence analysis of the amplified *bcr-abl* fragments showed that in both patients a deletion of exon a2 of the *abl* gene had occurred in the chimeric *bcr-abl* mRNA.

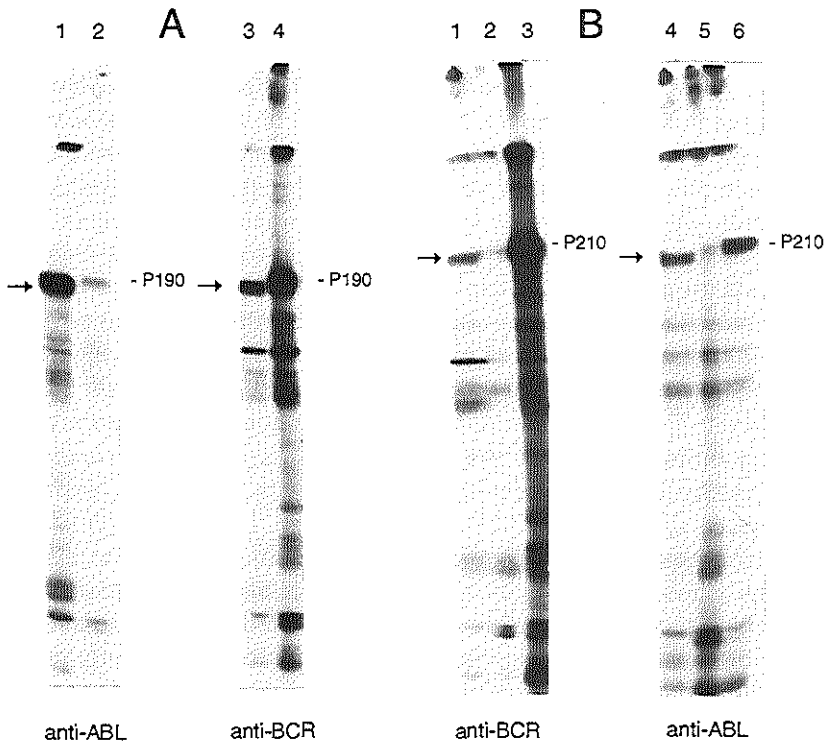

Figure 6

A Immunoprecipitation analysis followed by autophosphorylation of blast cells isolated from patient 1; $1x1\text{-}10^7$ blast cells were isolated from peripheral blood and precipitated with 50µl Na_2SO_4 precipitated anti-ABL antiserum (lane 1) and anti-BCR antiserum (lane 3). As a positive control, $1x10^7$ blast cells from an ALL patient with an e1-a2 BCR-ABL junction were precipitated with anti-ABL antiserum (lane 2) and anti-BCR antiserum (lane 4).

B Immunoprecipitation analysis follwed by autophosphorylation of blast cells isolated from patient 2; $1x10^7$ blast cells were isolated from peripheral blood and precipitated with 50µl Na_2SO_4 precipitated anti-BCR antiserum (lane 1) and anti-ABL antiserum (lane 4). As positive controls, $1x10^7$ blast cells from a CML patient with an b2-a2 BCR-ABL junction (lanes 2 and 5) and $1x10^7$ BV173 cells (lanes 3 and 6) were precipitated, respectively.

Consequently, the joining-configuration in patient 1 was e1-a3 and in patient 2 b2-a3. This is a remarkable finding since all patients with Ph-positive leukemia, molecularly investigated so far, always seem to include *abl* exon a2 in their chimeric *bcr-abl* mRNA. In addition, Southern blotting data from patient 1 (Figure 4) and chromosomal cloning and sequencing data from patient 2 (Figures 5A and 5B) showed that the deletion of *abl* exon a2 from the chimeric *bcr-abl* mRNA was due to the position of the breakpoint in the *abl* gene on

chromosome 9. In both cases the breakpoint maps in the 0.6-kb intron, separating *abl* exons a2 and a3. Since breakpoints in *abl* can be scattered over an area of more than 200 kb (4,6,33) and assuming that the position of the breakpoint would be random, the small target size of the intron between *abl* exons a2 and a3 predicts that in theory less than 0.3% of the Ph translocations will take place here (0.6kb:200kb = 0.003). Unfortunately there are no large scale data available on the position of *abl* breakpoints to check this prediction.

From both cDNA sequencing and the protein studies we conclude that deletion of the *abl* exon a2 results in the generation of in-frame *bcr-abl* chimeric mRNAs which in both patients give rise to the expression of a chimeric BCR-ABL protein with enhanced tyrosine kinase activity. Although the tyrosine kinase assay is not quantitative, the signals on the autoradiogram suggest that the tyrosine kinase activity of the proteins missing a2 sequences is not significantly different from the ones containing a2 sequences. Therefore we expected that the tumorigenicity of the *bcr-abl* proteins without *abl* exon a2 sequences will not be different from their counterparts containing exon a2 sequences.

The finding of the novel BCR-ABL variant with deletion of ABL exon a2 and increased tyrosine kinase activity is of interest, since it may help to clarify the mechanism by which the c-ABL tyrosine kinase is activated. The kinase activity of ABL seems to be crucial in tumorigenesis, as the transforming activity of the v-*abl* gene in the Abelson Murine Leukemia Virus (AMuLV) is directly dependent on this activity (34). Evidence is accumulating that disruption of the *abl* gene (either by viral transduction as occurring in AMuLV, or by translocation as in Ph-positive leukemia) and concomitant deregulation of its kinase domain could play a pivotal role in the origin of the leukemia (35-38). As has been published, the kinase domain of the *abl* gene shows strong homology with the kinase domain of the c-*src* oncogene and is called the SRC-homology 1 region (SH1) (39). Amino-terminal of this region two additional homology regions have been described in the *abl* gene: the SH2 and the SH3 region (Figure 7). The SH2-region is suggested to be a positive regulatory region immediately N-terminal of the kinase domain (39) and this region is needed by the v-ABL kinase for its

Figure 7 The orientation of the various SH-regions in the c-ABL protein is given. SRC-homology 1 region (SH1) contains the kinase domain of c-ABL. SH2 is a positive regulator and SH3 is a negative regulator of SH1.

transforming activity (37). The SH3-region is located directly N-terminal of SH2 and comprises 50 amino acids (40). Deletions in this particular region of the c-SRC (41, 42) as well as deletions in the homologous domains encoding mouse ABL type IV proteins, activate the tyrosine kinase (43,44).

The mutant proteins show transforming abilities in NIH3T3 cells. Therefore, a negative regulatory influence on the kinase domain of the *abl* gene has been proposed for the SH3-region (43,44). Jackson and Baltimore (43) suggested that the SH3 inhibiting function on the catalytic domain may be modulated by interaction of the SH3 region with an as yet unidentified cellular factor. Deletions in SH3 would prevent the interaction with this factor, resulting in the loss of the blocking function and concomitant unleashing of the tyrosine kinase activity. Supporting evidence for this mechanism already exists in vivo since in the v-ABL protein, which has heightened tyrosine kinase activity, the SH3-region has been deleted.

The two patients described in this article also show a deletion in the SH3-region, since the SH3-region is partly encoded by *abl* exon a2 which is missing in both patients. Due to this deletion 17 N-terminal amino acids of the SH3-region are removed from the BCR-ABL fusion proteins in both patients. This includes amino acids, which upon deletion potentiate the c-SRC transforming activity and in this way may give rise to a BCR-ABL transforming protein with heightened tyrosine kinase activity. However, in published data of patients with Ph-positive leukemia, so far, an e1-a2, b2-a2 or b3-a2 joining has been found at the mRNA level. This implicates that *abl* exon a2 is present and that the SH3-region is still intact in the BCR-ABL fusion proteins of these patients. Since these proteins show enhanced tyrosine kinase activity we assume that the inhibiting function of the SH3-region is impaired by the BCR-moiety of the proteins by an unknown mechanism.

Probably the 5'BCR-sequences downregulate SH3-function either by steric hindrance or by binding to the cellular factor, which regulates SH3. Thus, negative regulation of the kinase domain of ABL can be disturbed either by deletion of the SH3-region (as in v-ABL or in both patients described in this article) or by interfering 5' sequences that impair the function of SH3 (as in most patients with Ph-positive leukemia). In this respect no difference exists between the two patients described in this article and the majority of patients with Ph-positive leukemia.

Understanding of the regulation of the kinase domain of ABL and identification of substrate proteins for the tyrosine kinase activity will elucidate its significance for the origin and development of Ph-positive leukemias. Patients as described in this paper may help in unravelling this issue.

This paper is dedicated to the memory of André Hermans.

Acknowledgments

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Immunologic characterization of the tumor specific BCR-ABL junction in Philadelphia chromosome-positive acute lymphoblastic leukemia

Janneke van Denderen[1], Toon Meeuwsen[1], Paulien ten Hacken[1], Dorien van der Plas[1], Netty Zegers[2], Wim Boersma[2], Gerard Grosveld[1] and Willem van Ewijk[1].

[1]Departments of Immunology and Genetics, Erasmus University, Rotterdam, The Netherlands, [2]Medical Biological Laboratory TNO, Rijswijk, The Netherlands

Summary

Philadelphia (Ph)-positive acute lymphoblastic leukemia (ALL) is highly associated with two forms of chimeric BCR-ABL proteins: $P190^{bcr\text{-}abl}$ and $P210^{bcr\text{-}abl}$. Whereas $P210^{bcr\text{-}abl}$ also occurs in chronic myeloid leukemia, $P190^{bcr\text{-}abl}$ is uniquely expressed in Ph-positive ALL. As a consequence, $P190^{bcr\text{-}abl}$ is preeminently a tumor-specific marker in leukemic cells of ALL patients. Since $P190^{bcr\text{-}abl}$ is composed of the normal BCR and ABL proteins, the major part of the $P190^{bcr\text{-}abl}$ molecule comprises non-tumor-specific determinants. Exclusively the joining region between BCR and ABL, newly generated during the Ph translocation, is a tumor-specific epitope on the $P190^{bcr\text{-}abl}$ molecule. Therefore, only antibodies against the BCR-ABL joining region will detect the tumor-specificity of $P190^{bcr\text{-}abl}$.

In this study a polyclonal antiserum, termed BP-ALL, was raised against a synthetic peptide corresponding to the BCR-ABL junction in $P190^{bcr\text{-}abl}$. The reactivity of BP-ALL with native $P190^{bcr\text{-}abl}$ derived from a Ph-positive ALL cell line (TOM-1), was tested using immunoprecipitation analysis. BP-ALL reacted highly specifically with $P190^{bcr\text{-}abl}$ but not with $P210^{bcr\text{-}abl}$ isolated from CML cell lines. Peptide inhibition studies further confirmed the fine specificity of BP-ALL. Next, we tested the reactivity of BP-ALL with a panel of 16 selected cALL patients. Fifteen of these patients expressed *bcr-abl* mRNA rearrangements, whereas one patient was *bcr-abl*-negative. $P190^{bcr\text{-}abl}$ was precipitated from 13 patients. Two out of the three $P190^{bcr\text{-}abl}$-negative patients expressed $P210^{bcr\text{-}abl}$. As expected, the third patient, i.e. the *bcr-abl* negative patient, expressed no chimeric protein at all.

Our data indicate that the tumor-specific BCR-ABL junction domain is exposed in an antigenic fashion on the $P190^{bcr\text{-}abl}$ molecule. Moreover, we show that BP-ALL in principle can be used in ALL diagnosis.

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Introduction

The Philadelphia (Ph) chromosome, a minute chromosome 22, is found in 90% of all patients with chronic myeloid leukemia (CML) (1). 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 (AML) the Ph chromosome can be detected (2). Moreover in childhood acute lymphoblastic leukemia (ALL) a 2-5% 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% (3,4).

Although the Ph chromosomes in CML and acute leukemias are cytogenetically indistinguishable, molecular analyses have shown important differences (5,6). In all cases the Ph chromosome is the result of a reciprocal translocation between chromosomes 9 and 22 (7,8,9). Here, the *abl* oncogene has moved from chromosome 9 into the *bcr* gene on chromosome 22. On the *bcr* gene 2 regions are defined in which breakpoints occur. These regions are termed major breakpoint cluster region (M-bcr) and minor breakpoint cluster region (m-bcr) (10). In all Ph-positive CML patients the *abl* oncogene has moved from chromosome 9 into M-bcr, localized in the middle of the *bcr* gene on chromosome 22. The result of this particular translocation is a chimeric *bcr-abl* gene, encoding a 8.5-kb mRNA which is translated into a 210-kD protein, $P210^{bcr\text{-}abl}$ (11,12,13). With respect to the Ph translocation process, Ph-positive ALL patients are heterogeneous (14): Kurzrock et al. mentioned 50% of adults and 10% of children with Ph-positive ALL to show the same type of *bcr-abl* rearrangement as found in CML. Accordingly, these patients express $P210^{bcr\text{-}abl}$ (14). In contrast, a $P190^{bcr\text{-}abl}$ protein is demonstrated in the other group of ALL patients, including almost all cases of childhood ALL (15,16,17). We have previously shown a different *bcr-abl* rearrangement in such patients (5,6). Here, breakpoints were noticed in the first intron of the *bcr* gene, termed m-bcr. The first *bcr* exon (e1) is then spliced to exon 2 of the *abl* gene (termed a2), resulting in a 7.0-kb *bcr-abl* mRNA. Therefore, in the chimeric $P190^{bcr\text{-}abl}$ protein the e1-a2 BCR-ABL junction is present.

Since $P210^{bcr\text{-}abl}$ as well as $P190^{bcr\text{-}abl}$ are expressed exclusively in malignant cells in CML and ALL, these proteins are by definition tumor-specific. However, $P210^{bcr\text{-}abl}$ and $P190^{bcr\text{-}abl}$ are composed of parts of BCR and 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 BCR and ABL. Recently we have shown that the BCR-ABL junction in $P210^{bcr\text{-}abl}$ is antigenically exposed on this protein (18).

In the present study we investigated the expression of the e1-a2 BCR-ABL junction in $P190^{bcr\text{-}abl}$ in cell lines and in leukemic cells of ALL patients. Our data indicate that also the BCR-ABL junction in $P190^{bcr\text{-}abl}$ can be recognized by

antibodies. These experiments indicate that the tumor-specific joining regions in the BCR-ABL fusion proteins are new immunological markers for CML and ALL diagnosis.

Materials and methods

Cell lines

TOM-1 is a cell line derived from a Ph-positive ALL patient (19). The cell line was kindly provided by Dr. Okabe (Sapporo, Japan). K562 and BV173 are cell lines derived from patients during blast crisis of CML (20,21). Cells were cultured in RPMI medium supplemented with 10% fetal calf serum, 100 µg/ml penicillin and 60 µg/ml streptomycin.

Patients

Presence of BCR-ABL proteins was analyzed in 16 patients: 5 men and 12 women, varying in age from 20 to 73. Diagnosis common ALL was based on hematologic and immunophenotypic criteria. Blast cells, either from peripheral blood or from bone marrow were isolated by Ficoll Hypaque centrifugation. After cryopreservation the cells were stored under liquid nitrogen.

Peptide synthesis, purification and conjugation

Peptides were synthesized using solid phase synthesis as described previously (18). Purification of the peptides was performed by gelfiltration and reverse phase HPLC. A cysteine residue was added to the amino terminus during synthesis. Peptides were cross-linked through free sulfhydryl groups to the carrier protein chicken gamma globulin (CGG) using m-maleimidobenzoyl-sulfosuccinimide-ester (MBS) (22).

Immunizations, antisera and purification of immunoglobulins

Antiserum BP-ALL Flemish rabbits (MBL-TNO, Rijswijk, the Netherlands) were primed intracutaneously with 250 µg protein complex consisting of peptide e1-a2 coupled via MBS to CGG, emulsified in an equal volume of Complete Freund's Adjuvans. Rabbits were boosted twice after intervals of four weeks; the first time with 250 µg of protein in CFA, the second time with the same dose in Incomplete Freund's 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 (18). The immunoglobulin fraction of both antisera was purified by precipitation with 16% Na_2SO_4.

Anti-ABL antiserum Anti-ABL antiserum is a polyclonal antiserum raised in rabbits against a synthetic peptide with proven antigenicity, comprising the amino acids: CSISDEVEKELGK (23). For immunization the peptide was coupled to the carrier molecule CGG as described above. The same immunization procedure was followed as described above.

Immunoprecipitation and protein kinase reaction

Immunoprecipitation and protein kinase reaction were performed as described previously (18), except that, in the present study, the tyrosine kinase reaction was performed for 10 min. at 30^0 C instead of 10 min. at 37^0 C.

Amplification of cDNA by the PCR method

Total RNA was extracted according to the LiCl method (24), followed by cDNA synthesis by reverse transcriptase. The cDNA amplification was performed using the polymerase chain reaction as described previously (5). For the amplification *bcr* primers from exon e1 and b2 and an *abl* primer from exon a3 were used. In Figure 1 the primers on the respective cDNA molecules are shown. Twenty four cycles of the polymerase chain reaction were performed using Taq polymerase. The samples were electrophoresed, and transferred to nylon filter (Zeta probe). Filters were hybridized to ^{32}P end-labeled oligonucleotides, spanning the e1-a2, b2-a2 and b3-a2 junctions (6). After hybridization the blots were washed and exposed to Fuji XR films.

Results

Peptides corresponding to the bcr-abl junction in $P190^{bcr-abl}$

Based on the previously published nucleotide sequence (5) a peptide was synthesized corresponding to the BCR-ABL junction in $P190^{bcr-abl}$. 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:

cys :-ala-phe-his-gly-asp-ala-g ¦ lu-ala-leu-gln

e1 a2

As a control peptide b3-a2 (SP b3-a2) was constructed, corresponding to the b3-a2 joining region in the CML specific protein $P210^{bcr-abl}$. SP b3-a2 has the following amino acid sequence:

cys :-lys-gln-ser-ser-l ¦ ys-ala-leu-gln-arg-pro

b3 a2

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

Since fusion of the *bcr* and *abl* genes both in $P190^{bcr-abl}$ and in $P210^{bcr-abl}$ occurs in a coding triplet, the glu in SP e1-a2 and the second lys in SP b3-a2

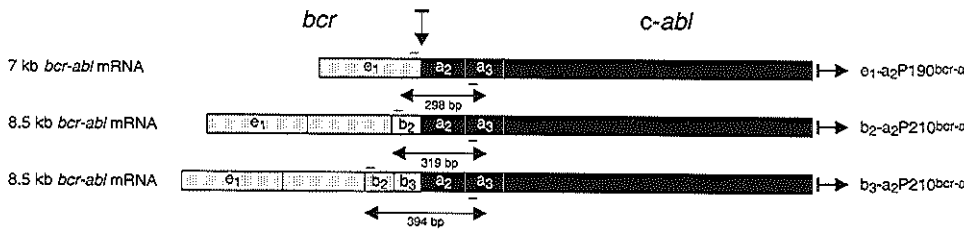

Figure 1 Schematic representation of the chimeric *bcr-abl* mRNAs and proteins. The small horizontal bars indicate the localization of the primers used in the PCR. The vertical arrow indicates the *bcr-abl* fusion point.

are newly generated by the translocation process (5,12).

Figure 1 schematically shows the respective chimeric *bcr-abl* mRNAs 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 differ also at their amino terminus and have the same ABL terminal amino acids.

cDNA analysis of the BCR-ABL joining in TOM-1 cells

As a source of $P190^{bcr\text{-}abl}$ protein TOM-1 cells were used. First, presence of $P190^{bcr\text{-}abl}$ mRNA comprising the e1-a2 BCR-ABL junction in our TOM-1 cells had to be confirmed. To this purpose the highly sensitive polymerase chain reaction (PCR) technique was performed. The *bcr-abl* cDNA was amplified and

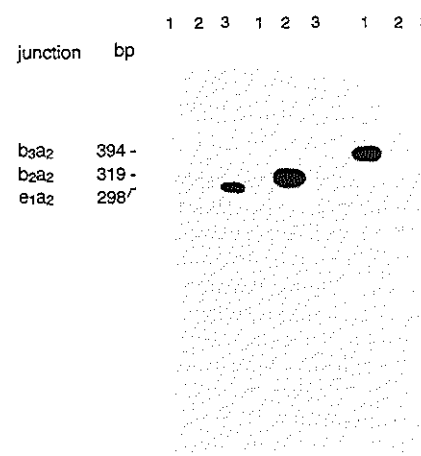

Figure 2 Amplification of the *bcr-abl* junction from K562 (lanes 1), BV173 (lanes 2) and TOM-1 (lanes 3) cDNA. Hybridization was performed to oligonucleotides spanning the e1-a2, b2-a2 and b3-a2 *bcr-abl* junction.

hybridized to oligonucleotides specific for the e1-a2, b2-a2 and the b3-a2 joining in the chimeric cDNA. As shown in Figure 2 a 298-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 (Figure 2 lane 1, b3-a2 probe), while a 319-bp fragment, specific for a b2-a2 joining, was detected in BV173 cells (Figure 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 $P190^{bcr\text{-}abl}$ and $P210^{bcr\text{-}abl}$ in different cell lines was tested with an immunoprecipitation assay followed by an autophosphorylation reaction. As shown in Figure 2, TOM-1 cells contain mRNA comprising

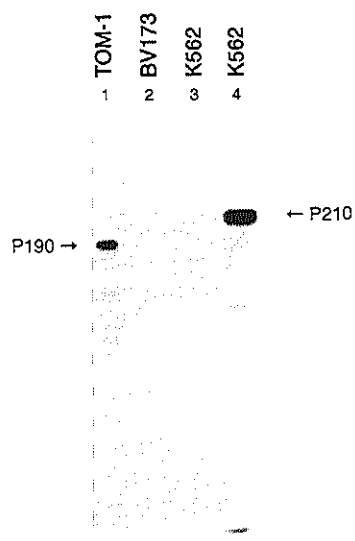

Figure 3 Immunoprecipitation analysis of BCR-ABL chimeric proteins from TOM-1, K562 and BV173 cells with a polyclonal antiserum directed against the amino-terminus of the BCR protein. The immunoprecipitation was followed by autophosphorylation of the proteins. Cells were lysed and proteins were precipitated with 25 µl Na_2SO_4-precipitated normal rabbit serum (lanes 1,3 and 5) and with 25 µl Na_2SO_4-precipitated anti-BCR antiserum (lanes 2,4 and 6).

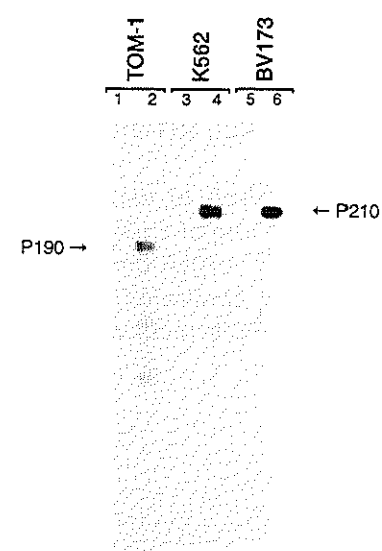

Figure 4 Immunoprecipitation analysis of the specificity of antiserum BP-ALL. TOM-1, BV173 and K562 cells were lysed and proteins were precipitated with 25 µl Na_2SO_4 precipitated serum BP-ALL (lanes 1, 2, and 3). In lane 4 proteins from a lysate of K562 cells were precipitated with 25 µl Na_2SO_4 precipitated anti-BCR antiserum. The immunoprecipitation was followed by autophosphorylation of the proteins.

the e1-a2 BCR-ABL junction. However, in order to confirm that this mRNA was indeed translated into protein, we searched for the presence of $P190^{bcr\text{-}abl}$ in TOM-1 cells. Figure 3 shows that $P190^{bcr\text{-}abl}$ 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 $P210^{bcr\text{-}abl}$ after immunoprecipitation with the same anti-BCR antiserum (Figure 3, lanes 4 and 6).

Next, we tested the reactivity of antiserum BP-ALL with $P190^{bcr\text{-}abl}$. Incubation of BP-ALL with a lysate of TOM-1 cells resulted in a clear precipitation of $P190^{bcr\text{-}abl}$ (Figure 4, lane 1). In contrast, BP-ALL did not precipitate $P210^{bcr\text{-}abl}$ from either K562 or BV173 (Figure 4, lanes 2 and 3). These results imply that antiserum BP-ALL specifically recognizes the e1-a2 junction domain in $P190^{bcr\text{-}abl}$.

To confirm this notion we performed peptide blocking studies. 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 Figure 5, pre-incubation of BP-ALL with cognate peptide SP e1-a2 prevented precipitation of $P190^{bcr\text{-}abl}$ completely (lane 2). However, precipitation of background bands was not inhibited. Pre-incubation 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 $P190^{bcr\text{-}abl}$ is precipitated by antibodies in BP-ALL which are specifically directed against the

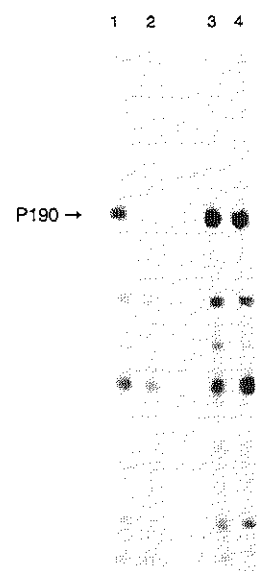

Figure 5 Immunoprecipitation analysis of TOM-1 cells with 25 µl Na_2SO_4 precipitated antiserum BP-ALL (lanes 1 and 3), after pre-incubation of BP-ALL with 0.25 mM SP e1-a2 (lane 2) and after pre-incubation with 0.25 mM SP b3-a2 (lane 4).

e1-a2 junction.

Precipitation of P190$^{bcr-abl}$ from leukemic cells of ALL patients

Finally we investigated whether P190$^{bcr-abl}$ from leukemic cells of ALL patients was also precipitated by BP-ALL. To this aim we tested a selected group of 16 common ALL patients. Fifteen of these patients expressed *bcr-abl* mRNA rearrangements. Age, sex and diagnosis of the respective patients are summarized in Table 1. Ficoll Hypaque enriched blood or bone marrow cells were lysed and incubated with either BP-ALL or as control, with anti-ABL antiserum. The immunoprecipitation was followed by the autophosphorylation assay. As shown in Figure 6, P190$^{bcr-abl}$ proteins were precipitated from 13 samples, both with BP-ALL (lanes 1) and with the control antiserum anti-ABL (lanes 2). From

Table 1. Protein, molecular and cytogenetic data on 16 cALL patients.

number	age	sex	material	BP-ALL	anti-ABL	pcr	cytoge-netics	South-ern[a]
1	73	F	pb	+++	+++	e1-a2	nd	nd
2	53	F	pb	+++	+++	e1-a2	Ph	nd
3	52	F	bm	-	++210	b3-a2, e1-a2	nd	nd
4	60	F	bm	++	++	e1-a2	Ph	-
5	50	F	pb	+	+	e1-a2	nd	-
6	60	F	bm	++	++	e1-a2	na	nd
7	20	F	pb	++	++	e1-a2	na	-
8	27	M	bm	-	-	-	nd	nd
9	58	F	pb	++	++	e1-a2	nd	nd
10	48	M	pb	-	++210	b2-a2, b3-a2	nd	nd
11	29	M	bm	++	++	e1-a2	nd	nd
12	46	F	bm	++	++	e1-a2	nd	nd
13	46	F	pb	+	+	e1-a2	nd	nd
14	63	F	bm	+	++	e1-a2	nd	nd
15	33	F	pb	++	++	e1-a2	na	nd
16	55	M	pb	+	+	e1-a2	nd	nd

Abbreviations:
pb: peripheral blood; bm: bone marrow; na: not analysed, no metaphases obtained; nd: not done
Southern[a]: results of Southern blot analysis, with respect to breakpoint in M-bcr

leukemic cells of three ALL patients $P190^{bcr\text{-}abl}$ proteins could not be precipitated, respectively from patient 3, 8 and 10. However, two of these particular patients, i.e. patient 3 and patient 10, were found to express $P210^{bcr\text{-}abl}$, since a clear P210 band is visible after precipitation with anti-ABL antiserum (patients 3 and 10, lanes 2). From the other $P190^{bcr\text{-}abl}$ negative patient (patient 8) chimeric proteins could not be precipitated, neither with BP-ALL nor with anti-ABL antiserum.

The results of the immunoprecipitation analysis are summarized in Table 1, together with results of PCR, Southern blot and cytogenetic analysis of the same patients. In individual patients the results of the PCR and immunoprecipitation analysis are highly corresponding to each other. In most cases amplification of the e1-a2 junction corresponds with precipitation of e1-a2 $P190^{bcr\text{-}abl}$, whereas from patients with amplified b2-a2 or b3-a2 junctions, $P210^{bcr\text{-}abl}$ proteins were precipitated. However there is one exception: immunoprecipitation analysis of patient 3 shows only precipitation of P210 molecules, whereas by performing the PCR technique both e1-a2 and b3-a2 junctions were amplified.

In some cases immunoprecipitation and PCR results were confirmed by either cytogenetic and Southern blot analysis. Here, in agreement with the immunoprecipitation and PCR data, no breakpoints in the M-bcr were observed in $P190^{bcr\text{-}abl}$ positive patients (patients 4, 5 and 7). From 5 patients cytogenetic analysis was performed. The evaluable cases (patients 2 and 4) were both Ph-positive.

These results indicate that the e1-a2 BCR-ABL junction is exposed as a antigenic determinant on the chimeric protein both in leukemic cells of ALL patients and in cell lines. Moreover, these results show the possibility to perform patient studies with BP-ALL.

Discussion

Chromosomal abnormalities in ALL are frequently observed and have prognostic significance (25). 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 (4,26). Especially in childhood ALL the Ph chromosome implicates a bad prognosis (26). It is obvious that for clinical diagnosis antibodies directed against the tumorspecific 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 $P210^{bcr\text{-}abl}$ (18). Now, we report on the production of an antiserum, BP-ALL, specifically recognizing the e1-a2 BCR-ABL joining region in $P190^{bcr\text{-}abl}$. The specificity of the polyclonal antiserum, raised against a synthetic peptide corresponding to the e1-a2 junction, was tested in

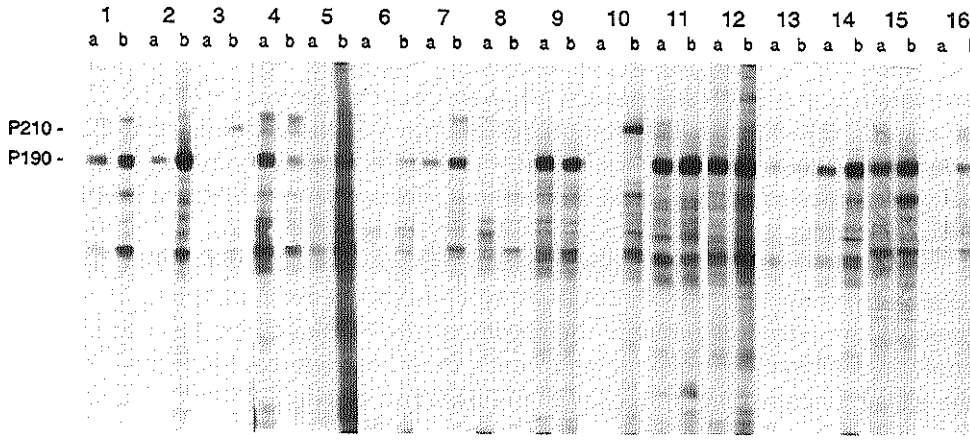

Figure 6 Immunoprecipitation analysis of Ficoll-Hypaque enriched peripheral blood or bone marrow cells of 16 common ALL patients. $10x10^6$ blast cells were lysed and proteins were precipitated with 25 µl Na_2SO_4 precipitated BP-ALL (lane 1) and with 25 µl Na_2SO_4 precipitated anti-ABL antiserum (lane 2).

an immunoprecipitation assay, followed by an autophosphorylation. As a source of Ph-positive cells TOM-1 cells were used. 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 e1-a2 *bcr-abl* junction, indicating a breakpoint in the m-bcr. Presence of $P190^{bcr\text{-}abl}$ in these cells could therefore be expected. We confirmed this notion by immunoprecipition of $P190^{bcr\text{-}abl}$ from TOM-1 cells using an antiserum directed against the amino-terminus of the BCR protein.

We subsequently showed that antiserum BP-ALL, raised against a peptide corresponding to the e1-a2 junction, reacted in a highly specific way with the native protein $P190^{bcr\text{-}abl}$ in TOM-1 cells. We argue that $P190^{bcr\text{-}abl}$ is precipitated by antibodies in BP-ALL which specifically recognize the e1-a2 junction. Antibodies reacting with e1 and/or a2 sequences only do not play a role, since no other BCR-ABL chimeric proteins, b2-a2 and b3-a2 $P210^{bcr\text{-}abl}$, which also contain e1 and a2 sequences, were immunoprecipitated by BP-ALL. Moreover, the reaction of BP-ALL with $P190^{bcr\text{-}abl}$ was specifically inhibited blocking with cognate peptide. Thus, precipitation of $P190^{bcr\text{-}abl}$ was completely abrogated after pre-incubation of BP-ALL with peptide e1-a2. Pre-incubation with a related peptide SP b3-a2, which eliminates antibodies from BP-ALL directed against a2 sequences, had no effect on the immunoprecipitation of $P190^{bcr\text{-}abl}$. These observations provide strong evidence that the tumor specific BCR-ABL junction e1-a2 is expressed in an antigenic way on the $P190^{bcr\text{-}abl}$ molecule.

The e1-a2 joining region in $P190^{bcr\text{-}abl}$ is similarly exposed in leukemic cells of ALL patients as in the TOM-1 cell line. This observation confirms the idea that the e1-a2 epitope can be used as an immunological marker for Ph-positive ALL.

In the present study 13 out of 16 adult ALL patients expressed e1-a2 $P190^{bcr\text{-}abl}$ chimeric proteins. Immunoprecipitation results were confirmed either by PCR, cytogenetic or Southern blot analysis. Due to the relatively low sensitivity of the protein analysis, compared to the PCR technique, false negative protein data could be expected. However, false negative samples were not observed. Still, one contradictory result was observed. In one particular patient both the b3-a2 and the e1-a2 junction was amplified using the PCR technique, whereas only e1-a2 $P190^{bcr\text{-}abl}$ was detected at the protein level. In theory, this phenomenon can be explained in two ways. (*i*) The leukemia in this patient could be bi-clonal. The e1-a2 PCR signal was rather weak compared to the b3-a2 amplification signal. Therefore, this patient could have a large clone comprising b3-a2 mRNA and a smaller one expressing e1-a2 mRNA. In this particular case the e1-a2 $P190^{bcr\text{-}abl}$ molecules could be behind detection by the tyrosine kinase detection. (*ii*) The discrepancy between the PCR and protein studies can also be caused by the fact that both b3-a2 and e1-a2 mRNA are expressed at the mRNA level, but b3-a2 $P210^{bcr\text{-}abl}$ only at the protein level. Probably, as a result of an unknown regulatory mechanism only b3-a2 $P210^{bcr\text{-}abl}$ is able to be translated translated into protein. Simultaneous expression of two *bcr-abl* transcripts has been described previously (27,28,29). The present paper is the first which reports on protein expression in such a particular case.

The data presented in this paper have implications for ALL diagnosis and classification. Here we have demonstrated the antigenic expression of the tumor-specific e1-a2 BCR-ABL junction in $P190^{bcr\text{-}abl}$. 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 $P210^{bcr\text{-}abl}$, 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 (9). Ph-positive ALL patients expressing $P190^{bcr\text{-}abl}$ would then be de novo ALL patients. Without necessarily subscribing to this hypothesis, it is of interest to investigate whether Ph-positive ALL with $P190^{bcr\text{-}abl}$ differ from the cases of Ph-positive ALL expressing $P210^{bcr\text{-}abl}$ 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 polyclonal and monoclonal antibodies directed against the tumor specific BCR-ABL junction in $P190^{bcr\text{-}abl}$ and $P210^{bcr\text{-}abl}$ is therefore invaluable for accurate diagnosis of ALL and CML.

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CHAPTER 3.5

Recognition of the ALL-specific BCR-ABL junction in $P190^{bcr\text{-}abl}$ by monoclonal antibody ER-FP1.

Janneke van Denderen[1], Paulien ten Hacken[1], Paul Berendes[1], Netty Zegers[2], Wim Boersma[2], Gerard Grosveld[3], Willem van Ewijk[1]

[1]Department of Immunology, Erasmus University, Rotterdam, [2]Laboratory of Medical Biology, TNO, Rijswijk, [3]Department of Genetics, Erasmus University, Rotterdam, The Netherlands

Summary

The Ph chromosome, resulting from the t(9;22) translocation, is one of the most frequently observed cytogenetic aberrations in acute lymphoblastic leukemia (ALL). Two genes, *bcr* and *abl*, are involved in this translocation. As a consequence, parts of the *bcr* and *abl* genes are fused resulting in chimeric *bcr-abl* genes, encoding chimeric BCR-ABL proteins. Three *bcr-abl* genes and proteins have been identified: e1-a2 $P190^{bcr\text{-}abl}$, b2-a2 $P210^{bcr\text{-}abl}$ and b3-a2 $P210^{bcr\text{-}abl}$. Since these chimeric proteins only occur in Ph chromosome positive- leukemic cells, they are by definition *tumor-specific* markers.

Ph chromosome-positive ALL is correlated with a poor prognosis, therefore the detection of chimeric BCR-ABL proteins is of prime importance in early ALL diagnosis. In the present study we report on the generation of a monoclonal antibody termed ER-FP1, raised against the tumor-specific e1-a2 BCR-ABL junction in $P190^{bcr\text{-}abl}$. We show that ER-FP1 reacts highly specifically with e1-a2 $P190^{bcr\text{-}abl}$ in different assays. The reactivity of ER-FP1 with e1-a2 $P190^{bcr\text{-}abl}$ in soluble form was analyzed in an immunoprecipitation assay; specificity was confirmed by peptide inhibition studies. Binding of ER-FP1 to e1-a2 $P190^{bcr\text{-}abl}$ at the single cell level was detected by using indirect immunofluoresence techniques. To this aim chimeric BCR-ABL proteins were highly expressed in COS cells, after transfection with the respective *bcr-abl* cDNAs. Immunological double staining experiments using ER-FP1 and a monoclonal antibody recognizing all ABL proteins confirmed the specificity of ER-FP1 for the e1-a2 fusion-point.

Introduction

The Philadelphia (Ph) chromosome ($22q^-$) occurs in more than 90% of all patients with chronic myeloid leukemia (CML) (1). Although a marker for CML,

accepted for publication in Leukemia

the Ph chromosome is not exclusively found in CML. Also in acute lymphoblastic leukemia (ALL) the Ph chromosome is observed (2). Here, the Ph chromosome is the most frequently occurring chromosomal aberration with an incidence of 25-30% in adult ALL and 2-5% in childhood ALL (3). Moreover, presence of a Ph chromosome in leukemic cells of ALL patients is associated with a poor prognosis (4,5). Therefore accurate diagnosis of Ph-positive ALL is of utmost importance.

The Ph translocation is the result of a reciprocal translocation between the chromosomes 9 and 22 t(9;22)(q3;q11) (6,7). Two genes are involved in the translocation: the *abl* gene on chromosome 9 and the *bcr* gene on chromosome 22 (8). During the translocation process both genes are interrupted and exchanged, resulting in a functional chimeric gene on the Ph chromosome comprising *bcr* and *abl* sequences. Breakpoints on chromosome 9 are scattered over a 200-kb distance but are almost always found 5' of *abl* exon a2 (8,9,10). In contrast, the chromosome 22 breakpoints are found to occur in two well defined areas of the *bcr* gene, either in the minor breakpoint cluster region (m-bcr) or in the major breakpoint cluster region (M-bcr) (11). The m-bcr is localized in the intron between the first (e1) and the second (e2) *bcr* exon (12,13). A break in the m-bcr results in a chimeric gene encoding a 190-kD protein with an e1-a2 *bcr-abl* junction, e1-a2 $P190^{bcr\text{-}abl}$ (14,15,16). The M-bcr spans a 5.8-kb region in the middle of the *bcr* gene including 4 exons (b1-b4). M-bcr breakpoints occur either between exon b2 and b3 or between exon b3 and b4 (8,9,10). As a consequence two different chimeric genes are generated encoding 210-kD proteins with different BCR-ABL junctions, b2-a2 $P210^{bcr\text{-}abl}$ and b3-a2 $P210^{bcr\text{-}abl}$ respectively (17). B2-a2 $P210^{bcr\text{-}abl}$ and b3-a2 $P210^{bcr\text{-}abl}$ differ only in 25 amino acids encoded by the bcr exon b3.

As described previously, the majority of the ALL patients with *bcr-abl* rearrangements express the e1-a2 *bcr-abl* mRNA i.e. 85% of all children and 68% of all adults (18). This means that the e1-a2 $P190^{bcr\text{-}abl}$ protein is a powerful tumor-specific marker for antibody diagnosis of Ph chromosome-positive ALL. However, the chimeric molecule is composed of parts of the non-tumorspecific proteins, BCR and ABL. The only tumor-specific epitope of the protein is formed by the junction between BCR and ABL (19).

In the present study we demonstrate the clinical and diagnostic potential of a monoclonal antibody, termed ER-FP1, which was raised against the e1-a2 BCR-ABL junction in e1-a2 $P190^{bcr\text{-}abl}$. Our data indicate that ER-FP1 specifically reacts with e1-a2 $P190^{bcr\text{-}abl}$ in different testsystems: using the tyrosine kinase assay, e1-a2 $P190^{bcr\text{-}abl}$ is recognized in cell lysates; immunofluorescence analysis allows detection of e1-a2 $P190^{bcr\text{-}abl}$ at the single cell level.

Materials and methods

Cell lines

TOM-1, BV173 and K562 are Ph positive cell lines derived from ALL (TOM-1) and CML patients respectively (20,21,22). Mouse SP2/0 cells were used as fusion partner for the production of monoclonal antibodies. All cell lines were cultured in RPMI medium supplemented with 5% fetal calf serum.

Patients

Patient L. is a 52-year-old male ALL patient. He carried a standard Philadelphia translocation, t(9;22)(q34;q11). Diagnosis precursor B-ALL was based on immunologic and hematologic criteria at presentation. Blast cells were isolated by Ficoll-Hypaque centrifugation and after cryopreservation stored under liquid nitrogen. Patient W. is a 23 year old male CML patient without evidence of a Philadelphia chromosome, but with rearranged *bcr* and *abl* genes, resulting in a chimeric b2-a2 *bcr-abl* gene, as localized on chromosome 9. (23, patient 1). Clinical and laboratory data are extensively described by Hagemeijer *et al.* (23). After T-cell depletion the blood cells were cryopreserved and stored under liquid nitrogen.

Peptide synthesis, purification and conjugation

The following peptides were synthesized corresponding to the respective BCR-ABL junctions:

SP e1-a2 : H G D A E* A L Q R P V
SP b2-a2 : C - I N K E E* A L Q R P
SP b3-a2 : C - F K Q S S K* A L Q

Residues indicated with an asterisk are newly formed as a result of the translocation.

SP e1-a2 was synthesized using Fmoc chemistry on an automated Milligen 9050 continuous Synthesizer (Millipore Co, Milford, MA). SP b2-a2 and SP b3-a2 were synthesized as described previously (24). All peptides were purified as described previously (24). Peptides were coupled to carrier molecules either via 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) or glutaraldehyde.

Immunization and production of ER-FP1 hybridoma

Balb/c mice were immunized with 25µg SP e1-a2 coupled via EDC to the carrier keyhole limpet hemocyanin. The peptide-carrier complex was emulsified in Complete Freund Adjuvant and injected in the hind footpad of the mice (25). After four weeks the mice were boosted i.p. with the same dosis of antigen in Incomplete Freund's Adjuvant. Three days after the booster the popliteal lymph nodes were removed and cell suspensions were made. For fusion, SP2/0 cells were mixed with immune lymph node cells at a ratio of 1:2. Cell fusion was induced with PEG-4000 (72% w/v in RPMI). Next, the cells were spun down and resuspended in RPMI supplemented with 10% fetal calf serum, 40 U IL-6/ml, hypoxanthine (10^{-4} M), azaserine (1 µg/ml) 2-mercapthoethanol (5.10^{-5} M) and antibiotics and plated in 96 wells culture plates at a density of 8.10^4 cells per well. The ER-FP1 clone was selected after screening of the supernatants for specific reactivity with the cognate peptide in an ELISA. Cell culture supernatant of ER-FP1 was

affinity purified using rat-anti-mouse-κ immunoglobulins coupled to Sepharose beads. ER-FP1 is a IgG2a type antibody.

ELISA

Reactivity of hybridoma supernatant with synthetic peptides was tested in a sensitive micro ELISA system as previously described (24). ELISA trays were coated with 10 µl of a dilution of 10 µg/ml SP e1-a2 conjugated to BSA through glutaraldehyde.

Antisera

BP-ALL is a polyclonal antiserum raised in rabbits against the e1-a2 BCR-ABL junction in e1-a2 $P190^{bcr\text{-}abl}$ (19). The *anti-BCR* antiserum is a polyclonal antiserum raised against sequences encoded by the first *bcr* exon (24). *8E9* is a monoclonal antibody (IgG1) directed against ABL proteins (26). 8E9 is a generous gift of Dr J. Wang.

Protein tyrosine kinase reaction

Reactivity of monoclonal antibodies with native proteins was tested by an immunoprecipitation followed by an autophosphorylation reaction as described previously (24). Briefly, $5\text{-}10x10^6$ cells were lysed and incubated with monoclonal or polyclonal antibodies. To precipitate the antigen-antibody complexes, protein-G Sepharose beads (Pharmacia, Sweden) were used instead of protein-A Sepharose beads. The precipitation was followed by an autophosphorylation reaction using 20 µCi (γ-[^{32}P]) ATP. Next, the beads were washed, boiled in sample buffer and run at 6% polyacrylamide gels.

Subsequently, the gels were dried and autoradiographed using Fuji (RX-NIF) films.

Transfection of COS cells

COS cells were grown on sterilized microscope glasses at a density of 1×10^4 cells/cm^2. Cells were transfected with pCDX plasmids comprising full lengths cDNA encoding e1-a2 $P190^{bcr\text{-}abl}$, b2-a2 $P210^{bcr\text{-}abl}$ or b3-a2 $P210^{bcr\text{-}abl}$. Transfection was mediated by calcium-phosphate (27).

Immunofluorescence analysis

Transfected COS cells were fixed for 20 min. by using a 3% paraformaldehyde solution diluted in PBS at room temperature. Next, the cells were washed 3 times in phosphate buffered saline (PBS) and permeabilized with 100% ice cold methanol for 20 minutes. After washing the cells twice in PBS, cells were blocked for 5 min. in PBS-0.5% BSA and incubated with first stage antibodies. All following incubations were carried out for 30 min. at room temperature. Between the various incubations cells were thoroughly washed 3 times with PBS-0.5% BSA. The permeabilized cells were first incubated with the anti-ABL monoclonal antibody 8E9 (IgG1). Subsequently the cells were incubated with an optimal dilution of a fluorescein isothyocyanate (FITC)-labeled goat anti-mouse IgG1 (Southern Biotechnology). Next, ER-FP1 (IgG2a) was applied followed by an incubation with an optimal dilution of tetramethylrodamine isothiocyanate (TRITC)-labeled goat-anti-mouse IgG2a (Southern Biotechnology). Fluorescence was evaluated using a Zeiss fluorescence microscope. Two filter combinations were used: Zeiss filter combination 14 (BP 510-560; FT 580; LP 590) for the evaluation of TRITC labeling and

Zeiss filtercombination 19 (BP485/20; FT 510; LP 515) for evaluation of FITC labeling.

Results

Binding of ER-FP1 to junction specific synthetic peptides

ER-FP1 was raised against the e1-a2 junction in e1-a2 $P190^{bcr\text{-}abl}$. Reactivity with the cognate peptide SP e1-a2 was determined by using a micro ELISA system. To detect in the same experiment any cross-reactivity of ER-FP1 with ABL derived a2 amino acids, ER-FP1 (2 µg/ml) was preincubated overnight at 4^0C with or without various concentrations of the free peptides, SP b3-a2 or SP b2-a2. As a positive control ER-FP1 was preincubated with SP e1-a2. The

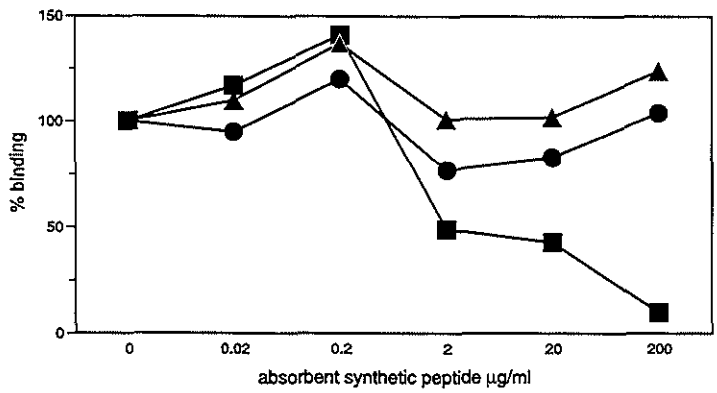

Figure 1 Relative binding inhibition of ER-FP1 to BSA-glutaraldehyde-SP e1-a2 in an ELISA after preincubation of 2 µg/ml ER-FP1 with various concentrations SP e1-a2 (■), SP b3-a2 (●) or SP b2-a2 (▲).

following day the samples were tested for residual anti-SP e1-a2 activity in the ELISA system, in which the Terasaki trays were coated with SP e1-a2 conjugated to BSA. Figure 1 shows that a peptide dose related inhibition of the antibody binding to the peptide conjugate was observed. In samples preincubated with the highest dose cognate peptide, i.e. 200 µg/ml the ER-FP1 reactivity is reduced to 10% (■). In contrast, preincubation with either SP b3-a2 (●) or SP b2-a2 (▲) does not significantly influence binding of ER-FP1 in the ELISA, indicating that ER-FP1 does not recognize ABL derived a2 residues. These data indicate that ER-FP1 has a specific affinity for the junction representing peptide SP e1-a2.

Binding of ER-FP1 to native BCR-ABL chimeric proteins

Binding of ER-FP1 to native proteins was determined in an immunoprecipitation assay followed by autophosphorylation. As source for BCR-ABL proteins ALL and CML cell lines were used, harboring the respective chimeric proteins e1-a2 $P190^{bcr\text{-}abl}$ (TOM-1), b2-a2 $P210^{bcr\text{-}abl}$ (BV173) and b3-a2 $P210^{bcr\text{-}abl}$ (K562). In Figure 2 a representative immunoprecipitation analysis with ER-FP1 is shown. ER-FP1 clearly precipitates a 190-kD protein from the lysate of TOM-1 cells, whereas no precipitation of either b2-a2 $P210^{bcr\text{-}abl}$ or b3-a2 $P210^{bcr\text{-}abl}$ can be observed in the lanes containing immunoprecipitations of K562 and BV173 lysates. These results indicate that also in the native protein the e1-a2 BCR-ABL junction is recognized by ER-FP1.

In order to exclude any reactivity of ER-FP1 with ABL derived a2 sequences we performed peptide inhibition studies with SP e1-a2 and other a2 comprising sequences such as SP b2-a2 and SP b3-a2. ER-FP1 (1,8 µg/ml) was incubated overnight with the respective peptides in various concentrations (200 µg/ml and 20 µg/ml). Next, lysates of TOM-1 cells were precipitated with the pre-absorbed ER-FP1. As positive control TOM-1 cells were precipitated with ER-FP1 without addition of peptides. As shown in Figure 3 reactivity of ER-FP1 with e1-a2 P $190^{bcr\text{-}abl}$ was abrogated completely after incubation with 200 µg/ml SP e1-a2 (SP e1-a2, lane 1). After incubation with 20 µg/ml SP e1-a2 only a faint

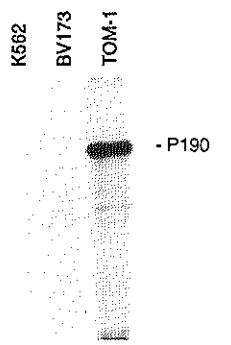

Figure 2

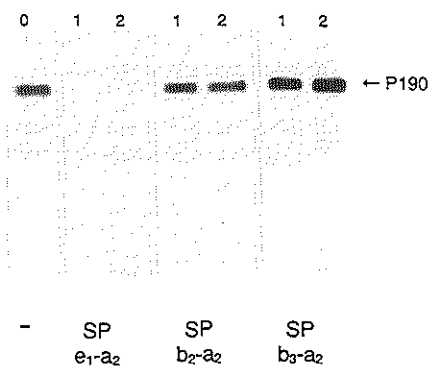

Figure 3

Figure 2 Junction specificity analysis of ER-FP1. $10x10^6$ TOM-1, K562, or BV173 cells were lysed and precipitated with 30µg/ml ER-FP1. TOM-1 cells express e1-a2 $P190^{bcr\text{-}abl}$, K562 cells express b3-a2 $P210^{bcr\text{-}abl}$ and BV173 cells express b2-a2 $P210^{bcr\text{-}abl}$.

Figure 3 Epitope specificity analysis of ER-FP1. $5x10^6$ TOM-1 cells (e1-a2 $P190^{bcr\text{-}abl}$) were lysed and precipitated with either 1,8 µg/ml ER-FP1 (lane 0) or with 1,8 µg/ml ER-FP1 after pre-incubation with synthetic peptides (SPe1-a2, SPb2-a2 or SPb3-a2) in various concentrations. ER-FP1 was pre-incubated with either 200 µg/ml (lanes 1) or with 20 µg/ml (lanes 2).

$P190^{bcr\text{-}abl}$ band is visible (SP e1-a2, lane 2). However, preincubation with 200 µg/ml SP b2-a2 or SP b3-a2 had no inhibiting effect on the precipitation of e1-a2 $P190^{bcr\text{-}abl}$ by ER-FP1 (SP b2-a2 and SP b3-a2, lanes 1 and 2).

From these results we conclude that ER-FP1 reacts highly specifically with native protein e1-a2 $P190^{bcr\text{-}abl}$. Moreover, the reactivity is specific for the e1-a2 junction and not directed against a2 sequences, since binding of ER-FP1 to e1-a2 $P190^{bcr\text{-}abl}$ is not inhibited after preincubation with other peptides comprising a2 derived amino acids.

Reactivity of ER-FP1 with leukemic cells of an ALL and a CML patient

In order to determine whether ER-FP1 reacted with e1-a2 $P190^{bcr\text{-}abl}$ from freshly obtained leukemic cells we incubated lysates of blast cells from patient L. with ER-FP1. Blast cells were isolated from the peripheral blood of this

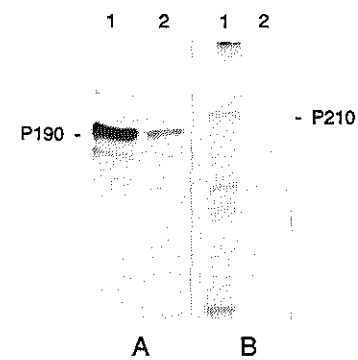

Figure 4 Immunoprecipitation analysis of ALL and CML patients. $10x10^6$ Fycoll-Hypaque enriched leukemic blast cells of patient L. were lysed and precipitated with 30 µg/ml ER-FP1 (panel A, lane 1) or 50 µl polyclonal antiserum BP-ALL (panel A, lane 2). $20x10^6$ T-cell depleted blood cells of patient W. were lysed and precipitated with 30 µg/ml ER-FP1 (panel B, lane 2) or 50 µl polyclonal anti-BCR antiserum (panel B, lane 1).

patient by Fycoll-Hypaque centrifugation. Next, cells were lysed and incubated either with ER-FP1 or, as control, with the e1-a2 junction specific polyclonal antiserum BP-ALL. As a control T cell-depleted blood cells derived from a Ph-negative CML patient with a b2-a2 rearranged *bcr-abl* mRNA were analysed. Figure 4 clearly shows that ER-FP1 reacts with e1-a2 $P190^{bcr\text{-}abl}$ derived from leukemic blast cells of ALL patient L. (panel A, lane 1). The reaction pattern obtained after precipitation with ER-FP1 or with BP-ALL (panel A, lane 2) is highly similar. After incubation of cell lysates of CML patient W. with ER-FP1 no proteins were precipitated (Figure 4, panel B, lane 2). However, patient W. actually expressed $P210^{bcr\text{-}abl}$ protein, since a strong $P210^{bcr\text{-}abl}$ band was observed after precipitation with an antiserum directed against the amino-terminal part of BCR (Figure 4, panel B, lane 1). These data indicate that ER-FP1 reacts also highly specifically with the e1-a2 BCR-ABL junction in leukemic

cells. As such, ER-FP1 can be applied in ALL diagnosis using the tyrosine kinase assay.

Immunofluorescence analysis of ER-FP1

Immunofluorescence analysis of ER-FP1 was carried out on COS cells transfected with an expression plasmid (pCDX) containing a full length cDNA encoding e1-a2 $P190^{bcr\text{-}abl}$. As controls, COS cells were transfected with expression plasmids containing cDNAs encoding either b2-a2 $P210^{bcr\text{-}abl}$ or b3-a2 $P210^{bcr\text{-}abl}$. To investigate whether e1-a2 $P190^{bcr\text{-}abl}$ was recognized by ER-FP1 double immunological staining experiments were carried out using ER-FP1 and the anti-ABL antibody 8E9. Binding of ER-FP1 was visualized by applying a TRITC-labeled goat-anti mouse IgG2a. For the detection of anti-ABL binding, FITC-labeled goat-anti mouse IgG1 was used. Figure 5 shows fluorescence photographs of e1-a2 $P190^{bcr\text{-}abl}$ transfected cells after incubation with ER-FP1 (a) and anti-ABL (b). These micrographs show a striking overlapping staining pattern indicating that the same molecules are detected by both monoclonal antibodies in a highly specific way. Most of the antibody binding is localized in the cytoplasm associated with the cytoskeleton. Also the inner surface of the cell membrane is intense stained. Moreover, next to the nucleus a brightly fluorescent area is visible, probably indicating binding of the antibodies to the Golgi apparatus. Binding of ER-FP1 to untransfected cells is at the background level. These cells also fail to show specific staining by the anti-ABL antibody 8E9 (Figure 5b).

As a final specificity control, immunological double labeling studies were performed on $P210^{bcr\text{-}abl}$ transfected COS cells using ER-FP1 and the anti-ABL antibody 8E9 (Figure 5, panel c,d,e,f). ER-FP1 shows a weak background staining of all cells, either transfected or untransfected (Figure 5, panel c and e). The antibodies did not react with the $P210^{bcr\text{-}abl}$ proteins, since the pattern obtained after ER-FP1 incubation is completely different from the specific staining with 8E9 (Figure 5, panel d and f). Staining with 8E9 shows that $P210^{bcr\text{-}abl}$ and $P190^{bcr\text{-}abl}$ have both the same highly characteristic subcellular localization.

From these data we conclude that native e1-a2 $P190^{bcr\text{-}abl}$ is recognized by ER-FP1 in a highly specific way at the single cell level.

Discussion

The Philadelphia chromosome is the most frequently occurring chromosomal aberration in ALL (3). Because this type of leukemia has a bad prognosis in comparison with Philadelphia negative ALL, accurate diagnosis is highly important. To this purpose, several techniques are now available, either

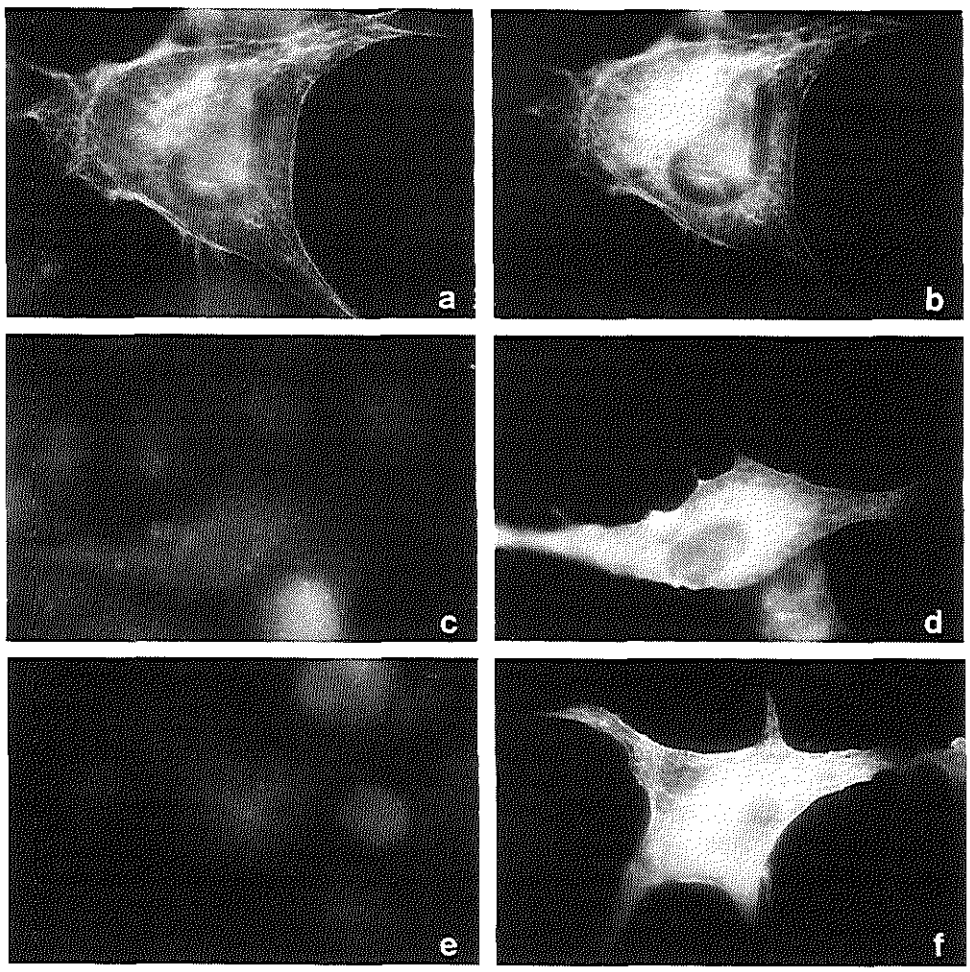

Figure 5 Immunofluorescence analysis of ER-FP1. COS cells were transfected with pCDX plasmids comprising full lengths cDNA encoding e1-a2 P190$^{bcr-abl}$ (a and b), b2-a2 P210$^{bcr-abl}$ (c and d) and b3-a2 P210$^{bcr-abl}$ (e and f). Transfected cells were double labeled with ER-FP1, detected with TRITC-labeled goat-anti mouse IgG2a (a, c and e) and with anti-ABL antibody 8E9, detected with FITC-labeled goat-anti mouse (b, d and f). Magnification: 200x.

detecting the Ph chromosome or the molecular rearrangements caused by the translocation.

In the past years we have focussed on the detection of the various BCR-ABL chimeric proteins i.e. e1-a2 P190$^{bcr-abl}$, b2-a2 P210$^{bcr-abl}$ and b3-a2 P210$^{bcr-abl}$. We have demonstrated that the BCR-ABL junctions in all three chimeric proteins are expressed as antigenic determinants on the respective

proteins (19,24,28). In these studies we raised polyclonal antisera directed against synthetic peptides corresponding to the respective BCR-ABL junctions.

Now we report on the reactivity of a monoclonal antibody, ER-FP1, raised against a synthetic peptide corresponding to the e1-a2 BCR-ABL junction in e1-a2 $P190^{bcr\text{-}abl}$. We show evidence that ER-FP1 reacts specifically with e1-a2 peptides in an ELISA as well as with native e1-a2 $P190^{bcr\text{-}abl}$ molecules in immunoprecipitation experiments. Specificity of ER-FP1 was further confirmed in both systems by peptide inhibition studies. ER-FP1 could also be applied in immunofluorescence analyses. COS cells transfected with cDNA encoding the complete e1-a2 $P190^{bcr\text{-}abl}$ protein, were stained with ER-FP1. The staining pattern of ER-FP1 was strikingly similar to the pattern obtained after staining transfected COS cells with a monoclonal antibody against ABL, indicating that the same molecules were recognized. Our results on the localization of ABL proteins correspond closely to earlier reports by van Etten (29) and Wang (30,31). These authors describe association of activated ABL proteins with the cytoskeleton, resulting in a fine reticular staining pattern. For the first time our data show specific reactivity of a BCR-ABL junction specific antibody with native chimeric protein at the single cell level. This study indicates that the antigenic determinant formed by the e1-a2 junction, is not lost by our fixation procedure.

We have performed immunofluorescence and flow cytometry studies with ER-FP1 with CML and ALL cell lines using this protocol as well (data not shown). Unfortunately, in these experiments we only observed a very strong background staining in all cells, either expressing $P190^{bcr\text{-}abl}$ or $P210^{bcr\text{-}abl}$. A feasible explanation for this phenomenon is that the expression of e1-a2 $P190^{bcr\text{-}abl}$ is to low in ALL cell lines, where specific staining does not rise above the background. Alternatively it is possible that the same epitope formed by the e1-a2 junction in $P190^{bcr\text{-}abl}$ is also expressed by other, as yet unknown, intracellular proteins. This latter idea finds support in recently performed Western blotting experiments where we observed binding of ER-FP1 to a range of proteins with various molecular weights, which may account for the background immunofluorescence staining (data not shown). Refined immunofluorescence analysis, e.g. using confocal laserscan microscopy, might resolve this background staining problem. Preliminary results of such an experiment in which we performed double labeling of $P190^{bcr\text{-}abl}$ positive and negative cells with ER-FP1 and anti-actin, suggest that e1-a2 $P190^{bcr\text{-}abl}$ co-localizes with actin (data not shown), confirming earlier published data by Wang (31). However, also in $P190^{bcr\text{-}abl}$ negative cells such co-localization was observed. Albeit that, the frequency of double positive cells and the intensity of the fluorescence was higher in the $P190^{bcr\text{-}abl}$ positive cells compared to the control $P190^{bcr\text{-}abl}$ negative cells. Both in the $P190^{bcr\text{-}abl}$ positive and in the $P210^{bcr\text{-}abl}$ positive cells the

background staining revealed a fine granular staining pattern in the nucleus and in the cytoplasm, whereas the double stained material was present closely under the cell membrane. In the future further analysis of the co-localization pattern may provide the answer to a specific detection of $P190^{bcr\text{-}abl}$ in routine immunofluorescence studies of malignant cells.

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CHAPTER 4

General discussion

Contents

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General discussion

Tumor-specific proteins (TSPs) are exclusively expressed by tumor cells. Normal cells do not harbor these particular proteins. Logically, the question arises what their significance might be in tumorigenesis and tumor rejection. Moreover, the question can be raised whether TSPs are clinically relevant with respect to tumor diagnosis and tumor therapy. This chapter addresses these questions in more detail.

4.1 Tumor-specific proteins and tumorigenesis

Structural changes in normal proteins generated by genetic alterations may have consequences for the biological function of the proteins in question. As described in chapter 2 of this thesis, in tumor cells several types of proteins are are modified as a result of chromosomal aberrations or point mutations in the DNA of tumor cells. Based on their function in the cell the altered proteins, termed 'tumor-specific proteins' (TSPs), can be classified in two groups. The major group consists of TSPs which are involved in transcription regulation; either directly by binding to DNA and/or indirectly through complexing with other DNA binding proteins. The other, minor group is formed by growth factor receptors or proteins involved in signal transduction. Both groups differ from each other with respect to their putative role in tumorigenesis. The transcription factor type of TSPs cause deregulation of the cell metabolism from *inside* of the cell. The altered transcription factor itself is functioning improperly, resulting in abnormal transcription of target genes, which may cause autonomous growth of tumor cells. Alterations in growth factor receptors and in molecules involved in signal transduction result in deregulation of the cell metabolism, because the cell is not able to react adequately on signals from *outside* the cell. Communication with and control by the environment is disturbed, resulting in independent growth of tumor cells.

TSPs themselves are however not the only originators of cancer. In general, tumorigenesis is thought to be a multistep process. Several observations support this hypothesis.

- As described in chapter 2.2 of this thesis, point mutations in RAS and P53 proteins are frequently observed in multiple types of tumors. However, RAS or P53 mutations are not expressed in all tumors of the same type (Bos, 1989). This observation indicates that both types of TSPs are not exclusively required for tumorigenesis, because, in that particular case all tumors of the same type would harbor mutations in RAS and P51, repectively. Other, yet unknown factors are probably also important for tumor formation.
- Additional chromosomal aberrations, suggesting abnormal activation of yet

unknown proteins, are often observed in tumor-progression. Moreover, the risk of developing cancer increases with age. Both observations suggest that multiple hits may accumulate and finally cause cancer.

However, several studies show clearly a direct involvement of TSPs in tumorigenesis (Heisterkamp *et al.*, 1990; Daley *et al.*, 1990; Kelliher *et al.*, 1990; Kamps and Baltimore, 1993; Dedera *et al.*, 1993). Studies with $P190^{bcr\text{-}abl}$ transgenic mice show that an aggressive leukemia is generated due to introduction of $P190^{bcr\text{-}abl}$ cDNA *alone* (Heisterkamp *et al.*, 1990). Likewise, a CML-like disease has been induced in mice transplanted with $P210^{bcr\text{-}abl}$ positive bone marrow cells (Daley *et al.*, 1990; Kelliher *et al.*, 1990). Studies with E2A-PBX1 transgenic mice and with mice transplanted with E2A-PBX1 positive bone marrow cells indicate that also E2A-PBX1 on its own is able to induce leukemia (Kamps and Baltimore, 1993; Dedera *et al.*, 1993). Thus, in these cases a direct relationship exist between the presence of the TSP alone and the onset of the tumor. Interestingly, as mentioned above, in man both respective TSPs are associated with a more severe form of leukemia compared to the TSP negative leukemias. Whether BCR-ABL and E2A-PBX1 form exceptions on the rule that tumorigenesis is a multistep process, or that additional mutations in other genes in the tumors have been arisen has to be investigated.

4.2 Tumor-specific proteins and tumor rejection

TSPs can be generated by alterations in the DNA, induced by for example toxic chemicals or UV radiation. However, to prevent DNA damage and the subsequent forming of TSPs, the cell is equipped with appropriate protection mechanisms. First, intracellular enzymes, such as the cytochrome P450 enzymes, are able to change toxic, DNA damage-inducing chemicals into a nontoxic form (Korzekwa and Jones, 1993). Also the multidrug resistance protein may be considered as a mechanism through which the cell is protected against DNA damage: toxic drugs will be eliminated from the cell by the MDR pump before the DNA can be harmed (reviewed by Ferguson and Baguley, 1993). Thus, both systems prevent DNA damage, and as a consequence prevent the generation of TSPs. In case that the DNA has been damaged, the cell disposes of a DNA repair mechanism (Hoeijmakers, 1993; Palumbo, 1994). Damaged DNA, which is in principle able to encode a TSP, is repaired to prevent the formation of a TSP and the subsequent tumor. Therefore, the intracellular DNA repair mechanism can be considered as the second step to prevent the formation of TSPs.

Whenever TSPs are formed, either as a result of unrepaired DNA or due to less subtle chromosomal aberrations which are beyond repair, tumor formation

might be restrained by a third system, i.e. the immune system. In contrast to the above mentioned intracellular control mechanisms, the immune system regulates tumor growth from outside the malignant cell. In this particular process TSPs might play an important role. After all, the immune system will recognize the newly formed TSPs as non-self antigens. As discussed in Chapter 2, the majority of all TSPs are intracellular proteins. Thus, tumor control through a *humoral immune response* does not seem obvious. However, MHC class I and class II molecules are able to present intracellular proteins, in the form of processed peptides, to the immune system to evoke a *cellular immune response*. Over the last years immunological studies showed evidence that also intracellular TSPs can elicit a class I or class II restricted immune response. CD4-positive T-cells directed against mutated RAS proteins have been cloned, after both *in vivo* immunization of mice with $P21^{ras}$-derived peptides and after *in vitro* simulation of human T cells with $P21^{ras}$ peptides. (Peace *et al.*, 1991; Jung and Schluesener, 1991). Similarly, BCR-ABL specific CD4-positive T-cells, were obtained either after in vivo immunization of mice, or after in vitro stimulation of human T cells with peptides corresponding to the tumor-specific fusion-points in BCR-ABL proteins (Chen *et al.*, 1992; ten Bosch pers. comm.). Human CD8-positive, P53 specific, T-cells have been isolated after in vitro stimulation of T-cells with P53 derived peptides (Houbiers *et al.*, 1993). These studies indicate that TSP specific T-cells can be induced. One might predict therefore, that a proportion of human tumors does not persevere in vivo, due to the control or the complete eradication of the immune system.

These studies offer the possibility to speculate about the role of TSPs as targets for immunotherapy. In this respect, studies by Feltkamp *et al.* (1993) have shown promising results. They have shown that vaccination of mice with a peptide corresponding to a putative CTL inducing epitope of human papillomavirus type 16, was able to generate a virus specific CTL response. As a result, mice were protected against a challenge with papillomavirus type 16 transformed tumor cells. Possibly, in the future, vaccination with TSP-specific peptides will be a new form of protective immunotherapy in human cancer.

4.3 Tumor-specific proteins and tumor diagnosis

Tumor-specific proteins are pre-eminently tumor markers, since they are only expressed in tumor cells and absolutely not in normal cells. Therefore, in principle TSPs are excellent targets for antibody diagnosis of TSP positive tumors. Nevertheless, as described in Chapter 2, only a minority of TSPs have detected at the protein level using TSP specific antibodies. Thus far, antibodies against respectively mutations in P21-RAS, P53 and against the internal fusion-point in the EGFR have been described (Wong *et al.*, 1986; Pullano *et al.*, 1989; Gannon *et al.*, 1990; Yewdel *et al.*, 1986; Bartek *et al.*, 1991; Humphrey *et al.*,

1990).

It is likely, that technical difficulties have so far prevented the generation of TSP-specific antibodies. The production of monoclonal antibodies directed against peptides corresponding to tumor-specific sequences in TSPs, is laborious and time consuming. We experienced that the production of *peptide-specific* antibodies was relatively easy. However, when we tested the reactivity of such peptide-specific antibodies with *native protein*, we frequently observed either no reactivity at all, or a positive reaction with a large range of other proteins. This cross reactivity hampers accurate diagnosis at the single cell level. Several explanations may explain the non- and multireactivity of peptide-specific monoclonal antibodies.

- No reactivity will be found when the protein concentration in the cell is too low.
- No reactivity of anti-peptide antibodies with native proteins will be observed when the linear peptide structure does not correspond to the tertiary structure of the native protein at the site of the junction.
- The peptide-specific antibodies may have a too low avidity or affinity for the native protein to allow binding.
- Reactivity with different proteins may be occur when the antibody recognizes a linear sequence which also prresent in other proteins. Although our peptides were screened for homologous structures in known proteins and none were found, the possibility remains that the sequence recognized by the monoclonal antibodies also occurs proteins not yet described.
- Specific reaction with other proteins will also be observed when the recognized peptide-sequence is also present in a tertiary structure of non-homologous proteins .

Since we were able to isolate a monoclonal antibody ER-FP1, we conclude that it is possible to raise a fusion-point-specific monoclonal antibody (see Chapter 3.5).

The question is legitimate whether the production of monoclonal antibodies directed against TSPs is rational. Other methods have been developed to detect TSPs, such as the PCR technique. As described in Chapter 3, the PCR technique allows detection of small amounts of junction specific *bcr-abl* mRNA. However, several reasons argue in favor of the use of TSP-directed antibody diagnosis.

- As discussed in Chapter 3, the PCR technique is more sensitive compared to immunofluorescence diagnosis. However, the chance to obtain false positive results in PCR is considerable. Moreover, to perform PCR, advanced and highly controlled laboratory equipment is required.
- Detection of minimal residual disease in situ, at the single cell level is highly successful using antibodies in immunocytochemical or immunofluorescence analyses of tumor material.

- Double labeling techniques using TSP specific antibodies and multiple other markers may give insight in the nature and the differentiation stage of the malignant cell.
- From the economical point of view, antibody diagnosis is far less expensive and time consuming than PCR diagnosis. In general, immunofluorescence analysis allows diagnosis within one day, whereas PCR results are available at first after 4 to 5 days.

In summary, tumor-specific monoclonal antibodies are valuable tools in tumor diagnosis.

Therefore, other techniques, may overcome the difficulties we experienced in generating monoclonal antibodies. Other immunization methods can be tried in conjunction with different types of adjuvantia. We have performed one experiment in which mice were immunized with various types of peptides and various adjuvantia (i.e. Specol, an oil in water emulsion (Solvey-Duphar, Weesp), carbomer 934P (commercially availabe) and Freund's adjuvant). We observed an extensive variation in peptide specific antibody responses, depending on both the type of adjuvant and the structure of the peptide. This particular experiment suggests that the choice of adjuvant depends on the structure of the peptide. The tertiary structure in which the peptide is presented during the immunization, may also be important in raising an antibody response. In our experiments aimed to obtain monoclonal antibodies, we have immunized the mice with peptides coupled to carrier molecules, such as chicken-γ-globulin and keyhole limpet hemocyanin. Recently, a new method using multi-antigenic-peptides (MAP), mimicking the tertiary structure of the native protein, has been shown to generate peptide- and protein-specific antibodies (Berendes *et al.*, manuscript in prep.). Also peptides coupled to a T-cell epitope, providing direct T-cell help in the immune response are interesting new variants as antigens in the hybridoma technology (Zegers *et al.*, 1993).

In spite of the technical problems we experienced, our work, as described in Chapter 3 of this thesis, shows that antibody recognition of the tumor-specific BCR-ABL fusion-points in respectively e1-a2 $P190^{bcr\text{-}abl}$, b2-a2 $P210^{bcr\text{-}abl}$ and b3-a2 $P210^{bcr\text{-}abl}$ is possible. We have demonstrated the possibility to raise both polyclonal and monoclonal fusion-point specific antibodies which also recognize the native proteins. From the clinical point of view, all respective antisera are able to be applied in diagnosis of Ph-positive CML and ALL using the tyrosine kinase assay.

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Summary

The human body consists of innumerable numbers of cells. Each cell has its own, genetically programmed function. To operate correctly, a cell has to its disposal an internal, highly regulated machinery which controls growth and differentiation. Protein molecules are important regulators of both processes. Changes in DNA, either caused by chromosomal alterations or by mutations within the DNA, may yield structurally altered proteins. Such alterations may result in loss or gain of the biological activity of the proteins. As a consequence the processes regulated by the proteins are disturbed. The overall effect is a tumor caused by the unbridled, deregulated growth or differentiation of cells comprising the altered protein.

The structurally altered protein is a so called 'tumor-specific protein' (TSP), because the molecule is only expressed in the tumor cell and not in normal cells. Thus, TSPs distinguish tumor cells from normal cells. TSPs are therefore of clinical importance.

Our own experimental work was aimed at the detection and characterization of one type of TSPs, i.e. the BCR-ABL chimeric proteins, occurring in chronic myeloid leukemia (CML) and acute lymphoblastic leukemia (ALL). BCR-ABL proteins are the first known TSPs. They were first detected ten years ago. Since then, more TSPs have been discovered and the number of identified TSPs is still growing.

In the present thesis we made an inventory of the TSPs known thus far. In the Chapters 1 and 2 we describe the state of art within the field of TSPs, focusing on the following questions.

- How are TSPs generated?
- In which type of tumors, and in which frequency, do TSPs occur?
- Is the structure of a TSP in one type of tumor always the same or varies the structure from tumor to tumor and from patient to patient?
- Which role play TSPs in the generation of a tumor?

With respect to the first question, TSPs are able to arise by two processes. One group of TSPs may be generated by a translocation of two chromosomes, which results in the fusion of two proteins. The other group may be formed by subtle point mutations in the DNA.

In the second chapter we summarize the tumors in which TSPs occur. The frequencies are given in a detailed description of each particular TSP. The incidences of TSPs within the various types of tumors are highly varying. For example, in acute myeloid leukemia only a small minority of all cases carries the

t(6;9) translocation and probably express the TSP generated by this particular translocation. However, in other tumors almost all patients express the TSP: more than 90% of all patients with chronic myeloid leukemia express the t(9;22) translocation and the TSP generated by the t(9;22) translocation. In this context, it is relevant to mention that large scale studies aimed at the detection of tumor-specific *proteins* have been performed not yet. Statements about the incidence of TSPs are commonly based on incidence of altered chromosomes or genes.

The results of our literature search show that there is a broad variety in homo- or heterogeneity of TSPs within one type of tumor. Highly homogeneous TSPs are the BCR-ABL fusion proteins occurring in CML. Here, only three variant BCR-ABL proteins exist. The most heterogeneous type of TSP is formed by mutated P53; the variety of mutations in P53 is almost as high as there are tumors.

Homogeneous TSPs form the best targets for tumor diagnosis. In Chapter 2 we have indicated which TSPs are or are not appropriate targets for tumor diagnosis.

Finally we describe the putative role TSPs play in the generation of cancer. Based on their function, two groups of TSPs can be distinguished. The first group consists of structurally altered 'transcription factors' whereas the second group consists of structurally altered molecules involved in 'signal transduction'.

Transcription factors control the decoding process from DNA into protein. Altered transcription factors function incorrectly, resulting in aberrant decoding of DNA, which is then followed by aberrant expression of proteins. In turn, abnormal expression of these particular proteins may cause deregulation of the cell metabolism and unbridled growth or differentiation of the affected cells.

The second group of TSPs consists of proteins involved in signal transduction. Normal cells are able to communicate with each other by sending out and receiving signals. When proteins active in this communication process, are disfunctioning due to structural alterations, also the communication of the affected cell with other cells may be disturbed. This may result in isolated, autonomous growth of (cancer)-cells carrying the TSPs.

In Chapter 3 we describe the results of our own experimental work aimed at the specific recognition of the tumor-specific BCR-ABL chimeric proteins in chronic myeloid leukemia (CML) and acute lymphoblastic leukemia (ALL). These particular chimeric proteins are generated by the so called 'Philadelphia translocation', a reciprocal translocation between chromosome 9 and chromosome 22. The abnormal chromosome 22, which is generated by the translocation, is called the Philadelphia (Ph) chromosome.

As a result of the Philadelphia translocation, the *abl* gene is translocated from chromosome 9 into the *bcr* gene on chromosome 22, resulting in a chimeric *bcr-abl* gene, encoding the tumor-specific BCR-ABL proteins. Depending on the localization of the breakpoint within the *bcr* gene, three types of chimeric genes and proteins occur. The respective proteins are termed e1-a2 $P190^{bcr\text{-}abl}$, b2-a2 $P210^{bcr\text{-}abl}$ and b3-a2 $P210^{bcr\text{-}abl}$, respectively. The fundamental question of our experimental work was: 'Are these particular TSPs useful targets for antibody-mediated diagnosis of Ph chromosome-positive CML and ALL?'

To answer this question we investigated whether it was possible to raise antisera against the three types of BCR-ABL proteins. To this aim, peptides were synthesized, corresponding to the tumor-specific sites on the proteins, i.e. the BCR-ABL junctions. In order to obtain polyclonal antisera rabbits were immunized with these 'tumor-specific' peptides. Moreover, in order to generate tumor-specific monoclonal antibodies mice were immunized with these particular peptides.

We tested the reactivity of the antibodies with the synthetic peptides in a highly sensitive peptide ELISA. The reactivity with native BCR-ABL proteins was investigated using an immunoprecipitation assay, followed by autophosphorylation of the precipitated proteins. We obtained the following results.

- Three polyclonal rabbit antisera termed BP-1, BP-2 and BP-ALL, each recognizing one type of chimeric BCR-ABL proteins, were isolated.
- These polyclonal antisera specifically recognize the respective chimeric proteins, isolated from CML and ALL cell lines, in an immunoprecipitation assay.
- The polyclonal antisera did not specifically recognize the native chimeric proteins in situ, in intact leukemic cells.
- One particular monoclonal antibody, termed ER-FP1, was isolated recognizing specifically e1-a2 $P190^{bcr\text{-}abl}$.
- This monoclonal antibody reacted with e1-a2 $P190^{bcr\text{-}abl}$ both in an immunoprecipitation assay and in cells highly expressing e1-a2 $P190^{bcr\text{-}abl}$.

From these results we conclude that it is possible to generate both polyclonal and monoclonal antisera against the chimeric BCR-ABL proteins and that the tumor-specific BCR-ABL fusion points are expressed on the proteins as tumor-specific epitopes. To test whether the antisera could be used in diagnosis of CML and ALL patients, we analyzed with our antisera several blood and bone marrow samples of CML and ALL patients. We observed that our antisera were able to detect the chimeric proteins in fresh, patient derived material. However, to this aim only the immunoprecipitation assay was suitable.

Therefore the overall conclusions from this work can be summarized as follows.

- Antisera can be raised which specifically react with BCR-ABL chimeric proteins.
- The BCR-ABL fusion points are expressed as antigenic determinants on the respective proteins.
- These antisera can be applied in the diagnosis of Ph chromosome-positive CML and ALL.

In Chapter 4 we discuss the role of TSPs in tumorigenesis and tumor rejection in general terms. We focus also on the results of our own experimental work. In addition, we summarize the difficulties we experienced in the production of fusion-point specific monoclonal antibodies. Finally we give technical suggestions to improve the rate of success in the production of tumor-specific monoclonal antibodies.

List of abbreviations

AF-4	ALL-1 fused gene on chromosome 4
ALL	acute lymphoblastic leukemia
ALL-1	acute lymphoblastic leukemia-1 protein
AML	acute myeloid leukemia
AML-1	acute myeloid leukemia-1 protein
AML-M2	subtype of acute myeloid leukemia
AML-M4Eo	subtype of acute myeloid leukemia
AmuLV	Abelson murine leukemia virus
ANLL	acute non lymphoblastic leukemia
APL	acute promyelocytic leukemia
ATP	adenosine triphosphate
AUL	acute undifferentiated leukemia
bcr	BCR encoding gene or RNA
BCR	breakpoint cluster region protein
bHLH	basic helix loop helix
BP-1	polyclonal antiserum recognizing b2-a2 $P210^{bcr\text{-}abl}$
BP-2	polyclonal antiserum recognizing b3-a2 $P210^{bcr\text{-}abl}$
BP-ALL	polyclonal antiserum recognizing e1-a2 $P190^{bcr\text{-}abl}$
BSA	bovine serum albumin
BV173	cell line derived from a CML patient
(c-)*abl*	(cellular) Abelson gene or RNA
(c-)ABL	(cellular) Abelson protein
cALL	common ALL
CBF	core binding factor
cDNA	complementary DNA
CGG	chicken γ globulin
CML	chronic myeloid leukemia
CSF-1	colony stimulating factor-1
DNA	desoxyribonucleic acid
E2A	E2-box binding protein
EAP	Eber associated protein
EGF(R)	epidermal growth factor (receptor)
ER-FP1	monoclonal antibody recognizing e1-a2 $P190^{bcr\text{-}abl}$
ETO	eight twenty one protein
EWS	Ewing sarcoma protein
FCS	fetal calf serum
FITC	fluorescein isothyocyanate
Fli-1	Friend leukemia integration site-1 protein
GAP	GTPase activating protein
GDP	guanosine diphosphate
G_{i2}	adenylate cyclase inhibitory protein G
G_s	adenylate cyclase stimulating protein G

GTP	guanosine triphosphate
H-RAS	Harvey rat sarcoma viral oncogene homologue
HLF	hepatic leukemia factor
HRX/HTRX	human thritorax protein
icb	intron containing the breakpoint
Ig	immunoglobulin
IgH	immunoglobulin heavy chain
ins	insertion
inv	inversion
K-RAS	Kirsten rat sarcoma viral oncogene homologue
K562	cell line derived from a CML patient
kb	kilo base
kD	kilo Dalton
KLH	keyhole limpet hemocyanin
LAMA-84	cell line derived from a CML patient
m-bcr	minor breakpoint cluster region
M-bcr	major breakpoint cluster region
MBS	m-maleimido-benzoyl-sulfosuccinimide ester
MDR	multi drug resistance
MDS	myelodysplastic syndrome
MHC	major histocompatibility complex
MLPS	mixed liposarcoma
mRNA	messenger ribonucleic acid
MW	molecular weight or molecular mass
N-RAS	neuroblastoma RAS viral oncogene homolog
NRG	protein encoded by the non rel gene
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Ph	Philadelphia
PLZF	promyelocytic leukemia zinc finger protein
PML	promyelocytic leukemia protein
RARα	retinoic acid receptor α
RB	retinoblastoma susceptibility protein
RET/PTC	RET protein in papillary thyroid carcinoma
RLF	rearranged L-myc fusion protein
SH1/2/3	SRC homology region 1/2/3, resp
SMMHL	smooth muscle myosin heavy chain
SP	synthetic peptide
TcR	T cell receptor
TLS	protein translocated in liposarcoma
TOM-1	cell line derived from an ALL patient
TRITC	tetramethylrhodamine isothiocyanate
TSA	tumor-specific antigen
TSPs	tumor-specific proteins
Zn finger	zinc finger

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TUMOR-SPECIFIEKE EIWITTEN BIJ KANKER

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Het in dit proefschrift beschreven onderzoek werd uitgevoerd op de afdeling Immunologie van de Faculteit der Geneeskunde en Gezondheidswetenschappen, Erasmus Universiteit Rotterdam.

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voor iedereen die wil weten
waar dit proefschrift over gaat

Inhoud

VOORWOORD

Dit Nederlandstalige gedeelte is geschreven voor iedereen die wil weten waar dit proefschrift over gaat. Het bestaat uit twee delen.

In het eerste hoofdstuk wordt uitgelegd wat tumor-specifieke eiwitten zijn, hoe ze ontstaan en wat ze doen. Het tweede hoofdstuk bevat een samenvatting van ons eigen onderzoek. Het gaat over tumor-specifieke eiwitten, die voorkomen bij twee vormen van bloedkanker, namelijk bij chronische myeloïde leukemie en acute lymfatische leukemie.

Ik heb mijn best gedaan om in begrijpelijk Nederlands te schrijven. Misschien is het verhaal op sommige punten te uitgebreid en op andere punten weer te beknopt. Toch hoop ik dat iedereen na het lezen van deze twee hoofdstukken iets meer te weten is gekomen over 'tumor-specifieke eiwitten bij kanker'.

Janneke van Denderen
mei 1994

HOOFDSTUK 1

Normale en tumor-specifieke eiwitten

1.1 De cel, chromosomen, genen, DNA en eiwitten

Het menselijk lichaam is opgebouwd uit cellen. Een cel kan vergeleken worden met een ei met een zachte, sponsachtige schaal. Elke cel wordt omgeven door een celwand (overeenkomend met de eierschaal). Verder bevat de cel een kern, 'de dooier', en cytoplasma, 'het eiwit', een vloeibare massa die de ruimte tussen kern en celwand opvult.

In de mens bevat de celkern 23 paar chromosomen die het erfelijk materiaal, het DNA, bevatten. Elk paar chromosomen heeft zijn eigen karakteristieke vorm. Cytogenetici, chromosomen-deskundigen, hebben ieder paar chromosomen een nummer gegeven, van 1 t/m 22. Het 23^{e} paar bestaat bij de vrouw uit twee zgn. X chromosomen en bij de man uit één X en één Y chromosoom.

Chromosomen zien er in ieder mens hetzelfde uit, maar de samenstelling van het DNA ín de chromosomen verschilt van mens tot mens. In het DNA ligt de genetische code vast. Die code wordt geprogrammeerd d.m.v. 'genen', dit zijn kleine afgebakende stukjes DNA. Sommige genen zijn in alle mensen hetzelfde terwijl andere genen juist sterk verschillen. 'Haar' hebben we bijvoorbeeld allemaal, maar bij de ene mens is het rood en bij de ander zwart. Dat ligt vast in de genen. Het zijn echter niet de genen zèlf die zorgen dat het haar rood of zwart wordt. Dat gebeurt door de eiwitten, die gecodeerd worden door de 'haarkleur'-genen.

Meer in het algemeen gesteld nemen eiwitten deel aan allerlei processen in de cel. Want een cel is geen statische eenheid. Een cel kan zich door deling vermenigvuldigen. Op deze manier vermeerdert het aantal cellen van dezelfde soort en is er sprake van celgroei. Sommige cellen kunnen ook veranderen van het ene celtype naar het andere, dit heet differentiëren. Zo kunnen b.v. bepaalde cellen die vrij rond zwemmen in het bloed, differentiëren tot cellen die door de wand van het bloedvat heen kruipen om in het omringende weefsel te infiltreren.

Zowel het proces van celgroei als van celdifferentiatie wordt gecontroleerd door eiwitten. Het is dus voor te stellen dat, als er iets mis gaat met die eiwitten, de cel, waarin die verandering optreedt, ongecontroleerd gaat groeien. Op zo'n manier kan een tumor van de cellen in kwestie ontstaan. De patiënt heeft dan kanker.

1.2 Tumor-specifieke eiwitten

Dit proefschrift gaat over eiwitten die alleen voorkomen in kankercellen, de zgn. tumor-specifieke eiwitten. Dit zijn eiwitten waarmee 'iets is misgegaan' en die alleen voorkomen bij kanker.

Waarom is wetenschappelijk onderzoek van deze eiwitten belangrijk? Twee redenen zijn hiervoor aan te geven.

1. Het is nog steeds niet duidelijk op welke manier precies kanker ontstaat. Omdat deze eiwitten een 'foutje' bevatten en alleen voorkomen bij kanker kan verondersteld worden dat ze direct betrokken zijn bij het kankerproces. Onderzoek naar de *werking* en de *effecten* van deze eiwitten op de groei en differentiatie van cellen kan dus wellicht inzicht verschaffen in dit proces.
2. De 'foute' eiwitten waar het hier om gaat, komen alleen voor in tumorcellen en niet in normale cellen in het menselijk lichaam. Daarom worden ze 'tumor-specifieke eiwitten' of 'tumor-specifieke proteïnen', afgekort TSPs, genoemd. TSPs zijn van belang voor de diagnostiek van kanker. Wanneer een van kanker verdacht stukje weefsel, dat uit een patiënt is weggenomen, cellen blijkt te bevatten die een TSP in zich dragen, is de diagnose kanker gerechtvaardigd. Onderzoek naar methoden om TSPs aan te tonen, is dus uiterst belangrijk voor de diagnostiek van kanker.

Ons eigen onderzoek is de afgelopen jaren gericht geweest op het aantonen van TSPs die voorkomen bij 2 soorten van bloedkanker, chronische myeloïde leukemie (CML) en acute lymfatische leukemie (ALL). Eén van deze TSPs was ongeveer tien jaar geleden het eerste tumor-specifieke eiwit dat werd beschreven in de wetenschappelijke literatuur. Sindsdien zijn er nog vele andere TSPs ontdekt. Om inzicht te krijgen in de feiten die op dit moment bekend zijn over TSPs, hebben wij literatuuronderzoek gedaan. Daarbij hadden we de volgende vragen voor ogen:

1. Hoe ontstaan TSPs?
2. Bij welke vormen van kanker komen TSPs voor? Hoe frequent komen ze voor?
3. Zien TSPs in één vorm van kanker er altijd hetzelfde uit of verschilt de structuur van de TSP van tumor tot tumor en van patiënt tot patiënt? Met andere woorden, hoe bruikbaar zijn TSPs bij de diagnose van kanker?
4. Dragen TSPs bij aan het ontstaan van kanker, en zo ja, op welke manier?

In de hoofdstukken 1 en 2 van het Engelstalige gedeelte van dit proefschrift staan de resultaten van dit literatuuronderzoek uitgebreid beschreven. Hieronder volgt een korte samenvatting.

1.2.1 Hoe ontstaan TSPs?

TSPs kunnen in twee groepen worden ingedeeld, naar de manier waarop ze ontstaan. De ene groep bestaat uit zgn. 'fusiepunt-TSPs', die ontstaan door een fusie van twee eiwitten. Zo'n fusie kan worden veroorzaakt door een zgn. chromosomale translocatie die vaak in kankercellen voorkomt. Bij een chromosomale translocatie zijn twee verschillende chromosomen betrokken, die beide op een bepaald punt breken. Een voorbeeld van een chromosomale translocatie staat in Figuur 1 schematisch weergegeven. Dit is de translocatie tussen chromosoom 9 en chromosoom 22. Een stukje van chromosoom 9 verhuist (transloceert) naar chromosoom 22 en vice versa. Als gevolg van de translocatie ontstaan 2 abnormale chromosomen. Als beide chromosomen breken op de plaats van een gen, breken ook de twee betrokken genen. Bij de (9;22) translocatie zijn dat het zgn. *abl* gen op chromosoom 9 en het *bcr* gen op chromosoom 22. De afgebroken stukjes van elk gen verhuizen met de chromosomen mee en worden aan het achtergebleven stukje van het andere gen geplakt. Het gevolg is dat er zgn. fusie-genen worden gevormd die een voor- en achterkant hebben afkomstig van verschillende genen.

Op chromosoom 9 ontstaat het *abl-bcr* gen en op chromosoom 22 het *bcr-abl* gen. Zoals al in paragraaf 1.1 is vermeld, coderen genen voor eiwitten. De fusie-genen in kwestie coderen dus voor eiwitten, die een BCR-kop en een ABL-staart hebben of vice versa. Een dergelijk eiwit wordt een chimeer of hybride eiwit genoemd.

Chimere eiwitten komen alleen voor in kankercellen en niet in normale cellen. Het zijn daarom tumor-specifieke eiwitten. Ze bestaan echter uit stukjes van de

Figure 1 De 'Philadelphia translocatie'; translocatie tussen chromosoom 9 en 22. Een stukje van chromosoom 9 verhuist naar chromosoom 22 en een stukje van chromosoom 22 verhuist naar chromosoom 9. Hierbij ontstaan een abnormaal chromosoom 9 en een abnormaal chromosoom 22. Het abnormale chromosoom 22 wordt het Philadelphia chromosoom genoemd.

'gewone' eiwitten, die niet tumor-specifiek zijn. Het enige tumor-specifieke stukje in een dergelijk eiwit is het 'plak'-of fusiepunt van de beide betrokken eiwitten. Vandaar dat deze TSPs 'fusiepunt-TSPs' worden genoemd.

De andere groep TSPs wordt gevormd door eiwitten die worden gecodeerd door genen die als geheel intact gebleven zijn, maar op één punt veranderd zijn. Het resulterende eiwit is dus ook maar op één punt veranderd. Alleen dit punt is het tumor-specifieke stukje binnen het eiwit, omdat de rest van het eiwit hetzelfde is gebleven.

1.2.2 Bij welke vormen van kanker komen TSPs voor?

In het tweede hoofdstuk van het Engelstalige gedeelte van dit proefschrift staan alle tumoren, waarbij TSPs voorkomen, beschreven. Opvallend is dat er in de literatuur veel beschrijvingen zijn van TSPs die voorkomen bij leukemie. Er zijn nog maar weinig TSPs geïdentificeerd die voorkomen bij 'solide tumoren' (knobbels). Een mogelijke reden hiervan kan zijn dat er van oudsher veel onderzoek is gedaan op het gebied van leukemie, omdat het tumor materiaal (bloed) uiterst gemakkelijk te isoleren is uit de patiënt. Omdat de methoden waarmee TSPs ontdekt kunnen worden steeds verbeteren, zullen er in de toekomst waarschijnlijk nog meer TSPs worden geïdentificeerd, ook in solide tumoren.

Hoe frequent komen TSPs voor? Het voorkomen van TSPs bij de respectievelijke kankers is zeer variërend. Er zijn vormen van kanker waarbij TSPs sporadisch voorkomen. Zo komt bij minder dan 1% van alle patiënten met acute myeloïde leukemie (AML) een tumor-specifieke chromosomale translocatie voor tussen chromosoom 6 en chromosoom 9. Het fusiepunt-TSP dat ten gevolge van deze translocatie ontstaat, zal dus ook maar bij 0,01% van alle AML patiënten kunnen worden aangetoond. Er zijn echter ook vormen van kanker waarbij in bijna alle tumoren het TSP voorkomt. Bij chronische myeloïde leukemie (CML) heeft meer dan 90% van alle patiënten een chromosomale translocatie tussen de chromosomen 9 en 22 in de kankercellen. Het fusiepunt-TSP zal hier dus waarschijnlijk bij bijna alle patiënten kunnen worden aangetoond. In dit verband is het belangrijk om te vermelden dat grootschalige studies op het gebied van tumor-specifieke eiwitten nog niet zijn uitgevoerd. Uitspraken over het voorkomen van TSPs zijn in het algemeen gebaseerd op het voorkomen van chromosomale veranderingen of veranderingen in het DNA.

1.2.3 Zien de TSPs in één vorm van kanker er altijd hetzelfde uit?

TSPs zijn bruikbaar bij diagnostiek van kanker als ze er in één vorm van kanker altijd hetzelfde uitzien. Dergelijke TSPs worden homogene TSPs genoemd. Er zijn echter ook TSPs, waarvan de structuur verschilt van tumor tot tumor en van patiënt tot patiënt. Dit zijn heterogene TSPs.

Uiterst homogene TSPs zijn b.v. de 'BCR-ABL fusiepunt-TSPs' die voorkomen bij de al eerder genoemde (9;22) translocatie in chronische myeloïde leukemie. Er ontstaan hierdoor slechts drie verschillende typen BCR-ABL eiwitten. Het meest heterogene type TSP is het zogenaamde 'P53 eiwit'. De plaats én de manier van verandering variëren hier nagenoeg van tumor tot tumor.

1.2.4 Dragen TSPs bij aan het ontstaan van kanker?

Of en hoe alle TSPs bijdragen aan het ontstaan van kanker is nog niet bekend. Er bestaat een theorie die stelt dat kanker niet ontstaat door één verandering in de cel (b.v. een TSP), maar door een opeenstapeling van veranderingen. Vóór deze theorie pleit het feit dat de kans om kanker te krijgen groter wordt naarmate men ouder wordt. De cellen worden ouder en de kans dat er fouten in voorkomen ook.

De bevinding dat er identieke tumoren bestaan mét en zonder TSP pleit ervoor, dat TSPs niet per se noodzakelijk zijn om een tumor te veroorzaken. Er zijn echter ook studies die aantonen dat de aanwezigheid van één TSP in de cel wel degelijk kanker kan veroorzaken. Dergelijke proeven zijn uitgevoerd met muizen. Nadat het *bcr-abl* gen via genetische manipulatie was ingebracht in de muizen, kregen deze leukemie. Hiermee is dus wél een direct verband aangetoond tussen het TSP en het ontstaan van de leukemie.

Op grond van hun vermoedelijke werking zijn TSPs in 2 groepen in te delen. De ene groep bestaat uit abnormale 'transcriptiefactoren'. Deze factoren controleren het decoderingsproces van genen naar eiwitten. Wanneer transcriptiefactoren abnormaal gaan werken, wordt het decoderingsproces verstoord. Hierdoor komen weer andere eiwitten abnormaal tot expressie. Waarschijnlijk heeft dit tot gevolg dat de stofwisseling van de cel wordt verstoord en er ongecontroleerde groei of differentiatie (kanker) optreedt.

De andere groep van TSPs bestaat uit eiwitten die betrokken zijn bij het doorgeven van signalen van de ene cel naar de andere cel. Normale cellen zijn in staat om met elkaar te communiceren door signalen uit te zenden en te ontvangen. Op deze manier ontstaat een evenwicht in groei en differentiatie van cellen. Wanneer eiwitten die bij de communicatie betrokken zijn door een fout van samenstelling veranderen, raakt ook de communicatie tussen de cellen verstoord. Het gevolg kan zijn dat cellen geïsoleerd en ongecontroleerd gaan groeien en kanker ontstaat.

Eigen onderzoek naar tumor-specifieke BCR-ABL eiwitten

2.1 Inleiding

Ons eigen werk heeft zich de afgelopen jaren gericht op TSPs, die voorkomen bij chronische myeloïde leukemie (CML) en acute lymfatische leukemie (ALL).

Chronische myeloïde leukemie *(CML) is een kanker van witte bloedcellen. De patiënten hebben abnormaal veel witte bloedcellen in hun bloed en vaak een vergrote lever en milt. De gemiddelde leeftijd waarbij deze ziekte optreedt ligt tussen de 50 en 60 jaar. CML verloopt in twee fasen. De ziekte start met een chronische fase, die ongeveer 1 tot 4 jaar kan duren. In deze fase is de ziekte onder controle te houden met geneesmiddelen, zoals cytostatica. Ook krijgen chronische fase-patiënten beenmergtransplantaties. De tweede fase wordt blast crisis genoemd. Er komen dan cellen in het bloed voor die er helemaal niet in thuis horen. Gewoonlijk reageren patiënten in deze fase slecht op therapie en sterven zij binnen 3 - 6 maanden. Slechts 50-60% van alle behandelde patiënten heeft een 5-jaars overleving.*

Acute lymfatische leukemie *(ALL) wordt gekenmerkt door een groot aantal cellen in het bloed, die normaliter in het beenmerg thuis horen. Deze cellen verdringen de normale bloedcellen. Als gevolg hiervan lijden ALL patiënten, door een gebrek aan rode bloedcellen, aan bloedarmoede. Zij kunnen ook problemen krijgen met de bloedstolling doordat ze te weinig bloedplaatjes hebben. Verder zijn ze uiterst gevoelig voor infecties omdat de witte bloedcellen, die bij de afweer betrokken zijn, verdrongen zijn uit het bloed. Door deze oorzaken kunnen ALL patiënten binnen enkele weken tot maanden na het begin van de ziekte overlijden. De therapie voor deze patiënten is chemotherapie of beenmergtransplantatie. ALL komt op alle leeftijden voor. Bij kinderen van twee tot drie jaar is het de meest voorkomende vorm van kanker. Van alle kinderen met ALL die met chemotherapie worden behandeld, blijft ongeveer 75% langdurig ziekte-vrij. Bij volwassenen met ALL ligt dit percentage op 35%.*

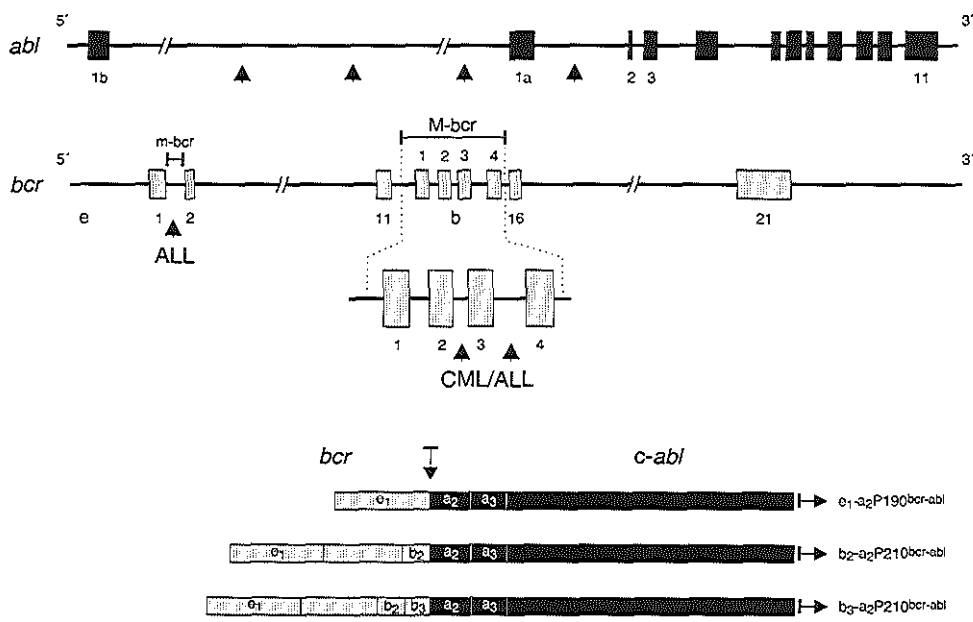
De TSPs die wij bestudeerd hebben zijn de BCR-ABL fusiepunt-TSPs die zijn ontstaan door de translocatie tussen chromosoom 9 en 22. Dit is de zgn. Philadelphia (Ph) translocatie, genoemd naar de plaats van ontdekking. In figuur 1 staat de Ph translocatie schematisch weergegeven.

Het Ph chromosoom, het abnormale chromosoom 22, komt voor bij 90% van alle CML patiënten, bij ongeveer een derde van alle volwassen ALL patiënten en bij 2-3% van alle kinderen met ALL. Het Ph chromosoom komt niet voor bij gezonde personen. Het is dus een tumor-specifiek chromosoom. Met speciale technieken kan dit chromosoom door cytogenetici aangetoond worden. Deze test is echter niet zo gevoelig en is (bij ALL) niet altijd succesvol. Daarom hebben wij nu onderzoek gedaan naar methoden om de tumor-specifieke eiwitten, die gecodeerd worden door genen op het Ph chromosoom, aan te tonen.

2.1.1 Hoe zien BCR-ABL eiwitten er uit?

Zoals al vermeld in het vorige hoofdstuk zijn bij de Ph translocatie twee genen betrokken, het *abl* gen op chromosoom 9 en het *bcr* gen op chromosoom 22. Als gevolg van de translocatie ontstaat er op chromosoom 9 een *abl-bcr* gen en op chromosoom 22 een *bcr-abl* gen. Wij hebben ons onderzoek gericht op het eiwit dat gecodeerd wordt door het *bcr-abl* gen. Het normale *bcr* gen kan op drie plaatsen breken. Als gevolg hiervan kunnen er drie verschillende *bcr-abl* fusie-genen ontstaan en drie verschillende BCR-ABL eiwitten. Deze worden resp. e1-a2 $P190^{bcr\text{-}abl}$, b2-a2 $P210^{bcr\text{-}abl}$ en b3-a2 $P210^{bcr\text{-}abl}$ genoemd. Het e1-a2 $P190^{bcr\text{-}abl}$ komt bijna uitsluitend bij ALL voor, terwijl de beide $P210^{bcr\text{-}abl}$ eiwitten zowel bij CML als ALL voorkomen.

De tumor-specifieke BCR-ABL eiwitten bestaan uit grote, niet tumor-specifieke gedeelten, die overeenkomen met de normale BCR en ABL eiwitten. Dit is schematisch weergegeven in Figuur 2. De enige tumor-specifieke gedeelten in



Figuur 2 Schematische weergave van het *bcr* en het *abl* gen. Met de pijlen zijn de breukpunten op de respectievelijke genen aangegeven. Het *abl* gen breekt altijd voor 'a2'. Het *bcr* gen kan op verschillende plaatsen breken. De chimere eiwitten die ontstaan ten gevolge van de translocatie zijn in deze figuur ook schematisch weergegeven. Afhankelijk van de plaats van de breuk binnen het *bcr* gen kunnen 3 verschillende eiwitten gevormd worden: e1-a2 $P190^{bcr\text{-}abl}$, b2-a2 $P210^{bcr\text{-}abl}$ of b3-a2 $P210^{bcr\text{-}abl}$. Het enige tumor-specifieke gedeelte op deze eiwitten wordt gevormd door het fusiepunt tussen het BCR en het ABL deel.

deze eiwitten zijn de fusiepunten tussen het BCR en het ABL gedeelte. Dus als een dergelijk fusiepunt in een cel van een patiënt kan worden aangetoond, is het zeker dat het een tumorcel betreft.

2.2 Doel en resultaten van ons onderzoek

Het doel van ons onderzoek was het aantonen van tumor-specifieke BCR-ABL fusiepunten in CML en ALL cellen, Om dit doel te bereiken wilden wij reagentia maken, die alleen maar een reactie aangaan met de BCR-ABL fusiepunten en niet met stukjes van andere eiwitten. De reagentia in kwestie zijn 'antistoffen' of 'immunoglobulinen'.

Antistoffen *Na een vaccinatie, b.v. met tetanus vaccin, ontwikkelt het lichaam specifieke antistoffen tegen tetanus. Deze tetanus-specifieke antistoffen beschermen tegen een infectie met de tetanus bacterie. Antistoffen zitten in het bloed. De produktie van antistoffen gebeurt door speciale cellen in het bloed, de zgn. B-lymfocyten. Ook muizen en konijnen hebben B-lymfocyten. Als deze (proef)dieren gevaccineerd worden met een eiwit, zullen hun B-cellen specifieke antistoffen tegen dit eiwit gaan maken. Deze antistoffen kunnen worden geïsoleerd uit het bloed van de dieren. Dit worden polyklonale antistoffen genoemd. De oplossing die de antistoffen bevat wordt antiserum genoemd. Het is ook mogelijk om uit een muis de antistof producerende B-lymfocyten te isoleren. M.b.v. een speciale techniek, de zogenaamde hybridoma techniek, is het vervolgens mogelijk om die B-lymfocyten in het laboratorium verder te kweken. Deze B-lymfocyten produceren eiwit-specifieke, 'monoklonale' antistoffen.*

Met de produktie van antistoffen tegen tumor-specifieke BCR-ABL eiwitten zijn wij gedurende de hele onderzoeksperiode bezig geweest. Wij hebben muizen en konijnen geïmmuniseerd (=gevaccineerd) met kleine stukjes eiwit, te weten synthetische peptiden, die overeenkomen met de BCR-ABL fusiepunten. Daarna hebben we uit het bloed van de konijnen polyklonale antistoffen geïsoleerd. Uit de muizen hebben we de B-lymfocyten gehaald voor de produktie van monoklonale antistoffen. We hebben vervolgens gekeken of de polyklonale en monoklonale antistoffen specifiek reageerden met de peptiden waartegen ze waren opgewekt. Over het algemeen was dit het geval.

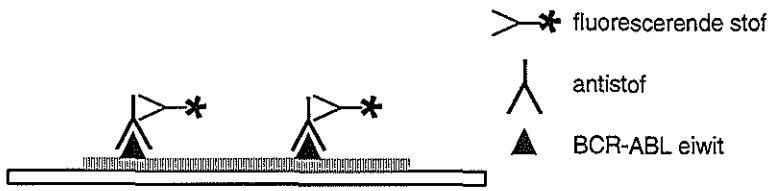
De volgende vraag was of de antistoffen ook zouden reageren met de volledige, intacte, tumor-specifieke BCR-ABL eiwitten, zoals die in de cellen van patiënten voorkomen. Dit bleek een groot probleem. We moesten hiervoor eerst een goede test ontwikkelen. Hiermee zijn we ruim een jaar bezig geweest. Bij deze test, de zogenaamde 'tyrosine kinase assay', worden de BCR-ABL eiwitten geïsoleerd uit cellijnen afkomstig van CML en ALL patiënten. Wanneer de antistoffen de BCR-ABL eiwitten herkennen, is aan het eind van de test een zwart bandje op een Röntgen film te zien.

Toen de test werkte, konden we de reactie van de antistoffen testen met de drie bovengenoemde tumor-specifieke BCR-ABL eiwitten. Het resultaat van één van die testen is weergegeven in Figuur 3. Dit is een foto van een Röntgen film.

Figuur 3 Resultaat van een tyrosine kinase assay met antiserum BP-ALL. De reactie van antiserum BP-ALL met 3 verschillende cellijnen is getest. TOM-1 cellen bevatten het e1-a2 $P190^{bcr\text{-}abl}$, BV173 cellen bevatten b2-a2 $P210^{bcr\text{-}abl}$ en K562 cellen bevatten b3-a2 $P210^{bcr\text{-}abl}$.

Op deze foto staat het reactiepatroon van het antiserum 'BP-ALL'. Dit antiserum is geïsoleerd uit een konijn dat was geïmmuniseerd met het BCR-ABL fusiepunt dat voorkomt in e1-a2 $P190^{bcr\text{-}abl}$. TOM-1, K562 en BV173 zijn namen van cellijnen afkomstig van CML en ALL patiënten. TOM-1 cellen bevatten het e1-a2 $P190^{bcr\text{-}abl}$, terwijl K562 en BV173 cellen respectievelijk b3-a2 $P210^{bcr\text{-}abl}$ en b2-a2 $P210^{bcr\text{-}abl}$ eiwitten bevatten. Op de foto is alleen een zwart bandje te zien in de 'TOM-1 laan' en niet in de lanen van K562 en BV173. Dit betekent dat het antiserum alleen reageert met het $P190^{bcr\text{-}abl}$ eiwit en niet met de $P210^{bcr\text{-}abl}$ eiwitten uit K562 en BV173. Dit antiserum herkent dus specifiek het e1-a2 $P190^{bcr\text{-}abl}$ eiwit. Op een vergelijkbare manier hebben we ook antisera getest die wel met $P210^{bcr\text{-}abl}$ eiwitten reageren en niet met $P190^{bcr\text{-}abl}$. Uiteindelijk hebben we drie typen antisera verkregen: BP-1, dat alleen reageert met b2-a2 $P210^{bcr\text{-}abl}$; BP-2, dat alleen reageert met b3-a2 $P210^{bcr\text{-}abl}$ en het hierboven beschreven BP-ALL.

Uit verder onderzoek bleek dat we de antistoffen ook konden gebruiken in testen met 'verse' cellen afkomstig van CML en ALL patiënten. De tyrosine kinase assay is echter té bewerkelijk om ook routinematig cellen te testen. Daarom wilden wij een eenvoudiger en snellere test ontwikkelen waarmee we patiëntencellen konden onderzoeken op aanwezigheid van tumor-specifieke BCR-ABL eiwitten. Wij kozen voor een test gebaseerd op de 'immunofluorescentie techniek'. In Figuur 4 staat die techniek schematisch weergegeven.



Figuur 4 Schematische weergave van de 'immunofluorescentie' techniek.Een cel met het tumor-specifieke BCR-ABL eiwit wordt op een glazen plaatje geplakt. Vervolgens worden er antistoffen op het glaasje gedruppeld. Deze antistoffen krijgen daarna de tijd om te reageren met en te binden aan de BCR-ABL eiwitten. Daarna worden de glaasjes gewassen. Uiteindelijk wordt er een fluorescerend stofje opgebracht dat reageert met de gebonden antistoffen. Dus daar waar de BCR-ABL eiwitten zich bevinden zit nu ook het fluorescerende stofje. Met behulp van een speciale microscoop kan dit waargenomen worden.

Wij verwachtten dat onze antistoffen in de immunofluorescentie alleen zouden reageren met de cellijnen waarmee ze ook in de tyrosine kinase assay hadden gereageerd. Helaas reageerden alle antistoffen positief met alle cellijnen. We hebben heel veel variaties aangebracht in de proeven om toch het gewenste reactiepatroon te verkrijgen, maar dat is helaas niet gelukt. Waarschijnlijk reageerden de antistoffen niet alleen met de tumor-specifieke BCR-ABL eiwitten, maar ook nog met andere niet-tumor-specifieke eiwitten. Het komt vaker voor dat antistoffen in de ene test wel specifiek reageren en in de andere test niet.

Uiteindelijk hebben we één *monoklonaal* antilichaam gemaakt, dat wel m.b.v. de immunofluorescentietechniek alleen het e1-a2 $P190^{bcr\text{-}abl}$ en niet de beide typen $P210^{bcr\text{-}abl}$ eiwit herkent. Dit antilichaam, ER-FP1, reageert echter alleen met cellen die we kunstmatig hebben voorzien van grote hoeveelheden e1-a2 $P190^{bcr\text{-}abl}$. Met CML en ALL cellen, die maar kleine hoeveelheden van het eiwit bevatten, reageert ER-FP1 niet specifiek.

2.3 Wat heeft het onderzoek opgeleverd?

De resultaten van ons onderzoek kunnen in het kort als volgt worden samengevat. Wij hebben drie typen antistoffen gemaakt, die elk heel specifiek één type BCR-ABL eiwit herkennen. Met het feit dat we er antistoffen tegen hebben kunnen maken, tonen we aan dat de BCR-ABL fusiepunten zich op een voor antistoffen herkenbare manier in de BCR-ABL eiwitten bevinden. We hebben ook laten zien dat wij met behulp van de antistoffen cellen van leukemie patiënten kunnen testen op aanwezigheid van BCR-ABL eiwitten.

'Verklarende woordenlijst'

abl	Abelson gen
ABL	Abelson eiwit
ALL	acute lymfatische leukemie
AML	acute myeloïde leukemie
bcr	breakpoint cluster region gen
BCR	breakpoint cluster region eiwit
BP-1	antiserum dat met b2-a2 $P210^{bcr-abl}$ reageert
BP-2	antiserum dat met b3-a2 $P210^{bcr-abl}$ reageert
BP-ALL	antiserum dat met e1-a2 $P210^{bcr-abl}$ reageert
BV173	cellijn afkomstig van een CML patiënt, bevat b2-a2 $P210^{bcr-abl}$
cellijn	groep identieke cellen die generaties lang in het laboratorium gekweekt kan worden
chimeer eiwit	eiwit dat uit stukjes van twee verschillende eiwitten bestaat
chromosoom	structuur in de kern van een cel, bevat het DNA
CML	chronische myeloïde leukemie
DNA	deoxyribonucleic acid (genetische code, erfelijk materiaal)
ER-FP1	monoklonale antistof die met e1-a2 $P190^{bcr-abl}$ reageert
gen	afgebakend stukje DNA; bevat de code voor een eiwit
K562	cellijn afkomstig van een CML patiënt, bevat b3-a2 $P210^{bcr-abl}$
leukemie	bloedkanker
$P190^{bcr-abl}$	tumor-specifiek BCR-ABL eiwit met een molecuulgewicht van 190 kilo Dalton
$P210^{bcr-abl}$	tumor-specifiek BCR-ABL eiwit met een molecuulgewicht van 210 kilo Dalton
Ph	Philadelphia chromosoom, abnormaal chromosoom 22
synthetisch peptide	klein stukje eiwit dat in het laboratorium is gemaakt
TOM-1	cellijn afkomstig van een ALL patiënt, bevat e1-a2 $P190^{bcr-abl}$
transcriptiefactor	eiwit dat het decoderingsproces van DNA naar eiwit regelt
translocatie	verhuizing van een stukje van het ene chromosoom naar het andere
TSP	tumor-specifiek proteïne of tumor-specifiek eiwit

Curriculum vitae

naam : Adriana Cornelia van Denderen (Janneke)

22 april 1955 : geboren te 's-Gravenhage

juni 1973 : eindexamen gymnasium β; Coornhert Gymnasium, Gouda

juni 1977 : examen hoger beroepsonderwijs (HBO-B), medisch biologisch analist; van 't Hoff Instituut, Rotterdam

sept. 1977 : aanvang studie biologie; Vrije Universiteit, Amsterdam

juni 1980 : kandidaats examen biologie, medisch biologische richting (B5')

aug. 1983 : doctoraal examen biologie; Vrije Universiteit, Amsterdam
hoofdvak: Immunologie, dr. L.A. Aarden; Centraal Laboratorium voor de Bloedtransfusiedienst, Amsterdam
bijvakken: Antropogenetica, dr. F. Arwert; Vrije Universiteit, Amsterdam
Virologie, dr. J. Buitenwerf; Universiteit van Amsterdam

aug. 1983 - juni 1985 : wetenschappelijk onderzoek o.l.v. dr. L.A. Aarden op de afdeling Autoimmuunziekten van het Centraal Laboratorium voor de Bloedtransfusiedienst, Amsterdam
project: Autoantistoffen tegen de TSH receptor op schildklierweefsel

juni 1985 - jan. 1994 : promotie onderzoek o.l.v. Prof. dr. W. van Ewijk op de afdeling Celbiologie en Genetica en de afdeling Immunologie van de Erasmus Universiteit, Rotterdam
project: Monoklonale antistoffen voor serotherapie van patienten met B-cel maligniteiten
project: Monoklonale antistoffen gericht tegen breukpunt epitopen bij leukemie

Puzzel

Het is vaak een gepuzzel om de goede mensen te vinden om mee samen te werken. Sommige mensen vallen je direct op, naar anderen moet je goed zoeken.

Q	S	D	V	A	X	A	H	U	D	M	C	Z	K
Y	K	B	M	A	R	E	L	L	A	D	K	I	P
O	T	E	G	A	N	N	E	M	S	I	E	V	B
O	P	T	R	E	R	H	A	C	L	A	U	S	A
E	D	A	E	Y	O	G	K	O	E	G	K	L	H
B	O	B	U	N	S	R	R	J	A	N	E	W	J
K	A	L	N	L	W	W	G	E	L	O	N	E	A
S	W	O	U	W	I	M	Y	E	E	S	C	L	G
F	G	A	J	L	H	E	N	K	P	T	P	L	D
U	P	C	L	S	P	E	N	M	I	I	R	E	N
G	A	E	E	E	M	A	R	L	E	E	N	I	L
N	M	V	T	E	N	O	A	T	T	K	B	N	S
B	G	A	J	E	S	T	T	E	E	O	S	A	K
F	G	E	R	A	R	D	I	O	R	I	O	D	I
J	S	E	E	J	C	P	C	N	B	A	R	N	W
F	H	K	N	R	O	Q	A	A	A	I	I	V	O
W	M	J	B	E	T	U	U	U	R	L	A	N	D
L	F	O	T	E	T	J	N	E	L	Y	E	S	C
T	G	O	D	F	R	I	E	D	S	M	W	V	M
K	S	P	T	L	K	T	C	K	R	F	G	J	R
D	O	R	I	E	N	C	H	A	I	U	Y	H	R
T	W	T	Y	U	L	V	C	H	J	G	E	O	N

Ik heb ze gevonden. Bedankt allemaal! Janneke.