RECOGNITION OF TUMOR-SPECIFIC PROTEINS IN HUMAN CANCER

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RECOGNITION OF TUMOR-SPECIFIC PROTEINS IN HUMAN CANCER

Herkenning van tumor-specifieke eiwitten bij kanker

PROEFSCHRIFT

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Dit proefschrift is tot stand gekomen binnen de afdeling Immunologie van de Erasmus Universiteit Rotterdam.

Omslag: fluorescentieopnamen van twee getransfecteerde COS cellen. De COS cel aan de bovenkant werd getransfecteerd met cDNA coderend voor E2A-PBX1 eiwitten, de onderste COS cel werd getransfecteerd met cDNA coderend voor $e_1a_2P190^{BCR\text{-}ABL}$ eiwitten. De COS cellen werden aangekleurd met respectievelijk ER-GO4 of ER-FP1 antilichamen (voor een gedetailleerde beschrijving, zie hoofstukken 6 en 7).
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This is what we know about leukemia: during normal blood cell proliferation the cells differentiate into specific types. In a pathological situation the differentiation into specific cells is blocked. This disturbance of normal differentiation - so called leukemia - is a disease *sui generis*. We know the sequela of this disease, but we don't know its origin.

Virchow 1849

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Chapter 1

General Introduction

General Introduction

Cancer:

At present, approximately 20% of people living in the developed countries of the world, will die of cancer.

Cancer is a disease of all ages and even though cancer is not a common disease in younger people, cancer is recognized as one of the most important causes of death at any age. In fact, when considering the main death causes in people younger than thirty years, cancer is second only to accidents. Beyond the age of thirty years, the number of deaths from cancer increases upon aging, gradually at first, rising sharply later on. Apart from cancer causing death, the disease is feared because patients who suffer from cancer are often condemned to a long and painful terminal illness.

Based on their frequency of occurrence, cancers are traditionally categorized as either epithelial cancers (approximately 85% of all human cancers) or non-epithelial cancers (approximately 15% of all human cancers). Cancers arising from epithelial cells (i.e. cells lining the body cavities and skin) are called carcinomas (Latin: karkinos - crab or lobster; oma - swelling), while those arising from non-epithelial cells are further subdivided according to the tissue and cell type from which they originate. Thus, sarcomas are derived from connective tissue or muscle cells (Greek: sarx - meat), while leukemias are derived from hematopoietic cells (Greek: leucos - white; haima - blood). Other non-epithelial cancers include the ones derived from cells of the nervous system and germinal or embryonal cells.

Cancer is often traced to a primary site in the human body where cancer cells have started to grow initially. Since most individual cancer cells in a primary tumor frequently display very similar morphological characteristics, cancer is thought to originate from one single, autonomously growing cell that withdrew from environmental growth regulating signals (i.e. clonal growth). The transition from a healthy cell to a malignant cancerous cell is caused by a 'multi-step' process, that often starts with a certain change in the cell's genome (i.e. the entire genetic information needed for a human body to survive). While a single genomic change is usually not enough to cause cancer, the genomic change may be irreversible and consequently transmitted to a cell's progeny. From this point on, cells encounter other genomic changes, of which a certain combination eventually causes a cell to proliferate autonomously.

While autonomous proliferation is one characteristic of cancer cells, it is not the sole reason that cancer is life-threatening. In fact, certain autonomously growing tumors, the so-called 'benign tumors', are often relatively harmless as they remain localized to a particular place in the human body. In contrast, malignant tumors, known collectively as cancer, are feared, as their malignant cells invade surrounding tissues, burrow through local blood and lymphatic vessels and spread to other parts of the body where they give rise to secondary tumors, or metastases.

If left untreated, a patient with cancer eventually dies when vital tissues or organs are involved (e.g. lung, liver or brain). Although striking reductions in death from cancers have been achieved over the past few years, approximately 50% of patients with cancer still die. Depending on the origin and spread of a cancer, therapeutic outcomes differ enormously. While most patients with skin cancer can be cured completely, almost all patients suffering from anaplastic thyroid carcinoma, a fortunately rare type of cancer, die within five years from diagnosis.
The term 'cancer' comprises a heterogeneous group, in which each type has its own characteristics when considering its malignant potential and its response to therapy. It is because of this variability that a simple, universal treatment, effective for all cancers, is illusive. The kind of therapy chosen depends on characteristics of the cancer type and is usually based on three modalities:

1. Surgical treatment is, despite recent progress in other fields of cancer treatment, still the primary choice in cancer treatment for most solid tumors. Most people with cancer are cured through the surgical removal of a tumor. Yet, an important limitation of surgery is that it is often difficult to remove the microscopic invasions of malignant cells in surrounding tissues.
2. Radiation therapy is preferred over surgery in many instances. Cancer cells are often more vulnerable to radiation than normal cells. The genetic damage, inflicted through radiation, causes cancer cells to die, including the ones that have microscopically spread. Yet, like surgery, radiation therapy is not enough to cure a patient with cancer that has already metastasized throughout the body.
3. Chemotherapy provides a means to destroy metastasized tumors. Tumors, that cannot be removed by using surgery or radiotherapy (e.g. leukemias) are treated with chemotherapeutic drugs as well. It has been verified that, when different chemotherapeutic drugs are combined into one therapeutic protocol, they are often more effective in destroying cancer cells, although not all cancers respond equally well. Yet, chemotherapeutic drugs tend to destroy all proliferating cells: cancer cells, but unfortunately also normal healthy dividing cells.

The effectiveness of current treatment protocols is empirically determined. It has been demonstrated that, depending on the origin and spread of the cancer and the physical condition of a patient, the three basic cancer treatments prove more effective when they are joined in one protocol. Although current treatment protocols have improved the chance of survival from most types of cancer, they are in a way still dissatisfactory. It is realized that current cancer treatment often gives rise to side-effects that compromise the benefits of treatment enormously.
Despite recent progress in our understanding of the developmental issues of cancer, tailor made therapies, that allow the specific eradication of certain cancer cells without concurrently affecting healthy cells, are still not available. One of the major limitations in reaching this goal is basically due to inaccurate diagnosis. If one cannot identify a cancer correctly and distinguish it from other cancer types, one cannot dis-

cover its causes, predict its outcome, select (or develop) the most appropriate treatment for a given patient, and make trials on a population of patients to judge whether a proposed treatment is effective.

Scope of the thesis

Cancer is a heterogeneous disease regarding both its biology as well as its prognosis. Years of cancer research have paved the way for understanding the essential processes involved in the malignant change of previously healthy, normal cells (i.e. carcinogenesis). It was only recently that researchers clearly demonstrated that most cancers have a genetic basis. This new insight was paralleled by the development of new (diagnostic) techniques. These techniques are not only beneficial for a correct tumor-diagnosis but they may also contribute to the development of new therapeutic protocols.

This thesis is designed as a guide to understanding our experimental work that aimed at the development of a new kind of tumor-specific, immunological diagnosis.
Chapter 2 covers the theoretical basis of cancer, starting with the description of the normal cellular processes involved in cell growth and division, concluding with the chromosomal, the genetic and the protein basis of cancer in general.
Chapter 3 represents an overview of the genetic basis of leukemia. When considering the current status of our understanding of the processes involved in carcinogenesis, it can be stated that at present, the greatest achievements have been realized in leukemia. Recent discoveries indicate that leukemia is a heterogeneous disease. Several examples of its heterogeneity and their implications considering diagnosis, treatment and prognosis of the leukemic disease are discussed. Special emphasis will be placed on two of the most important aberrant genes involved in acute lymphoblastic leukemia: *BCR-ABL* and *E2A-PBX1*.
Chapters 4, 5, 6, 7 and 8 describe our experimental work. The presence of consistent chromosomal and molecular aberrations in leukemic cells provides the opportunity to screen patients at diagnosis for the presence of these abnormalities. Our experimental work reports on the *immunologic* identification of two of these abnormalities: the tumor-specific BCR-ABL and E2A-PBX1 proteins. We focus on both the generation as well as the application of both polyclonal and monoclonal antibodies directed against both types of tumor-specific proteins.
In the general discussion in *Chapter 9* we discuss several aspects that concern the immunologic recognition of cancer cells. When considering an antibody based tumor diagnosis, it is essential to understand the background of the reagents used in diagnostic, immunological assays. We focus on antibodies specifically recognizing cancer cells and we present new ideas on how to develop new immunological reagents that are useful for tumor diagnostic purposes.

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Chapter 2

The theoretical basis of cancer

The theoretical basis of cancer

Fundamental to the origin of cancer is loss of responsiveness to normal growth controls. Cancer cells divide continuously, apparently unaware of the various regulatory mechanisms that control normal cell growth. To place the abnormal neoplastic cellular proliferations in perspective of the normal, non-neoplastic controlled proliferations are considered first.

Normal cell proliferation

Cell proliferation is an ongoing process in the healthy human body. Cells are initiated to grow and divide primarily to compensate for ongoing cell loss (e.g. skin, gut and blood). To maintain the steady state of the adult human body (i.e. homeostasis), overgrowth must be prevented. Therefore, cell proliferation is, on its turn, also down-regulated (1).
The complex processes of cell proliferation are controlled by ingenious networks of growth regulatory pathways (2, 3). Growth control is realized by regulatory processes known as normal growth regulation and normal cell cycle regulation. Although both processes are intrinsically related, they are discussed separately below.

Normal growth regulation

Growth regulation is controlled by several molecules associated with growth stimulatory and growth inhibitory relay systems. These relay systems are activated by incoming growth regulating signals, either produced by cells located in close proximity to the target cell (i.e. cytokines) or by cells located at distant sites from the target cell (i.e. hormones). Unless growth inhibitory signals have been provided concurrently, growth stimulatory signals trigger a cell to prepare itself for cell division.
It has been demonstrated that most growth stimulatory molecules activate a cell to differentiate (i.e. the transition from a primitive to a more sophisticated status). Although in some cases the fully differentiated cell simply divides to produce daughter cells of the same type (e.g. endothelial cells and hepatocytes), most cells, especially those with a rapid turnover (e.g. epithelial cells and blood cells), develop through differentiating from primitive dividing 'stem' cells (4). The more differentiated a cell becomes, the less likely it is for such a cell to proliferate.
As long as such differentiated cells serve a particular purpose in the human body they are tolerated. However, when such cells impede other developmental processes (e.g. embryonal development), are irreversibly damaged (e.g. radiation), or become potentially harmful to the human body (e.g. cells from the immune system), they are sacrificed by a process known as apoptosis (5, 6). Similar to the stimulatory

and inhibitory processes that govern cell proliferation, apoptosis is controlled by a delicate balance between both positively and negatively regulating factors. Thus, a cell's intracellular death program will be activated by the appearance of activating signals or disappearance of inhibiting signals (6).

Normal cell cycle regulation

Cell growth usually precedes cell division. During cell growth a cell duplicates its contents (e.g. its organelles and DNA) and prepares itself to divide. While the division of most of the cellular contents among daughter cells does not have to occur precisely on a 1:1 basis, chromosomes are, after duplication, equally distributed. The events involved in cell proliferation occur in four defined successive stages, termed G_1, S, G_2 and M.

In the G_1 (gap 1) phase, the cell increases in size and prepares to duplicate its DNA. This DNA duplication occurs in the next stage, termed S (for synthesis). Then, after the DNA is copied, a second gap period (G_2) follows, in which the cell prepares itself for M (mitosis). During mitosis the enlarged cell divides in half to produce its two daughter cells, each of them endowed with a complete set of chromosomes. On their turn, these daughter cells start a new cell cycle by entering G_1, or they may withdraw into a quiescent state, termed G_0 (1).

Although each respective time period, needed for a cell to complete a particular phase of the cell cycle, varies to some extent, by far the greatest variation occurs in the duration of G_1. In the G_1 phase of the cell cycle, both growth stimulatory as well as growth inhibitory signals determine whether a cell progresses through the cell cycle or whether a cell remains quiescent. It appears that most cells have a critical time point in the G_1 phase, termed: the restriction point. Once beyond this point, growth factors are of no further influence and DNA synthesis usually starts one to three hours later (i.e. the G_1 to S transition). During the next steps, the cell checks the integrity of its DNA and monitors whether the different steps in the cell cycle are completed successfully. The cell may be provoked to commit suicide when essential intra-cellular checkpoints within the cycle are not reached in time. Otherwise, the cell completes the subsequent steps of the cycle according to a rigid timetable (7).

Thus, while *extra-cellular* signals are required to enter the cell cycle (i.e. G_1), *intra-cellular* signals determine whether the cycle can be completed (i.e. succeeding through S, G_2 and M).

The basics of carcinogenesis

The chromosomal basis of cancer

Genes carry the genetic information which is needed for a human body to survive. Genes are located on enormously long DNA molecules, of which each of them forms the basic structure of one chromosome. Virtually all human cells carry 23 pairs of chromosomes: half a pair inherited from the mother, the other half from the father.

As microscopic techniques improved in the early 1970s, both numerical chromosomal changes (e.g. more than 23 pairs of chromosomes) as well as structural chromosomal changes (e.g. a reciprocal exchange of chromosome fragments between non-homologous chromosomes) were revealed in human cancer. A causal relationship of chromosomal changes with neoplasm was suggested when specific chromosomal aberrations were identified in characteristic tumor types. Yet, until the 1980s, investigators lacked the tools to determine whether chromosomal aberrations were among the causes of cancer or, were a by-product of cancer development (8).

The genetic basis of cancer

The causal relationship between chromosomal aberrations and cancer became clear in the early 1980s through the discovery that foreign DNA introduced into cells (e.g. by a tumor virus) changes these cells into a malignant state (9). This experimental breakthrough paved the way for the discovery of two new human gene classes that have become known as: proto-oncogenes and tumor-suppressor genes (2, 3). These genes are localized at different places in human chromosomes and are involved in the offset of cancer. However, as long as these genes are not damaged, they play central roles in normal regulatory processes of cell growth and division. In such regulatory processes, cell growth is stimulated by the products of proto-oncogenes and inhibited by the products of tumor-suppressor genes (2, 3).

A proto-oncogene may change into a cancerous oncogene when it is mutated as a result of either carcinogens (e.g. tar from cigarette smoke, causing lung cancer), radiation (e.g. ultraviolet rays in sunlight, causing skin cancer) or tumor viruses (e.g. papilloma virus, causing cervical tumors). Gene mutations cause the (proto-)oncogene to encode a growth stimulatory factor that is either abundantly expressed or simply overly active. However, one altered (proto-)oncogene does not necessarily lead to cancer (10). As has been discussed before, cells turn cancerous through the accumulation of various oncogenic events (i.e. the multi-step theory). In order to prevent cancer development, a cell is provided with complex fail-safe systems that take care of any disturbance in the regulatory processes of normal cell growth. When a number of these fail-safe systems are damaged, a cell may eventually turn cancerous (11).
One of the most obvious ways for a cell to prevent the initiation of cancer is to restore the DNA damage inflicted by cancer causing agents. Therefore, a cell is provided with so-called 'gene repairing' systems: these systems recognize and repair damaged DNA at various stages of the cell cycle (12). Moreover, even if these gene repairing systems fail, the oncogene's growth promoting stimuli are still not necessarily brought into effect. As long as their growth stimulatory effects are reduced by the growth inhibitory products of tumor suppressor genes, a cell is protected from autonomous growth. When tumor suppressor genes are damaged in such a way that they are not able to counteract the growth-stimulatory signals of (proto-) oncogenes, endogenously produced growth stimulatory factors are given a free hand and eventually cause a cell

to become cancerous. In contrast to oncogenes, where the presence of only one mutated allele is sufficient to contribute to cancerous growth, gene mutations must have been afflicted by cancer causing agents on both alleles of a tumor suppressor gene. Only if none of both tumor suppressor genes produce active growth inhibitory signals, cell growth and proliferation result from ongoing growth stimulatory signaling.

From the aforementioned successive stages it is clear that gene mutations are the basis on which a cell changes into a cancer cell. Although these gene mutations do not necessarily have to occur in this proposed order, they contribute to a cancerous behavior as each of them causes either: 1) ectopic expression of a gene product, 2) an altered function of an altered gene product or, 3) the inactivation of a gene product (2, 3).

The protein basis of cancer

Genes encode for proteins through processes known as transcription and translation. It is not difficult to imagine that gene mutations can cause severe changes in protein structure. Because proteins are involved in various vital cellular processes (e.g. cell proliferation, cell differentiation and cell metabolism), they are generally considered as the true working elements in a cell.

How proteins are involved in cancer is best exemplified through tracing the pathway that is followed by incoming growth regulatory signals, once they have reached the cell's surface. The signaling relay system is therefore characterized by successive stages, occurring at three different levels of a cell: 1) the cellular membrane, 2) the cytoplasm and 3) the nucleus.

The cellular membrane, or plasma membrane, defines the cell's boundaries and maintains essential differences between its contents and the environment. This membrane encloses the cell's interior, which comprises the cytoplasm and the nucleus. The cytoplasm on its turn is compartmentalized into various cell organelles, 'floating' in a common space, termed the cytosol. The cell nucleus is regarded as the most significant cellular structure as it contains nearly all of the cellular DNA molecules; providing the coding sequences of the various components needed for a cell to interact with the environment.

When (proto-)oncogenes force a cell to produce growth factors, they drive the proliferation of nearby cells or, in cancer cells, when these signals turn back, they drive proliferation of the cell that just produced them (i.e. autocrine growth stimulation model). These growth factors are captured by specific membrane proteins, or membrane receptors. As many membrane receptors extend across the membrane's lipid bilayer, they form communication bridges between the environment and the interior of a cell. When signals from the outside are captured by membrane receptors, these signals are switched to the interior of the cell by changing the chemical state of the intra-cellular part of the membrane receptor protein. At the inner side of the plasma membrane other signal transducing proteins are successively activated. Thus, by acti-

vating membrane receptor molecules a cascade of intra-cellular signaling is started, which usually ends in the nucleus.
A cancer cell may replace its signal transducing proteins by aberrantly activated proteins at each point in this signaling relay system. Such aberrantly activated membrane receptor proteins, cytoplasmic proteins or aberrantly activated nuclear proteins cause down-stream activation of the growth promoting relay system. As each of these aberrantly activated proteins result in abnormal growth stimulating stimuli, it is clear that the cancerous effects of the protein products of oncogenes is exerted at various cellular levels.
As mentioned earlier, cells must do more than overstimulate their growth promoting machinery to become truly malignant. Any tendency towards autonomous proliferation is tempered by growth inhibitory signals. Growth inhibitory signals are encoded by tumor suppressor genes and these genes provide a second fail-safe system that prevents cancerous behavior. However, growth inhibitory signals are delivered and relayed in a similar fashion as described for growth stimulatory signals. Any disruption of the growth inhibitory signaling cascade disables a cell to counteract growth stimulatory signals which results in loss of control on proliferation.

Other genetic alterations, increasingly implicated in cancer, are the ones that lead to an inappropriate expression of transcription factors. Transcription factors are nuclear proteins that recognize and bind specific regulatory DNA sequences. As a consequence, they stimulate or repress the mRNA transcription of their target genes.
The transcription factors that act on most mammalian genes can be divided in two types (13). The first category consists of general transcription factors that form part of a multi-protein complex (i.e. the RNA polymerase II initiation complex). This multi-protein complex recognizes specific DNA sequences immediately adjacent to a transcriptional start site. Binding of this multi-protein complex usually results in the active transcription of the involved gene. The rate of gene transcription is greatly influenced by the stability of the initiation complex and the frequency with which initiation complexes are formed. These variables are regulated by members of the second category of transcription factors: the specific transcription factors.
In contrast to general transcription factors, specific transcription factors recognize and bind specific DNA motifs, present in some genes but not in others. They regulate, through so-called transactivation domains, the RNA polymerase II activity by direct or indirect interaction with the initiation complex. Specific transcription factors are categorized in several families, based on homology of DNA-binding domains between various specific transcription factors (13).
Transcription factors are developmentally regulated (i.e. they are turned on or off at specific differentiational stages) and are usually involved in cell differentiation. These transcription factors may directly or indirectly influence the transcriptional rate of (proto-)oncogenes, tumor-suppressor genes or other genes involved in cell proliferation.

The 'master-gene' model:

The complexity of protein processes involved in carcinogenesis is best simplified by the 'master-gene' model, originally described by Rabbitts (14). According to the original model, master-genes are developmentally regulated and influence, although they do not necessarily encode transcription factors, the expression of one or several 'responder-genes' (14). The products of master-genes can act *positively* and up-regulate the expression of responder-genes or, they can act *negatively* by down-regulating the expression of responder-genes (14).

The master-gene model is important to understand the malignant transformation of previously normal, healthy cells. In cancer cells, aberrantly activated master-genes do not only upset a cell's transcriptional status, but they may also break the lineage specificity of transcription by activating responder-genes from different cellular origins. It is imaginable that, when master-genes and/or responder-genes are aberrantly expressed or activated, they cause the cell to respond to or neglect stimuli that are important for cell proliferation and differentiation control.

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Chapter 3

Cancer: one disease ?

Cancer: one disease ?

The traditional classification of cancer, which is based on morphological features, is inadequate. A close examination of the conventional categories reveals that most cancers consist of heterogeneous collections of disorders, each characterized by its own array of genetic lesions. Owing primarily to technical considerations, our knowledge of pathogenetic processes is most extensive in hematologic malignancies. Recent findings in a number of these hematologic malignancies not only exemplify the complexity and variability of human cancer, but also provide new tools that allow a precise diagnosis.

Leukemia

The formation of different types of blood cells (i.e. hematopoiesis) is essential for the development and survival of a healthy individual. New blood cells belonging to different cell lineages are formed from stem cells to replace cells that have completed their life span. These processes start early during embryogenesis and proceed throughout human life. Abnormalities in this normal developmental program for blood cell formation result in hematological diseases including leukemia (1).

Leukemic cells colonize the marrow of many of the bones. Like normal leukocytes, such neoplastic cells escape into the blood where they may be present in large numbers (hence 'leukemia'). Leukemic cells inhibit normal hemopoiesis, partly by replacing the bone marrow tissue, but also by secreting substances (e.g. cytokines) that inhibit the proliferation of (healthy) hematopoietic stem cells. As a consequence, death usually results from anemia, hemorrhage, or infection, resulting from a deficiency of red cells, platelets, or normal leukocytes, respectively (2).

Comparable to most malignant tumors, leukemias differ in the degree of differentiation of the tumor cells and, in general, their rate of neoplastic proliferation varies inversely with their degree of differentiation. According to clinical presentation, the leukemias are divided in acute leukemias which, if untreated, usually cause death in weeks or months and chronic leukemias which, if untreated, cause death in months or years (2, 3).

The acute leukemias are usually characterized by extensive cellular proliferation of abnormally differentiated, immature white blood cells in bone marrow. This kind of leukemia comprises a heterogeneous group of conditions that differ in both biology as well as prognosis. Depending on the cell lineage(s) involved in the leukemic process, acute leukemias are classified as acute lymphoblastic leukemias (ALL) and as acute non-lymphoblastic leukemias (ANLL) (2, 3). Acute leukemias are usually divided into those occurring in childhood (<15 years), with ALL as the most predominant type (80%) and those occurring in adults (> 15 years), of which ± 85% are ANLLs (3, 4).

Chronic leukemias are characterized as hematological malignancies in which the uncontrolled proliferating leukemic cells are capable of full maturation. Two subtypes of chronic leukemia are distinguished: chronic lymphocytic leukemia (CLL) and chronic myeloid leukemia (CML). The median age of patients with CLL is 65 years (5). Depending on complicating features such as anemia and thrombocytopenia, median survival rates between these patients vary from 24 months to 10 years (5). While CLL is rare below 40 years of age, CML may occur at any age, although the disease is commonest in adults (median age of CML patients is 50 years) (6). The course of CML is bi-phasic: initially starting as a chronic phase that lasts for 1-4 years, the disease changes into a second phase termed 'the blast crisis'. Blast crisis is characterized by the appearance of an unusually high number of immature lymphoid or myeloid blast cells in the peripheral blood. Usually, patients in blast crisis respond poorly to therapy (6, 7).

With the exception of CML, both ALL, AML, and CLL demonstrate a considerable heterogeneity in biology and prognosis. One of the first attempts to assess the prognosis and select the therapy more properly was made by a group of French, American, and British hematologists. This group developed a classification system (i.e. FAB classification) that was based on conventional morphological and cytochemical analysis of peripheral blood and bone marrow smears from patients with acute leukemia (8). Nowadays, according to redefined FAB criteria, the far majority (> 99%) of acute leukemias consists of three groups of acute lymphoblastic leukemias (i.e. L1 to L3) and eight groups of acute myeloid leukemias (i.e. M0 to M7), while only a small fraction (<1%) represents acute undifferentiated leukemias (AUL) (9-11). The FAB group also proposed morphologic criteria to classify CLL. Based on the proportion of atypical lymphocytes in blood, CLL is subdivided in: typical CLL, CLL-prolymphocytic leukemia and atypical CLL (5, 12). However, contrary to the FAB classification of acute leukemias, a FAB classification for CLL is still of much controversy (5, 13). The need to further subdivide ALL, AML and CLL is exemplified by worse treatment outcomes within subgroups. It is realized that a stratification that is primarily based on morphological features only partly reflects the biology of the malignancy and is, as such, limited to predict the prognosis of a leukemia patient.

Cytogenetics and leukemia

In 1960 Nowell and Hungerford detected a small marker chromosome, the so-called Philadelphia (Ph) chromosome, which was consistently found in CML (14). In 1973, Rowly (15) demonstrated that the Ph chromosome is identical to the 22q- chromosome and results from the reciprocal exchange of chromosomal fragments between chromosomes 9 and 22: t(9;22)(q34;q11). These findings initiated the field of cancer cytogenetics and it was realized that certain chromosomal aberrations were strongly associated with characteristic clinical features, response to treatment, and survival.

Nowadays, numerous chromosomal aberrations are detected by conventional cytogenetic techniques. In these techniques, cells are arrested at metaphase (i.e. one of the six phases in which the M phase is divided) and their chromosomes are stained with dyes, such as Giemsa or fluorescent dyes (e.g. atebrine and acridine orange). Typical chromosome bands are visualized; e.g. Giemsa staining distinguishes DNA rich in A-T nucleotide pairs (so-called Giemsa - or G-bands) from DNA rich in G-C nucleotide pairs (so-called reverse - or R-bands). Since chromosome bands are localized at specific places within chromosomes, it is possible to categorize each chromosome or part of the chromosome. Accordingly, aberrant chromosomes can be analyzed and classified (16).
Chromosomal aberrations are either numerical, meaning loss or gain of entire chromosomes (i.e. -7 or +8), or structural (e.g. translocations, deletions and inversions). There are two general types of structural aberrations: 1) balanced, primarily reciprocal translocations or inversions without visible gain or loss of chromosomal material and, 2) unbalanced aberrations, with gain or loss of parts of chromosomes. Furthermore, when referring to chromosome aberrations, a distinction is made between primary and secondary changes. Primary chromosome changes in leukemia are usually structural. Secondary (or subsequent) changes are often numerical and appear to correlate with tumor progression (17).

In childhood ALL, the group of patients with high hyperdiploidy (i.e. more than 50 chromosomes) proves to have the most durable responses to treatment, meaning that these patients have the least chance, when compared with children suffering from other types of childhood ALL, of reappearing leukemic blasts ensuing complete remission (18). While the hyperdiploid group accounts for about 25% of childhood cases, the largest group is formed by the group of patients with a pseudo diploid karyotype (± 40%). This group is characterized by a chromosome number of 46 per cell with structural abnormalities, most often translocations (18, 19).
The t(9;22) and the t(4;11) have both been shown to identify groups of patients with a particular poor therapeutic outcome (20, 21). The most frequently observed structural abnormality upon conventional cytogenetic analysis of childhood ALL cases is the t(1;19), which identifies a group of patients with several adverse prognostic features (e.g. high white blood cell count and lack of hyperploidy) (18, 19). However, as will be discussed later on, one must realize that conventional cytogenetic analysis is limited by technical difficulties and does not detect all chromosomal abnormalities (e.g. the t(12;21)(p12;q22) in childhood ALL) (22).
While remarkable improvements in the cure rate of children with ALL have occurred over the past 30 years, the results in treatment of adults with ALL remain poor, despite adoption of strategies used successfully in children (23). However, as was also observed in childhood ALL, when leukemia cells have a hyperdiploid karyotype, adult ALL patients usually have a more favorable prognosis than patients suffering from ALL with different karyotypes. Presence of t(9;22) hallmarks a large group of ALL patients (± 30% of adult ALL) with an unusual poor prognosis (23).

Numerical chromosomal changes in AML, in particular +8 or -7 are typical examples of secondary changes and are the major components that characterize a type of leukemia with a very poor prognosis. Approximately one third of AML cases is associated with specific translocations. The t(8;21), t(15;17) and t(9;22) are the most frequently observed cytogenetic abnormalities in AML. While patients with t(8;21) or t(15;17) are generally regarded as having a favorable prognosis, AML patients with the t(9;22) are associated with poor prognosis and short survival (24).
About 50% of patients with CLL has karyotypic abnormalities, particularly trisomy 12, del(13)(q12-14) and 14q+. Trisomy 12, which accounts for ± 35% of all CLL cases, is the best known karyotypic abnormality in CLL that is associated with a poor prognosis. Although other cytogenetic aberrations, such as t(11;14)(q13;q32), t(14;19)(q32;q13) have been reported, their impact on the prognosis of CLL is still unclear. However, as is also observed in other leukemias, the more cytogenetic aberrations are detected in CLL, the greater the likelihood of a short survival (13, 16).
Both ALL, AML, and CLL, are heterogeneous regarding their diversity of chromosomal aberrations. In contrast, the leukemic cells of more than 90% of CML patients are characterized by a typical chromosomal aberration: the t(9;22)(q34;q11) or, the Philadelphia translocation. Additionally, 5% of CML patients can be identified by a variant Ph translocation, seemingly not affecting chromosome 22. In these variant cases, part of the Philadelphia chromosome appears to have moved to other chromosomes (6). In CML blast crisis, additional chromosomal abnormalities, such as +8, i(17q) and +22q-, are often detected.
Although 5% of CML patients lack characteristic or variant Philadelphia chromosomes, it is common practice to consider the presence of a Philadelphia chromosome as a hallmark for CML diagnosis (7).

In summary, certain (characteristic) chromosomal aberrations are strongly related with typical clinical features, response to treatment and survival and it appears that a number of these chromosomal aberrations is causally related to the malignant properties of the leukemic (sub-)type. By using cytogenetic analysis, it is possible to further classify the major subtypes of leukemia, especially ALL and AML, according to their characteristic chromosomal aberrations.

Molecular genetics and leukemia

The resolution of conventional cytogenetic techniques is not more than a single chromosome band. When one realizes that even the thinnest chromosomal band that is visible through the microscope, comprises an enormous amount of DNA, it is obvious that chromosomes of leukemia cells may appear normal upon microscopic evaluation even though (submicroscopic) aberrations are present (16). Furthermore, metaphase analysis using banding techniques depends on the number of metaphases, and hence, also on the proliferative rate of leukemic cells (25).

New molecular techniques prove independent of the cell cycle and they provide a means to discover even the smallest genetic change imaginable. Typical examples of genetic changes that can be identified upon molecular analysis are given below:

I. **Point mutations:** one of the four bases (i.e. A, T, C, and G) in the DNA molecule is replaced by any of the other three bases. Point mutations may either activate (proto-) oncogenes (e.g. the *RAS* oncogene in AML) (26) or they may inactivate tumor-suppressor genes (e.g. the *P53* tumor-suppressor gene in CML) (27).

II. **Gene rearrangements:** part of the gene is lost and replaced by another part of the DNA molecule. This part is either derived from the same gene (see: '*immunoglobulin or T-cell receptor gene rearrangements*') or from another gene. When this replacement involves a gene that is different from the original gene, the substituting gene is either derived from the same chromosome (see: '*deletions*') or from another chromosome (see: '*translocations*').

a. Immunoglobulin or T-cell receptor gene rearrangements:
Immunoglobulin (*IG*) and T-cell receptor (*TCR*) gene rearrangements are normal physiological processes that are required to generate the enormous diversity of the antigen receptor molecules which typify the lymphoid cell population (28). Immunoglobulin (Ig) molecules, characteristic antigen receptors located on the surface of the B-lymphoid cell population, are composed of two heavy chain molecules, encoded by the *IGH* gene located on chromosome 14, and two light chain molecules. These light chains are either encoded by the *IGκ* gene located on chromosome 2, or by the *IGλ* gene located on chromosome 22 (28). T-cell receptor (TcR) molecules are, as the name implies, characteristic for the T-lymphoid cell population. In mature T-cells, two different types of TcR molecules have been identified: TcRαβ and TcRγδ. The TcRαβ molecule is composed of two different glycoproteins, encoded by two genes *TCRα* and *TCRβ*, located on chromosomes 14 and 7, respectively. The TcRγδ molecule consists of two glycoproteins encoded by the *TCRγ* gene located on chromosome 7 and, the *TCRδ* gene located within the *TCRα* gene on chromosome 14 (28).
These antigen-receptor genes comprise several discontinuous germline segments (i.e. V-variable, D-diversity, J-joining and C-constant regions). The V-, D-, and J-segments are successively rearranged during differentiation from stem cell to mature lymphoid B- or T-cell, creating a diversity of antigen receptor genes large enough to provide (in principle) each lymphocyte in the human body with its own specific antigen receptor. Because such *IG* and *TCR* gene rearrangements are unique for the individual cell and its progeny, they are particularly useful in leukemia diagnosis. Leukemia is a clonal disease that is, especially in ALL and CLL patients, featured by abundant presence of a characteristic *IG*- and / or *TCR* gene rearrangement at diagnosis (25).

Thus, although antigen-receptor gene rearrangements are by no means causative for the malignant transformation, such gene rearrangements provide excellent markers for clonality that can be extremely useful in both leukemia diagnosis as well as monitoring residual tumor-cells during and after treatment (25, 29, 30).

b. Deletions:

The complete gene or part of the gene is removed from the genome. Probably the best example of a *sub*-microscopic deletion in leukemia is the so-called '*TAL1* deletion'. This deletion, del(1)(p32), is found in approximately 15-20% of patients with T-cell ALL. The del(1)(p32) is characterized by the juxtaposition of all coding exons of the *TAL1* gene to the first nine non-coding exons of the *SIL* gene (31). This gene rearrangement results in deletion of the coding sequences of *SIL*, a gene that is, in contrast to the *TAL1* gene, normally expressed in T-cells. As a result of the deletion, the *TAL1* gene is transcriptionally controlled by the *SIL* promotor and, as a consequence, aberrantly transcribed into TAL1 mRNA. Aberrantly expressed transcription factor encoding genes, such as *TAL1* in T-cells, are supposed to contribute, either directly or indirectly, to malignant transformation (32, 33).

Table 1. TRANSLOCATIONS AND LEUKEMIA

Typical *IG* and *TCR* gene rearrangements associated with leukemia[a]

Translocation	**genes involved**	**disease**
t(2;8)(p12;q24)	*IGK; MYC*	B-ALL
t(8;22)(q24;q11)	*MYC; IGL*	B-ALL
t(8;14)(q24;q32)	*MYC; IGH*	B-ALL
t(11;14)(q13;q32)	*BCL1; IGH*	CLL[b]
t(14;18)(q32;q21)	*IGH; BCL2*	CLL[b]
t(14;19)(q32;q13)	*IGH; BCL3*	CLL
t(8;14)(q24;q11)	*MYC; TCRαδ*	T-ALL
t(1;7)(p32;q35)	*TAL1; TCRβ*	T-ALL
t(1;7)(p34;q35)	*LCK; TCRβ*	T-ALL
t(7;9)(q35;q34)	*TCRβ; TAN1*	T-ALL
t(7;9)(q35;q32)	*TCRβ; TAL2*	T-ALL
t(7;10)(q35;q24)	*TCRβ; HOX4*	T-ALL
t(7;11)(q35;p13)	*TCRβ; RBTN2*	T-ALL
t(7;19)(q35;p13)	*TCRβ; LYL1*	T-ALL
t(1;14)(p32;q11)	*TAL1; TCRδ*	T-ALL
t(10;14)(q24;q11)	*HOX11; TCRδ*	T-ALL
t(11;14)(p13;q11)	*RBTN2; TCRδ*	T-ALL
t(11;14)(p15;q11)	*RBTN1; TCRδ*	T-ALL

[a] see references: 16, 17, 18, 19 and references therein

[b] both translocations are more commonly detected in lymphomas (see reference 34)

c. *Translocations:*

The discovery of the *IG* and *TCR* genes provided new tools that allowed the identification of new genes involved in leukemia (34). The availability of molecular probes that fitted the antigen receptor genes, facilitated the discovery of adjacently located genes. When *IG* or *TCR* genes are involved in chromosomal translocations in lymphoblastic leukemia, they are often juxtaposed to genes that encode transcription factors (Table 1) (34, 35). Moreover, especially in T-ALL, these transcription factors are frequently ectopically expressed; meaning that these transcription factors are normally not expressed in T-cells or, if expressed, at least at far lower levels (35). The transcriptional control elements of the antigen receptor gene (i.e. *IG* or *TCR*) are thought to regulate the (inappropriate) expression of the juxtaposed (onco-)gene (36). As noted above (see '*TAL1* deletion'), it is assumed that aberrantly expressed oncogenes are causative, either directly or indirectly, for the malignant transformation of normal cells (35, 36).

Yet, various molecular genetic studies have indicated that *IG* or *TCR* genes are not involved in the majority of chromosomal translocations in hematopoietic malignancies found to date (Table 2). New genes have been identified and a number of them suggest a fundamentally different mechanism of oncogenic conversion, of which the *BCR-ABL* and the *E2A-PBX1* genes are prototypes (37).

Table 2. TRANSLOCATIONS AND LEUKEMIA

Most frequently observed chromosomal translocations within leukemic sub-groups[a]

Childhood ALL	**frequency**	**fusion-genes**
t(12;21)(p12;q22)	25-30%	*TEL-AML1*[b]
t(1;19)(q23;p13)	5-6%	*E2A-PBX1*
t(9;22)(q34;q11)	3-5%	*BCR-ABL*
Adult ALL		
t(9;22)(q34;q11)	30%	*BCR-ABL*
AML		
t(8;21)(q22;q22)	5-8%	*ETO-AML1*
t(15;17)(q22;q12-q21)	4-7%	*PML-RARα*
t(9;22)(q34;q11)	1-3%	*BCR-ABL*
CLL		
t(11;14)(q13;q32)	< 5%	no fusion-gene
CML		
t(9;22)(q34;q11)	> 95%	*BCR-ABL*

[a] see references: 16, 17, 18, 19, 22 and references therein.

[b] the t(12;21)(p12;q22) is more reliably detected by FISH than by conventional cytogenetic analysis

BCR-ABL genes

The *c-ABL* proto-oncogene was first identified in the mammalian genome (38) by its homology to the oncogene of the Abelson murine leukemia virus (A-MuLV) (39). In the early 1980s, the *c-ABL* gene was implicated in human cancer for the first time, when it was shown that this proto-oncogene was translocated to chromosome 22q- (the Philadelphia chromosome) in t(9;22)(q34;q11) positive CML (Figure 1) (40, 41). It was demonstrated that the *c-ABL* gene loses part of its coding sequence as a result of the t(9;22)(q34;q11) and becomes truncated. In the majority of CML patients, breakpoints in the *c-ABL* gene were found, scattered over a distance of approximately 200 kb, nearly always 5' of exon 2 (termed a2) (41). From this point on, in quick succession, the gene (termed *BCR* - from breakpoint cluster region) and its breakpoints on chromosome 22 were identified (42-45). In contrast to the *ABL* gene, breakpoints in *BCR* are clustered within two regions, defined as: the major breakpoint region (M-BCR) and the minor breakpoint region (m-BCR) (Figure 2).

The M-BCR spans five exons, termed b1-b5; corresponding to exons 12-16 of the *BCR* gene. In CML, almost all breakpoints occur within the M-BCR, either between exon b2 and b3 (50% of Ph^+ CML) or between exon b3 and b4 (50% of Ph^+ CML). Breakpoints outside the M-BCR are extremely rare in CML (46-48).

In ALL, the majority of *BCR* breakpoints is found within the m-BCR region, localized between the first (e1) and the second (e2) exon of the *BCR* gene (49). Within the population of ALL patients carrying a *BCR-ABL* gene translocation, 85% of all children and almost 70% of all adults show m-BCR breakpoints (50). 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 (50-52).

The reciprocal exchange between the telomeric ends of chromosome 9 and 22 in t(9;22)(q34;q11) positive leukemias, results in a shortened 22q- chromosome containing the *BCR-ABL* gene and a 9q+ derivative harboring the reciprocal *ABL-BCR* gene (Figure 1). Although reports on the *ABL-BCR* fusion-gene are still limited, it appears

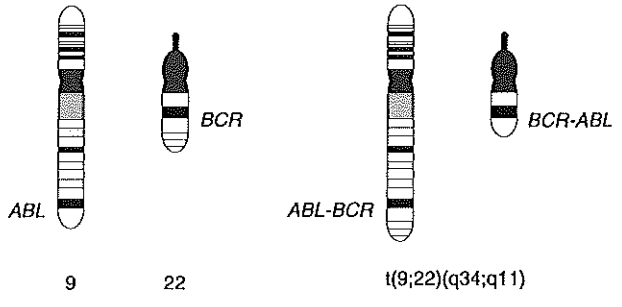

Figure 1. Schematic representation of the Philadelphia translocation, a reciprocal translocation between chromosome 9 and chromosome 22. As a result of the transocation a chimeric *ABL-BCR* gene has been generated on the 9q+ chromosome and a chimeric *BCR-ABL* gene has been generated on the 22q- chromosome,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

that in approximately two thirds of CML patients the *ABL-BCR* fusion-gene is transcribed into its corresponding ABL-BCR fusion transcript (53). Despite of the in-frame fusion of the two open reading frames of both truncated genes, ABL-BCR fusion-proteins have not been detected (53).
The *BCR-ABL* fusion-gene on the other hand, is transcribed into fusion mRNA and subsequently translated. While the 7 kb e1a2 BCR-ABL fusion mRNA gives rise to a 190 kDa fusion-protein designated $e_1a_2P190^{BCR\text{-}ABL}$, 8.5 kb BCR-ABL fusion mRNAs comprising b2a2 or the b3a2 BCR-ABL junctions are translated into 210 kDa fusion proteins, termed $b_2a_2P210^{BCR\text{-}ABL}$ or $b_3a_2P210^{BCR\text{-}ABL}$, respectively (54-57) (Figure 2).

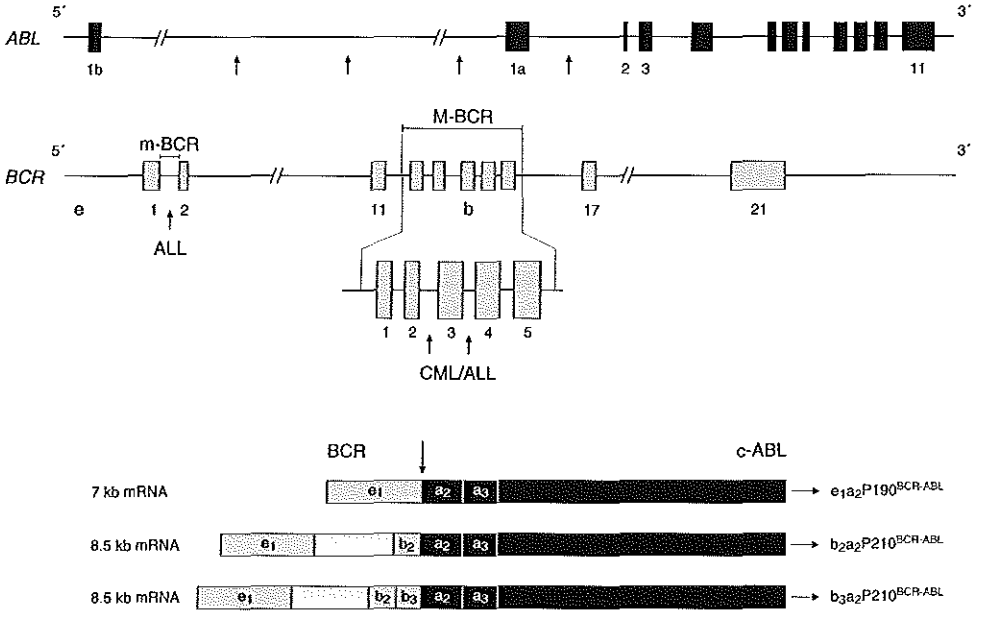

Figure 2. Schematic representation of the normal *ABL* and *BCR* genes and of the chimeric BCR-ABL mRNAs and proteins. Black boxes and black bars represent *ABL* exons and *ABL* derived mRNA and protein sequences. Grey boxes and bars symbolize *BCR* exons and *BCR* derived mRNA and protein sequences,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

BCR-ABL mediated leukemogenesis: chimeric proteins, such as BCR-ABL proteins observed in t(9;22)(q34;q11) positive leukemias, combine different domains that are normally only present in one of both wild-type proteins.
The wild-type *c-ABL* proto-oncogene encodes a 145 kDa protein; a non-receptor protein kinase with autokinase activity (i.e. the capacity to phosphorylate itself). The cytoplasmic c-ABL protein is as a non-receptor protein kinase, probably involved in intracellular signal transduction pathways. The c-ABL protein contains at least four functional domains: 1) an N-terminal domain, encoded by two alternative exons termed Type 1a and 1b (54, 58), 2) a kinase regulatory domain, including two SRC-homology

regions (SH3 and SH2), 3) the tyrosine kinase domain (SH1) and, 4) a large C-terminal region which is unique to the ABL family (59). The autokinase activity of the c-ABL protein is tightly regulated, a feature that is mediated by the kinase regulatory domains SH2 and SH3.
It has been demonstrated that all three types of BCR-ABL proteins have an enhanced tyrosine kinase activity in comparison with that of the normal c-ABL protein (56, 57, 60). BCR sequences that are encoded by the first exon of the *BCR-ABL* gene, interfere with the kinase regulatory domains through binding to the SH2 domain (61). The enhanced tyrosine kinase activity found in the chimeric BCR-ABL proteins is likely caused through shielding the SH3 domain by N-terminal BCR sequences, through which the inhibitory effect of the SH3 domain on the tyrosine kinase domain is neutralized (61, 62).
However, the enhanced tyrosine kinase activity is probably not the sole reason that BCR-ABL proteins are oncogenic. N-terminal BCR sequences do not only bind to the SH2 domain found in BCR-ABL, but also bind to the SH2 domain of another SH2-SH3 containing protein, termed GRB2. The GRB2 protein has been described to link tyrosine kinase activity to activation of the signaling pathway in which RAS proteins are involved (61, 63, 64).
According to the master-gene model introduced by Rabbitts (65), it is estimated that BCR-ABL proteins are the products of a master-gene. In this model, the uncontrolled cellular proliferation of tumor-cells is explained by the disturbance of normal cell cycle regulating networks. When considering BCR-ABL proteins, it is possible that normal cell cycle regulation is directly affected by BCR-ABL master-genes or, indirectly via responder genes such as *RAS* (66). Recently, interactions between c-ABL and the Retinoblastoma protein (pRB), the 'master brake' of the cell cycle clock, have been described (67, 68).

Deciphering these master-responder gene networks contributes to our understanding of *how* the BCR-ABL proteins are involved in leukemogenesis processes. *That* BCR-ABL proteins are involved in the malignant transformation of hematopoietic cells was recently demonstrated *in vivo*. Animal models for human Ph positive leukemia, whether generated through retroviral (69-72) or transgenic technologies (73-75), demonstrate that 'BCR-ABL positive' animals develop leukemias resembling the leukemic disease observed in humans. The longer latency time of $P210^{BCR-ABL}$ associated tumorigenesis in transgenic mice seems to reflect the intrinsic biochemical differences between $P210^{BCR-ABL}$ on the one hand and $P190^{BCR-ABL}$ on the other (75).

Prognostic and therapeutic implications: the t(9;22) provides markers that are applicable for both tumor-specific diagnostic purposes as well as the specific detection of residual tumor-cells during and after treatment. Yet, conventional cytogenetic analysis of leukemic cells is frequently hampered by a lack of sufficient metaphases as it largely depends on the proliferative rate of the leukemic cells. Molecular techniques, such as fluorescent in situ hybridization (FISH) (76, 77) or reverse transcriptase

polymerase chain reaction (RT-PCR), often detect *BCR-ABL* gene rearrangements in cases in which no Philadelphia chromosome are detected by conventional cytogenetic techniques (78, 79).
Patients suffering from a t(9;22)(q34;q11) positive leukemia have a poor prognosis. Especially in ALL, it is important to distinguish Ph positive ALL from Ph negative ALL. Whether children or adults, patients with Ph positive leukemias often relapse during or after their first round of therapy with chemotherapeutic drugs (i.e. induction therapy) (20, 23, 80). To allow an early intervention with more aggressive and experimental therapies (e.g. bone marrow transplantation), it is not only important to diagnose Ph positive leukemias as accurately as possible, but it is also important to diagnose these Ph positive leukemias as quickly as possible (20, 23, 80).

E2A-PBX1 genes

The transcriptional control of *IG* and *TCR* genes is dominated by specific nucleotide sequences, termed 'enhancers'. The *IGH* and *IGK* gene enhancers were the first to be identified and were found in the introns that separate the J and C regions. These enhancers are known as E-boxes, named after Anna Ephrussi, the first author on the initial report (81). E-boxes bind multiple nuclear proteins, some of which are tissue specific while others are ubiquitously expressed (82).
In 1989, Murre reported on the isolation of two enhancer binding proteins E12 and E47 (83). These two proteins, together with the E2-5 protein, are derived from a single gene termed *E2A* (84). Shortly after the *E2A* gene was discovered, it was found to be located within chromosome band 19p13: the breakpoint of the t(1;19)(q23;p13) observed in childhood ALL (Figure 3) (85).
Breakpoints in the *E2A* gene almost invariably occur within the 3.5 kb intron located between exons 13 and 14 (86). In both balanced t(1;19)(q34;q11) and unbalanced der(19)t(1;19) positive leukemias the *E2A* gene is fused on chromosome 19 to a previously unknown gene *PBX1* located on chromosome 1 (87, 88). Although the genomic organization of *PBX1* has not been fully determined, it is assumed that the breakpoints occur in a single, large intron (> 50 kb) of the *PBX1* gene (Figure 4) (86).
The fusion-point detected in the E2A-PBX1 transcript is remarkably consistent (89), although several groups detected a 27 additional nucleotide insert at the junction between *E2A* and *PBX1* that seems to co-exist with the conventional E2A-PBX1 transcripts in 5-10% of t(1;19) positive ALL (90, 91). These additional 27 nucleotides are identical in each case and appear to arise from a differentially spliced *E2A* or *PBX1* exon, but their exact derivation is unknown (90). The t(1;19) is predominantly found in pre-B-ALL, of which 25% carries this particular translocation (18).
Unlike t(9;22)(q34;q11) positive leukemias in which reciprocal chimeric ABL-BCR transcripts are detected together with the 'conventional' BCR-ABL mRNAs, reciprocal PBX1-E2A transcripts are not observed in t(1;19)(q23;p13) positive leukemias.

Apart from loss of the *PBX1-E2A* gene on derivative chromosome 1 in unbalanced t(1;19) positive leukemias (i.e. more than 80% of all t(1;19) positive leukemias), transcriptional regulation of *PBX1-E2A* would be mediated by the PBX1 promotor (see Figure 3). This promotor is silent in pre-B and mature B-cells (88).
The E2A-PBX1 mRNA was calculated to give rise to protein products with a molecular mass of 85 kDa (i.e. E2A-PBX1a) and 77 kDa (i.e. E2A-PBX1b) (87, 88). Due to differential splicing in the PBX1 part of the E2A-PBX1 primary transcript, different E2A-PBX1 proteins are *simultaneously* expressed by single t(1;19) positive cells (Figure 4) (92). This is in contrast to BCR-ABL proteins in Ph positive leukemias. Although different BCR-ABL proteins have been described, they are usually *not* simultaneously expressed by a single t(9;22) positive cell (Figure 2).

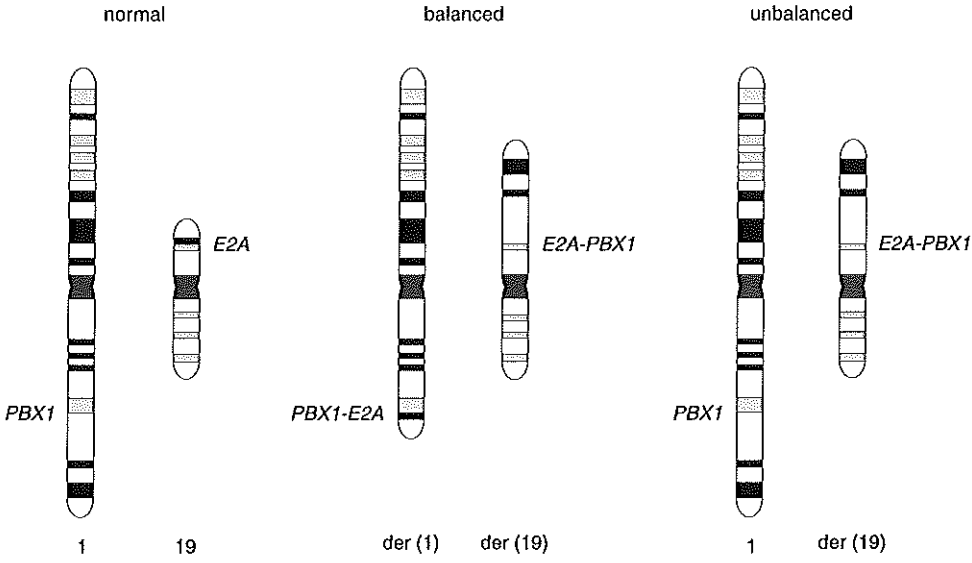

Figure 3. Schematic representation of the t(1;19)(q23;p13) and its unbalanced variant. In the unbalanced form, the derivative chromosome 1 is probably lost by nondisjunction and is replaced by duplication of the normal chromosome 1 homoloque. The unbalanced variant accounts for more than 80% of all t(1;19)(q23;p13) translocations and does not harbor a reciprocal *PBX1-E2A* gene

E2A-PBX1 mediated leukemogenesis: chimeric E2A-PBX1 proteins observed in t(1;19)(q23;p13) positive pre-B-ALL, combine two different transcription factor domains that are normally only present in one of both wild-type proteins.
E2A proteins belong to the so-called 'basic Helix-Loop-Helix' family of transcription factors (35). Specific DNA binding is mediated by the basic region located at the amino-terminus of the Helix-Loop-Helix domain, the latter domain is involved in dimerization processes that mediate binding of E2A proteins to identical or related transcription factor proteins. It has been demonstrated that protein dimers, but not monomers, can bind DNA in a sequence specific manner (93). RNA polymerase II

activity is regulated by direct or indirect interaction with the initiation complex through two transactivation domains (i.e. ADI and ADII) present on the E2A proteins (94). In the normal situation, E2A proteins are involved in the regulation of a diverse array of cellular processes including myogenic differentiation (95, 96), and transcription of *IG* genes (96-98) and pancreatic genes (99).

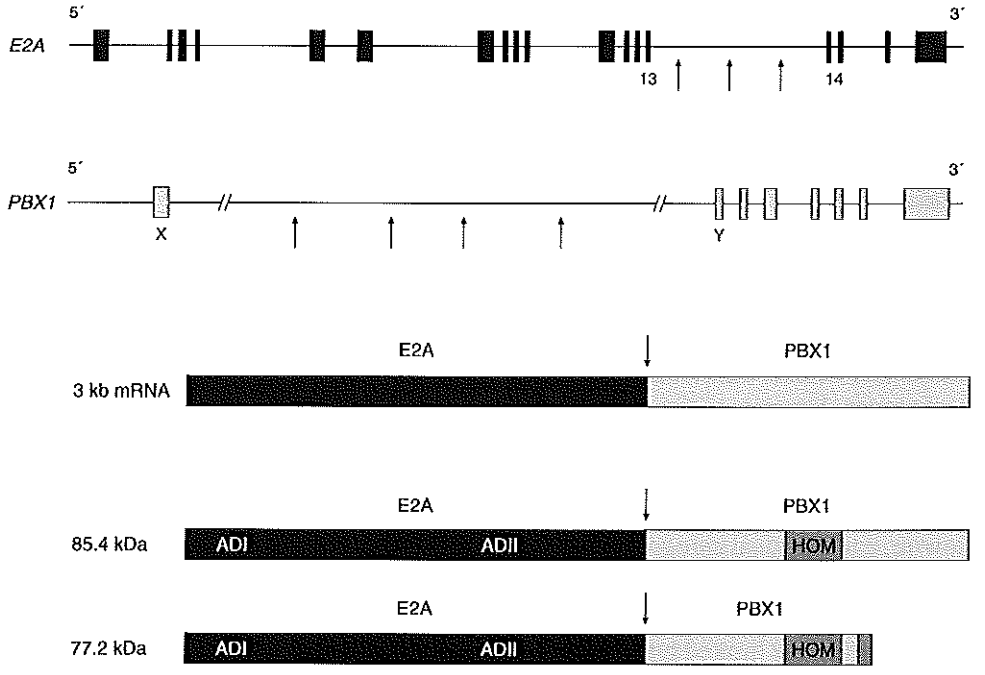

Figure 4. Schematic representation of the normal *E2A* and *PBX1* genes and of the chimeric E2A-PBX1 mRNA and proteins. Black boxes and bars represent *E2A* exons and *E2A* derived mRNA and protein sequences. Grey boxes symbolize *PBX1* exons and *PBX1* derived mRNA and protein sequences. Abbreviations: AD I and AD II, transcriptional activation domains 1 and 2; HOM, homeodomain.

In E2A-PBX1 proteins, the basic Helix-Loop-Helix domain of E2A proteins is replaced by the homeobox DNA-binding and protein interaction domains of PBX1 proteins (87, 88). Contrary to the wild-type proteins, i.e. E2A and PBX1, the chimeric E2A-PBX1 protein not only contains the two transactivation domains from E2A but also contains the homeobox DNA-binding and protein interaction domains from PBX1. Recent experiments have demonstrated that, in contrast to the wild-type proteins, the chimeric E2A-PBX1 protein is capable to activate transcription of a reporter gene which contains the PBX1 consensus binding site (100-102).

The E2A-PBX1 proteins perfectly fit the 'master-gene' model described by Rabbitts (65). The altered function of the chimeric E2A-PBX1 'master' protein is believed to cause activation of 'responder' genes that are normally not expressed, or expressed at far lower levels (103-105). These responder genes probably interact directly or indi-

rectly with the cell cycle regulating network involved in cell proliferation. Recently, it has been demonstrated that E2A proteins have growth-inhibitory properties at the G_1-S transition point when over-expressed in fibroblasts (106).

The causative role of E2A-PBX1 proteins in the malignant transformation of hematopoietic cells has been demonstrated *in vivo* as well. From both 'simple' fibroblast transformation assays to the more elaborate animal models in which chimeric *E2A-PBX1* genes are expressed, it was concluded that *E2A-PBX1* functions as a potent oncogene (92, 107, 108). Surprisingly, cells from *E2A-PBX1* transgenic mice suffering from T-cell lymphomas displayed a concomitant increase in cells undergoing programmed cell death or apoptosis, a feature that seems in paradox with the malignant character of the *E2A-PBX1* positive tumor-cell (108).

Prognostic and therapeutic implications: the t(1;19) provides markers that can be used for specific diagnosis and specific detection of residual disease during and after treatment. As already noted in BCR-ABL positive leukemias, molecular genetic analysis frequently detects translocations that are not observed with conventional cytogenetic techniques. Several studies suggest that 25% to 50% of leukemias with *E2A-PBX1* gene rearrangements are missed upon standard karyotypic analysis (109, 110).
While false-negative results are a common feature of cytogenetic techniques (often due to the lack of sufficient metaphases of leukemic cells), false-positive results following conventional cytogenetic analysis also occur. At least one case originally described as a t(1;19) positive early pre-B-ALL was, when re-analyzed using molecular techniques such as FISH, determined to be a t(1;22) (111). Another example, that demonstrates the limited resolution of conventional cytogenetic analysis, is provided by t(1;19) positive common-B-ALL (i.e. cytoplasmic Ig *negative*). In this immunological leukemic subtype, neither *E2A* nor *E2A-PBX1* gene rearrangements are detectable, despite presence of the t(1;19). These observations, which are attributable to the limited resolution of conventional cytogenetic banding techniques, suggest that the t(1;19) observed in common-B ALL (also known as t(1;19) positive *early* pre-B-ALL) involves other, yet to be identified genes (91, 112, 113). These variant t(1;19) positive, but *E2A-PBX1* gene negative common-B-ALL, appear to have a better prognosis than $t(1;19)^{E2A\text{-}PBX1}$ positive pre-B-ALL (114). It seems therefore, that the detection of the chimeric *E2A-PBX1* gene, rather than the detection of the aberrant karyotype, is of real prognostic value.
Although some investigators still claim presence of the *E2A-PBX1* gene in t(1;19) positive common-B-ALL, Troussard *et al.* recently demonstrated that the cytoplasmic Ig negativity found in previously diagnosed $t(1;19)^{E2A\text{-}PBX1}$ positive common-B-ALL was due to technical limitations of conventional slide immunofluorescence analysis (115). Cytoplasmic Ig presence was revealed in all cases upon re-examination by flow cytometric analytical techniques (one case lacked sufficient material to be re-examined), indicating that *E2A-PBX1* gene rearrangements are typical for t(1;19) translocations in ALL with a pre-B immunophenotype (115).

Approximately 25% of childhood ALL has a pre-B immunophenotype. Within the pre-B-ALL group of patients, a similar proportion can be identified by the presence of the t(1;19) translocation (116, 117). Initially, patients with a t(1;19) positive pre-B-ALL had a poorer prognosis than pre-B-ALL patients without this cytogenetic abnormality (117, 118). In the early 1990s, when the curative effects of new treatment protocols on patients with pre-B-ALL ALL were evaluated, it was demonstrated that the prognosis of all pre-B-ALL patients had significantly improved (119, 120). It was realized that a combination therapy of various chemotherapeutic drugs, when administered according to intensive treatment protocols to patients with pre-B-ALL, reduced the difference in prognosis between patients with a t(1;19) positive pre-B-ALL and pre-B-ALL patients without this cytogenetic abnormality significantly (119, 120).

Aim of the study

The genetic basis of cancer is generally accepted. As exemplified by the prognostic and therapeutic implications of certain tumor-specific genetic abnormalities, such as the poor prognosis of *BCR-ABL* positive leukemias (20, 23, 80) versus the improved treatment outcome of *E2A-PBX1* positive leukemias (119, 120), it is obvious that accurate diagnostic techniques are extremely important. It is evident that an accurate identification of such tumor-specific genes is not only important for primary diagnosis and therapy planning, but is also important for detecting residual tumor cells during and after treatment (25, 29). At present, a number of such diagnostic techniques exists (16, 25). Both indications as well as limitations of three of the most important diagnostic techniques are briefly summarized below:

a. *Conventional cytogenetic techniques* are still the methods of first choice for identifying and analyzing various chromosomal abnormalities in leukemic cells (25). The practical use of conventional cytogenetic techniques relies on its broad applicability. In contrast to molecular genetic techniques, cytogenetic techniques are not restricted by specific probes of which their use is limited to the identification of a single chromosomal aberration. However, cytogenetic techniques are not very sensitive since results depend on both the number of metaphases investigated as well as the limited resolution of chromosome banding. Moreover, the number of metaphases amenable to be analyzed varies with the type and origin of the leukemic clone. In this respect, it is well acknowledged that ALL samples are more difficult to analyze than ANLL samples, especially when these samples are obtained from children (6). Only institutions with special experience in ALL cytogenetics achieve successful karyotype analysis in almost every patient. Even then, some cryptic rearrangements escape detection by conventional cytogenetic analysis, which is exemplified by Ph chromosome negative, but BCR-ABL positive leukemias (121).

b. Contrary to conventional cytogenetic analysis, *fluorescent in situ hybridization* (FISH) techniques are not limited to a laborious analysis of metaphases (25). By using differently labeled probes, each directed against characteristic parts of a tumor-gene, specific chromosome abnormalities are identifiable in interphase cells. Yet, only a small number of such specific probes is presently available. Furthermore, depending on the co-localization of differently labeled hybridization signals to one spot, the sensitivity of the FISH technique is limited because artifactual co-localization may occur in normal cells.

c. The *polymerase chain reaction* (PCR) is currently the most sensitive method for detecting genetic abnormalities (25). In fact, by using PCR techniques, molecular genetic analysis frequently detects chromosomal aberrations that are not observed karyotypically. Especially during anti-cancer therapy, PCR analysis seems particularly useful to detect (low numbers of) residual tumor cells. However, some genetic aberrations are not easily detected by direct amplification of genomic DNA (e.g. BCR-ABL positive leukemias). This problem can be solved after reverse transcription of mRNA into cDNA, although strict precautions are required to prevent false positive (due to cross-contamination) or false negative (due to RNase) results.

While current tumor-diagnostic techniques are based on the detection of either an aberrant chromosome, aberrant genes or aberrant mRNA, only few diagnostic techniques exist that exclusively identify the aberrant protein product of a tumor-specific gene. In this respect, immunologic techniques seem particularly useful to detect such tumor-specific proteins. Although present immunologic techniques do not (yet) rely on the specific detection of tumor-specific proteins, improved, *non* tumor-specific immunologic methods (e.g. double staining techniques and flow cytometry) have already increased the precision of tumor-diagnosis. The immunologic classification of leukemia, that is primarily based on the co-expression of certain differentiation markers provides an eminent example.
It is estimated that the exclusive detection of tumor-specific proteins will further improve the diagnostic potential of currently used immunologic methods.
The discovery of chimeric tumor-genes together with the introduction of the hybridoma technology in 1975 by Köhler and Milstein (122), provided new tools for generating antibodies directed towards tumor-specific antigens. From an immunological point of view it was assessed that only a certain region of the tumor antigen would be unique for the tumor-specific protein. It is assumed that this so-called 'tumor-specific epitope' is not expressed on other cellular proteins and might serve as a tumor-specific marker. The ability to produce peptides with an amino acid sequence, identical to the tumor-specific epitope, offers an opportunity to direct the immune response, thereby diminishing the chance to raising antibodies towards *non*-tumor-specific epitopes.

The generation of antibodies, specifically recognizing tumor cells:
One of the first reports on a (polyclonal) antiserum, of which its generation is based on a peptide based immunization strategy, describes a (polyclonal) antiserum that specifically reacts with the tumor-specific epitope found in Ph chromosome positive leukemias (123). This antiserum, which was termed 'BP-1' (from 'Break-Point-1'), was derived by immunizing rabbits with a peptide conjugated to a protein-carrier complex. The peptide that was used had an amino acid sequence identical to the (predicted) amino acid sequence of the b_2a_2 fusion-point epitope present in $b_2a_2P210^{BCR\text{-}ABL}$ proteins. The BP-1 rabbit antiserum reacts with the fusion-point epitope present on the cognate $b_2a_2P210^{BCR\text{-}ABL}$ protein. Its specific reactivity with the b_2a_2 fusion-point epitope was confirmed as the BP-1 antiserum did not detect related BCR-ABL proteins, such as $b_3a_2P210^{BCR\text{-}ABL}$. The $b_3a_2P210^{BCR\text{-}ABL}$ proteins are identical with the $b_2a_2P210^{BCR\text{-}ABL}$ proteins at their amino- and carboxyl-termini, but differ at the tumor-specific fusion-point epitope.
Based on a similar methodology, two other polyclonal antisera have been reported, each of them specifically reacting with the tumor-specific fusion-point epitope present in cognate BCR-ABL proteins; i.e. BP-ALL specifically recognizes $e_1a_2P190^{BCR\text{-}ABL}$ proteins (124), and BP-2 recognizes $b_3a_2P210^{BCR\text{-}ABL}$ proteins (125).

From these experiments, it was concluded that *tumor-specific* fusion-point epitopes, as present on the chimeric BCR-ABL proteins, are *antigenically* exposed and can serve as a *new* means for tumor-*specific* diagnosis.

Diagnostic problems and questions to be solved:
Even though the 'anti-BCR-ABL fusion-point' specific polyclonal antibodies specifically detect the tumor-specific fusion-point epitopes on tumor-specific BCR-ABL antigens, their intended use; i.e. the *microscopic* identification of intact, Ph chromosome positive tumor cells, had not been realized (123, 124, 125). It appeared that specific problems needed to be solved before a tumor-specific diagnosis of leukemias by immunologic means was achievable. In the next chapters of this thesis, we attempt to answer specific questions that are related to the generation of antibodies useful for the tumor-specific, immunological diagnosis of leukemias. A brief introduction to these chapters is given below.

In **chapter 4** we question whether E2A-PBX1 chimeric proteins can be specifically detected by using a peptide based immunization strategy. These chimeric proteins are predominantly found in $t(1;19)^{E2A\text{-}PBX1}$ positive pre-B-ALL. We demonstrate, by using essentially the same strategy as had been applied for the generation of the anti-BCR-ABL fusion-point antibodies, that chimeric proteins, such as tumor-specific E2A-PBX1 proteins, are also specifically detected. The antiserum, which is termed BP 1/19, was raised towards the tumor-specific fusion-point of E2A-PBX1 proteins. This BP 1/19 antiserum specifically recognizes the tumor-specific E2A-PBX1 fusion-point;

both at the peptide as well as at the protein level. This antiserum can also be used for the microscopic analysis of leukemias, since it specifically detects t(1;19)$^{E2A\text{-}PBX1}$ positive cell lines. Only t(1;19)$^{E2A\text{-}PBX1}$ positive cell lines display a characteristic, granular, nuclear fluorescence pattern upon BP 1/19 immunostaining.

In **chapter 5** we examine the diagnostic use of BP 1/19 antiserum in tumor diagnosis. We tested the specificity of BP 1/19 immunostaining on 27 different hematopoietic cell lines. BP 1/19 immunostaining proved specific, since the characteristic, granular nuclear staining pattern was only observed in all t(1;19)$^{E2A\text{-}PBX1}$ positive pre-B cell lines but was undetected in any of the other cell lines. The sensitivity of the immunostaining technique was determined at one t(1;19)$^{E2A\text{-}PBX1}$ positive cell among 10,000 normal blood cells through artificial mixing experiments. The diagnostic potential of BP 1/19, considering tumor-specific recognition, was ascertained because antiserum BP 1/19 is capable to discriminate, at the cellular level, between previously diagnosed t(1;19) positive pre-B-ALL and t(1;19) negative ALL patients.

In **chapter 6** we investigate whether monoclonal anti-E2A-PBX1 fusion-point antibodies have the same diagnostic potential as the BP 1/19 antiserum. We demonstrate that monoclonal antibodies (which we termed ER-GO4), have similar characteristics as the polyclonal antiserum regarding the specific recognition of the tumor-specific E2A-PBX1 fusion-point epitope, at both the peptide as well as at the protein level. However, in contrast to the polyclonal BP 1/19 antibodies, ER-GO4 immunoglobulins are not capable to distinguish t(1;19)$^{E2A\text{-}PBX1}$ positive pre-B cells from t(1;19) negative pre-B cells. To prove that the limited use of ER-GO4 antibodies is a matter of relatively low expressed E2A-PBX1 antigens, we demonstrate that ER-GO4 antibodies are capable to specifically detect E2A-PBX1 fusion proteins in COS cells transfected with cDNA that encodes E2A-PBX1 proteins.

In **chapter 7** we examine whether monoclonal antibodies, directed towards the tumor-specific fusion-point epitope in $e_1a_2P190^{BCR\text{-}ABL}$ proteins can improve current diagnostic techniques used for diagnosing Ph chromosome positive ALL. By using a similar strategy as was used in chapter 6, we demonstrate that these monoclonal antibodies, which are termed ER-FP1, specifically detect an e1a2 fusion-epitope at both the peptide as well as at the protein level. To investigate whether the e1a2 fusion-point of $e_1a_2P190^{BCR\text{-}ABL}$ proteins is antigenically exposed in intact, albeit fixed cells, we performed immunostaining experiments with ER-FP1 antibodies on COS cells transfected with cDNA either encoding $e_1a_2P190^{BCR\text{-}ABL}$, $b_2a_2P210^{BCR\text{-}ABL}$ or $b_3a_2P210^{BCR\text{-}ABL}$ proteins. Since ER-FP1 antibodies specifically detect e1a2 BCR-ABL transfected COS cells, and not b2a2 or b3a2 BCR-ABL transfected COS cells we conclude that the fusion-point epitope of $e_1a_2P190^{BCR\text{-}ABL}$ proteins remains intact despite cell fixation. However, analogous to ER-GO4 antibodies, ER-FP1 antibodies are also not capable to specifically distinguish leukemic tumor cells from normal, healthy cells, at the microscopic level.

In **chapter 8** we question whether alternative immunological techniques can improve current diagnostic techniques that are used to specifically detect particular tumor types. We describe the development of a new, simple and rapid technique: the 'BCR-ABL dipstick' assay. The principle behind the BCR-ABL dipstick assay is based on the successive detection of two distinct antigenic sites on the BCR-ABL proteins by the combined specificity of two different antibodies. The combination of different antibodies in this assay allows the *exclusive* detection of *tumor-specific* BCR-ABL proteins within 24 hours. Moreover, by choosing the proper combination of antibodies, this assay discriminates between e_1a_2BCR-ABL proteins and $b_2a_2P210^{BCR\text{-}ABL}$ or $b_3a_2P210^{BCR\text{-}ABL}$ proteins. The poor prognosis of Ph chromosome positive leukemias, especially Ph positive ALL, could improve by an early adjustment of currently used induction therapies. Yet, early therapy adjustment requires both a specific as well as a quick diagnosis, both of which could be provided by the BCR-ABL dipstick assay.

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Chapter 4

Specific immunologic recognition of the *tumor-specific* E2A-PBX1 fusion-point antigen in t(1;19)-positive pre-B-cells

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Specific immunologic recognition of the *tumor-specific* E2A-PBX1 fusion-point antigen in t(1;19)-positive pre-B-cells

The t(1;19) translocation is one of the most commonly observed chromosomal translocations in childhood acute lymphoblastic leukemia (ALL). Its presence among pre-B-ALL cases, has been associated with a poor prognosis. Two genes, E2A and PBX1, are involved in this t(1;19) translocation. As a consequence, parts of the E2A and PBX1 genes are fused, resulting in a chimeric E2A-PBX1 gene, encoding chimeric E2A-PBX1 proteins. As such, the amino acids sequence at the fusion site represents a unique tumor-specific determinant. We report on the generation of a polyclonal antiserum, termed BP 1/19, raised against the tumor-specific E2A-PBX1 junction of E2A-PBX1 proteins. The specificity of antiserum BP 1/19 for the E2A-PBX1 fusion-point is demonstrated at the peptide and at the protein level. Furthermore, specific binding of antiserum BP 1/19 to t(1;19) positive cells was shown using immunofluorescence techniques. This study shows that: (1) the tumor-specific fusion-point epitope on E2A-PBX1 proteins is presented in an antigenic fashion and, (2) this particular fusion-point epitope can be used in immunological marker analysis by using fluorescence microscopy.

Introduction

The t(1;19)(q23;p13) translocation is, with approximately 5-6% of childhood ALL cases, one of the most common cytogenetic aberrations observed in childhood ALL (1). This particular translocation is found in 1% of all children with an early pre-B-ALL (cytoplasmic Ig negative) and in 25% of all children with pre-B-ALL (cytoplasmic Ig positive) (2).

Two different transcription factor encoding genes are involved in this translocation: the *PBX1* gene, derived from chromosome 1, and the *E2A* gene derived from chromosome 19 (3, 4). As a result of the translocation, both genes are interrupted and translocated, resulting in a chimeric *E2A-PBX1* fusion gene on chromosome 19.

The E2A-PBX1 fusion gene leads, after transcription and translation, to different E2A-PBX1 proteins (5). Differences between those proteins probably result from alternative splicing. Since E2A-PBX1 proteins are only found in t(1;19)$^{+}$ pre-B-ALL, E2A-PBX1 proteins are per definition *tumor-specific*.

However, chimeric E2A-PBX1 proteins consist of parts of normal cellular proteins, i.e. E2A and PBX1 proteins. Therefore, the actual determinants on the chimeric E2A-PBX1 protein that are truly *tumor-specific*, occur exclusively at the joining region between E2A and PBX1. Due to the translocation process, a new amino acid sequence is created at this joining region, not occurring in normal E2A or in the normal PBX1 proteins.

In the present study we investigate whether the E2A-PBX1 junction is antigenically exposed on the chimeric protein. Our data show that the joining region of E2A-PBX1 proteins can indeed be recognized by polyclonal antibodies, both at the protein and at the cellular level.

Materials and methods

Cell lines
Sup-B27 (a generous gift from Dr S Smith, Department of Pediatric Hematology, University of Chicago, IL, USA) and 697 (a generous gift from Dr JJM van Dongen, Department of Immunology, Erasmus University, Rotterdam, the Netherlands) are cell lines (6) derived from patients formerly diagnosed as t(1;19) positive pre-B-ALL. Nalm-6 and ALL/MIK are cell lines (7) derived from t(1;19) negative pre-B-ALL patients. Since pre-B cells do not express PBX1 proteins, we used the K562 cell line as a PBX protein containing positive cell line (8). The K562 cell line was derived from a patient during blast crisis of CML (9).
Cells were cultured in RPMI medium supplemented with 10% fetal calf serum.

Peptides
A multiple antigenic peptide (MAP) was constructed, essentially as described by Tam (10). This MAP consists of a small immunogenically inert core matrix of lysine residues with α- and ε-amino groups for anchoring eight copies of the E2A-PBX1 peptide. Monomeric peptides corresponding to parts of the amino acid sequences of the E2A , E2A-PBX1 and PBX1 proteins, were elongated during synthesis with a cysteine residue at their carboxyl terminal ends. These peptides were coupled to sulfosuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC) activated bovine serum albumin (BSA) (Pierce, Rockford, IL, USA) via their cysteine residues.

Immunization
Mice (12-week-old female BALB/c) were immunized by intraperitoneal injection of 200 µl of an emulsion of 50 µg MAP antigen in 0.083 M sodium phosphate buffer, 0.9 M NaCl, pH 7.2 and an equal volume of 'Oil in Water' adjuvant (Solvay-Duphar, Weesp, The Netherlands). Mice were given booster injections after 4, 24 and 38 weeks with the same dose of antigen in the same type of adjuvant. Test and final bleeds were taken from the retro-orbital plexus, 2 weeks after each injection.
In this paper we show the results which were obtained with serum of one of these mice (BP 1/19).

Enzyme-linked immunosorbant assay (ELISA)
Antibody capture assay: Wells of polivinylchloride microtiter plates (Falcon 3911; Becton Dickinson, Oxnard, CA, USA) were coated with 150 µl of BSA-peptide conjugate (1 µl/ml) in phosphate buffered saline (PBS), overnight at 4 °C (the composition of PBS in this and in further experiments is: 9.38 mM Na_2HPO_4, 0.88 mM KH_2PO_4, 2.68 mM KCl, 137 mM NaCL, pH 7.8). Free sites were blocked with 0.2% gelatine and 0.05% Tween-20 in PBS (30 min 37 °C). Dilutions of either pre-immune serum or BP 1/19 in PBS supplemented with 0.05% Tween-20 were incubated for 1h at 37 °C. Bound antibodies were detected with

goat anti-mouse Ig conjugated to horseradish peroxidase (Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands) and 3,5,3,5'-tetramethyl-benzidine GR (Merck, Darmstadt, Germany) as substrate. After 15 min at room temperature, the reaction was stopped by the addition of 2M HCl and absorbance was measured at 450 nm.

Antigen competition assay: Due to the hydrophobic nature of the peptides (especially E2A-PBX1), the peptides were dissolved in 6 M guanidine-HCl, 0.01 M phosphate buffer, pH 7.0. Peptides concentrations were determined, using Ellman's reagent (Pierce), by comparing the peptide solutions against a standard curve prepared from cysteine.
Peptides were diluted in the 6 M guanidine-HCl, 0.01 M phosphate buffer, pH 7.0. BP 1/19 in a 10^{-5} dilution was added to different peptide concentrations and allowed to react for 1 h at room temperature. These mixtures were tested on E2A-PBX1-BSA conjugate (1 µg/ml) coated plates. After 1 h incubation at 37 °C, bound antibodies were detected as described above.

Immunoblot analysis
Cells were harvested and washed with PBS. Next, the cells were solubilized (2 x 10^7 cells/ml) in sodium dodecyl sulfate (SDS) sample buffer (60 mM Tris-HCl, pH 6.8, 10% glycerol, 10 mM EDTA, 2% SDS, 2% β-mercaptoethanol, and 0.03% bromophenol blue) and boiled for 10 min. These samples (10 µl/well) were subjected to 10% SDS-PAGE and transferred to nitrocellulose (Mini Protean; Bio Rad, Richmond, CA, USA) in 25 mM Tris, 190 mM glycine and 20% methanol. Nitrocellulose sheets were blocked in low-fat milk for 60 min and subsequently washed three times with 0.5% Tween-20 in PBS. All further washes were performed in this particular buffer.
Sheets were then incubated for 2h at room temperature in the presence of a 10^{-3} dilution of BP 1/19 in 0.5% Tween-20 in PBS. Following three washes, goat anti-mouse Ig conjugated to alkaline phosphatase (Tago, Burlingame, CA, USA) was added to a 10^{-3} dilution and incubation was allowed to proceed for 1h. The blot was washed twice with PBS-Tween and finally with 0.15 M veronal-acetate, pH 9.6. For visualization of antibody-antigen complexes, we used the alkaline phosphatase substrate nitro blue tetrazolium/5-bromo-4-chloroindoxyl phosphate (NBT/BCIP) as described by Blake *et al.* (11).

Specificity controls: Anti-E2A moAb (G98-271.1.3, a generous gift from Drs G Bain and C Murre, Department of Biology, University of California, San Diego, CA, USA), 0.2 µg/ml in 0.5% Tween-20 in PBS was added with or without 10^{-3} diluted BP 1/19 to blots containing proteins from cell lysates of 697 and Nalm-6. After 2h incubation the blots were further treated as described above.
Bacterially expressed PBX1a was biotinylated using N-hydroxy-succinimido-biotin (NHS-biotin; Sigma) to determine its respective molecular weight through electrophoresis and blotting. Biotinylated PBX1a proteins were visualized using alkaline phosphatase labeled streptavidin (South. Biotechn. Ass., Birmingam, AL, USA) and NBT/BCIP as substrate.
In vitro translated E2A-PBX1a, E2A-PBX1b and PBX1a proteins were used, together with bacterially expressed PBX1a proteins to determine the specificity of BP 1/19. The protein samples were boiled in the sample buffer mentioned above, electrophoretically separated, and blotted. After blocking, the blot was incubated with 10^{-3} diluted BP 1/19 in 0.5% Tween-20 in PBS for 2 h at room temperature. Visualization of proteins recognized by BP 1/19 was established by using the same protocol as described above.

Immunofluorescence
697 and Nalm-6 cells were separately washed twice with ice-cold PBS. To discriminate these two cell lines from one another, membrane proteins of 697 (respectively Nalm-6 cells) were biotinylated with N-hydroxy-succinimido-biotin (NHS-biotin; Sigma) as previously described (12), distinguishing them from non-biotinylated Nalm-6 (respectively 697 cells). Biotinylated cells were washed twice in ice-cold PBS and mixed at a 1:1 ratio with non-biotinylated cells. Cells were attached to poly-L-lysine (Sigma) coated slides (10 min room temperature). Next, the cells were fixed using 4% paraformaldehyde (10 min room temperature) and permeabilized with methanol (2 min room temperature). E2A-PBX1 proteins were identified by incubation with antiserum BP 1/19 diluted (10^{-3}) in PBS containing 3% BSA. After 45 min incubation and three subsequent washes in PBS containing 0.025% Tween-20, incubation was proceeded with 10^{-2} diluted FITC-conjugated to goat anti-Ig antibodies (DAKO, Denmark) and 5 x 10^{-5} diluted streptavidin-Cy3 conjugate (Sigma) in PBS containing 2% NHS and 3% BSA. As a specificity control we used the anti-E2A moAb Yae (Santa Cruz Biotechn.,Santa Cruz, CA, USA). Yae recognizes the N-proximal part of E2A proteins, and therefore also recognizes E2A-PBX1 proteins. Two micrograms of Yae/ml were used on a slide containing biotinylated 697 cells and non-biotinylated Nalm-6 cells. Fluorescence was analyzed using confocal microscopy on a Bio Rad MRC-600 confocal system.

Results

Peptides
Based on the previously published nucleotide sequence (3-5), a peptide was constructed corresponding to the E2A-PBX1 junction in E2A-PBX1 proteins. The MAP approach was chosen because of its ability of raising anti-peptide sera which recognize the protein of which the peptide forms a part (10,13).
The MAP system we constructed, consists of a small immunogenically inert (10) core matrix of lysine residues with α- and ε-amino groups capable of anchoring eight copies of the E2A-PBX1 peptide (Figure 1a). This macromolecule has a molecular weight of 16 kDa, which is large enough to serve as an immunogen on its own.
As controls, we synthesized peptides corresponding to the sequences in the normal E2A and PBX1 protein, which occur just around the respective fusion-point of E2A-PBX1 (3, 4, 8) (Figure 1b).

Antibody binding to E2A-PBX1 at the peptide level
Antiserum BP 1/19 was raised against the E2A-PBX1 MAP. This antiserum is liable to contain different antibodies with specificities to different epitopes expressed at the E2A-PBX1 peptide. To elucidate these specificities, PVC plates were coated with maleimide-activated BSA conjugated to either E2A, E2A-PBX1 or PBX1-peptides. BP 1/19 showed strong reactivity against E2A-BSA and E2A-PBX1-BSA conjugates (Figure 2a). This latter observation indicates that BP 1/19 hardly contains any antibodies specific for either PBX1 or BSA. Therefore, we conclude that the strong reac-

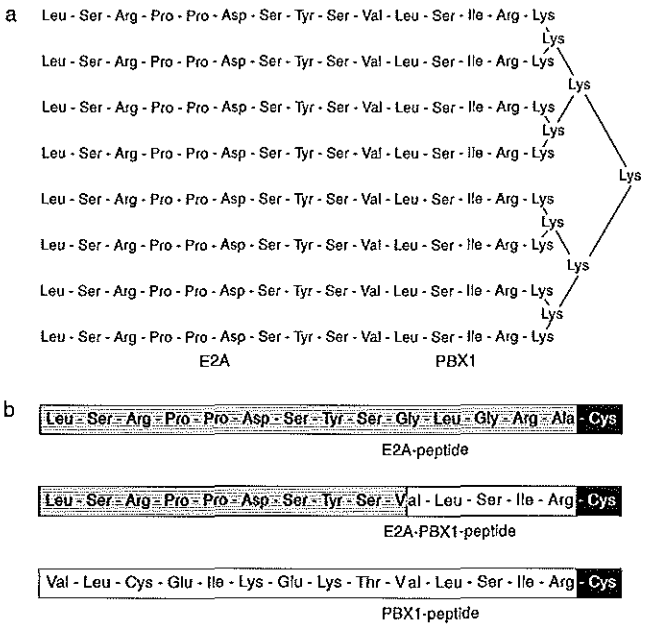

Figure 1 (a) Schematic representation of the multiple antigenic peptide (MAP), consisting of eight fusion-point E2A-PBX1 peptides connected to each other via a lysine core matrix. The molecular weight of MAP is approximately 16 kDa. (b) Schematic representation of the E2A, E2A-PBX1 and PBX1 synthetic peptides. The amino acid sequence of E2A-PBX1 is identical to the sequence of the E2A peptide up to the fusion-point, thereafter the amino acid sequence resembles PBX1. For coupling to a carrier protein each peptide was elongated with a cysteine

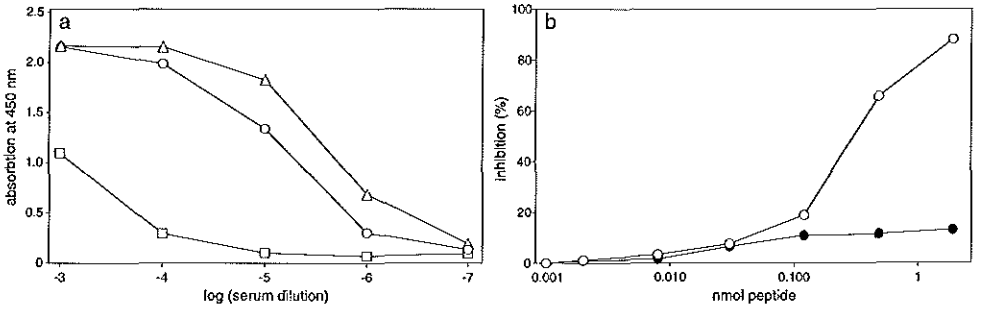

Figure 2 (a) Binding characteristics in an ELISA system of graded dilutions of antiserum BP 1/19 to E2A peptides (○), E2A-PBX1 peptides (△) and PBX1 peptides (□). All peptides were conjugated to maleimide activated BSA. (b) Inhibition study of the binding of 10^{-5} diluted BP 1/19 to either graded concentrations of E2A and PBX1 free peptides (●) or to equimolar graded concentrations of E2A-PBX1 peptide (○). Inhibition is expressed as: [1-{(absorbtion in the presence of peptide)/(absorbtion in the absence of peptide)}] x 100%. Each point represents the mean of a duplicate

tivity against the E2A-PBX1 peptide is caused by antibodies directed against E2A and likely also by antibodies against the fusion-point epitope.
To further elucidate whether antiserum BP 1/19 contains antibodies specific for a E2A-PBX1 fusion-point epitope, we performed inhibition experiments. Figure 2b shows that preincubation of BP 1/19 with the cognate E2A-PBX1 peptide completely abrogated binding of BP 1/19 to E2A-PBX1 peptides conjugated to BSA. Preincubation of BP 1/19 with a mixture of E2A and PBX1 peptides (equimolar to the E2A-PBX1 dilutions) did not show any inhibition at all (Figure 2b).
These data indicate that antiserum BP 1/19 indeed contains antibodies which specifically recognize an E2A-PBX1 fusion-point epitope present on the E2A-PBX1 peptide.

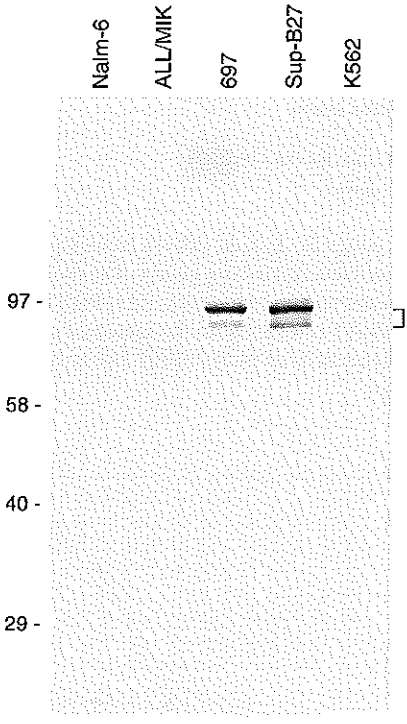

Figure 3

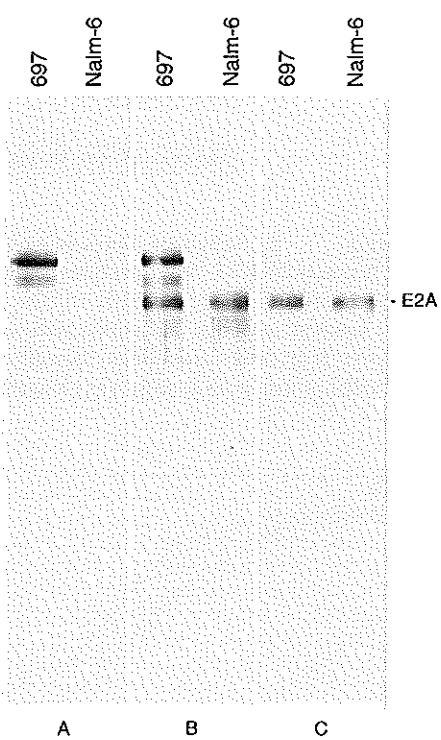

Figure 4

Figure 3 Immunoblot analysis of antiserum BP 1/19. Nalm-6, ALL/MIK, 697 and Sup-B27 are of human pre-B lymphoid cellular origin. Nalm-6 and ALL/MIK are t(1;19)$^-$ cell lines, while 697 and Sup-B27 are t(1;19)$^+$ cell lines. K562 is a t(1;19)$^-$ human erythroid cell line. Each lane represents 2 x 10^5 of the respective cells. Molecular weight in kDa is indicated left, E2A-PBX1 proteins are indicated at the right side of the lanes

Figure 4 Immunoblot analysis of E2A cross-reactivity of antiserum BP 1/19. Blots were incubated with BP 1/19 (a), with a mixture of BP 1/19 and anti-E2A (moAb G98-271.1.3) antibody (b) or with this anti-E2A antibody alone (c). Each lane represents 2 x 10^5 of the respective cells. E2A-PBX1 proteins are indicated left, E2A proteins are indicated at the right side of the lanes

Antibody binding to E2A-PBX1 at the protein level

Binding of antiserum BP 1/19 to E2A-PBX1 proteins was tested by immunoblotting lysates of several cell lines. For the detection of the chimeric E2A-PBX1 proteins, lysates of t(1;19) positive pre-B cell lines, i.e. 697 and Sup-B27, were employed. Lysates of Nalm-6 and ALL/MIK served as t(1;19) negative pre-B cell controls. As the source of normal PBX proteins the K562 cell line (erythroid) was employed (8). Figure 3 clearly shows the detection of E2A-PBX1 proteins in t(1;19) positive cell lines (lanes 3 and 4) but not in t(1;19) negative cell lines (lanes 1, 2 and 5).

Since E2A-PBX1 proteins were recognized by BP 1/19, we conclude that antiserum BP 1/19 does contain antibodies which recognize E2A-PBX1 proteins.

To elucidate whether antiserum BP 1/19 contains antibodies specifically recognizing a *fusion-point* epitope on the E2A-PBX1 proteins and not its counterparts, i.e. E2A or PBX1 proteins, two control experiments were performed.

First, we investigated whether E2A proteins were recognized by BP 1/19. We used moAb G98-271.1.3, an anti-E2A antibody recognizing the C-terminal part of E2A proteins as our control antibody (14). Blots were incubated with BP 1/19 (Figure 4a), moAb G98-271.1.3 (Figure 4c) or a mixture of both (Figure 4b). The data from this experiment indicate that E2A proteins are not recognized by BP 1/19. Only blots incubated with moAb G98-271.1.3 (as single incubation or in combination with BP 1/19) show E2A proteins at approximately 75 kDa. This protein band is clearly missing in

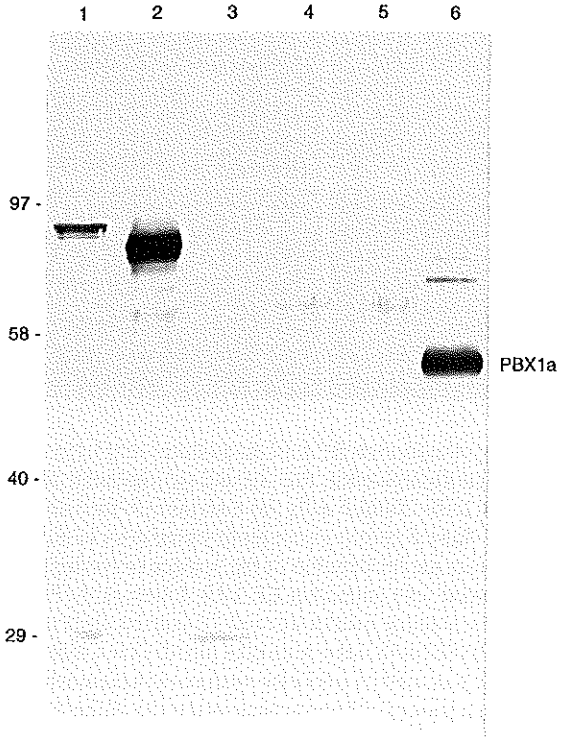

Figure 5 Immunoblot analysis of PBX1 cross-reactivity of antiserum BP 1/19. Blot containing 1 µl *in vitro* translated E2A-PBX1a protein (lane 1), 1 µl *in vitro* translated E2A-PBX1b protein (lane 2), 1 µl *in vitro* translated PBX1a protein (lane 3), bacterially expressed PBX1a protein: 100 ng (lane 4), 40 ng (lane 5). Proteins were detected through BP 1/19 incubation. Alkaline phosphatase-conjugated streptavidin was used to detect the biotinylated bacterially expressed PBX1a protein (40 ng, lane 6). Molecular weight in kDa is indicated left

the blot incubated with BP 1/19 alone. Therefore, we conclude that antiserum BP 1/19 does not cross-react with E2A proteins.
Since PBX1 proteins are not expressed in lymphocytes (8) we had to design another experimental set-up to investigate if BP 1/19 would cross-react with PBX1 proteins. Instead of using anti-PBX1 antibodies as a control, we now used *in vitro* translated PBX1 proteins or bacterially expressed PBX1a proteins. *In vitro* translated E2A-PBX1a proteins and E2A-PBX1b proteins were used as positive controls. Bacterially expressed PBX1a proteins were biotinylated to indicate the molecular weight of recombinant PBX1a proteins. The data from Figure 5 indicate that only *in vitro* translated E2A-PBX1 proteins are recognized by BP 1/19. *In vitro* translated PBX1a and bacterially expressed PBX1a are not recognized by BP 1/19. Therefore, we conclude that BP 1/19 does not recognize PBX1 proteins. (These data are in agreement with our ELISA results.)

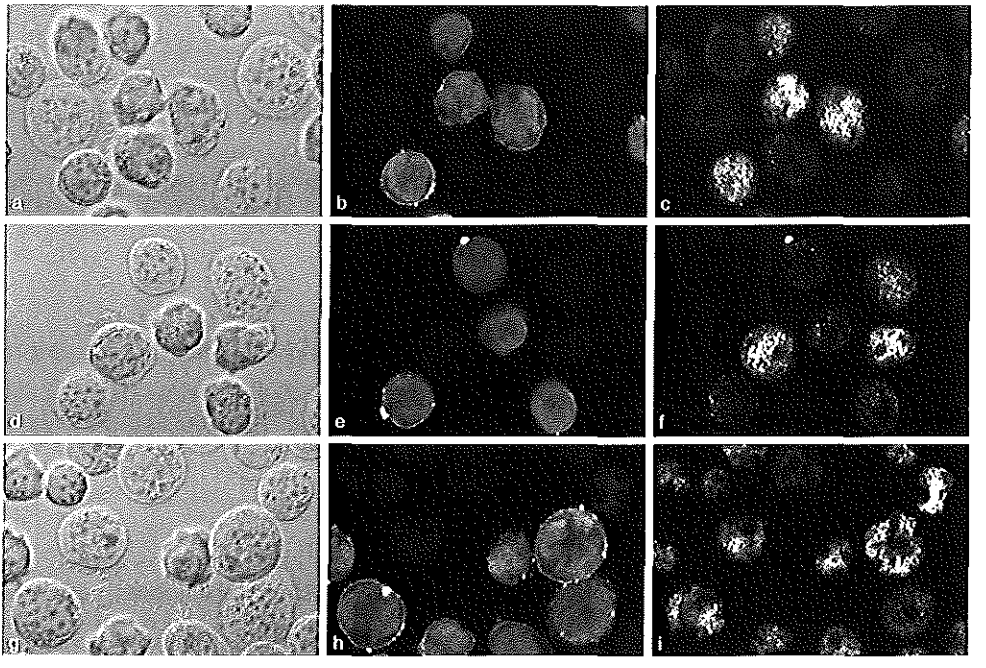

Figure 6 Specificity analysis of the reactivity of antiserum BP 1/19 on mixtures of t(1;19)$^+$ 697 cells and t(1;19)$^-$ Nalm-6 cells using double immunofluorescence. (a), (d) and (g) represent phase photographs of mixtures of t(1;19)$^+$ 697 cells and t(1;19)$^-$ Nalm-6 cells. To discriminate the two cell lines from each other, either 697 cells (b) and (h) or Nalm-6 cells (e) were biotinylated prior to mixing both cell lines. Streptavidin Cy-3 was used to indicate the localization of the respective biotinylated cell line. Cellular mixtures (a) and (d) were incubated with antiserum BP 1/19, followed by FITC-labeled goat anti-mouse immunoglobulins. A nuclear staining pattern ((c) and (f)) can be observed in either biotinylated (b) or non-biotinylated (e) t(1;19)$^+$ 697 cells only. Anti-E2A moAb (Yae), followed by FITC-labeled goat anti-mouse immunoglobulins, was used on a cellular mixture (g) to demonstrate that biotinylated t(1;19)$^+$ 697 cells (h) show the same pattern of fluorescence as t(1;19)$^-$ Nalm-6 cells (i) upon incubation with this non-fusion-point specific antibody

Altogether, BP 1/19 is also at the protein level specific for E2A-PBX1 proteins, as neither E2A nor PBX1 proteins are recognized by BP 1/19.

Antibody binding at the cellular level

We investigated whether BP 1/19 can be used to discriminate t(1;19)$^+$ cells from t(1;19)$^-$ cells, at the single cell level. A mixture of both cell types, i.e. t(1;19)$^+$ 697 cells mixed with t(1;19)$^-$ Nalm-6 cells, was examined. However, before using this cell mixture as a tool to validate the specificity of BP 1/19, we first needed to distinguish the two cell populations from one another by means other than immunostaining with BP 1/19. Therefore, before mixing both cell populations, we biotinylated only one particular cell line. Subsequent incubation with streptavidin Cy-3 allowed us to discriminate biotinylated cells from non-biotinylated cells.

First, we incubated a mixture of biotinylated t(1;19)$^+$ 697 cells and non-biotinylated t(1;19)$^-$ Nalm-6 cells with BP 1/19. Subsequent incubation with a mixture of streptavidin Cy3 and FITC-labeled goat anti-mouse immunoglobulins allowed us to evaluate the specificity of antiserum BP 1/19. Cells in Figure 6c, which correspond to the biotinylated t(1;19)$^+$ 697 cells in Figure 6b, show fluorescent granular nuclei with non-fluorescent nucleoli. In contrast, nuclei from those cells which correspond to the t(1;19)$^-$ Nalm-6 cells are negative.

Thus, these data show that antiserum BP 1/19 is able to discriminate t(1;19)$^+$ cells from t(1;19)$^-$ cells at the single cell level.

However, to exclude the possibility that the biotinylating process on itself influences the detection of t(1;19)$^+$ cells among t(1;19)$^-$ cells through non-specific binding of BP 1/19 to biotin, a control experiment was performed. In this experiment we biotinylated t(1;19)$^-$ Nalm-6 cells instead of t(1;19)$^+$ 697 cells (Figure 6e). Binding of BP 1/19 is visualized in Figure 6f: cells with fluorescent nuclei are clearly detected among non-fluorescent cells. As expected, the fluorescent cells in Figure 6f correspond to the non-biotinylated cells in Figure 6e. Thus, this experiment shows that the detection of t(1;19)$^+$ cells among t(1;19)$^-$ cells, using BP 1/19, is not affected by the biotinylation process and is exclusively caused by binding of BP 1/19 to t(1;19)-positive cells.

Finally, to show that t(1;19)-positive cells can only be specifically detected through recognition of the tumor-specific *fusion-point* of E2A-PBX1 proteins, and not by detecting E2A-PBX1 protein expression alone, we used another anti-E2A (Yae) antibody (15). This antibody recognizes both E2A and E2A-PBX1 proteins. Figure 6i clearly shows that biotinylated 697 cells can not be discriminated from non-biotinylated Nalm-6 cells through the use of Yae antibody.

Therefore, we conclude that BP 1/19 is able to distinguish t(1;19)$^+$ cells from t(1;19)$^-$ cells through a specific nuclear staining in t(1;19)$^+$ cells only.

Discussion

In this study we have shown that the fusion-point in E2A-PBX1 proteins, as it is expressed in t(1;19)$^+$ pre-B-ALL, can act as an accurate target for immuno-diagnostic purposes. Immunization of mice with the appropriate peptide led to the generation of a new polyclonal antiserum BP 1/19, which specifically detects the fusion-point epitope present on E2A-PBX1 proteins in t(1;19)$^+$ pre-B cell lines. The specificity of BP 1/19 for the E2A-PBX1 fusion-point epitope was confirmed at the peptide, the protein and at the single cell level.

BP 1/19 shows high avidity towards E2A-PBX1 peptides and proteins. However, BP 1/19 also recognizes E2A *peptides*. Our peptide inhibition studies using cognate E2A, E2A-PBX1 and PBX1 peptides, indicate that the reactivity of BP 1/19 towards E2A-PBX1 *fusion-point* epitopes can be totally blocked by E2A-PBX1 peptide pre-incubation. In contrast, preincubation of BP 1/19 with a mixture of E2A and PBX1 peptides does not inhibit the reactivity of BP 1/19 towards E2A-PBX1 peptides at all. Therefore, the avidity of BP 1/19 towards E2A-PBX1 *fusion-point* epitopes is likely to be higher than its avidity towards E2A epitopes.

By comparing protein bands on Western blots visualized after incubation with BP 1/19 with or without an anti-E2A antibody (G98-271.1.3), we clearly demonstrate that the E2A proteins are not recognized by BP 1/19. Therefore, although BP 1/19 cross-reacts with E2A *peptides*, E2A *proteins* are not detected by BP 1/19. In this respect, it is acknowledged that anti-peptide sera do not always react with the cognate protein (16). Likewise, reactivity against PBX1 was excluded in our immunoblot experiments using recombinant PBX1a proteins.

Together, these results demonstrate that BP 1/19 contains antibodies which recognize the E2A-PBX1 fusion-point epitope specifically.

Most importantly, BP 1/19 enables us to specifically detect the *tumor-specific* fusion-point epitopes on E2A-PBX1 proteins in t(1;19)$^+$ cells. Cells harboring the t(1;19), i.e. 697, incubated with BP 1/19 show a specific nuclear staining pattern, whereas t(1;19)$^-$ cells, i.e. Nalm-6, do not. If BP 1/19 were to contain anti-E2A and/or anti-PBX1 reactivity, both cell types would have shown a similar staining pattern. We were also able to distinguish other t(1;19)$^+$ cells from t(1;19)$^-$ cells, i.e. t(1;19)$^+$ Sup-B27 cells could be discriminated from t(1;19)$^-$ ALL/MIK cells and normal blood cells (data not shown). The specific staining pattern we noticed, a granular nuclear fluorescence that spares the nucleoli, is similar to the staining pattern observed by Kamps *et al.* (4, 5). In those reported experiments, NIH-3T3 cells infected with virus encoding p77$^{E2A\text{-}PBX1}$, showed a nuclear fluorescence of E2A-PBX1 proteins upon staining with anti-PBX1 polyclonal antibodies. However, due to sensitivity problems of the anti-PBX1 antibodies, Kamps *et al.* (5) were only able to demonstrate strict nuclear localization of E2A-PBX1 proteins in virus (encoding E2A-PBX1) infected cells, but not in t(1;19)$^+$ cells. Likewise, anti-E2A antibodies can not be used to discriminate t(1;19)$^+$ cells from t(1;19)$^-$ cells.

Although the amino acid sequence we used as a t(1;19) *tumor-specific* marker is rela-

tively consistent in t(1;19)$^+$ pre-B-ALL (17), variants in fusion-point sequences have been described. In a minority of t(1;19)$^+$ pre-B cell cases, Izraeli *et al.* (18) reported three patients with a 27-bp insert at the E2A-PBX1 junction, while Numata *et al.* (19) reported a new fusion-point of E2A-PBX1 located downstream of the expected E2A-PBX1 fusion-transcript. We do not expect the 27-bp insert described by Izraeli *et al.* (18) to impair the use of BP 1/19 as a tool to detect t(1;19)$^+$ cells, since these transcripts seem to co-exist with the conventional E2A-PBX1 proteins which are recognized by BP 1/19. Furthermore, it remains to be elucidated whether these aberrant chimeric mRNAs lead to translation into their respective chimeric proteins.

The relatively simple use of antibodies specifically recognizing t(1;19)$^+$ pre-B-ALL cases using immunofluorescence techniques is potentially useful as a supplement to existing techniques (eg. cytogenetic analysis and reverse transcriptase polymerase chain reaction). Children with a t(1;19) positive pre-B-ALL appear to have a worse prognosis as compared to t(1;19) negative pre-B-ALL cases (20, 21). This poorer prognosis can be improved by an intensified chemotherapeutic protocol (22). Accurate diagnostic techniques, such as tumor-specific antibodies, may be helpful when choosing the most appropriate therapy.

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Chapter 5

Specific recognition of t(1;19)$^{E2A\text{-}PBX1}$ positive ALL by polyclonal antibody BP 1/19

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Specific diagnosis of t(1;19)$^{E2A\text{-}PBX1}$ positive ALL by polyclonal antibody BP 1/19

The t(1;19) is one of the most frequently occurring chromosomal translocations in childhood acute lymphoblastic leukemia (ALL). This translocation results from the reciprocal exchange of chromosomal material between the two chromosomes involved. As a consequence, a new chimeric gene, termed E2A-PBX1 is generated. The close association of this particular chromosomal translocation with pre-B-ALL provides several tumor-specific markers for both diagnosis as well as detection of residual t(1;19)$^{E2A\text{-}PBX1}$ leukemic cells during and after treatment. At diagnosis, conventional cytogenetics and reverse transcriptase polymerase chain reaction (RT-PCR) are used for identifying the tumor-specific chromosomal aberration or the tumor-specific E2A-PBX1 mRNA, respectively. The RT-PCR technique can also be used for detection of small numbers of residual leukemic cells during and after leukemia treatment.
Here, we report on the specific diagnosis of t(1;19)$^{E2A\text{-}PBX1}$ positive pre-B-ALLs by using immunofluorescence microscopy. The specific detection of the chimeric E2A-PBX1 proteins at the cellular level with our recently described polyclonal antiserum BP 1/19, enables us to distinguish t(1;19)$^{E2A\text{-}PBX1}$ positive pre-B-ALLs from t(1;19) negative precursor-B-ALLs. The specificity and sensitivity of the BP 1/19 immunostaining technique are evaluated and discussed in view of currently used diagnostic methods.

Introduction

The t(1;19) is one of the most common cytogenetic aberrations observed in childhood acute lymphoblastic leukemia (ALL) and is associated with approximately 25% of cases with a pre-B immunophenotype (1). Molecular studies have shown that t(1;19) results in fusion of the *E2A* gene, a basic Helix Loop Helix (b-HLH) coding gene on chromosome 19, with *PBX1*, a homeobox coding gene on chromosome 1 (2, 3). The *E2A-PBX1* fusion-gene, located on chromosome 19, expresses fusion mRNAs composed of 5' E2A sequences and 3' PBX1 sequences. The fusion-site between E2A and PBX1 mRNA sequences is highly conserved in different t(1;19) positive leukemias (4). This conserved fusion-site has been successfully utilized to detect t(1;19) positive pre-B-ALLs by reverse transcriptase polymerase chain reaction (RT-PCR) (5, 6, 7). In a previous study we have shown that this fusion-point in mRNA leads to a consistent fusion-point epitope at the protein level, which can be used as an immunological target. We demonstrated that BP 1/19, a polyclonal antiserum from mice immunized with a peptide resembling the amino-acid sequence of the E2A-PBX1 fusion-point epitope, is capable of recognizing the E2A-PBX1 fusion-point specifically, both at the peptide, the protein and at the microscopic level (8).

In the present study, we evaluate the application of BP 1/19 immunostaining for specific diagnosis of t(1;19)$^{E2A-PBX1}$ positive leukemias. Specificity and sensitivity of the BP 1/19 staining technique were both determined on various human hematopoietic cell lines. Moreover, blood or bone marrow samples from patients previously diagnosed as t(1;19) positive pre-B-ALL revealed the characteristic nuclear staining of E2A-PBX1 proteins upon BP 1/19 immunostaining.

Materials and methods

Cell samples

Cell lines. Twenty-seven human cell lines were used to study the expression of E2A-PBX1 proteins and E2A proteins (see also Table I): two precursor-B-cell lines derived from patients with severe combined immunodeficiency (SCID) (RF and YF); 12 precursor-B-cell lines (REH; HAL-01; BV173; TOM-1; NALM-6; 207; SMS-SB; MIK-ALL; 697; SUP-B27; RCH-ACV and UoC-B5); two Burkitt cell lines (NAMALWA and RAJI); two mature B-cell lines (KCA and CAN-1); two plasma cell lines (L363 and LP-1); three T-cell lines (MOLT-4; HUT-78 and DND-41); two myeloid cell lines (KCL-22 and KYO-1) and two erythroid cell lines (LAMA-84 and K562) (9, 10, 11). All cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS).

Leukemic cell samples. Cell samples were obtained from 14 patients with precursor-B-ALL. Mononuclear cells (MNC) were isolated from peripheral blood (PB) or bone marrow (BM) by Ficoll-Paque (density: 1.077 g/ml; Pharmacia, Uppsala, Sweden) density centrifugation. Immunologic and cytogenetic analysis were performed as part of the diagnostic procedure. Remaining cells were stored in liquid nitrogen.

Immunofluorescence staining technique

Cell lines. Cell lines were harvested in their logarithmic growth phase and washed twice with ice-cold phosphate buffered saline (PBS: 9.38 mM Na_2HPO_4, 0.88 mM KH_2PO_4, 2.68 mM KCl, 137 mM NaCl, pH 7.8). Cells were attached to poly-L-Lysine (Sigma, St Louis, MO, USA) coated slides (10 min, room temperature, RT). Next, the cells were fixed using 4% paraformaldehyde (10 min, RT) and permeabilized with methanol (2 min RT). Cells were either incubated with antiserum BP 1/19 (1:1000 in PBS containing 3% BSA) or with anti-E2A monoclonal antibody (moAb) G98-271.1.3 (a generous gift from Drs G Bain and C Murre, Department of Biology, University of California, San Diego, CA, USA) at a concentration of 2 µg / ml PBS containing 3% BSA. After 45 min incubation and three subsequent washes with PBS containing 0.025% Tween-20, incubation was proceeded (30 min, RT) with 10^{-2} diluted fluorescein isothiocyanate conjugated to goat anti-mouse immunoglobulin antiserum (GαM-FITC; DAKO, Glosstrup, Denmark). Excess reagent was washed off and slides were mounted in glycerol / PBS (9:1), containing 1 mg phenylenediamine / ml.

Artificial mixing. Cryopreserved PB-MNC cells were carefully thawed and resuspended in ice-cold RPMI-1640 medium supplemented with 10% FCS. After washing with ice-cold PBS, PB-MNC were mixed with biotinylated t(1;19) positive 697 cells. Cells were bio-

tinylated as described previously (12). Biotinylated cells were detected by 5 x 10^{-5} diluted streptavidin-Cy3 conjugate (Sigma, St Louis, MO, USA).

Leukemic cell samples. Cryopreserved leukemic cell samples were carefully thawed and resuspended in RPMI-1640 medium supplemented with 10% FCS. Next, samples were washed twice in ice-cold PBS; fixation, permeabilization and immunostaining were performed as described above.

Microscopes. Immunofluorescence was evaluated using a Zeiss Axioscop fluorescence microscope (Carl Zeiss, Oberkochen, Germany), equipped with a 100 Watt mercury lamp and phase contrast facilities and contains a x63, NA 1.4, Planapochromat oil immersion objective (Zeiss). Micrographs were recorded by using a confocal laser scanning microscope, equipped with an air-cooled 488 nm argon laser (CLSM 410 Invert Confocal Microscope, Zeiss). The sensitivity of the CLSM (i.e. gain and offset) was set at a constant level. From this point on, no further readjustments were made and t(1;19) positive and t(1;19) negative samples were recorded successively.

Results

Determination of the specificity of the BP 1/19 immunofluorescence staining technique

To investigate whether BP 1/19 can be used as a diagnostic tool, allowing specific diagnosis of t(1;19)$^{E2A-PBX1}$ positive pre-B-ALL by immunofluorescence microscopy, we evaluated the specificity of BP 1/19 immunostaining on an extended panel of hematopoietic cell lines representing various differentiation stages.

Table I presents the data of the immunofluorescence microscopic evaluation of E2A-PBX1 protein expression in twenty-seven cell lines. E2A-PBX1 proteins were detected by antiserum BP 1/19 followed by GαM-FITC. Only cell lines with previously reported t(1;19) translocations (i.e. 697, SUP-B27, RCH-ACV and UoC-B5) (11) showed a speckled nuclear staining that spared the nucleoli. Nuclear staining was not observed upon BP 1/19 immunostaining in cells with chromosomal aberrations other than t(1;19) (11).

However, the negative BP 1/19 nuclear staining in t(1;19) negative cells could also be explained by inefficient fixation and permeabilization procedures. Since cellular membranes are in principle impermeable to antibodies, intra-nuclear staining is only possible when cells are properly fixed and permeabilized. To assure immunoglobulin accessibility to the nuclei, we examined the nuclear staining patterns of E2A proteins by using immunofluorescence microscopy. As E2A proteins are ubiquitously expressed in the nuclei of various cell types (13, 14), immunofluorescence analysis of anti-E2A protein staining would address our contentions considering nuclear permeability. MoAb G98-271.1.3 was chosen for its unique reactivity towards E2A proteins (8, 14).

Table I presents the data of the immunofluorescent microscopic evaluation of E2A protein expression in twenty-seven cell lines. Almost all cell lines tested showed an anti-E2A protein staining pattern, comparable to the speckled nuclear staining pattern

Table I.
Immunostaining of E2A-PBX1 and E2A proteins in Human Hematopoietic Cell Lines

Hematopoietic cell lines	t(1;19)(q23;p13)[a]	BP 1/19[b]	G98-271.1.3[c]
B-cell lines			
SCID cell lines			
RF	–[1]	–	+
YF	–[1]	–	+
precursor-B-cell lines			
REH (Cμ-)	–[1]	–	++
HAL-01 (Cμ-)	–[1,2]	–	++
BV173 (Cμ-)	–[1,2]	–	++
TOM-1 (Cμ-)	–[1,2]	–	++
NALM-6 (Cμ+)	–[1,2]	–	++
207 (Cμ+)	–[1]	–	++
SMS-SB (Cμ+)	–[1]	–	++
MIK-ALL (Cμ+)	–[1,2]	–	++
697 (Cμ+)	+[1,2]	++	++
SUP-B27 (Cμ+)	+[1,2]	++	++
RCH-ACV (Cμ+)	+[1,2]	++	++
UoC-B5 (Cμ+)	+[2]	+	+
Burkitt cell lines			
NAMALWA	–[1,2]	–	+++
RAJI	–[1]	–	++
Mature cell lines			
KCA (IgM+)	–[1]	–	++
CAN-1 (IgG+)	–[1]	–	(–)
Plasma cell lines			
L363	–[1]	–	++
LP-1	–[1]	–	++
T-cell lines			
MOLT-4 (SmCD3-)	–[3]	–	++
HUT-78 (SmCD3+)	–[1]	–	++
DND-41 (SmCD3+)	–[1]	–	+
Myeloid cell lines			
KCL-22	–[1,2]	–	+
KYO-1	–[1,2]	–	+
Erythroid cell lines			
LAMA-84	–[1,2]	–	+
K562	–[1,2]	–	+

[a] chromosomal aberrations other than t(1;19) are not presented by this table.

[b] BP 1/19 polyclonal antibody, recognizing the tumor-specific E2A-PBX1 fusion-point epitope (8).

[c] G98-271.1.3 monoclonal antibody, recognizing the carboxyl terminus of E2A proteins (14).

[1] as documented by the Department of Cell Biology and Genetics, Erasmus University Rotterdam.

[2] as documented by Drexler *et al.* (11).

[3] as documented in ATCC, CELL LINES and Hybridomas, 8th edition, 1994.

observed upon anti-E2A-PBX1 protein staining by BP 1/19 antiserum. However, depending on the cell line investigated, the intensity of nuclear fluorescence varied from a strong fluorescence, as observed in the NAMALWA cell line, to practically non-detectable in the CAN-1 cell line. Since almost all cell lines (except CAN-1) displayed the expected nuclear anti-E2A fluorescence patterns, we conclude that intra-nuclear staining by antibodies is, in principle, possible upon the fixation and permeabilization procedures we used.
Therefore, we conclude that among the twenty-seven hematopoietic cell lines tested, BP 1/19 exclusively detects the t(1;19)$^{E2A\text{-}PBX1}$ positive cell lines.

Determination of the sensitivity of the BP 1/19 immunofluorescence staining technique
To determine the sensitivity of the BP 1/19 immunofluorescence staining technique, artificial mixtures of 697 cells (i.e. a t(1;19)$^{E2A\text{-}PBX1}$ positive cell line) and normal PB-MNC were examined. To exclude false positive staining by BP 1/19 antibodies, we decided to label t(1;19) positive cells also in a different way. Therefore, t(1;19) positive cells were biotinylated before mixing them with normal PB-MNC. Subsequent incubation with Cy-3 conjugated to streptavidin permitted us to discriminate between biotinylated t(1;19) positive cells and normal PB-MNC, independent of the immunostaining results with BP 1/19.

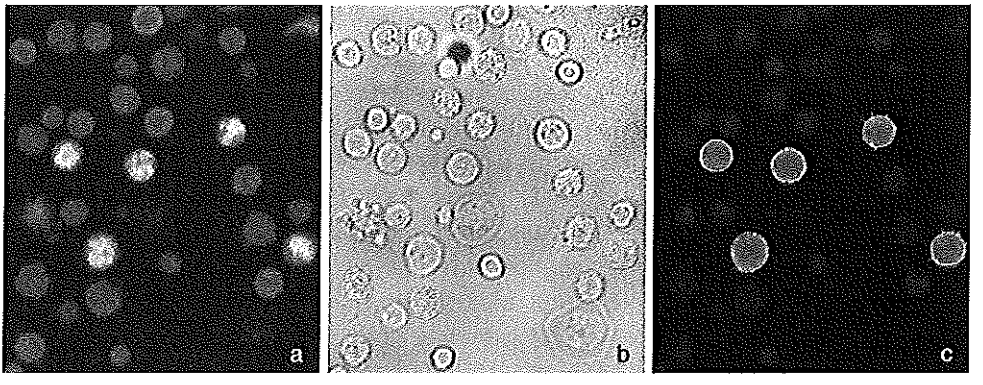
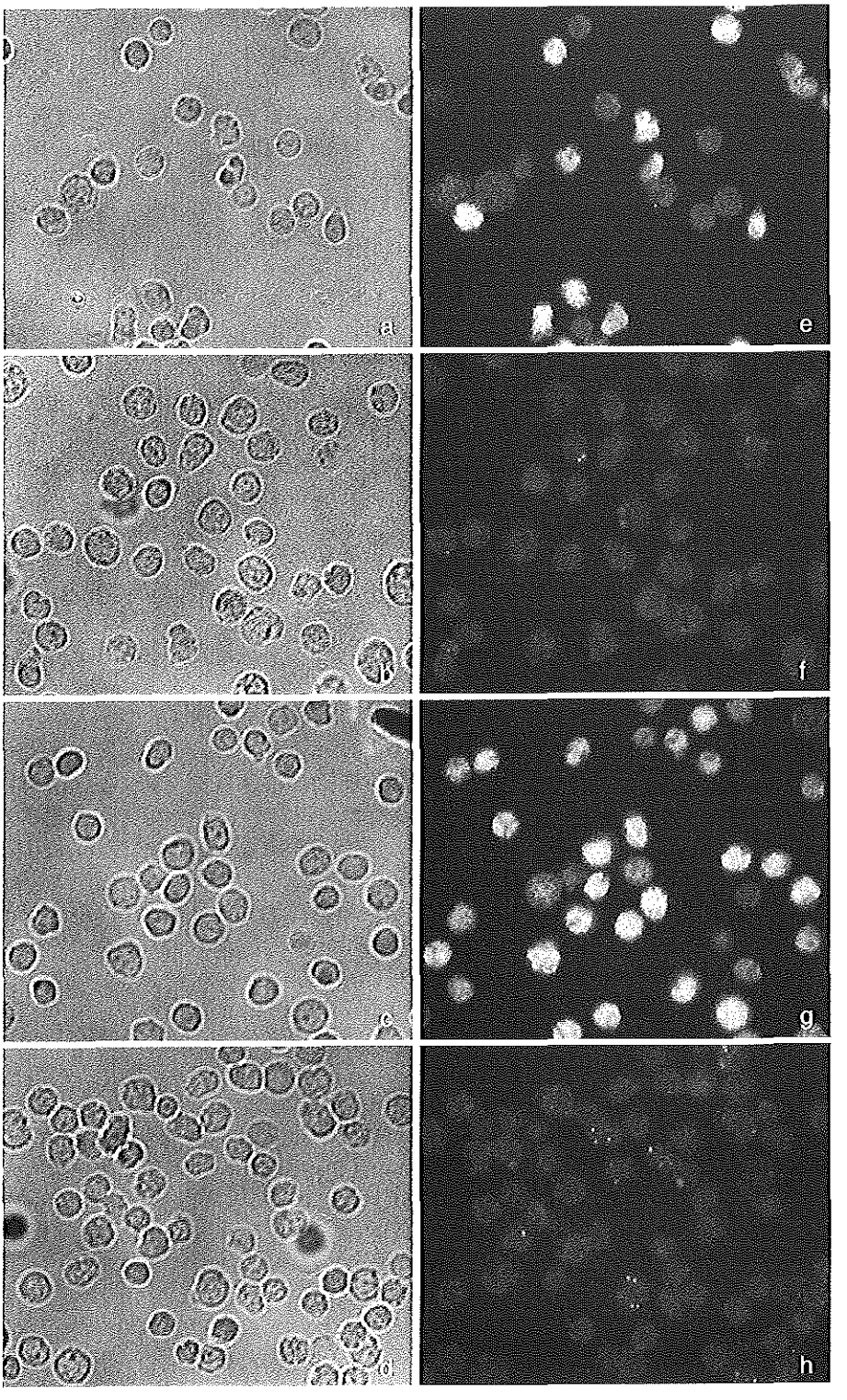


Figure 1 A typical example of the sensitivity analysis of the BP 1/19 staining technique. The sensitivity was determined by evaluating BP 1/19 nuclear staining of t(1;19) positive cells mixed at different ratios with normal PB-MNC, by using double immunofluorescence microscopy. Bound BP 1/19 antibodies were detected with FITC-conjugated to second step reagent. To identify all t(1;19) positive cells independent of BP 1/19 staining, t(1;19) positive cells were biotinylated before mixing with normal PB-MNC. Biotinylated cells were detected by a Cy-3 fluorochrome conjugated to streptavidin. (a), (b) and (c) represent micrographs of the same field of observation. In these micrographs: (a) a typical nuclear staining can be observed in five cells upon evaluating BP 1/19 staining, (b) represents the bright-field micrograph of the mixture of biotinylated t(1;19) positive cells and normal peripheral blood cells, (c) identifies all biotinylated t(1;19) positive cells by using Cy-3 conjugated to streptavidin. Comparing figures (a) and (c) demonstrates that all biotinylated t(1;19) positive cells are identified by BP 1/19.

Figure 1 shows an example from the sensitivity analysis of the BP 1/19 immunostaining technique. Typical intranuclear fluorescent speckled nuclei are observed in all biotinylated t(1;19)$^{E2A\text{-}PBX1}$ positive cells upon evaluating BP 1/19 immunostaining. This result indicates that antiserum BP 1/19 specifically detects t(1;19)$^{E2A\text{-}PBX1}$ positive cells. False positive staining is excluded since nuclei of normal PB-MNC are not stained by BP 1/19 (Figure 1).
To determine the maximum sensitivity of the BP 1/19 staining technique, artificial mixtures of different ratios between t(1;19)$^{E2A\text{-}PBX1}$ positive cells and normal PB-MNC were examined. An almost perfect correlation between biotinylated t(1;19)$^{E2A\text{-}PBX1}$ positive cells and BP 1/19 staining was observed. Although not all t(1;19)$^{E2A\text{-}PBX1}$ positive cells were identified upon evaluating BP 1/19 immunostaining (i.e. BP 1/19 immunostaining was observed in approximately 80% of all biotinylated t(1;19)$^{E2A\text{-}PBX1}$ positive cells), E2A-PBX1 protein staining was still detected in almost all biotinylated t(1;19)$^{E2A\text{-}PBX1}$ positive cells upon examination of 10^{-4} mixtures of biotinylated t(1;19)$^{E2A\text{-}PBX1}$ positive cells and normal PB-MNC.

Can BP 1/19 be applied for specific diagnosis of t(1;19)$^{E2A\text{-}PBX1}$ positive pre-B-ALL?
To investigate the value of antiserum BP 1/19 for diagnosing t(1;19)$^{E2A\text{-}PBX1}$ positive pre-B-ALL, cryopreserved leukemic PB or BM samples from thirteen children at diagnosis were examined by using fluorescence microscopy. Immunologic and cytogenetic data were available from all samples: four t(1;19) positive pre-B-ALL; four t(1;19) negative pre-B-ALL; four t(1;19) negative common-ALL and one blast crisis in chronic myeloid leukemia (CML). All samples were simultaneously processed and E2A-PBX1 protein expression was evaluated by using a CLSM.
As can be expected in PB or BM samples from patients examined at first diagnosis; 50% to 70% of cells from t(1;19)$^{E2A\text{-}PBX1}$ positive samples demonstrated the characteristic nuclear fluorescence of E2A-PBX1 proteins upon BP 1/19 immunostaining (Figures 2e and 2g). Comparable results were found in all four investigated t(1;19) positive pre-B-ALL. In contrast, control samples derived from leukemic patients without cytogenetically detectable t(1;19) did not demonstrate the characteristic nuclear fluorescence staining pattern. Typical BP 1/19 staining of t(1;19) negative PB samples and t(1;19) negative BM samples are represented by Figures 2f and 2h, respectively. Although a low level of background immunofluorescence can be observed in t(1;19)

Figure 2 BP 1/19 staining of t(1;19) positive and t(1;19) negative blood or bone marrow samples from patients suffering from acute lymphoblastic leukemia. The bright-field micrographs (a-d) represent the same microscopic field as observed in adjacent fluorescence micrographs (e-h). A typical BP 1/19 staining is observed in samples derived from patients with a t(1;19) positive pre-B ALL: approximately 50% of peripheral blood cells (a and e) and approximately 70% of bone marrow cells (c and g) demonstrate the typical nuclear fluorescence. In contrast, BP 1/19 staining of peripheral blood (f) or bone marrow (h) cells, derived from patients suffering from respectively t(1;19) negative c-ALL or t(1;19) negative pre-B ALL does not result in the typical BP 1/19 nuclear staining, although few cytoplasmic fluorescent dots can be observed in either peripheral blood cells (b and f) or bone marrow cells (d and h).


a
e
f
g
h

negative cells, sometimes superimposed by a bright cytoplasmic fluorescent dot (Figures 2f and 2h), the typical nuclear fluorescence observed in t(1;19) positive samples is clearly absent (cf Figures 2e and 2f or 2g and 2h).
Thus, while the characteristic speckled nuclear pattern is detected in all t(1;19) positive pre-B-ALL, none of the t(1;19) negative samples demonstrated this particular nuclear fluorescence upon BP 1/19 staining. These results indicate that BP 1/19 can be applied for specific diagnosis of t(1;19)$^{E2A\text{-}PBX1}$ positive ALLs, by using immunofluorescence microscopy.

Discussion

The t(1;19) was recognized as a non-random recurring translocation in childhood ALL in the early 1980s (15, 16). The aberrant t(1;19) karyotype has been identified in 25% of pediatric pre-B-ALL (15, 17). Initially, the detection of t(1;19) proved to be of prognostic value, as the t(1;19) was associated with poor prognosis (18). Later on, when treatment protocols improved, the prognosis of the t(1;19) positive pre-B-ALL improved concurrently (19, 20).
The close association of the t(1;19) or its products (i.e. the chimeric fusion-gene, fusion-mRNA, or chimeric fusion-protein) with leukemia, provides excellent tumor-markers for both diagnosis of the t(1;19)$^{E2A\text{-}PBX1}$ positive leukemias as well as monitoring residual disease during or after treatment. To date, a number of diagnostic techniques are available to distinguish between leukemic cells and their normal hematopoietic counterparts (21). When considering applicabilty and sensitivity of the assay in tumor-diagnosis, it is realized that each of these techniques has its own specifications. At primary diagnosis, conventional cytogenetics is generally the method of first choice, as it permits unambiguous identification of the various chromosomal abnormalities occurring in different leukemias (1). However, the success of conventional cytogenetics largely depends on the number of metaphases that can be examined. Thus, if leukemic cells die in short-term culture or do not enter mitosis, a false negative result may be obtained; a phenomenon likely to occur when small numbers of leukemic cells are present. In contrast to the relatively low sensitivity of cytogenetic analysis, the PCR technique is extremely sensitive: allowing detection of a single leukemic cell among 10^5 to 10^6 normal cells (21). Yet, the PCR cannot be used for detecting the genetic t(1;19) abnormality at the DNA level. As fusion-points are scattered in a large *E2A-PBX1* fusion intron, PCR amplification of the tumor-specific fusion-point at the DNA level would result in sequences of too variable length. The PCR procedure can be applied after reverse transcription (RT) of mRNA into cDNA. Since fusion-points are highly consistent at the mRNA level in most t(1;19)$^{E2A\text{-}PBX1}$ positive cases (4), the RT-PCR method has been applied successfully for both specific diagnosis as well as the specific detection of minimal residual disease (5, 6, 7). Residual t(1;19)$^{E2A\text{-}PBX1}$ positive tumor cells are sometimes still detected by RT-PCR 84 months after chemotherapy had been started (7). It is difficult to verify reduction of residual disease during

or after treatment. Premature mRNA degradation and/or variable efficiency of reverse transcription are among the causes that hamper accurate quantification of residual tumor cells by using RT-PCR techniques (22).

Contrary to conventional cytogenetics and RT-PCR techniques, immunologic techniques appear to identify more clearly patients at a high risk of relapse (22). Moreover, immunofluorescence techniques are commonly available in clinical settings, are relatively simple, and require only a few hours to obtain a result. However, single immunological marker analysis by tumor-specific antibodies is still not suitable for distinguishing neoplastic cells from normal hematopoietic cells. The current immunologic strategy for detecting residual disease takes advantage of the observation that combinations of single leukocyte markers may be found on malignant cells that are normally not observed in PB or BM (22). Although double immunological marker analysis gives excellent results in T-ALL and a large part of acute myeloid leukemias (AML), this technique is of limited value in ALL of B-cell lineage (21, 22).

In the present study we show that t(1;19)$^{E2A\text{-}PBX1}$ positive ALL with a pre-B immunophenotype are specifically detected through specific E2A-PBX1 protein staining with our BP 1/19 antiserum, by using single immunological marker analysis.

Specificity analysis on a large panel of cell lines, representing various hematopoietic differentiation stages, showed that t(1;19)$^{E2A\text{-}PBX1}$ positive cells *exclusively* display the characteristic nuclear fluorescence upon BP 1/19 immunostaining. None of the t(1;19) negative cell lines stained by BP 1/19 showed noticeable staining beyond background. In contrast, almost all hematopoietic cell lines tested demonstrated, upon staining with anti-E2A antibodies, a nuclear granular fluorescence pattern that spared the nucleoli. Although E2A proteins are considered to be ubiquitously expressed among various cell types (13, 14), anti-E2A protein staining of the mature B cell line CAN-1 by moAb G98-271.1.3, unexpectedly did not result in a noticeable nuclear fluorescence. Our recent Western blotting experiments (according to the method described previously (8)) confirmed the absence of E2A proteins in CAN-1 cells (results not shown).

The sensitivity of the BP 1/19 immunostaining technique was determined as one t(1;19)$^{E2A\text{-}PBX1}$ positive cell among 10,000 normal PB-MNC. However, one should realize that, due to variable expression of E2A-PBX1 proteins, not all t(1;19) positive cells are detected. This variable protein expression not only concerns E2A-PBX1 proteins in t(1;19) positive cells, but also E2A proteins in all cell lines tested when stained for E2A protein expression. The noticed variability of E2A-PBX1 and E2A protein expression between investigated cell lines probably accounts for differences in cell cycle progression (23). Indeed, mitotic cells are difficult to identify upon BP 1/19 or G98-271.1.3 immunostaining. The absence of nuclear staining in mitotic cells can be explained by breakdown of the nuclear envelope in cells entering mitosis, causing E2A-PBX1 proteins and E2A proteins to disperse throughout the cytoplasm. Pulford *et al.* found similar results upon immunostaining for TAL1 protein expression (24). TAL1 proteins belong to the same b-HLH family of transcription factors as E2A proteins (24).

Final proof of specific immunostaining by BP 1/19 antibodies was provided by analyzing the immunofluorescence patterns of samples from t(1;19) positive and t(1;19) negative leukemic patients. A clear granular nuclear staining was observed in t(1;19) positive ALL PB and BM samples, but not in t(1;19) negative ALL PB or BM samples. Although nuclear staining was absent in t(1;19) negative samples, we did observe a diffuse background staining that was sometimes superimposed by a bright cytoplasmic fluorescent dot. These cytoplasmic fluorescent dots probably explain our failure to distinguish $t(1;19)^{E2A\text{-}PBX1}$ positive cells from t(1;19) negative cells when using BP 1/19 in preliminary flow cytometric studies (data not shown).
Although BP 1/19 is a powerful reagent (1:1000 dilutions are optimal) and small aliquots can be used for fluorescence microscopy (50 µl of diluted BP 1/19 per sample), monoclonal antibodies directed towards the tumor-specific fusion-point of E2A-PBX1 proteins would be favored. Moreover, such fusion-point specific antibodies should allow double or even triple labeling studies on the same PB sample or BM sample.

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Chapter 6

Recognition of the *tumor-specific* E2A-PBX1 fusion-point epitope by monoclonal antibody ER-GO4

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Recognition of the *tumor-specific* E2A-PBX1 fusion-point epitope by monoclonal antibody ER-GO4

The t(1;19) chromosomal translocation is observed in 25% of pediatric pre-B-cell acute lymphoblastic leukemias (ALL) and results in the expression of chimeric E2A-PBX1 proteins. As such, E2A-PBX1 proteins can be considered as tumor-specific markers that could be useful for diagnosing t(1;19)$^{E2A\text{-}PBX1}$ positive ALL and monitoring residual t(1;19)$^{E2A\text{-}PBX1}$ positive leukemic cells during and after leukemia treatment. However, since the tumor-specific E2A-PBX1 proteins consist of amino-acid sequences also present in non tumor-specific E2A and PBX1 proteins, the exclusive identification of E2A-PBX1 proteins using antibodies is hampered by non tumor-specific epitopes also present on E2A- and PBX1 proteins. One of the most obvious antigenic determinants that can be applied as a tumor-specific target is located at the fusion-point of E2A-PBX1 proteins. In a previous study we have demonstrated that the E2A-PBX1 fusion-point is presented in an antigenic fashion which allows specific identification of t(1;19)$^{E2A\text{-}PBX1}$ positive cells by using fluorescence microscopy. We now report on the generation of an anti E2A-PBX1 fusion-point specific monoclonal antibody, termed ER-GO4. Its reactivity towards the fusion-point epitope of E2A-PBX1 proteins was confirmed at the peptide, the protein, and at the cellular level. We show that this antibody is not only of practical interest considering diagnosis of t(1;19)$^{E2A\text{-}PBX1}$ positive ALL, but is also of academic interest, since moAb ER-GO4 recognizes novel intranuclear structures in E2A-PBX1 transfected COS-cells.

Introduction

The chromosomal translocation t(1;19), found in 5% to 6% of pediatric acute lymphoblastic leukemia, results in the expression of chimeric E2A-PBX1 fusion proteins (1). The pathogenetic relation of t(1;19) with neoplasia was suggested in the early 1980s, as t(1;19) was recognized as a non-random recurring translocation in childhood acute lymphoblastic leukemia (2, 3). Immunophenotyping has shown this particular translocation to be present in 20-25% of pediatric pre-B-ALL (4, 5, 6).
The *E2A* gene (encoding transcription factors: E12, E47 (7) and E2-5 (8), collectively known as E2A proteins), located within chromosome band 19p13, was found to be rearranged in t(1;19) positive pre-B-cell leukemias (9). Nourse *et al.* and Kamps *et al.* discovered that the t(1;19) results in replacement of the 3' terminus of *E2A* with exons from the *PBX1* gene (a homeobox gene) on chromosome 1 (10, 11). Since its discovery, the involvement in leukemogenesis of *E2A-PBX1* and its products have been implicated by several studies (12, 13, 14).
It has been suggested that the detection of the *E2A-PBX1* gene or its products, rather than the detection of the aberrant karyotype is of prognostic value (15). Variant t(1;19) positive leukemias, lacking *E2A* or *PBX1* aberrations, appear to have a better progno-

sis than t(1;19) positive leukemias with rearranged *E2A-PBX1* genes, when treated with less intensive chemotherapy (15).
The fusion of E2A and PBX1 genes in t(1;19)$^{E2A\text{-}PBX1}$ positive leukemias results in a remarkable consistent fusion-point at the mRNA level (16). The specific detection of the E2A-PBX1 fusion-point by reverse transcriptase polymerase chain reaction (RT-PCR) has been shown to be a powerful tool, at both diagnosis as well as remission of t(1;19)$^{E2A\text{-}PBX1}$ positive leukemias (17, 18).
We have recently demonstrated that the fusion-point of the chimeric E2A-PBX1 protein is presented in an antigenic fashion (19). Our BP 1/19 polyclonal antiserum, specifically detects t(1;19)$^{E2A\text{-}PBX1}$ positive ALLs by immunofluorescence microscopy (submitted).
We now report on the establishment of a new hybridoma cell line, producing monoclonal antibodies directed towards the fusion-point epitope of E2A-PBX1 proteins. The reactivity of this monoclonal antibody (moAb), which we termed ER-GO4 was tested at the peptide, the protein and at the cellular level. We show that this antibody is not only of practical interest considering diagnosis of t(1;19)$^{E2A\text{-}PBX1}$ positive ALL, but is also of academic interest since moAb ER-GO4 recognizes novel intranuclear structures in E2A-PBX1 transfected COS-cells.

Materials and methods

Cell lines

RCH-ACV (a generous gift from Dr M Cleary, Laboratory of Experimental Pathology, Department of Pathology, Stanford University School of Medicine, Stanford, CA, USA), SUP-B27 (a generous gift from Dr S Smith, Department of Pediatric Hematology, University of Chicago, IL, USA) and 697 are cell lines formerly diagnosed as t(1;19) positive pre-B-ALL (10, 20, 21). NALM-6 and REH cell lines were used as t(1;19) negative pre-B-cell controls. HAL-01 (a generous gift from Dr K Ohyashiki (First Department of Internal Medicine, Tokyo Medical College, Tokyo, Japan) is derived from a patient diagnosed as t(17;19) pro-B-ALL (22). SP 2/0 cells were used as a fusion partner for hybridoma formation. The transformed monkey kidney epithelial cell line, COS, was used for transfection experiments.

Peptides

Peptides have been described previously (19). The MAP antigen consists of eight E2A-PBX1 peptides anchored to a branched core-matrix of lysine amino acids. The amino acid sequences of monomeric E2A, E2A-PBX1 and PBX1 peptides are given in Figure 1b.

Immunization and production of the ER-GO4 hybridoma

BALB/c mice were immunized by intraperitoneal (ip) injection of 200 µl of an emulsion of 50 µg MAP antigen in 0.083 M sodium phosphate buffer, 0.9 M NaCl, pH 7.2 and an equal volume of Complete Freund's Adjuvant. These mice were given repeatedly booster injections (minimal time interval of one month between booster injections) ip with the same dose of antigen in Incomplete Freund's Adjuvant. Seven days after the final booster the spleen from one mouse was removed and a cell suspension was prepared. For fusion, SP 2/0 cells were

mixed with spleen cells at a ratio of 1:5. Cell fusion was induced with PEG-4000 (72% w/v in RPMI). Next, 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), β-mercaptoethanol (5×10^{-5} M). Cells were distributed in 96-wells culture plates at a density of 4×10^4 cells/well. Initial screening of hybridomas was performed by evaluating the reactivity of hybridoma supernatants in ELISA (MAP antigen) and Western blot (recombinant E2A-PBX1[3]). Cell culture supernatant of ER-GO4 was affinity purified using Gammabind G Sepharose beads (Pharmacia Biotech AB, Uppsala, Sweden). ER-GO4, an IgG_1 type antibody, was conjugated to FITC according to Harlow and Lane (23).

Antigen competition assay (ELISA)

The antigen competition assay was performed essentially as described previously (19). First, peptide concentrations were determined using Ellman's reagent (Pierce, Rockford II, USA). Next, the peptide concentration of E2A-PBX1 fusion peptides was equilibrated to the peptide concentration of the mixture of E2A and PBX1 peptides, according to the formula:

x mol E2A-PBX1 peptide / y ml = (x mol E2A peptide + x mol PBX1 peptide) / y ml

Various amounts of peptides (as indicated in Figure 1) were pre-incubated with 5 ng ER-GO4 antibody for two hours at room temperature (RT). These mixtures were tested on MAP antigen coated PVC plates (1 µg/ml). Bound antibodies were detected with goat anti-mouse immunoglobulins conjugated to alkaline phosphatase (GαM-AP; Tago, Burlingame, CA, USA) (1:1000 v/v) and *p*-nitro phenyl phosphate (pNPP) as substrate (Sigma, St Louis, MO, USA).

Western blot

Nuclear extracts were prepared as described by Schreiber *et al.* (24), with some modifications. Briefly, 10^7 cells were washed once in Tris buffered saline. Next, cells were swollen in 1 ml hypotonic buffer (10 mM HEPES pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM DTT; 0.5 mM PMSF) at 4 °C. After the cell membranes were lysed through the addition of NP-40 to a final concentration of 0.5% nuclei were pelleted by centrifugation in an Eppendorf centrifuge. After removal of the supernatant, the pelleted nuclei were resuspended in 100 µl of high salt extraction buffer (20 mM HEPES pH 7.9; 0.4 M NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM DTT; 1 mM PMSF). Nuclei were vigorously rocked for 30 min at 4 °C and subsequently centrifuged to remove the nuclei and particular matter. Nuclear extracts were collected, diluted (1:1 v/v) in sample buffer and boiled for 5 min. Western blotting was performed as described previously (19).

The following concentrations of antibodies were used: ER-GO4 at 2.5 µg/ml, moAb Yae (Santa Cruz Biotechn., Santa Cruz, CA, USA) at 0.5 µg/ml and rabbit polyclonal antibody PBX 1/2/3 (Santa Cruz Biotechn.) at 0.5 µg/ml. Bound antibodies were detected by either, peroxidase conjugated goat anti-mouse immunoglobulins (DAKO, Glostrup, Denmark) (1:2000 v/v), GαM-AP (Tago) (1:2000 v/v) or alkaline phosphatase conjugated goat anti-rabbit immunoglobulins (DAKO) (1:1000 v/v). For visualization of antibody-antigen complexes, we either used the enhanced chemoluminescent (ECL) Western blotting system, as instructed by the manufacturer (Amersham Int., Little Chalfont, UK), or the NBT/BCIP method as described previously (19).

Plasmids

E2A-PBX1[3] plasmid

E2A-PBX1[3] plasmid contains three copies of an E2A-PBX1 fusion-point oligonucleotide (GATCCCGGCCTCCCGACTCCTACAGTGTTTTGAGTATCCGAGGAGCCCAGGAGATC) inserted in the *Bam*HI site of pET21B (Invitrogen BV, Leek, The Netherlands). The recombinant E2A-PBX1[3] fusion-protein was expressed and purified as instructed by the manufacturer. Recombinant E2A-PBX1[3] fusion-protein is expressed as a 10 kDa protein and contains three copies of the amino acid sequence SRPPDSYSVLSIRGAQEI.

E2A, E2A-PBX1 (a or b) and PBX1a SV-40-plasmids

The E2A SV-40 plasmid contains full length E12 inserted downstream of the transcription start site of the SV-40 promotor pJ3Ω (25) (a kind gift of Dr G Bain, Department of Biology, University of California, San Diego, CA, USA). E2A-PBX1a, E2A-PBX1b and PBX1a cDNAs, cloned downstream of the SV-40 promotor in the pJ3Ω vector, were described previously (26). Plasmids were purified by equilibrium centrifugation in CsCl-ethidium bromide density gradients.

E2A-PBX11a and E2A-PBX1b CMV-plasmids

The E2A-PBX1 (a or b) CMV-plasmids contain the *Eco*RI/*Hind*III fragment from previously described E2A-PBX1 (a or b) effector plasmids pJ3Ω (26). The complete coding sequence of E2A-PBX1 (a or b) was cloned downstream of the transcription start site of the CMV promotor in pcDNAI/Amp (Invitrogen).

In vitro translation

Translations were performed using RNA transcribed *in vitro* from E2A-PBX1 (a or b) CMV plasmids and previously described PBX1 effector plasmids using SP6 polymerase (Promega, Madison WI, USA) (26). RNA was translated *in vitro* using a nuclease treated rabbit reticulocyte lysate (Promega) under conditions recommended by the supplier. The *in vitro* translation products were diluted in sample mix (1:9 v/v) and analyzed by Western blotting.

Transfection

One µg of either plasmid was introduced into COS cells using 10 µl LipofectAMINE reagens (GIBCO BRL), according to the manufacturer's instructions. Two days after transfection, COS cells were trypsinized and distributed on fibronectin coated 4-wells slides (Nutacon). Each well on the fibronectin coated 4-wells slide contained either E2A, E2A-PBX1a, E2A-PBX1b or PBX1a transfected COS cells.

Double immunofluorescence staining

Three days after transfection, COS cells cultured on fibronectin coated 4-wells slides were washed in ice-cold PBS, fixed with 4% paraformaldehyde in PBS (10 min, room temperature (RT)) and permeabilized with methanol (2 min, RT). Next, slides were rinsed in PBS containing 0.025% Tween-20, followed by a 45 min incubation in blocking solution (2% BSA, 0.1% sodium azide, 0.2% Tween-20, 6.7% glycerol). Slides were either incubated with a mixture of biotinylated anti-E2A moAb G98-271.1.3 (1 µg/ml) (moAb G98-271.1.3, a generous gift from Drs G Bain and C Murre, Department of Biology, University of California, San Diego CA, USA) and FITC-labeled ER-GO4 (5 µg/ml) or with a mixture of polyclonal rabbit antibody PBX 1/2/3 (2 µg/ml) (Santa Cruz) and FITC-labeled ER-GO4 (5 µg/ml).

After 45 min incubation and three subsequent washes in PBS containing 0.025% Tween-20, incubation was proceeded with either 10^{-5} diluted streptavidin-Cy-3 conjugate (Sigma, St Louis MO, USA) or 1:30 (v/v) diluted TRITC-conjugated to anti-rabbit immunoglobulins (GαR-TRITC; Supertechs, Bethesda MD, USA). Fluorescence was evaluated using a confocal laser scanning microscope, equipped with an air-cooled 488 nm argon laser (CLSM 410 Invert Confocal Microscope, Zeiss, Oberkochen, Germany).

Results

Specificity analysis of moAb ER-GO4 at the peptide level

Initial screening of hybridomas was performed by evaluating the reactivity of hybridoma supernatants with E2A-PBX1 peptides in ELISA (MAP antigen) and recombinant E2A-PBX1[3] protein, using Western blotting. After seven fusions, one hybridoma, which we termed ER-GO4 (Greek: ergon - labor, phorus - carrying: carrying a specific activity), was selected for further subcloning, Ig-purification and subsequent analysis.

To determine the specificity of moAb ER-GO4, an antigen competition assay was performed within an enzyme-linked immunosorbant assay (ELISA), as described previously (19). To this purpose, ER-GO4 immunoglobulins (Ig) were either preincubated with E2A-PBX1 peptides, or with a mixture of E2A and PBX1 peptides (equimolar to E2A-PBX1 peptides). After preincubation, the peptide/ER-GO4 Ig mixtures were allowed to react with E2A-PBX1 peptide (MAP antigen) coated on PVC plates.

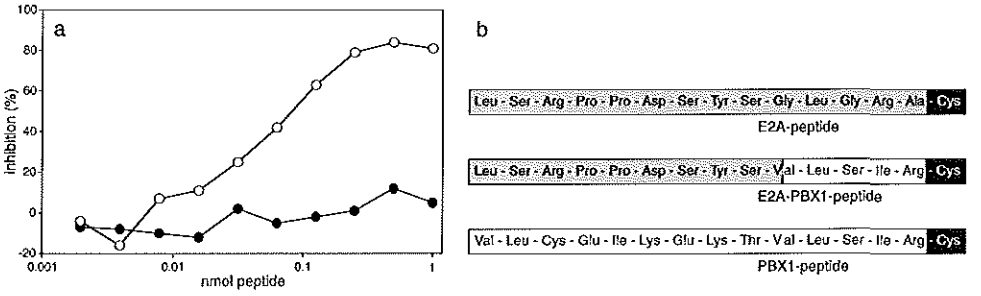

Figure 1a Peptide inhibition study to delineate the antigenic determinant recognized by moAb ER-GO4. To this purpose either graded concentrations of a mixture of monomeric E2A- and PBX1 peptides (●) or graded concentrations of monomeric E2A-PBX1 peptides (○) were used to examine their respective capacity to inhibit binding of ER-GO4 antibodies to the octameric E2A-PBX1 (MAP antigen) peptide complex, immobilized on the solid phase. Inhibition is expressed as: [1-{(absorbtion in the presence of peptide)/(absorbtion in the absence of peptide)}] x 100%. Each point represents the mean of a duplicate

Figure 1b Schematic representation of the amino acid sequence of monomeric E2A, E2A-PBX1 and PBX1 synthetic peptides. The amino acid sequence of E2A-PBX1 peptide is identical to the amino acid sequence of E2A peptide up to the fusion-point, thereafter the amino acid sequence of E2A-PBX1 peptides resembles the amino acid sequence of the PBX1 peptide.

Figure 1 shows that preincubation of moAb ER-GO4 with cognate E2A-PBX1 peptides completely abrogates binding of ER-GO4 antibodies to E2A-PBX1 peptide coated plates. In contrast, preincubation of moAb ER-G)4 with a peptide mixture (1:1) of E2A and PBX1 peptides (equimolar to E2A-PBX1 peptide preincubations) barely affects binding of ER-GO4 to E2A-PBX1 peptides.
These data show that ER-GO4 reacts primarily with peptides containing an E2A-PBX1 fusion point amino acid sequence.

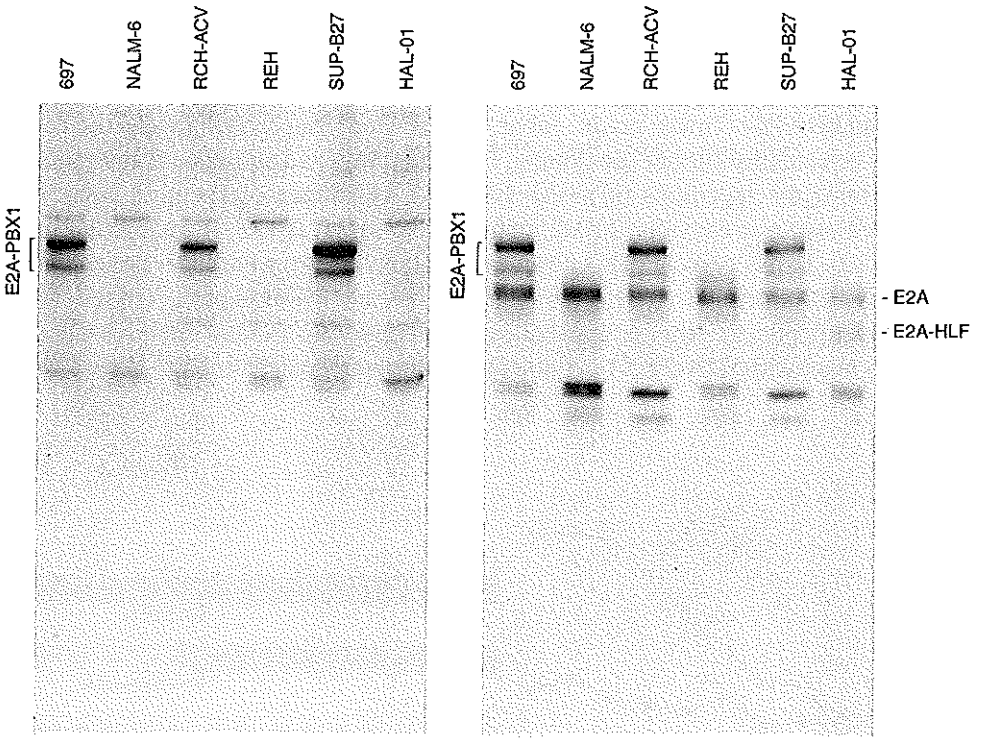

Figure 2a Immunoblot analysis of the reactivity of moAb ER-GO4. Nuclear extracts of t(1;19)$^{E2A-PBX1}$ positive human pre-B-cell lines; i.e. 697, RCH-ACV, SUP-B27 and, t(1;19) negative human pre-B-cell lines; i.e. NALM-6, REH and t(17;19)$^{E2A-HLF}$ positive human pro-B-cell line HAL-01, were used to examine the specificity of moAb ER-GO4. The localization of E2A-PBX1 proteins is indicated at the left.

Figure 2b Immunoblot analysis of the reactivity of anti-E2A moAb Yae. A similar blot as Figure 2a, containing the same nuclear extracts, was used to demonstrate the localization of respectively, E2A-PBX1 proteins, E2A proteins and E2A-HLF proteins. The localization of E2A-PBX1 proteins is indicated left, the localization of E2A proteins and E2A-HLF proteins are indicated at the right side of the blot.

Specificity analysis of ER-GO4 at the protein level

To elucidate whether moAb ER-GO4 can be used for specific recognition of the fusion-point epitope in E2A-PBX1 proteins, we analyzed the reactivity of moAb ER-GO4 on immunoblots derived from several hematopoietic cell lines. As a control for E2A-reactivity, Yae antibody was employed. Yae antibody is directed against the amino-proximal part of E2A proteins and recognizes both E2A proteins and E2A-PBX1 proteins (27). For the detection of E2A-PBX1 proteins we used a Western blotting procedure with nuclear extracts from t(1;19)$^{E2A\text{-}PBX1}$ positive cell lines (i.e. 697, RCH-ACV and SUP-B27). As t(1;19) negative controls, nuclear extracts from NALM-6, REH (both pre-B-cell lines) and HAL-01 cell line (t(17;19)$^{E2A\text{-}HLF}$ positive pro-B cell line were employed. The t(17;19) in the HAL-01 cell line creates a chimeric fusion gene, termed *E2A-HLF*, located on chromosome 19. In this rearrangement, *E2A* sequences are joined with those of a gene for hepatic leukemia factor (*HLF*). As a result of the t(17;19), chimeric E2A-HLF proteins are expressed in the nuclei of HAL-01 cells.

Figure 2a represents a Western blot incubated with ER-GO4 antibodies. E2A-PBX1 proteins are detected by moAb ER-GO4 in t(1;19)$^{E2A\text{-}PBX1}$ positive cell lines (lanes 1, 3, and 5), but not in t(1;19) negative cell lines (lanes 2, 4 and 6). A similar blot, now incubated with moAb Yae (Figure 2b) shows the localization of E2A-PBX1 proteins (lanes 1, 3 and 5), E2A proteins (Figure 2b: all lanes) and E2A-HLF proteins (Figure 2b: lane 6). Neither E2A, nor E2A-HLF proteins are recognized by ER-GO4 antibodies (cf Figures 2a and 2b).

From these data, we conclude that ER-GO4 recognizes E2A-PBX1 proteins and does not cross-react with E2A proteins nor with E2A-HLF proteins.

So far, reactivity of ER-GO4 to PBX1 proteins could not be excluded. Since PBX1 proteins are not expressed in lymphocytes (28), we decided to analyze, by Western blot, the reactivity of ER-GO4 towards *in vitro* translated PBX1a and PBX2 proteins. As positive control for the reactivity of moAb ER-GO4, both kinds of *in vitro* translated E2A-PBX1 (a and b) proteins were employed. The data presented in Figure 3a indicate that both *in vitro* translated E2A-PBX1 proteins (lanes a and b) are recognized by ER-GO4, whereas *in vitro* translated PBX1 proteins and *in vitro* translated PBX2 proteins are not recognized by ER-GO4 (lanes c and d).

To confirm the efficiency of the *in vitro* translation of PBX1a proteins and PBX2 proteins, we incubated a similar blot with a polyclonal antibody termed PBX 1/2/3 (Figure 3b). Antiserum PBX 1/2/3 is directed against the carboxyl terminus found in E2A-PBX1a, PBX1a and PBX2 proteins. E2A-PBX1b proteins contain a differentially spliced PBX1 carboxyl terminus (28), which is not recognized by polyclonal antibody PBX 1/2/3. Figure 3b shows the localization of E2A-PBX1a (lane a) and both PBX1a and PBX2 proteins (lanes c and d) upon incubation with PBX 1/2/3 polyclonal antibody. Thus, although PBX1a and PBX2 proteins were efficiently translated *in vitro*, they are not recognized by ER-GO4.

Together, the Western blotting experiments indicate that moAb ER-GO4 exclusively recognizes an E2A-PBX1 *fusion-point* epitope at the protein level.

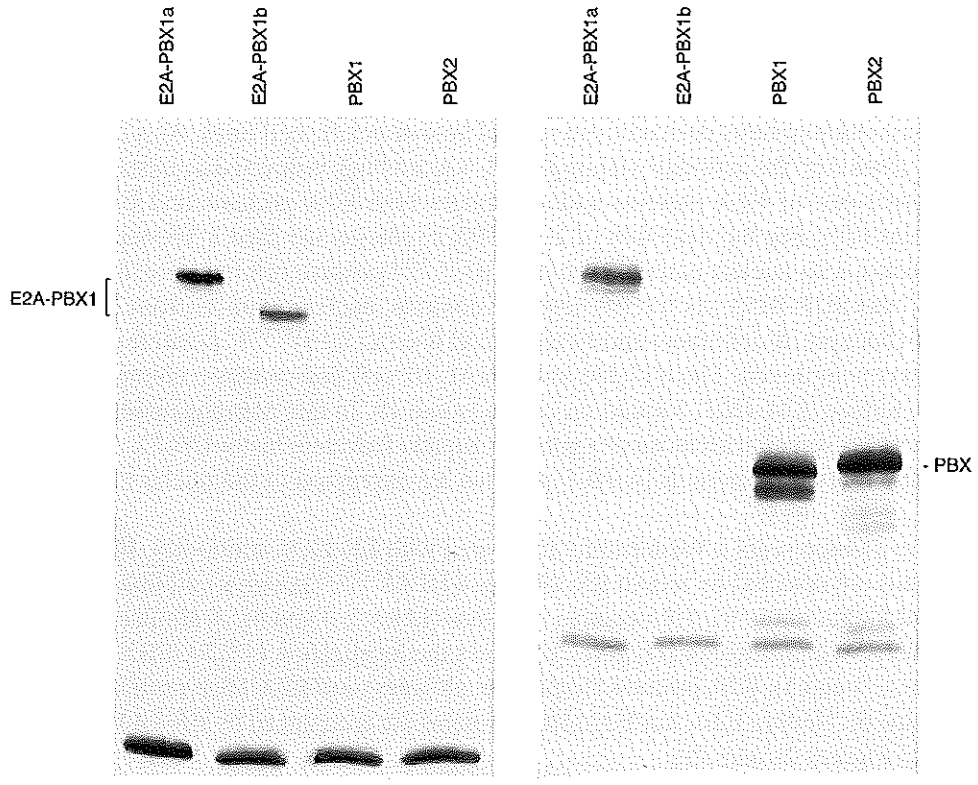

Figure 3a Immunoblot cross-reactivity analysis of moAb ER-GO4 to *in vitro* translated PBX1a and PBX2 proteins. Each lane represents, as indicated at the top if the bolt, either *in vitro* translated E2A-PBX1a proteins (1 µl), *in vitro* translated E2A-PBX1b proteins (1 µl), *in vitro* translated PBX1a proteins (1 µl) and *in vitro* translated PBX2 proteins (1 µl). As is demonstrated, MoAb ER-GO4 only recognizes *in vitro* translated E2A-PBX1 (a and b) proteins.

Figure 3b Immunoblot analysis of a similar blot as used in Figure 3a, containing the same amounts of *in vitro* translated E2A-PBX1a, E2A-PBX1b, PBX1a and PBX2 proteins. Rabbit polyclonal antibody PBX 1/2/3, recognizing the carboxyl-terminus of E2A-PBX1a, PBX1a and PBX2 proteins was employed to indicate the localization of *in vitro* translated E2A-PBX1a, *in vitro* translated PBX1a and *in vitro* translated PBX2 proteins.

Specificity analysis of ER-GO4 at the cellular level

We next examined whether moAb ER-GO4 distinguishes between $t(1;19)^{E2A\text{-}PBX1}$ positive pre-B-cell lines and t(1;19) negative cell lines, using fluorescence microscopy. Despite multiple attempts, using different fixation and staining protocols, we were unable to distinguish $t(1;19)^{E2A\text{-}PBX1}$ positive cell lines from t(1;19) negative cell lines. The characteristic nuclear staining pattern of $t(1;19)^{E2A\text{-}PBX1}$ positive cells, we usually observe upon incubating $t(1;19)^{E2A\text{-}PBX1}$ positive cell lines with our E2A-PBX1 fusion-point specific polyclonal antiserum BP 1/19 (19), was not noticed upon incubations with ER-GO4 antibodies. Both $t(1;19)^{E2A\text{-}PBX1}$ positive cell lines and t(1;19) negative cell lines show, besides a faint nuclear staining, reactivity of the ER-GO4 antibody in the cytoplasm.

At this point, it can be argued that: 1) the fusion-point epitope of E2A-PBX1 proteins is destructed as a result of fixation (29) or 2) E2A-PBX1 protein expression is too low to be detected by moAb ER-GO4. To investigate both possibilities, COS cells were transfected with an expression vector (pJ3Ω) containing full length E2A-PBX1a. Three days after transfection, COS cells were stained with ER-GO4 followed by GαM-FITC. Immunofluorescence was evaluated by using confocal laser scanning microscopy (CLSM).

To our surprise, CLSM analysis of ER-GO4 staining of E2A-PBX1a transfected COS cells revealed, at high magnification, strikingly different nuclear staining patterns, generally excluding the nucleoli (Figures 4a to 4c). By comparing different nuclear staining patterns of various E2A-PBX1a transfected COS cells, we noticed that all nuclei stained by moAb ER-GO4 displayed a diffuse nuclear pattern (Figure 4a), often superimposed by a punctate pattern (Figure 4b) or a patchy nuclear pattern (Figure 4c).

To ensure that these nuclear structures indeed represent E2A-PBX1a proteins, we next transfected COS cells with the pJ3Ω expression vector, now containing either full length E2A, E2A-PBX1a, E2A-PBX1b or PBX1a cDNAs. The four differently transfected COS cells were grown separately on wells of a 4-wells slide and analyzed by double immunofluorescence. To this purpose, 4-wells slides were either incubated with a mixture of FITC-labeled ER-GO4 and biotinylated anti-E2A moAb G98-271.1.3 (Figures 5a and 5b) or were incubated with a mixture of FITC-labeled ER-GO4 and rabbit polyclonal antibody PBX 1/2/3 (Figures 5c to 5h). MoAb G98-271.1.3, directed towards the carboxyl terminus of E2A proteins (19, 30), was chosen to confirm the effectiveness of the E2A-transfection procedure. PBX 1/2/3 antibodies were employed to ensure both specific E2A-PBX1a protein staining in E2A-PBX1a transfected COS cells as well as PBX1a protein expression in PBX1a transfected COS cells. PBX 1/2/3 antibodies do not recognize E2A-PBX1b proteins.

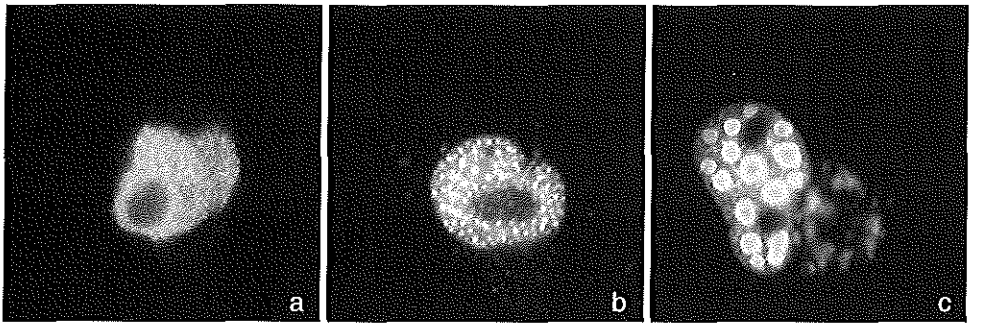

Figure 4 Immunofluorescence analysis of moAb ER-GO4 staining on COS cells transfected with an *E2A-PBX1a* containing pJ3Ω expression vector. Bound ER-GO4 antibodies were detected by FITC-conjugated to goat anti-mouse immunoglobulins.
Different nuclear staining patterns can be observed in E2A-PBX1a transfected COS cells (a, b, c). All E2A-PBX1a expressing COS cells, stained by moAb ER-GO4, display a diffuse nuclear pattern (a), often superimposed by a punctate nuclear pattern (b) or by a patchy nuclear pattern (c).

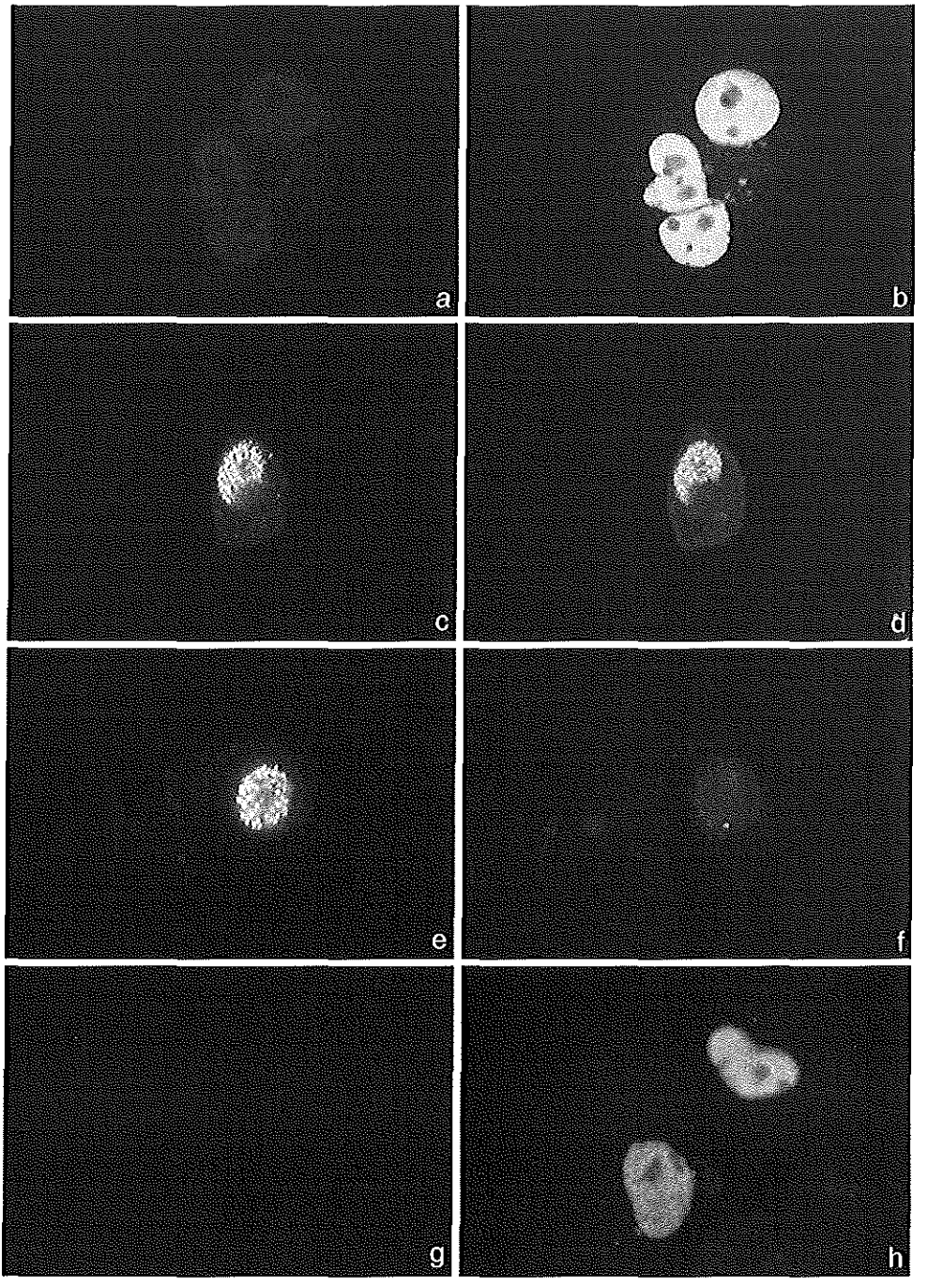

Figure 5 Specificity analysis of the reactivity of moAb ER-GO4. COS cells were transfected with either an *E2A* (a, b), an *E2A-PBX1a* (c, d), an *E2A-PBX1b* (e, f) or a *PBX1a* (g, h) containing pJ3Ω expression vector. Micrographs of cells depicted in the left panel (a, c, e, g) represent the same microscopic field as is observed in the right panel of micrographs (b, d, f, h).

FITC-labeled ER-GO4 antibody was used to demonstrate binding to E2A-PBX1a (c) and E2A-PBX1b proteins (e), but not to E2A (a) or PBX1a (g) proteins. Biotinylated anti-E2A moAb G98-271.1.3 was employed to verify E2A protein expression in E2A transfected COS cells (b). The Cy-3 fluorochrome conjugated to streptavidin was used to identify bound biotinylated G98-271.1.3 antibodies. Rabbit polyclonal antibody PBX 1/2/3 (recognizing E2A-PBX1a proteins but not E2A-PBX1b proteins), was used to confirm that E2A-PBX1 proteins are indeed localized in discrete nuclear structures in E2A-PBX1 transfected COS cells (d). Bound PBX 1/2/3 immunoglobulins were identified by TRITC-labeled goat anti-mouse immunoglobulins (GαR-TRITC). As E2A-PBX1b proteins are not recognized in E2A-PBX1b transfected COS cells, artefactual staining by either PBX 1/2/3 immunoglobulins or GαR-TRITC immunoglobulins is excluded (cf d and f). PBX 1/2/3 antibodies were also used to ensure PBX1a protein expression in PBX1a transfected COS cells (h).
The particular localization of E2A-PBX1 (a or b) proteins in discrete nuclear structures in E2A-PBX1 (a or b) transfected COS cells, is not observed in E2A transfected COS cells nor is it observed in PBX1 transfected COS cells, upon staining for E2A- or PBX1a protein expression, respectively. In contrast, both E2A and PBX1a transfected COS cells display a diffuse nuclear staining, sparing the nucleoli, when stained with the appropriate antibodies (G98-271.1.3 or PBX 1/2/3, respectively).

Figure 5 shows micrographs of double staining experiments of COS cells transfected with either E2A (Figures 5a and 5b), E2A-PBX1a (Figures 5c and 5d), E2A-PBX1b (Figures 5e and 5f) and PBX1a (Figures 5g and 5h) plasmids. First, to ensure that the particular nuclear structures observed in E2A-PBX1a transfected COS cells indeed represent E2A-PBX1 proteins, E2A-PBX1a and E2A-PBX1b transfected COS cells were analyzed upon double staining with FITC-labeled ER-GO4 antibodies and polyclonal PBX 1/2/3 antibodies. The staining patterns observed upon FITC-labeled ER-GO4 staining in E2A-PBX1a transfected COS cells (Figure 5c) were completely identical to the staining patterns observed upon PBX 1/2/3 immunoglobulin incubation, followed GαR-TRITC (Figure 5d). Any particular nuclear pattern detected upon FITC-labeled ER-GO4 antibody staining of E2A-PBX1a transfected COS cells (e.g. diffuse, punctate and patchy) was also observed upon PBX 1/2/3 double staining. Furthermore, the particular nuclear structures observed in E2A-PBX1a transfected COS cells were also detected in E2A-PBX1b transfected COS cells upon FITC labeled ER-GO4 staining (Figure 5e). In contrast, E2A-PBX1b transfected COS cells were not stained by PBX 1/2/3 antibodies (Figure 5f). These results, i.e. a positive PBX 1/2/3 antibody staining in E2A-PBX1a transfected COS cells (Figure 5d) versus a negative PBX 1/2/3 antibody staining in E2A-PBX1b transfected COS cells (Figure 5f) verify specific staining with PBX 1/2/3 antibodies: specifically reacting with E2A-PBX1a proteins, but not with E2A-PBX1b proteins. Thus, at the cellular level, E2A-PBX1a proteins are specifically detected as distinct nuclear structures in E2A-PBX1a transfected COS cells. Furthermore, the data from these double staining experiments indicate that E2A-PBX1 proteins are specifically recognized by moAb ER-GO4.
Specific binding of ER-GO4 antibodies towards the fusion-point epitope of E2A-PBX1 proteins was further investigated by double immunofluorescence studies of differently (i.e. E2A, E2A-PBX1a, E2A-PBX1b and PBX1a) transfected COS cells. The clear-cut nuclear fluorescence in E2A-PBX1a (Figure 5c) and E2A-PBX1b (Fig-

ure 5e) transfected COS cells upon FITC-labeled ER-GO4 staining, was not observed in FITC-labeled ER-GO4 stained E2A transfected COS cells (Figure 5a) nor was it observed in PBX1 transfected COS cells (Figure 5g). To confirm intracellular E2A or PBX1a protein expression, COS cells were double stained with biotinylated anti-E2A moAb G98-271.1.3 or rabbit polyclonal PBX 1/2/3 immunoglobulins (Figures 5b and 5h, respectively). To our surprise, while distinguishing transfected cells from non-transfected cells, we noticed that the particular nuclear structures observed in E2A-PBX1 (a or b) transfected COS cells, were not found in E2A transfected COS cells, nor were they found in PBX1a transfected COS cells. In fact, E2A transfected COS cells and PBX1a transfected COS cells stained with either biotinylated anti-E2A moAb G98-271.1.3 or polyclonal antibody PBX 1/2/3, both displayed a clear diffuse staining of the nuclei that spared the nucleoli (Figures 5b and 5h, respectively).
In conclusion, referring to the contentions stated above, these date indicate that the fusion-point epitope of E2A-PBX1 proteins, as it is recognized by ER-GO4, is not affected by the fixation procedures we used. Yet, under the present experimental conditions, ER-GO4 antibodies do not specifically recognize E2A-PBX1 proteins in $t(1;19)^{E2A\text{-}PBX1}$ positive tumor-cell lines. E2A-PBX1 protein expression in these $t(1;19)^{E2A\text{-}PBX1}$ positive tumor-cell lines is probably too low to be specifically recognized by a single antibody. In spite of this, moAb ER-GO4 revealed novel distinct subnuclear structures, consisting of E2A-PBX1 proteins, in E2A-PBX1 transfected COS cells. Similar distinct subnuclear structures were not detected in E2A transfected COS cells, nor were they detected in PBX1 transfected COS cells upon staining with the appropriate antibodies.

Discussion

In the present study we report on the generation of ER-GO4, a new monoclonal antibody directed towards the tumor-specific fusion-point of E2A-PBX1 proteins. The specificity of ER-GO4 was evaluated at the peptide, the protein and at the cellular level. Moreover, ER-GO4 allows detection of yet undefined nuclear structures in E2A-PBX1 transfected COS cells.
The specificity of ER-GO4 was evaluated by determination of the antigenicity of E2A-PBX1, E2A and PBX1 peptides. Peptide inhibitions are methods of first choice to assess the comparative antigenicity of the peptides analogues (31). Accordingly, considering the antigenicity of the investigated peptides for moAb ER-GO4, we found E2A-PBX1 peptides to be more antigenic than a mixture of E2A and PBX1 peptides. Therefore, the epitope recognized by ER-GO4 consists of amino acids located at both sides of the E2A-PBX1 fusion-point. However, we also found reactivity of ER-GO4 towards immobilized E2A peptides, whereas no reactivity of ER-GO4 towards immobilized PBX1 peptides could be detected (data not shown). Therefore, it is likely that E2A derived amino acids contribute to a large extent to the antigenicity of the fusion-point epitope of E2A-PBX1.

Yet, by comparing the reactivity of moAb ER-GO4 with other E2A-PBX1 recognizing antibodies (i.e. Yae and PBX 1/2/3) in Western blotting experiments, we clearly demonstrated that the fusion-pont epitope on E2A-PBX1 proteins is specifically recognized by moAb ER-GO4. Neither E2A proteins, E2A-HLF proteins nor PBX1 proteins are recognized by ER-GO4.

To this point the results of ER-GO4 are comparable with the ELISA data and Western blotting data found with our fusion-point specific polyclonal antibody BP 1/19 (19). However, in contrast to the polyvalent BP 1/19 immunoglobulins, ER-GO4 antibodies do not discriminate between $t(1;19)^{E2A\text{-}PBX1}$ positive cell lines and t(1;19) negative cell lines at the cellular level.

To eliminate epitope malformation (e.g. caused by fixation) (29), as a reason for the inability of ER-GO4 to distinguish $t(1;19)^{E2A\text{-}PBX1}$ positive cells from t(1;19) negative cells at the cellular level, we performed a series of transfection experiments. Subsequent double immunofluorescence studies on COS cells, transfected with either E2A, E2A-PBX1a, E2A-PBX1b or PBX1a plasmids, demonstrated that the E2A-PBX1 fusion-point eptitope, as it is recognized in E2A-PBX1 (a and b) transfected COS cells by moAb ER-GO4, is not destructed as a result of fixation or permeabilization procedures.

Yet, while moAb ER-GO4 specifically recognizes an E2A-PBX1 fusion-point epitope, ER-GO4 does not distinguish $t(1;19)^{E2A\text{-}PBX1}$ positive tumor-cell lines from t(1;19) negative cell lines. The exclusive detection of E2A-PBX1 proteins in $t(1;19)^{E2A\text{-}PBX1}$ positive tumor-cell lines is probably hampered by a relatively low concentration of E2A-PBX1 proteins. Cross-reactive epitopes present on non-related cellular proteins, likely obscure exclusive E2A-PBX1 protein detection in $t(1;19)^{E2A\text{-}PBX1}$ positive cells. In spite of this, E2A-PBX1a and E2A-PBX1b transfected COS cells show a unique discrete nuclear staining pattern upon ER-GO4 incubation. This particular pattern varied from a diffuse nuclear staining to a punctate or even to a patchy nuclear staining. The punctate and patchy patterns appear as bright foci superimposed on a diffuse nucleoplasmic staining. While punctate patterns were already detected two days after the transfections were started, patchy patterns were observed one day later. Since patchy patterns were detected three days post-transfection, we suggest that the appearance of patchy patterns is related to increasing E2A-PBX1 protein concentrations.

It is unlikely that the observed unique distribution of E2A-PBX1 proteins in COS cells transfected with E2A-PBX1 expression constructs, is caused by artefacts other than the ones caused by overexpression. As discussed above, as both ER-GO4 and PBX 1/2/3 antibodies recognize these structures through specific binding to E2A-PBX1 proteins, staining artefacts are excluded. Additional experiments, using other fixatives (methanol / acetone (1:1 v/v) 10 min -20 °C; acetone 2 min RT; 1% formaldehyde 2 min RT) resulted in identical structures upon incubation with ER-GO4 or PBX 1/2/3 antibodies (data not shown). Moreover, these particular E2A-PBX1 nuclear structures were also found in E2A-PBX1 transfected HeLa and E2A-PBX1 transfected HEp-2 cells (data not shown). In addition, identical structures in E2A-

PBX1 transfected COS cells have been observed by Cleary and co-workers (personal communication M Cleary, December 1996). These investigators also recognized differences in nuclear distribution: discrete nuclear structures of E2A-PBX1 (a or b) proteins on the one hand, a diffuse nuclear distribution of E2A and PBX1 proteins on the other.

Similar distinct subnuclear structures, termed 'nuclear bodies', have been recently described concerning immunofluorescence studies of other nuclear proteins, e.g. PML (32), WT-1 (33) and GATA (34). Larsson *et al.* showed that proteins derived from the tumor-suppressor gene *WT-1* (Wilms' tumor) either co-localize with splicing factors or co-localize with DNA in transcription factor domains (33). The nuclear protein PML (associated with acute promyelocytic leukemia) and the transcription factor GATA, each display a unique distribution. Neither PML proteins nor GATA proteins co-localize with proteins associated with either replication, transcription or post-transcriptional processing (32, 34).

Both Dyck *et al.* and Elefanty *et al.* demonstrated the presence of these novel class of nuclear bodies in leukemic cell samples and non-transfected cell lines (32, 34). Yet, particular nuclear E2A-PBX1 protein structures, identical to the ones detected in E2A-PBX1 transfected COS cells, are not observed in $t(1;19)^{E2A\text{-}PBX1}$ positive tumor-cell lines (19). The granular nuclear fluorescence detected upon antiserum BP 1/19 incubation in $t(1;19)^{E2A\text{-}PBX1}$ positive tumor-cells (19) may, however, reflect a condensed punctate nuclear pattern in the small nuclei of $t(1;19)^{E2A\text{-}PBX1}$ positive cells. Patchy structures on the other hand, are probably a prelude of cell death since they are not observed in $t(1;19)^{E2A\text{-}PBX1}$ tumor-cell lines. This notion finds support in the difficulties we encountered in trying to establish stable E2A-PBX1 transfected cell lines (unpublished observations).

The close association of E2A-PBX1 proteins and leukemogenesis is beyond doubt (13, 14). At present, one hopes to unravel the mechanism of E2A-PBX1 proteins in leukemia development by trying to identify target-genes of the transcriptional activator protein E2A-PBX1 (26, 35, 36). Detailed studies at the cellular level, e.g. co-localization of E2A-PBX1 proteins with proteins involved in either replication, transcription or post-transcriptional processes, might shed new light on the still obscure role of E2A-PBX1 proteins in leukemia development.

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Chapter 7

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

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

The Ph chromosome, resulting from the t(9;22) translocation, is the most frequently observed cytogenetic aberration in adult 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: $e_1a_2P190^{BCR-ABL}$, $b_2a_2P210^{BCR-ABL}$, and $b_3a_2P210^{BCR-ABL}$. Since these chimeric genes only occur in Ph-chromosome-positive leukemic cells, they are per definition tumor-specific markers. Ph-chromosome-positive ALL is correlated with a bad prognosis. Therefore, the detection of chimeric BCR-ABL proteins is of prime importance for ALL diagnosis. In the present study, we report on the generation of a monoclonal antibody termed ER-FP1, raised against the tumor-specific e_1a_2 BCR-ABL junction in $P190^{BCR-ABL}$. We show that ER-FP1 reacts highly specifically with $e_1a_2P190^{BCR-ABL}$ in different assays. The reactivity of ER-FP1 with $e_1a_2P190^{BCR-ABL}$ in soluble form was analyzed in an immunoprecipitation assay; specificity was confirmed by peptide inhibition studies. Binding of ER-FP1 to $e_1a_2P190^{BCR-ABL}$ at the single cell level was detected by using immunofluorescence techniques. Immunological double-staining experiments using ER-FP1 and a monoclonal antibody recognizing all BCR-ABL proteins confirmed the specificity of ER-FP1 for the e_1a_2 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, the Ph chromosome is not exclusively specific for CML. In acute lymphoblastic leukemia (ALL) the Ph chromosome is also 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 chromosomes 9 and 22, t(9;22)(q34;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 always found 5' of *ABL* exon a2 (8-10). 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-kDa protein with an e1a2 BCR-ABL junction, $e_1a_2P190^{BCR\text{-}ABL}$ (14-16). The M-BCR spans a 5.8-kb region in the middle of the BCR gene including five exons (b1-b5). M-BCR breakpoints occur either between exon b2 and b3 or between exon b3 and b4 (8-10). As a consequence, two different chimeric genes are generated encoding 210-kDa proteins with different BCR-ABL junctions, $b_2a_2P210^{BCR\text{-}ABL}$ and $b_3a_2P210^{BCR\text{-}ABL}$, respectively (17). $B_2a_2P210^{BCR\text{-}ABL}$ and $b_3a_2P210^{BCR\text{-}ABL}$ 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 e1a2 BCR-ABL mRNA, i.e. 85% of all children and 68% of all adults (18). This means that the $e_1a_2P190^{BCR\text{-}ABL}$ 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-tumor-specific 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 e_1a_2 BCR-ABL junction in $e_1a_2P190^{BCR\text{-}ABL}$. Our data indicate that ER-FP1 specifically reacts with $e_1a_2P190^{BCR\text{-}ABL}$ in different test systems: using the tyrosine kinase assay, $e_1a_2P190^{BCR\text{-}ABL}$ is recognized in cell lysates; immunofluorescence analysis allows detection of e_1a_2-$P190^{BCR\text{-}ABL}$ 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 (20-22). Mouse SP2/0 cells were used as fusion partner for the production of monoclonal antibodies. All cell 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. However, as described by Hagemeijer *et al.* (23) (patient 1) the *BCR* and *ABL* genes were actually rearranged resulting in a chimeric b2a2 *BCR-ABL* gene, localized on chromosome 9. 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:

SPe_1a_2:				H	G	D	A	E*	A	L	Q	R	P	V
SPb_2a_2:		C	-	I	N	K	E	E*	A	L	Q	R	P	
SPb_3a_2:	C	-	F	K	Q	S	S	K*	A	L	Q			

Residues indicated with an asterisk are newly formed as a result of the translocation process. SPe_1a_2 was synthesized using Fmoc chemistry on an automated Millgen 9050 continuous Synthesizer (Millipore, Milford, MA, USA). SPb_2a_2 and SPb_3a_2 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 25 µg SPe_1a_2 coupled via EDC to the carrier Keyhole Limpet Hemocyanin. The peptide-carrier complex was emulsified in Complete Freund's Adjuvant and injected in the hind footpad of the mice (25). After 4 weeks the mice were boosted i.p. with the same dose 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-mercaptoethanol (5×10^{-5} M) and antibiotics and plated in 96-well culture plates at a density of 8×10^4 cells per well. ER-FP1 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-immunoglobulins coupled to Sepharose beads. ER-FP1 is an 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 SPe_1a_2 conjugated to BSA through glutaraldehyde.

Antisera

BP-ALL is a polyclonal antiserum raised in rabbits against the e_1a_2 BCR-ABL junction in $e_1a_2P190^{BCR-ABL}$ (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 (University of California, San Diego, USA).

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, 5-10 x 10^6 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 glass plates at a density of 1 x 10^4 cells/cm^2. Cells were transfected with pCDX plasmids comprising full lengths cDNA encoding $e_1a_2P190^{BCR\text{-}ABL}$, $b_2a_2P210^{BCR\text{-}ABL}$ or $b_3a_2P210^{BCR\text{-}ABL}$. Transfection was mediated by calcium phosphate (27).

Immunofluorescence analysis

Transfected COS cells grown on glass plates were fixed for 20 min by using a 3% paraformaldehyde solution diluted in PBS at room temperature. Next, the cells were washed three times in phosphate-buffered saline (PBS) and permeabilized with 100% ice-cold methanol for 20 min. 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 reactions were carried out for 30 min at room temperature. Between the various incubations cells were thoroughly washed three 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 isothiocyanate (FITC) labeled goat anti-mouse IgG1 (Southern Biotechnology, Birmingham, USA). Next, ER-FP1 (IgG2a) was applied, followed by an incubation with an optimal dilution of tetramethylrhodamine 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 filter combination 19 (BP 484/20; 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 e_1a_2 junction in $e_1a_2P190^{BCR\text{-}ABL}$. Reactivity with the cognate peptide SPe_1a_2 was determined by using a micro-ELISA system. To detect in the same experiment any cross-reactivity of ER-FP1 with ABL derived a_2 amino acids, ER-FP1 (2 μg/ml) was preincubated overnight at 4 °C with or without various concentrations of the free peptides, SPb_2a_2 or SPb_3a_2. As a positive control ER-FP1 was preincubated with SPe_1a_2. The following day the samples were tested for residual anti-SPe_1a_2 activity in the ELISA system, in which the Terasaki trays were coated with SPe_1a_2 conjugated to BSA. Figure 1 shows that a peptide dose-related inhibition of the antibody was observed. The ER-FP1 reaction is reduced to 10% upon preincubation with the highest dose of peptide. In contrast, preincubation with either SPb_2a_2 or SPb_3a_2 does not significantly influence binding of ER-FP1 in the ELISA, indicating that ER-FP-1 does not recognize ABL derived a_2 residues.

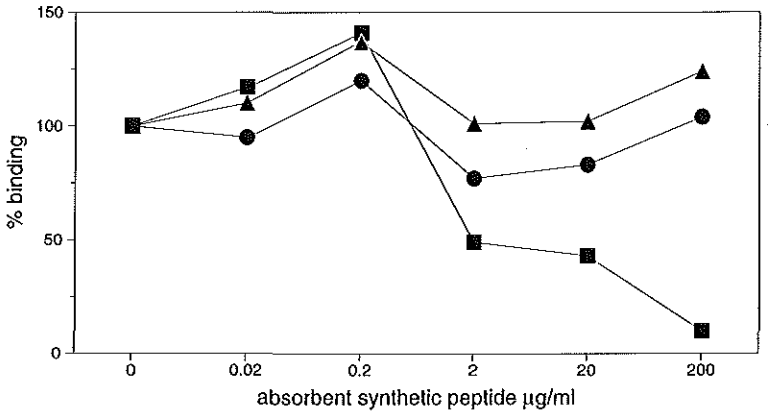

Figure 1 Relative binding of ER-FP1 to BSA-glutaraldehyde-SPe$_1$a$_2$ in an ELISA after preincubation of 2 µg/ml ER-FP1 with various concentrations SPe$_1$a$_2$ (■), SPb$_2$a$_2$ (▲) or SPb$_3$a$_2$ (●)

These data indicate that ER-FP1 has a moderate, but specific affinity for the junction representing peptide SPe$_1$a$_2$.

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

Binding of ER-FP1 to native proteins was determined in an immunoprecipitation reaction followed by autophosphorylation. As source for BCR-ABL proteins ALL and CML cell lines were used, harboring the respective chimeric proteins e$_1$a$_2$P190$^{BCR-ABL}$ (TOM-1), b$_2$a$_2$P210$^{BCR-ABL}$ (BV173), and b$_3$a$_2$P210$^{BCR-ABL}$ (K562). In Figure 2a re- presentative immunoprecipitation analysis with ER-FP1 is shown. ER-FP1 clearly precipitates a 190-kDa protein from the lysate of TOM-1 cells, whereas no precipitation of either b$_2$a$_2$P210$^{BCR-ABL}$ or b$_3$a$_2$P210$^{BCR-ABL}$ can be observed in the lanes containing immunoprecipitations of BV173 or K562 lysates. These results indicate that in the native protein the e$_1$a$_2$ BCR-ABL junction is recognized by ER-FP1.

In order to exclude any reactivity of ER-FP1 with ABL-derived a$_2$ sequences we performed peptide inhibition studies with SPe$_1$a$_2$ and other a$_2$ comprising sequences such as SPb$_2$a$_2$ and Spb$_2$a$_2$. 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 e$_1$a$_2$P190$^{BCR-ABL}$ was abrogated completely after incubation with 200 µg/ml SPe$_1$a$_2$ (SPe$_1$a$_2$, lane 1). After incubation with 20 g/ml SPe$_1$a$_2$ only a faint band is visible (SPe$_1$a$_2$, lane 2). However, preincubation with 200 µg/ml SPb$_2$a$_2$ or SPb$_3$a$_2$ had no inhibiting effect on the precipitation of e$_1$a$_2$P190$^{BCR-ABL}$ by ER-FP1 (SPb$_2$a$_2$ and SPb$_3$a$_2$, lanes 1 and 2).

From these results we conclude that ER-FP1 reacts specifically with native protein e$_1$a$_2$P190$^{BCR-ABL}$. Moreover, the reactivity is specific for the e$_1$a$_2$ junction and not di-

rected against a_2 sequences, since binding of ER-FP1 to $e_1a_2P190^{BCR\text{-}ABL}$ is not inhibited after preincubation with other peptides comprising a_2-derived amino acids.

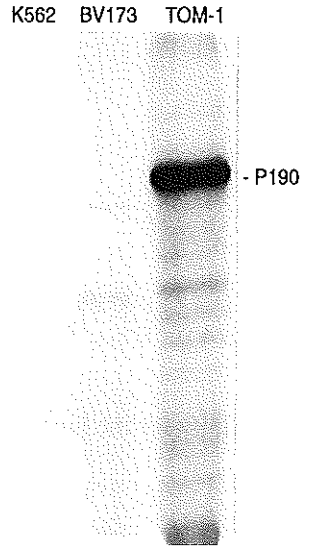

Figure 2

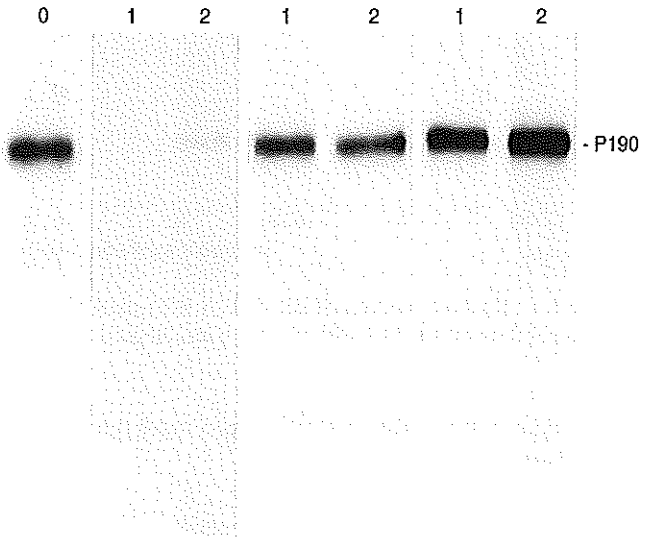

Figure 3

Figure 2 Junction specificity analysis of ER-FP1. 10 x 10^6 TOM-1, K562 or BV173 cells were lysed and precipitated with 30 µg/ml ER-FP1. TOM-1 cells express $e_1a_2P190^{BCR\text{-}ABL}$, K562 cells express $b_3a_2P210^{BCR\text{-}ABL}$, and BV173 cells express $b_2a_2P210^{BCR\text{-}ABL}$

Figure 3 Epitope specificity analysis of ER-FP1. 5 x 10^6 TOM-1 cells ($e_1a_2P190^{BCR\text{-}ABL}$) were lysed and precipitated with either 1.8 µg/ml ER-FP1 (lane 0) or with 1.8 µg/ml ER-FP1 after preincubation with synthetic peptides (SPe_1a_2, SPb_2a_2 or SPb_3a_2) in various concentrations. ER-FP1 was preincubated with either 200 µg/ml (lanes 1) or with 20 µg/ml (lanes 2)

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

In order to determine whether ER-FP1 reacted with $e_1a_2P190^{BCR\text{-}ABL}$ 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 patient by Ficoll-Hypaque centrifugation. Next, cells were lysed and incubated either with ER-FP1 or, as control, with the e_1a_2 junction-specific polyclonal antiserum BP-ALL. As a control T-cell depleted blood cells derived from a Ph-negative CML patient with a b_2a_2 rearranged *BCR-ABL* mRNA were analyzed. Figure 4a clearly shows that ER-FP1 reacts with $e_1a_2P190^{BCR\text{-}ABL}$ derived from leukemic blast cells of ALL patient L. (lane 1). The reaction pattern obtained after precipitation with ER-FP1 or with BP-ALL (lane 2) is highly similar. After incubation of cell lysates of CML patient W. with ER-FP1 no proteins were precipitated (Figure 4b, lane 2). However, patient W. actually expressed $P210^{BCR\text{-}ABL}$ proteins, since a strong $P210^{BCR\text{-}ABL}$ band was observed after precipitation

with an antiserum directed against the amino-terminal part of BCR (Figure 4b, lane 1). These data indicate that ER-FP1 also reacts highly specifically with the e_1a_2 BCR-ABL junction in leukemic cells. As such, ER-FP1 can be applied in ALL diagnosis using the tyrosine kinase assay.

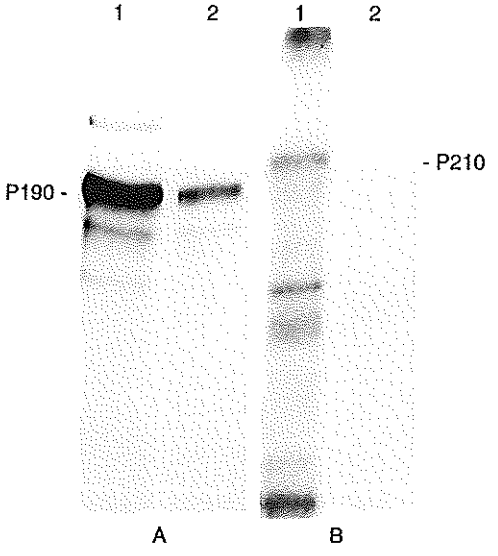

Figure 4 Immunoprecipitation analysis of ALL and CML patients. 10 x 10^6 Ficoll enriched leukemic blast cells of patient L. were lysed and precipitated with 30 µg/ml ER-FP1 (panel (A), lane 1) or 50 µl polyclonal antiserum BP-ALL (panel (A), lane 2), 20 x 10^6 T-cell depleted blood cells of patient W.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

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 $e_1a_2P190^{BCR-ABL}$. As controls, COS cells were transfected with expression plasmids containing cDNAs encoding either $b_2a_2P210^{BCR-ABL}$ or $b_3a_2P210^{BCR-ABL}$. To investigate whether $e_1a_2P190^{BCR-ABL}$ 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 was used. Figure 5 shows fluorescence photographs of $e_1a_2P190^{BCR-ABL}$ transfected COS 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 intensely 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 staining experiments were performed on $P210^{BCR-ABL}$ transfected COS cells using ER-FP1 and the anti-ABL antibody 8E9 (Fig-

ures 5c - 5f). ER-FP1 shows a weak background staining of all cells, either transfected or untransfected (Figure 5c, and 5e). The antibodies did not react with the $P210^{BCR-ABL}$ proteins, since the pattern obtained after ER-FP1 incubation is completely different from the specific staining pattern with 8E9 (Figure 5d, and 5f). Staining with 8E9 shows that $P210^{BCR-ABL}$ and $P190^{BCR-ABL}$ both have the same highly characteristic subcellular localization.
From these data we conclude that native $e_1a_2P190^{BCR-ABL}$ is recognized by ER-FP1 in a highly specific way at the single cell level.

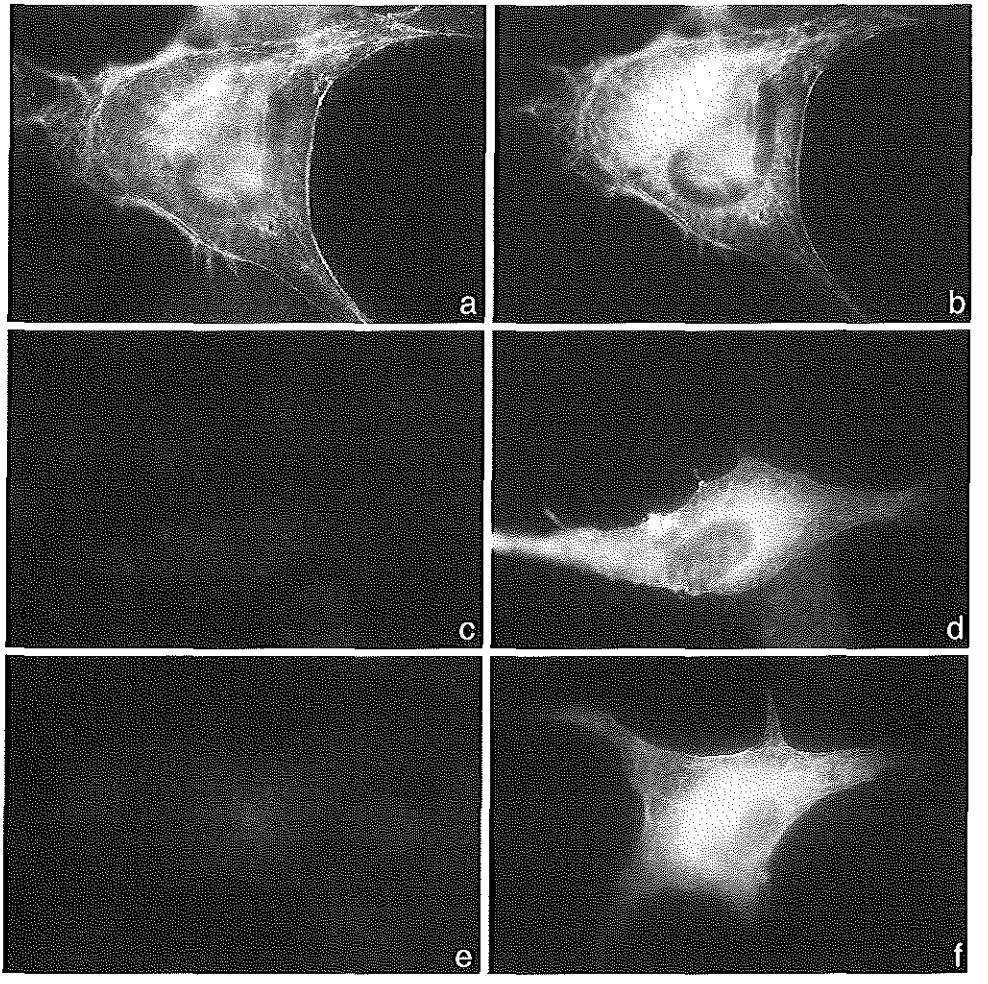

Figure 5 Immunofluorescence analysis of ER-FP1. COS cells were transfected with pCDX plasmids comprising full length cDNA encoding $e_1a_2P190^{BCR-ABL}$ (a) and (b), $b_2a_2P210^{BCR-ABL}$ (c) and (d) and $b_3a_2P210^{BCR-ABL}$ (e) and (f). Transfected cells were double-labeled with ER-FP1, detected with TRITC-labeled goat-anti mouse IgG2a (a), (c), and (e) and with anti-ABL antibody 8E9, detected with FITC-labeled goat-anti mouse IgG1(b), (d) and (f)

Discussion

The Philadelpia 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 detecting the Ph chromosome or the molecular rearrangement caused by the translocation.

In the past years we have focussed on the detection of the various BCR-ABL chimeric proteins, i.e. e_1a_2P190$^{BCR-ABL}$, b_2a_2P210$^{BCR-ABL}$ and b_3a_2P210$^{BCR-ABL}$. We have demonstrated that the BCR-ABL junctions in all 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 their respective BCR-ABL junctions.

Now we report on the reactivity of a monoclonal antibody ER-FP1, raised against a synthetic peptide corresponding to the e_1a_2 BCR-ABL junction in e_1a_2P190$^{BCR-ABL}$. We show evidence that ER-FP1 reacts specifically with e_1a_2 peptides in an ELISA as well as with native e_1a_2P190$^{BCR-ABL}$ 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 analysis. COS cells transfected with DNA encoding the complete e_1a_2P190$^{BCR-ABL}$ protein were stained with ER-FP1. The staining pattern of ER-FP1 was strikingly similar to the staining 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 *et al.* (29) and McWhirter 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 with native chimeric proteins at the single cell level. This study indicates that the antigenic determinant formed by the e_1a_2 junction, is saved 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 comprising P190$^{BCR-ABL}$ or P210$^{BCR-ABL}$. A feasible explanation for this phenomenon is that the expression of e_1a_2P190$^{BCR-ABL}$ is too low in ALL cell lines, where specific staining does not rise above background. Alternatively, it is possible that the same epitope formed by the e_1a_2 junction in P190$^{BCR-ABL}$ is also expressed by other, yet unknown, intracellular proteins. In Western blotting experiments we observed binding of ER-FP1 to many proteins, which could cause the background immunofluorescence (data not shown). However, comparing both the immunofluorescence and the Western blotting data is not quite correct, because e_1a_2P190$^{BCR-ABL}$ is not recognized by ER-FP1 on the Western blot. Nevertheless, whenever cross-reacting epitopes are specifically recognized by ER-FP1, diagnosis of Ph-positive ALL by using ER-FP1 in standard immunofluorescence techniques will be difficult. More refined im-

munofluorescence analysis by which specific staining patterns can be observed, e.g. using confocal laserscan microscopy, might resolve this 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, indicate that there is co-localization of $e_1a_2P190^{BCR\text{-}ABL}$ with actin (data not shown). Also in $P190^{BCR\text{-}ABL}$ negative cells we observed co-localization. However, the number of double-positive cells and the intensity of the fluorescence was higher in the $P190^{BCR\text{-}ABL}$ positive cells than in the $P190^{BCR\text{-}ABL}$ negative cells. The background staining both in the $P190^{BCR\text{-}ABL}$ positive and in the $P190^{BCR\text{-}ABL}$ negative cells revealed a fine granular staining pattern in the nucleus and in the cytoplasm, whereas the double stained material was closely related under the cell membrane. Further analysis of the co-localization pattern, which is present in almost all $P190^{BCR\text{-}ABL}$ positive and in a small number of $P210^{BCR\text{-}ABL}$ cells, may provide, in the future, the answer to the specific detection of $P190^{BCR\text{-}ABL}$ in routine immunofluorescence studies of tumor cells.

In summary, at present utilization of ER-FP1 in diagnosis of Philadelphia-chromosome-positive ALL is limited to the tyrosine kinase assay. To apply ER-FP1 in routine immunofluorescence diagnosis, more work is needed to solve the background problem and to selectively filter out and enhance the double-labeling signal.

Acknowledgements

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Chapter 8

The BCR-ABL dipstick assay: a simple and rapid method for detecting Ph$^+$ leukemias

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The BCR-ABL dipstick assay: a simple and rapid method for detecting Ph+ leukemias

The reciprocal translocation t(9;22)(q34;q11), observed in chronic myeloid leukemia (CML), acute lymphoblastic leukemia (ALL), and acute myeloid leukemia (AML), results from fusion between two genes: BCR and ABL. Depending on the localization of the breakpoint in the BCR gene, different tumor-specific BCR-ABL genes are generated. These BCR-ABL genes are transcribed and translated into tumor-specific BCR-ABL mRNAs and tumor-specific BCR-ABL proteins, respectively. Therefore, different diagnostic targets are available, each allowing for specific diagnosis of t(9;22) positive leukemias. While conventional cytogenetics relies on the detection of the characteristic chromosomal aberration (i.e. the Philadelphia chromosome: a minute chromosome 22q-), other techniques are used to specifically detect the BCR-ABL fusion-gene (e.g. fluorescent in situ hybridization) or the BCR-ABL fusion mRNA (e.g. reverse transcriptase polymerase chain reaction). Although all of the aforementioned techniques are well established and reliable, none of these techniques can be easily performed on a routine and short-term basis. Yet, especially in ALL, presence of the Ph chromosome is associated with poor prognosis. To improve the poor prognostic outcome, Ph positive ALLs require early identification to permit intensive induction regimens or alternative treatment protocols.

We present a new diagnostic technique, termed 'the BCR-ABL dipstick assay'. This technique is based on the exclusive detection of BCR-ABL proteins and is designed to identify Ph+ leukemias at first diagnosis in a rapid and simple fashion. The principle and the applications of the BCR-ABL dipstick assay are evaluated and discussed in view of current diagnostic methods.

Introduction

The Philadelphia chromosome (Ph): a minute chromosome 22q-, originally identified by Nowell and Hungerford in 1960 (1), was the first karyotypic aberration found to be tumor-related (1, 2). To date, the Ph chromosome is identified in various hematopoietic disorders, e.g. chronic myeloid leukemia (CML), acute lymphoblastic leukemia (ALL), and acute myeloid leukemia (AML), in both adults and children (3).

The Ph chromosome is generated by the reciprocal translocation between the long arms of chromosome 9 and 22: t(9;22)(q34;q11) and involves the *ABL* gene on chromosome 9 and the *BCR* gene on chromosome 22 (3). Both genes are interrupted and rearranged, resulting in a tumor-specific *BCR-ABL* fusion-gene on chromosome 22q- and a tumor-specific *ABL-BCR* fusion-gene on chromosome 9q+.

While reports on the *ABL-BCR* fusion-gene are still limited (4), *BCR-ABL* fusion-genes have been extensively studied over the past two decades. Depending on the chromosomal localization of the breakpoints, different *BCR-ABL* fusion-genes can be

identified. Although breakpoints are scattered over long distances in the *ABL* gene, they mostly occur 5' of exon a2 (5). In contrast, breakpoints in the *BCR* gene are clustered within two regions: a major breakpoint cluster region (M-BCR), comprising five exons termed b1 to b5 (6, 7, 8), and a minor breakpoint cluster region (m-BCR), located 5' of the M-BCR in the *BCR* gene (9).

In most CML patients and in approximately 30% of Ph^+ ALL patients (10, 11), breakpoints are evenly distributed in the M-BCR: either located between exon b2 and b3 or located between exon b3 and b4 (12, 13, 14). Breakpoints in Ph^+ ALL are in majority (±70%) found within the m-BCR region (10, 11), localized in an intron between exon e1 and e2. As breakpoints are scattered over long distances (especially in the *ABL* gene), different fusion-point introns in *BCR-ABL* genes are generated. Although *BCR-ABL* genes are highly variable between Ph^+ patients when considering the *BCR-ABL* fusion-point intron's length and nucleotide sequence, fusion-points of BCR-ABL transcripts are highly consistent. Thus, depending on the original *BCR-ABL* gene rearrangement, a single BCR-ABL mRNA is usually detected: a 7 kb mRNA comprising an e1a2 junction (9) or, a 8.5 kb mRNA that either comprises a b2a2 or a b3a2 junction (12, 14). Since the translational reading frames in the processed chimeric BCR-ABL mRNAs are maintained, characteristic chimeric BCR-ABL proteins are expressed by Ph^+ leukemic cells. Based on the variation of the BCR-ABL fusion-point, either $e_1a_2P190^{BCR\text{-}ABL}$, $b_2a_2P210^{BCR\text{-}ABL}$ or $b_3a_2P210^{BCR\text{-}ABL}$ proteins are detected (15, 16).

While Ph chromosomes are almost invariably present in CML cases, Ph chromosomes are less often detected in leukemic cells from patients suffering from AML or ALL. Still, 5% of AML cases, 25% to 30% of adults with ALL and 3% to 5% of children with ALL are diagnosed as Ph positive (17, 18). Reflected by a high rate of treatment failure and mortality in Ph^+ leukemias, in both adults and children, Ph chromosomes are hallmarked as significant risk-factors considering treatment failure (19, 20). The importance of identifying risk-features, such as the Ph chromosome, is beyond doubt. Current treatment protocols may be improved through the identification of the t(9;22) at an early time-point of the disease. At present, Ph^+ leukemias are identified by a number of techniques, either detecting the aberrant chromosome, the gene, the mRNA or the aberrant protein. Yet, each of these techniques is characterized by typical specifications and limitations which should be considered before diagnosing t(9;22)(q34;q11) positive leukemias (21).

In this study we report on a new diagnostic technique: the BCR-ABL dipstick assay. This assay was developed to discriminate between Ph^+ leukemias and Ph^- leukemias at diagnosis in a relatively rapid and simple fashion. The underlying principle of the BCR-ABL dipstick assay is based on the *exclusive* detection of tumor-specific BCR-ABL proteins. The principle and the applications of the BCR-ABL dipstick assay are evaluated and discussed in view of currently used diagnostic methods.

Materials and methods

Cell samples

Cell lines: Six Ph^+ cell lines were used to examine the specificity of both the sepharose-Western blotting procedure as well as the BCR-ABL dipstick assay: two b_3a_2 Ph^+ cell lines: LAMA-84 (22) and K562 (23), two b_2a_2 Ph^+ cell lines: KCL-22 (24) and BV-173 (25), and two e_1a_2 Ph^+ cell lines: TOM-1 (26) and ALL/MIK (27).
All cell lines were cultured in RPMI-1640 supplemented with 10% fetal calf serum.
Leukemic cell samples: Two leukemic cryopreserved peripheral blood samples from leukemic patients at diagnosis were used to examine the specificity of the BCR-ABL dipstick assay. Clinical and laboratory data of these patients have been described previously (28, 29): one patient suffered from a Ph^- CML, with rearranged b_2a_2 *BCR-ABL* genes, the other suffered from a Ph^+ precursor B-ALL, with rearranged e_1a_2 *BCR-ABL* genes.

Antibodies

All antibodies used were protein G purified and categorized as:
Catching antibodies: monoclonal antibody (moAb) 7C6 (a generous gift from Dr S Dhut), directed towards the b_2-epitope present in $b_2a_2P210^{BCR\text{-}ABL}$, $b_3a_2P210^{BCR\text{-}ABL}$, $P160^{BCR}$ and $P130^{BCR}$ (30); moAb ER-FP1, directed towards the e_1a_2 fusion-point in $e_1a_2P190^{BCR\text{-}ABL}$ (28) and; moAb Yae (Santa Cruz Biotechn., Santa Cruz, CA, USA) directed towards the amino-terminus of E2A proteins (31).
Detecting antibodies: moAb 8E9 (a generous gift from Dr J Wang), directed towards the SH2 domain present in $e_1a_2P190^{BCR\text{-}ABL}$, $b_2a_2P190^{BCR\text{-}ABL}$, $b_3a_2P190^{BCR\text{-}ABL}$ and $P145^{ABL}$ (32) and; moAb G98-271.1.3 (a generous gift from Dr G Bain) directed towards the carboxyl terminus of E2A proteins (33).
Both moAb 8E9 and moAb G98-271.1.3 were biotinylated according to a procedure described by Bayer (34).

Sepharose-Western blotting procedure

Cells were washed twice with ice-cold phosphate buffered saline (PBS) and lysed in ice-cold lysis buffer (1% Triton X-100, 0.05% sodium dodecyl sulfate (SDS), 150 mM NaCl, 5 mM EDTA in 10 mM sodium phosphate, pH 7.0), supplemented with 40 µl phenyl methyl sulfonyl fluoride (PMSF: 100 mM in 2-isopropanol) at a concentration of 1×10^7 cells/ml for 15 min. After the lysates were centrifuged in an Eppendorf centrifuge to remove insoluble material (5 min 4 °C), supernatants were split into equal volumes, each representing 1×10^7 cells.
Sepharose-Western blotting was performed by adding either 10 µg moAb 7C6 or 2 µg moAb ER-FP1 to the supernatant of lysed cells. Antigen-antibody reaction was allowed for two hours on a rotation device at 4 °C. Next, 40 µl of an 80% (v/v) suspension of GammaBind G sepharose beads (Pharmacia Biotech AB, Uppsala, Sweden) were added. After 30 min, beads were collected and washed three times in lysis-buffer without SDS. Beads were boiled for 5 min in 60 µl sample buffer (60 mM TRIS-HCl, pH 6.8, 10% glycerol, 10 mM EDTA, 2% SDS, 2% β-mercaptoethanol and 0.03% bromophenol blue). Proteins samples (10 µl/well) were subjected to 6% SDS-PAGE and transferred (Mini Protean; Bio Rad, Richmond, CA, USA) to nitrocellulose (0.45 µm pore size; Schleicher & Schuell, Dassel, Germany). Nitrocellulose sheets were blocked in 5% non-fat dry milk powder (Protifar, Nutricia, The Netherlands) in PBS supplemented with 0.05% Tween-20 (5% MPBS).

Next, sheets were incubated for two hours at room temperature in the presence of biotinylated moAb 8E9 (2 µg/ml) in 1% MPBS. Following three washes with PBS supplemented with 0.05% Tween-20, alkaline phosphatase conjugated to streptavidin (South. Biotechn. Ass., Birmingam, AL, USA) was added to a 1:1500 dilution and incubation was allowed to proceed for one hour. The blot was washed twice with PBS supplemented with 0.05% Tween-20 and finally with 0.15 M veronal acetate, pH 9.6. For visualization of antibody-antigen complexes we used the alkaline phosphatase substrate nitro blue tetrazolium / 5-bromo-4-chloroindoxyl phosphate (NBT/BCIP; Sigma, St Louis, MO, USA) as described by Blake *et al.* (35).

BCR-ABL dipstick method

Each catching antibody was applied as a single small spot to a (± 2 cm x 0.5 cm) nitrocellulose (0.45 µm pore size) strip and air dried. Each spot contained either 2 µg of moAb 7C6, 1 µg moAb ER-FP1 or 1 µg moAb Yae. Next, these nitrocellulose strips, called 'dipsticks', were rinsed in PBS supplemented with 0.05% Tween-20 and subsequently blocked in 5% MPBS (1 h, RT). At this point, dipsticks can be air dried and stored in an airtight container at 4 °C until further use.

Supernatants of cellular lysates (processed as described in the first paragraph of the above section), representing 1 x 10^7 cells, were added to the dipsticks. Antigen-antibody complex formation was allowed to proceed overnight at 4 °C on a rotation device. Next, dipsticks were rinsed three times in PBS supplemented with 0.05% Tween-20 and bound antigens were detected by incubating the dipstick with a mixture of biotinylated moAb 8E9 (2 µg/ml) and biotinylated moAb G98-271.1.3 (2 µg/ml), diluted in 1% MPBS. From this point on, dipsticks were further processed as described in the materials and method section of the sepharose-Western blotting procedure.

Results

Exclusive recognition of BCR-ABL proteins in a sepharose-Western blotting procedure

To determine whether the tumor-specific BCR-ABL fusion-proteins can be *exclusively* recognized by immunologic methods, we developed a sepharose-Western blotting procedure. A sepharose-Western blotting procedure is a combination of an immunoprecipitation reaction with a *catching* antibody, followed by a Western blotting procedure with *detecting* antibodies.

According to this procedure, we examined the combination of moAb 7C6 and moAb 8E9 as catching and detecting antibody, respectively. MoAb 7C6 specifically recognizes the b_2-epitope present in $b_2a_2P210^{BCR\text{-}ABL}$, $b_3a_2P210^{BCR\text{-}ABL}$, $P160^{BCR}$ and $P130^{BCR}$ proteins (30), while moAb 8E9 recognizes the SH2-domain present in both ABL proteins and BCR-ABL proteins (32). Given the specificity of both moAbs, one would expect that BCR-ABL proteins from cellular lysates of either $b_2a_2P210^{BCR\text{-}ABL}$ or $b_3a_2P210^{BCR\text{-}ABL}$ containing cell lines are recognized by the successive action of moAb 7C6 and moAb 8E9. To examine the specificity of the combination of moAbs 7C6 and 8E9, lysates from different cell lines, each harboring a distinctive BCR-ABL protein, were employed.

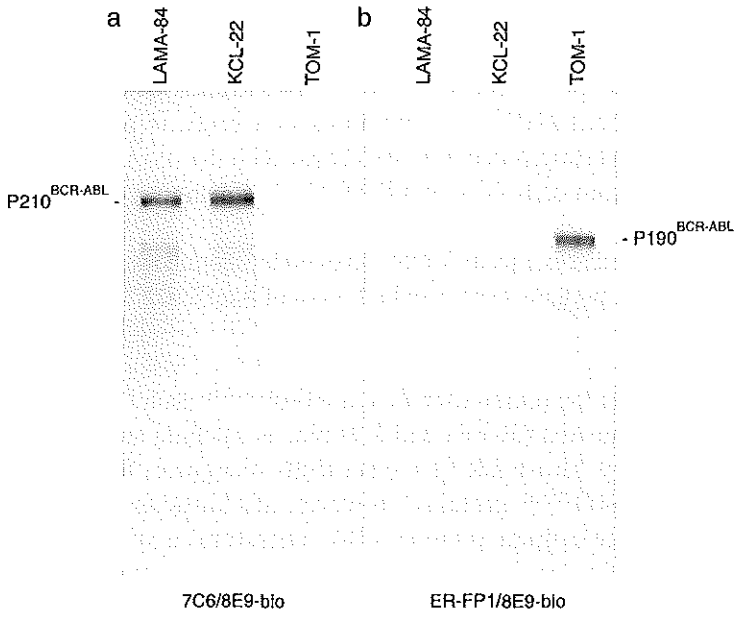

Figure 1 Sepharose-Western blotting experiments demonstrating the exclusive recognition of BCR-ABL proteins by the antibody combinations: moAbs 7C6/8E9-bio (a) and moAbs ER-FP1/8E9-bio (b), respectively. MoAbs 7C6 and ER-FP1 were used as *'catching'* antibodies, precipitating proteins from cellular lysates of LAMA-84 ($b_3a_2P210^{BCR-ABL}$ positive cell line), KCL-22 ($b_2a_2P210^{BCR-ABL}$ positive cell line) or TOM-1 ($e_1a_2P190^{BCR-ABL}$ positive cell line). Cell lines are indicated at the top of the blots. Following immunoblotting, precipitated proteins were detected by the successive use of biotinylated moAbs 8E9 as *'detecting'* antibodies and alkaline-phosphatase conjugated to streptavidin.
(a) Shows the exclusive recognition of b_3a_2BCR-ABL proteins and b_2a_2BCR-ABL proteins in the moAb 7C6 immuno-precipitates from cellular lysates of LAMA-84 and KCL-22, respectively. No proteins are detected in the 7C6 immuno-precipitate from the lysate of TOM-1 cells by biotinylated moAb 8E9.
(b) Shows the exclusive recognition of e_1a_2BCR-ABL proteins in the ER-FP1 immunoprecipitate from the TOM-1 cell line. No proteins are detected in the moAb ER-FP1 immunoprecipitates from the LAMA-84 nor from the KCL-22 cell lines by biotinylated moAb 8E9.

Figure 1a represents a Western blot of proteins from: (1) LAMA-84 ($b_3a_2P210^{BCR-ABL}$ positive cell line), (2) KCL-22 ($b_2a_2P210^{BCR-ABL}$ positive cell line) and (3) TOM-1 ($e_1a_2P190^{BCR-ABL}$ positive cell line), precipitated by moAb 7C6 and ensuingly detected by biotinylated moAb 8E9 and streptavidin-AP. As expected, this procedure leads to the detection of $P210^{BCR-ABL}$ proteins (Figure 1a; lanes 1 and 2).
However, besides the recognition of $P210^{BCR-ABL}$ proteins, the moAb 7C6/8E9-bio combination also detects proteins with a molecular weight of 190 kDa from cellular lysates of LAMA-84 and KCL-22 (Figure 1a; lanes 1 and 2). Dhut *et al.* and Guo *et al.* both reported on the detection of this aberrant P190 protein by moAb 7C6 and moAb 8E9, respectively (30, 32). Given the specificity of both antibodies: 1) moAb 7C6, exclusively recognizing the b_2-epitope present in $b_2a_2P210^{BCR-ABL}$, $b_3a_2P210^{BCR-ABL}$, $P160^{BCR}$

and $P130^{BCR}$ and 2) moAb 8E9, recognizing the SH2-domain present in BCR-ABL and ABL proteins, we deduce that the aberrant P190 represents a BCR-ABL protein that contains a b_2-epitope. Moreover, the aberrant $P190^{BCR-ABL}$, detected in lysates of $b_2a_2P210^{BCR-ABL}$ or $b_3a_2P210^{BCR-ABL}$ containing cell lines, is not observed upon a sepharose-Western blotting procedure performed with the moAb 7C6/8E9-bio combination in lysates from cells of an $e_1a_2P190^{BCR-ABL}$ containing cell line, i.e. TOM-1 (Figure 1a; lane 3).
Therefore, we conclude that the moAb 7C6/8E9-bio combination allows for the *exclusive* identification of BCR-ABL proteins from cellular lysates of $b_2a_2P210^{BCR-ABL}$ or $b_3a_2P210^{BCR-ABL}$ positive cell lines.

To determine whether the tumor-specific $e_1a_2P190^{BCR-ABL}$ proteins can be exclusively identified, we used moAbs ER-FP1 and 8E9-bio, as catching antibody and detecting antibody, respectively. MoAb ER-FP1 specifically recognizes the tumor-specific fusion-point of $e_1a_2P190^{BCR-ABL}$ proteins. However, this particular epitope is probably also present on other cellular proteins (28). Therefore, a specific recognition of $e_1a_2P190^{BCR-ABL}$ proteins can only be expected through a combination of the catching ER-FP1 antibody and the biotin labeled, detecting antibody moAb 8E9. We immunoprecipitated proteins from cellular lysates of LAMA-84 ($b_3a_2P210^{BCR-ABL}$ positive), KCL-22 ($b_2a_2P210^{BCR-ABL}$ positive) and TOM-1 ($e_1a_2P190^{BCR-ABL}$ positive) by moAb ER-FP1. The precipitated proteins were detected by biotinylated moAb 8E9 in a Western blotting procedure.
Figure 1b shows the detection of $e_1a_2P190^{BCR-ABL}$ proteins by the ER-FP1/8E9-bio antibody combination of antibodies (lane 3). Neither $b_3a_2P210^{BCR-ABL}$ (Figure 1b; lane 1) nor $b_2a_2P210^{BCR-ABL}$ (Figure 1b; lane 2) proteins are visible, nor is any other cross-reactive protein (either immunoprecipitate, Figure 1b; lanes 1 to 3).
Therefore, the combination of moAbs ER-FP1 and 8E9-bio allows for the exclusive recognition of $e_1a_2P190^{BCR-ABL}$ proteins. Moreover, the aberrant P190 protein, detected by the moAb 7C6/8E9-bio combination in $b_2a_2P210^{BCR-ABL}$ positive cell lines and $b_3a_2P210^{BCR-ABL}$ positive cell lines (Figure 1a; lanes 1 and 2), is not recognized by the moAb ER-FP1/8E9-bio combination. This further substantiates our notion that the aberrant $P190^{BCR-ABL}$ protein as detected by the 7C6/8E9-bio combination does not represent $e_1a_2P190^{BCR-ABL}$.
In conclusion, our sepharose-Western blotting data verify that tumor-specific BCR-ABL proteins are exclusively identified by the appropriate choice of monoclonal antibodies.

Exclusive recognition of BCR-ABL proteins in a dipstick assay
We next investigated whether the sepharose-Western blotting procedure could be simplified. By using the same sets of antibodies as were used in the sepharose-Western blotting experiments, we examined the possibilities of an alternative BCR-ABL detection system, termed: the 'BCR-ABL dipstick assay'.

The BCR-ABL dipstick assay was designed for the exclusive detection of BCR-ABL proteins, allowing detection and discrimination between $b_2a_2P210^{BCR\text{-}ABL}$ or b_3a_2-$P210^{BCR\text{-}ABL}$ proteins on the one hand and $e_1a_2P190^{BCR\text{-}ABL}$ proteins on the other. A control for proper assay development was also included in case a Ph^- sample would be investigated.

The BCR-ABL dipstick is made of nitrocellulose strips on which three different antibodies are immobilized: 1) moAb 7C6 (recognizing the b2 epitope present in $b_2a_2P210^{BCR\text{-}ABL}$, $b_3a_2P210^{BCR\text{-}ABL}$, $P160^{BCR}$ and $P130^{BCR}$), 2) moAb ER-FP1 (recognizing the e_1a_2 fusion-point epitope present in $e_1a_2P190^{BCR\text{-}ABL}$) and 3) moAb Yae (recognizing the amino-terminus of the ubiquitously expressed transcription factor E2A).

To investigate whether the BCR-ABL dipstick can be used for the specific detection of BCR-ABL proteins, BCR-ABL dipsticks were either incubated with cellular lysates from: 1) LAMA-84 ($b_3a_2P210^{BCR\text{-}ABL}$ positive cell line), 2) KCL-22 ($b_2a_2P210^{BCR\text{-}ABL}$ positive cell line) or 3) TOM-1 ($e_1a_2P190^{BCR\text{-}ABL}$ positive cell line). Cellular proteins that had been caught by the immobilized antibodies on the BCR-ABL dipstick were de-

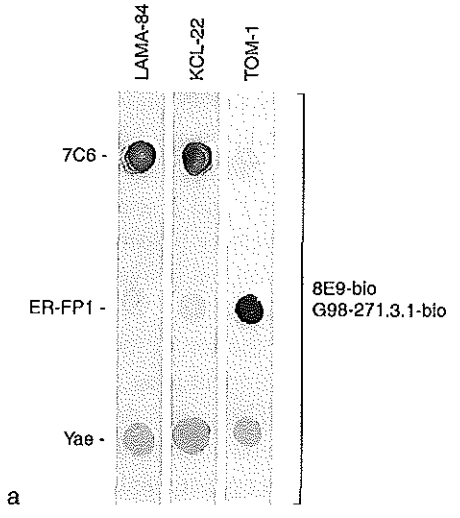

Figure 2a Specificity analysis of the BCR-ABL dipstick assay. *Catching* antibodies: moAb 7C6, moAb ER-FP1 and moAb Yae (indicated left, from top to bottom, respectively) were used to precipitate proteins from different cellular lysates. LAMA-84 ($b_3a_2P210^{BCR\text{-}ABL}$ positive cell line), KCL-22 ($b_2a_2P210^{BCR\text{-}ABL}$ positive cell line) and TOM-1 ($e_1a_2P190^{BCR\text{-}ABL}$ positive cell line), indicated on top of the BCR-ABL dipsticks, were used as BCR-ABL protein containing cellular lysates. Biotinylated moAb 8E9 was used as *detecting* antibody, *exclusively* identifying b_3a_2-BCR-ABL-, b_2a_2BCR-ABL- and e_1a_2BCR-ABL proteins from LAMA-84, KCL-22 or TOM-1 cellular lysates, respectively. The biotinylated *detecting* antibody G98-271.1.3 was used as a positive control, identifying ubiquitously expressed E2A proteins.

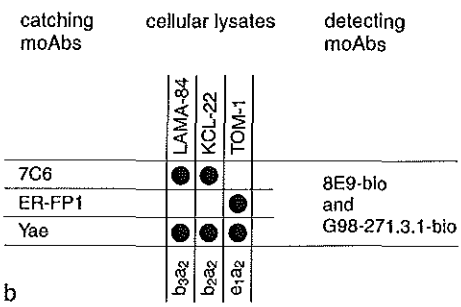

Figure 2b Schematic representation of the BCR-ABL dipstick assay incubation procedure. The first step, representing the immobilization of catching antibodies to the nitrocellulose strip, is indicated left. The second step, representing the incubation with the cellular lysate, is indicated in the middle. The third step, representing the detecting antibodies, is indicated at the right. BCR-ABL dipsticks are developed by the successive incubation with alkaline-phosphatase conjugated to streptavidin, followed by its sub-strate NBT/BCIP.

tected by subsequent incubation with a mixture of biotinylated moAb 8E9 (8E9-bio, recognizing the carboxyl terminus of both ABL and BCR-ABL proteins) and biotinylated moAb G98-271.1.3 (G98-271.1.3, recognizing the carboxyl terminus of E2A-proteins) followed by alkaline phosphatase conjugated to streptavidin.
Incubating a BCR-ABL dipstick with either cellular lysates from LAMA-84 or cellular lysates from KCL-22, results, upon successive incubation with biotinylated antibodies (i.e. 8E9-bio and G98-271.1.3-bio), streptavidin-AP and its substrate, in visible dots located at the moAb 7C6 spot (Figure 2; lanes 1 and 2, first spot). Considering the sepharose-Western blotting data described above, these dots represent bound $b_3a_2P210^{BCR\text{-}ABL}$ and $b_2a_2P210^{BCR\text{-}ABL}$ proteins, respectively. Confirmation of the specificity of the BCR-ABL dipstick assay is provided by another BCR-ABL dipstick, now incubated with a cellular lysate from TOM-1 cells ($e_1a_2P190^{BCR\text{-}ABL}$ positive). Subsequent development of this BCR-ABL dipstick through the successive incubation by the aforementioned molecules, does not result in a visible 7C6 spot (Figure 2; lane 3, first spot). These results indicate that $b_3a_2P210^{BCR\text{-}ABL}$ and $b_2a_2P210^{BCR\text{-}ABL}$ proteins present in cellular lysates from LAMA-84 and KCL-22 are recognized by the moAb 7C6/8E9-bio combination, but proteins from the cellular lysate of TOM-1 cells, especially $e_1a_2P190^{BCR\text{-}ABL}$ proteins, are not recognized by this antibody combination.
The tumor-specific $e_1a_2P190^{BCR\text{-}ABL}$ proteins are recognized by the second spot. This spot, representing the ER-FP1/8E9-bio antibody combination is clearly visible (Figure 2; lane 3, second spot), while second spots on the BCR-ABL dipsticks incubated with lysates from either LAMA-84 or KCL-22 (Figure 2, lanes 1 and 2, second spots) remain invisible. These results, together with the sepharose-Western blotting data described above, indicate that the ER-FP1/8E9-bio combination exclusively detects $e_1a_2P190^{BCR\text{-}ABL}$ proteins in the BCR-ABL dipstick assay.
The third spot, represents E2A proteins detected by the combination of moAbs Yae/G98-271.1.3-bio. This spot is visible upon subsequent development after incubation with either cellular lysate (Figure 2, lanes 1 to 3, third spot). These data demonstrate that the Yae/G98-271.1.3 moAb combination can be used as a positive control, assuring proper assay development in case a Ph negative sample is investigated.
Together, these data demonstrate that the BCR-ABL dipstick assay can be applied for the *exclusive* detection of the tumor-specific BCR-ABL proteins.

Sensitivity of the BCR-ABL dipstick assay

To investigate the sensitivity of the BCR-ABL dipstick assay, we performed a series of dilution experiments. To this purpose, cells from the $b_2a_2P210^{BCR\text{-}ABL}$ containing KCL-22 cell line and cells from the $e_1a_2P190^{BCR\text{-}ABL}$ containing TOM-1 cell line were mixed at various ratios. BCR-ABL dipsticks, represented by lane 1 to 12 in Figure 3, were incubated with the lysates of these various mixtures and were subsequently developed.

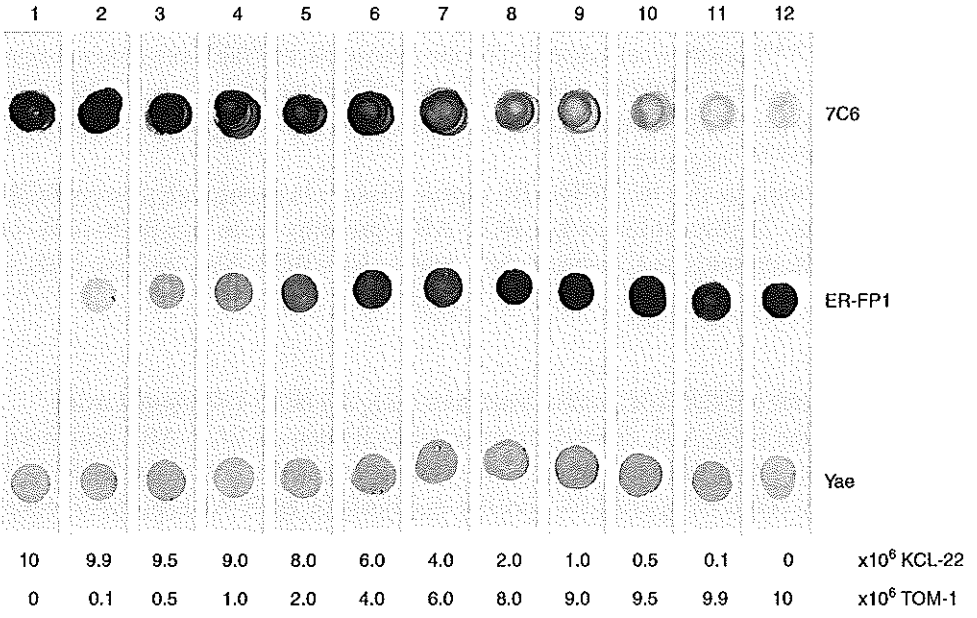

Figure 3 Sensitivity analysis of the BCR-ABL dipstick assay. Twelve BCR-ABL dipsticks were incubated with different mixtures of cellular lysates from KCL-22 ($b_2a_2P210^{BCR\text{-}ABL}$ positive cell line) and TOM-1 ($e_1a_2P190^{BCR\text{-}ABL}$ positive cell line). Each cellular lysate represents a total of 10^7 cells per dipstick. From left to right, each first spot represents declining amounts of b_2a_2BCR-ABL proteins detected by the moAb 7C6/8E9-bio combination. From right to left, each second spot represents declining amounts of e_1a_2BCR-ABL proteins detected by the ER-FP1/8E9-bio combination. The relations of the number of $b_2a_2P210^{BCR\text{-}ABL}$ positive cells to the number of $e_1a_2P190^{BCR\text{-}ABL}$ positive cells are depicted below.

The first spot on lane 9, represents the lowest $b_2a_2P210^{BCR\text{-}ABL}$ protein concentration that is clearly distinguishable from the background staining observed at the first spot on lane 12. Here, ten b_2a_2-$P210^{BCR\text{-}ABL}$ positive cells are detected among 90 $e_1a_2P190^{BCR\text{-}ABL}$ negative cells.

The second spot on lane 2, represents the lowest $e_1a_2P190^{BCR\text{-}ABL}$ protein concentration that is clearly distinguishable from the background staining of the second spot on lane 1. Here, one $e_1a_2P190^{BCR\text{-}ABL}$ positive cell is detected among 99 $b_2a_2P210^{BCR\text{-}ABL}$ negative cells.

As shown in Figure 3, the dot representing the 7C6/8E9-bio combination, is still clearly visible in lane 9. Here, ten $b_2a_2P210^{BCR\text{-}ABL}$ positive cells among 90 $e_1a_2P190^{BCR\text{-}ABL}$ negative cells are identified. The negative 7C6/8E9-bio control on lane 12 is hardly visible (Figure 3, lane 12). The 7C6/8E9-bio dots on lane 11 and 10 represent detection of 1% and 5% $b_2a_2P210^{BCR\text{-}ABL}$ positive cells, respectively. Unfortunately, staining of these dots is less pronounced and barely distinguishable from that of the negative 7C6/8E9-bio control on lane 12. Yet, when considering the sensitivity of the BCR-ABL dipstick assay for detecting presence of $b_2a_2P210^{BCR\text{-}ABL}$ positive cells, staining of the 7C6 spot on lane 9 is indisputably more intense than staining of the 7C6 spot on lane 12. Therefore, we conclude that the moAB 7C6/8E9-bio combination is capable to detect 10% $b_2a_2P210^{BCR\text{-}ABL}$ positive tumor cells, at least.

The spot in the middle of lane 2, representing one $e_1a_2P190^{BCR-ABL}$ positive cell among 99 $b_2a_2P210^{BCR-ABL}$ positive cells detected by the moAb ER-FP1/8E9-bio combination, is still visible in lane 2, while the same spot is not visible in lane 1. Thus, the ER-FP1/8E9-bio combination allows the detection of one $e_1a_2P190^{BCR-ABL}$ positive cell among 100 $b_2a_2P210^{BCR-ABL}$ negative cells.
These results indicate that the BCR-ABL dipstick assay: 1) exclusively detects BCR-ABL proteins, 2) discriminates e_1a_2BCR-ABL proteins from b_2a_2BCR-ABL or b_3a2BCR-ABL proteins and 3) specifically detects 10% BCR-ABL positive cells, at least.

Specific diagnosis of BCR-ABL positive leukemias using the BCR-ABL dipstick assay
At this point the BCR-ABL dipstick assay specifically detects BCR-ABL proteins in cellular lysates made from cell lines. Next, we investigated whether the BCR-ABL dipstick assay could be applied for specific diagnosis of BCR-ABL positive leukemias.

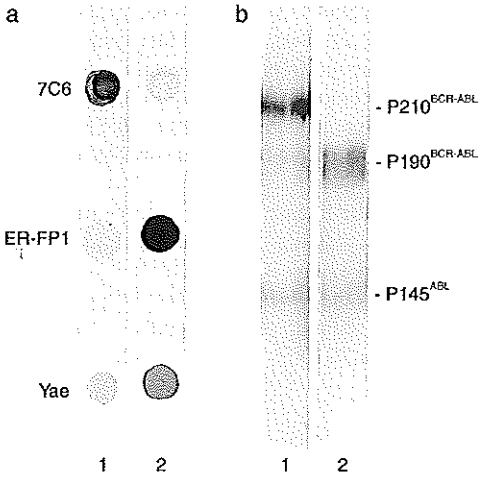

Figure 4a Represents the BCR-ABL dipstick after the analysis of blood samples from two patients suffering from either CML with rearranged b_2a_2 *BCR-ABL* genes (patient A, lanes 1) or ALL with rearranged e_1a_2 *BCR-ABL* genes (patient B, lanes 2). Consistent to the *BCR-ABL* gene rearrangements, dipstick spots are clearly visible: the first spot on dipstick 1 represents the 7C6/8E9-bio combination, While the second spot on dipstick 2 represents the ER-FP1/8E9-bio combination. The third spot, representing E2A protein detection by the Yae/G98-271.1.3-bio combination, is visible on both dipsticks.
Figure 4b Represents the sepharose-Western blotting analysis of the same patients by the 8E9/8E9-bio antibody combination. Consistent to Figure 4a, $b_2a_2P210^{BCR-ABL}$ proteins are detected in the cellular lysate of blood cells from patient A, while $e_1a_2P190^{BCR-ABL}$ proteins are detected in the cellular lysate of blood cells from patient B (lanes 1 and 2, respectively).

Two cryopreserved, Ficoll enriched blood samples from patient A and patient B, respectively, with previously diagnosed BCR-ABL positive leukemias were lysed and investigated by both the BCR-ABL dipstick assay as well as a sepharose-Western blotting procedure. The blood samples from patient A and patient B represent a Ph^- CML with cryptic rearranged b_2a_2 *BCR-ABL* genes and a Ph^+ ALL with rearranged e_1a_2 *BCR-ABL* genes, respectively.
Figure 4a, lane 1, shows that the first spot representing the 7C6/8E9-bio combination on the BCR-ABL dipstick is clearly positive upon incubation with the cellular lysate of the blood sample from patient A. The second spot of this BCR-ABL dipstick, representing the ER-FP1/8E9-bio combination is negative. These results indicate that either $b_2a_2P210^{BCR\text{-}ABL}$ proteins or $b_3a_2P210^{BCR\text{-}ABL}$ proteins are present in the cellular lysate from the blood sample of patient A. This result was verified by a sepharose-Western blotting procedure, performed with moAb 8E9 as both catching *and* detecting antibody, respectively (Figure 4b, lane 1). The observed $P210^{BCR\text{-}ABL}$ band confirms the specificity of the BCR-ABL dipstick; either detecting $b_2a_2P210^{BCR\text{-}ABL}$ or $b_3a_2P210^{BCR\text{-}ABL}$.
The BCR-ABL dipstick in Figure 4a, lane 2, shows that the second spot, representing the combination of moAbs ER-FP1/8E9-bio, is positive upon incubation with the cellular lysate of the blood sample from patient B. Since the first spot, representing the combination of moAbs 7C6/8E9-bio, is negative, it is obvious that leukemic cells from this patient contain $e_1a_2P190^{BCR\text{-}ABL}$ proteins. The presence of $e_1a_2P190^{BCR\text{-}ABL}$ proteins in the leukemic cells from this patient was confirmed by sepharose-Western blotting with the 8E9/8E9-bio combination (Figure 4b, lane 2).
The third spot, representing E2A proteins detected by the Yae/G98-271.1.3-bio combination, is positive on both BCR-ABL dipsticks (Figure 5a, lanes 1 and 2). Since the ubiquitously expressed E2A proteins are detected in the lysates of leukemic cells from both patients, we conclude that E2A protein detection in the BCR-ABL dipstick acts as a control for proper assay development.

Discussion

The presence of the Philadelphia chromosome in leukemic cells is associated with poor prognosis. Especially in ALL, it is important to distinguish Ph^+ leukemias from Ph^- leukemias, as the presence of the Ph chromosome identifies a large group of patients facing an insecure future (19, 20). Yet, this poor therapeutic outcome may be improved by an early start with more aggressive induction therapies. Therefore, sensitive and reliable diagnostic methods, identifying the Ph chromosome or its products at an early time-point of the disease, are extremely important in ALL diagnosis.
At present, conventional cytogenetic analysis is the method of first choice for identifying various chromosomal abnormalities in ALL. However, the results upon cytogenetic analysis are not always reliable since results largely depend on the number of metaphases investigated. Only institutions with special experience in ALL cytogenet-

ics achieve successful karyotype analysis in almost every patient. Even then, some cryptic BCR-ABL rearrangements escape detection by conventional cytogenetic analysis (36).

Contrary to conventional cytogenetic analysis, fluorescent in situ hybridization (FISH) techniques are not limited to a laborious analysis of metaphases. By applying probes directed against BCR and ABL genes, each labeled with a different fluorochrome, Ph^+ interphase cells can be identified. Yet, depending on the co-localization of the two hybridization signals to one spot, its sensitivity is limited, because artifactual co-localization in normal lymphocytes may be observed (37, 38).

The polymerase chain reaction (PCR) is at present the most sensitive method for detecting genetic abnormalities. In fact, molecular analysis frequently detects translocations that are not observed karyotypically (39). However, as breakpoints are scattered over long distances in the tumor-specific BCR-ABL fusion-point introns, the PCR procedure is only applicable after reverse transcription of BCR-ABL messenger RNA. Being very sensitive, strict precautions are required to prevent false positive (due to cross-contamination) or false negative (due to premature RNA degradation) results.

In this paper we describe the development of a new, simple and rapid technique: the BCR-ABL dipstick assay. The principle behind the BCR-ABL dipstick assay is based on the successive detection of two distinct antigenic sites on the BCR-ABL protein. The combined specificity of two different antibodies allows for the *exclusive* detection of *tumor-specific* BCR-ABL proteins within 24 hours. Moreover, provided that the right combination of antibodies is used, this assay can be applied to discriminate b_2a_2 and b_3a_2BCR-ABL proteins on the one hand from e_1a_2BCR-ABL proteins on the other.

Our assumptions concerning exclusive detection of BCR-ABL proteins by the proper antibody combinations proved correct as they were first tested in sepharose-Western blotting experiments. These experiments demonstrate that b_3a_2BCR-ABL proteins and b_2a_2BCR-ABL proteins are specifically identified by the moAb 7C6/8E9-bio combination, while e_1a_2BCR-ABL proteins are specifically identified by the moAb ER-FP1/8E9-bio combination. We next investigated whether the tedious and time consuming sepharose-Western blotting procedure could be simplified. The resulting BCR-ABL dipstick, a small nitrocellulose strip on which three different moAbs (i.e. moAb 7C6, moAb ER-FP1 and moAb Yae) are immobilized, was examined for both specificity and sensitivity by using different Ph^+ cell lines.

The specificity of the BCR-ABL dipstick assay was confirmed by the analysis of Ph positive cell lines; each cell line expressing a different type of BCR-ABL protein (i.e. the LAMA-84 cell line expresses $b_3a_2P210^{BCR\text{-}ABL}$; the KCL-22 cell line expresses $b_2a_2P210^{BCR\text{-}ABL}$ and the TOM-1 cell line expresses $e_1a_2P190^{BCR\text{-}ABL}$). These results are consistent and were also observed upon testing other Ph positive cell lines such as K562 ($b_3a_2P210^{BCR\text{-}ABL}$ positive cell line), BV-173 ($b_2a_2P210^{BCR\text{-}ABL}$ positive cell line) and MIK/ALL ($e_1a_2P190^{BCR\text{-}ABL}$ positive cell line) (results not shown).

The sensitivity of the BCR-ABL dipstick assay was determined to at least 10%. This

result indicates that this assay can act as an alternative screening method for detection of BCR-ABL positive leukemias at first diagnosis. Dipstick analysis of two leukemic cell samples with previously reported rearranged *BCR-ABL* genes (28) showed that initial diagnosis of BCR-ABL positive leukemias by the BCR-ABL dipstick assay is indeed feasible. Moreover, its surplus value considering conventional cytogenetic analysis was demonstrated by the analysis of patient A. Even though this patient suffered from a Ph *negative* CML with cryptic rearranged b_2a_2 *BCR-ABL* genes (29), BCR-ABL proteins were readily identified upon using the BCR-ABL dipstick method. In conclusion, the BCR-ABL dipstick is an easy and fast method that can be performed in any routine hematological laboratory, permitting diagnosis of Ph^+ samples in only a few steps. So far our results hold promise, although we are aware that larger studies are required to validate the use of this new assay for future diagnosis of Ph^+ leukemias.

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Chapter 9

General discussion

General discussion

Characteristic genetic abnormalities are strongly correlated with the prognosis of a patient suffering from cancer and, as a consequence, form a basis on which therapeutic decisions are made.
In this respect, it has been suggested that the poor prognostic features of BCR-ABL positive leukemias can be improved through the early identification of this characteristic genetic aberration. An aggressive kind of therapy, that is certainly not applicable to treat all leukemia subtypes and is only defensible by the otherwise poor prognosis of BCR-ABL positive leukemias, might, when given at an early time point of the disease, eradicate the tumor completely before it relapses during, or shortly after, the first rounds of therapy (1-3).
This was exemplified by E2A-PBX1 positive leukemias, of which the prognosis markedly improved in the early 1990s through the use of more intensive chemo-therapeutic protocols (4, 5). Probably the best example that demonstrates the clinical importance of an accurate diagnosis is provided by the t(15;17)(q21;q11-22). This translocation is particularly observed in acute promyelocytic leukemia (APL). In this translocation, the critical ligand- and DNA-binding domains of the retinoid acid receptor-α (*RARα*) gene from chromosome 17 are fused to sequences of the *PML* gene on chromosome 15 (6-8). By administering pharmacological doses of all-trans retinoid acid (i.e. the ligand for the RARα protein) to patients with t(15;17) positive APL, leukemic cells respond by differentiating into mature granulocytes, leading to morphological remissions (9-11).

The concept that tumor-cells are different from normal cells has prompted many investigators for many years to search for (tumor-specific) markers that are *exclusively* expressed by tumor-cells. At present, various tumor-specific markers, such as abnormal chromosomes, abnormal genes, and aberrant proteins, have been identified in characteristic tumor-types. The diagnostic value of current chromosomal and molecular genetic techniques in tumor diagnosis is well acknowledged: each diagnostic technique is characterized by its own specific, diagnostic applications and limitations. When considering an immunological tumor diagnosis, it is realized that immunologic, tumor-specific diagnostic techniques are still limited in their use. Yet, it is anticipated that the *immunologic* identification of tumor-specific proteins, or antigens, would not only contribute to an accurate, microscopic diagnosis of certain tumor types, but may also prove invaluable to develop new therapeutic strategies, such as immunotherapy (12, 13). In this chapter we discuss some of the considerations that emerged during our research aimed at generating antibodies specifically recognizing tumor-cells.

Tumor diagnosis and antigens

It is obvious that not every antigen which is expressed by tumor cells, is suitable for a tumor-*specific* diagnosis. Although many tumor-antigens have been discovered by monoclonal antibodies on characteristic tumor types, they are often not tumor-specific. In fact, most antigens are alternatively classified as 'tumor-related' or, 'shared' antigens because they are detected on both tumor-cells as well as on normal cells. This overlap, however, does not necessarily preclude the use of antibodies as diagnostic tools to recognize cancer cells, although their precise diagnostic applicability appears to depend on the methods through which these antibodies have been derived. Typical examples are discussed below.

Tumor cells as antigens:
Many tumor-antigens known today, have been discovered by evaluating the immune response of laboratory animals towards injected (tumor-)cells. Especially in leukemia research it was recognized that most tumor-related antigens reflect characteristic differentiation stages that typify the ongoing development from stem cells towards mature, differentiated blood cells. Even though these antigens are not exclusively expressed by tumor-cells and are also expressed by normal cells, they have become extremely important as differentiation markers. According to international agreements different antibodies have been categorized into antibody clusters, provided that identical antigens are recognized within each cluster. Antibody clusters are discriminated from one another by different CD (cluster of differentiation) codes (e.g. CD1, CD2, etc.). Owing to the clonal proliferation of malignant cells, it is nowadays customary to classify leukemic cells according to their degree of differentiation (14-16). While most 'CD antigens' were relatively easily discovered by evaluating antibody responses from laboratory animals (most often mice) towards injected (tumor)-cells, it has appeared that none of these CD-antigens is tumor-specific and that tumor-specific antigens are difficult to reveal (12-16).
Tumor-cells resemble normal cells to a large extent. Immunization strategies that are based on the injection of complete tumor-cells are, as a result of the antigenic resemblance between tumor-cells and normal cells, likely to result in the identification of antigens that are also expressed by the normal counterpart of the involved tumor-cell. Exceptions to this rule are two recently described monoclonal antibodies, both derived by injecting laboratory animals with (tumor-)cells. Since the reactivity of each of these antibodies exemplify some of the problems that are common to an immunological based tumor-specific diagnosis, they are briefly discussed below:

a. *MoAb KOR-SA3544:* identifies ALLs with the t(9;22)(q34;q11) (17). This monoclonal antibody was discovered through immunizing mice with a human, t(9;22)(q34;q11) positive, ALL derived leukemic cell line. The antibody was selected through its reactivity with 68 human cell lines, from which it appeared to

identify t(9;22)(q34;q11) positive lymphoid cell lines. Unfortunately, MoAb KOR-SA3544 cross-reacts with normal granulocytes, as well as with some leukemic cell lines with a 11q23 translocation.

b. *MoAb 7.1:* identifies childhood ALLs with the t(4;11)(q21;q23) and t(11;19)(q23;p13) as well as AMLs with abnormalities of chromosome band 11q23 (18, 19). This antibody was derived after the immunization of mice with a stromal cell line that descends from human bone marrow. The antibody was selected on the basis of its reactivity with various human cell lines, normal blood cells and bone marrow cells. It was found that this antibody specifically reacted with the human homologue of the rat chondroitin sulfate proteoglycan, NG2. This antigen is expressed on the cell surface of both AML and ALL blasts with abnormalities in chromosome band 11q23. Since the NG2 antigen is not expressed on normal hematopoietic tissue, it seems likely that the moAb 7.1 can be used as a tool to specifically identify leukemic cells with an abnormality in chromosome band 11q23.

The 'master-gene' model introduced by Rabbitts (20), essentially states that protein products of aberrantly expressed master-genes are, either directly or indirectly (by affecting the expression of 'responder genes'), involved in the malignant transformation of previously normally functioning cells. According to this model, one may expect that the antigenic profile of malignant cells is essentially changed by the aberrant expression of master-genes and differs from that of their normal counterparts.
When considering the reactivity of moAb KOR-SA3544 and moAb 7.1, it would appear that both antibodies recognize antigens that are encoded by so-called 'responder' genes. It is recognized that the 90 kDa antigen detected by moAb KOR-SA3544 is different from the tumor-specific $P190^{BCR\text{-}ABL}$ 'master' protein (17), while the NG2 molecule that is detected by moAb 7.1, differs from the MLL proteins (18, 19). MLL proteins are encoded by *MLL*, the 'master' gene that is located on chromosome band 11q23 (21, 22).
As exemplified by the 90 kDa antigen of moAb KOR-SA5344, it is probable that an immunization strategy that is based on the immunization with tumor cells, results in antibodies detecting tumor-related antigens of which both its function as well as its derivation are unknown (17). As a consequence further elaborate studies are required to examine the nature of the 90 kDa antigen and if possible, discover the encoding gene(s) (17-19).

Thus, an immunization approach intended for generating antibodies that *exclusively* recognize tumor cells, which depends on the injection of tumor cells, is likely to result in elaborate studies to deduce whether the detected antigens are *exclusively* expressed by tumor cells. Most of these studies are inclined to end with antibodies that recognize so called 'shared' antigens that are *also* expressed on normal cells.

Tumor (specific) proteins as antigens:
When considering generating antibodies that *exclusively* identify tumor-cells, it is obvious that an immunization approach should aim at *directing* the immune response, thereby diminishing the chance of generating antibodies that are reactive towards *non*-tumor-specific antigens. Recent molecular genetic findings in cancer research have identified new genes that are, as 'master' genes, directly involved in the malignant transformation of certain cells.
From the moment that the nucleotide sequence of such tumor genes has been revealed, it is easy to predict the complete amino acid sequence and, based on its sequence homology with other genes, predict the cellular function of the tumor-gene. A tumor gene's cDNA can be cloned, inserted in a suitable expression vector and used to express (recombinant) tumor-proteins (23). As immunogens, these recombinant tumor-proteins provide a new means to produce antibodies. Two sets of antibodies that have been generated by injecting recombinant proteins are depicted below.

a. *TAL1 proteins:* are potentially useful as tumor-antigens for diagnosing T-cell ALLs in which the *TAL1* gene is involved, such as in del(1)(p32), t(1;17)(p32;q35) and t(1;14)(p32;q11) positive T-cell ALLs. Four monoclonal antibodies (i.e. moAbs BTL73, 2TL75, 2TL170 and 2TL242) were derived after immunizing mice with recombinant TAL1 proteins (24). Antibodies were tested for their reactivity towards these recombinant proteins in appropriate assays, such as ELISA and Western blotting. Immunocytochemical studies indicate that these antibodies specifically react with cell lines expressing nuclear TAL1 proteins, including T-cell lines carrying tumor-specific *TAL1* gene aberrations. Although the *TAL1* gene is expressed in a variety of tissues, it is normally not expressed in T-cells (25, 26). Therefore, these antibodies are of particular interest to specifically diagnose T-cell ALLs in which TAL1 proteins are expressed.

b. *PML-RARα proteins:* are specifically expressed in t(15;17)(q21;q11-q22) positive APL. These proteins are truly tumor-specific since these chimeric proteins are not expressed in cells without the t(15;17) (q21;q11-q22). Immunohistochemical examination of APL cells reveals a unique abnormal distribution of anti-PML and anti-RARα antibody labeling. The PML pattern observed in normal cells consists of 5 to 10 discrete spherical nuclear bodies called PODs (for 'PML oncogenic domains'), whereas that of APL cells consists of smaller and far more numerous speckled patterns (27, 28). This abnormal PML staining pattern was demonstrated in cells from patients with t(15;17)-associated leukemias but not in patients with other neoplastic disorders (29). Moreover, reorganization of the speckled PML labeling pattern into PODs is observed in APL cells from patients receiving all-trans retinoid acid therapy (29, 30).

Ectopically expressed proteins, such as TAL1, are *not exclusively* expressed by tumor cells. Even though one estimates that the transition from a normal T-cell to a malig-

nant T-ALL cell is caused by the aberrant expression of TAL1 proteins in (TAL1 positive) T-cells, TAL1 proteins are themselves not tumor-specific. Since TAL1 proteins are also expressed by other cell types (e.g. erythroid, megakaryocytic, and epithelial cells), it is important when using the TAL1 antigen as a tumor marker, to distinguish T-cells from other hematopoietic cell lineages by using multiple marker analysis.

Chimeric fusion-proteins, such as PML-RARα, BCR-ABL and E2A-PBX1 are, as tumor-specific proteins, *exclusively* expressed by tumor cells. When considering microscopy or flow-cytometry as diagnostic read-out, it is important to recognize that not every antibody that reacts with a particular chimeric protein is suited for tumor-specific diagnostic purposes. A microscopic, tumor-specific diagnosis with antibodies recognizing chimeric and wild type proteins (e.g. normal PML or RARα proteins) might be feasible in some instances (e.g. PML speckles in t(15;17) positive cells versus PODs in normal cells upon anti-PML staining). However, one should anticipate that not every tumor cell that expresses chimeric proteins can be discriminated from normal cells by a characteristic (intra-)cellular localization of the chimeric protein. Immunolabeling procedures that recognize *both* chimeric proteins as well as wild type proteins are often not tumor-specific.
Thus, an immunization strategy aimed at generating antibodies that *exclusively* identify tumor-cells, which depends on the immunization with a complete (recombinant) tumor protein, is likely to result in antibodies that *also* recognize normal, wild type proteins expressed by normal cells.

Tumor specific epitopes as antigens:
Molecular genetic studies have clearly demonstrated that particular genetic aberrations, such as point mutations and gene rearrangements, generally result in the *exclusive* expression of *newly* generated, hence, tumor-specific proteins. Yet, sequence homology studies indicate that most of the predicted amino acid sequence of tumor-specific proteins is identical to (parts of the) amino acid sequence of the wild type protein counterpart(s). When considering tumor-specific proteins as antigenic targets to diagnose tumor cells specifically, it is evident that only certain regions of the protein are applicable as so called 'tumor-specific' epitopes.
According to the definition, tumor-specific epitopes are, as continuous and/or discontinuous epitopes, *exclusively* expressed on tumor-specific proteins. In this respect, continuous epitopes are defined as a stretch of contiguous amino acid residues in direct peptide linkage, while discontinuous epitopes consist of a unique group of residues that are brought together by folding of the polypeptide chain or by juxtaposition of two separate peptide chains (31).
It is difficult to predict the composition of discontinuous tumor-specific epitopes, but it is relatively simple to estimate the location and amino acid sequence of continuous tumor-specific epitopes upon comparing the predicted amino acid sequence of the

tumor protein with that of the wild type protein(s). Such tumor-specific continuous epitopes can be reproduced upon synthesizing peptides. Synthesized peptides with an amino acid sequence identical to that of a tumor-specific epitope are excellent tools to specifically direct the immune response, thereby diminishing the chance of generating antibodies that cross-react with normal proteins.

It is easily demonstrated that a certain region of the chimeric BCR-ABL fusion-protein, i.e. the protein region that includes the junction between *BCR* derived amino acids and *ABL* derived amino acids, forms a contiguous amino acid sequence that is not present in normal BCR or in normal ABL proteins. Depending on the localization of the fusion-site between *BCR* and *ABL* genes in Ph-chromosome positive leukemias, different BCR-ABL fusion-proteins are transcribed and translated: each BCR-ABL protein characterized by a specific amino acid sequence surrounding the fusion-point (i.e. $e_1a_2P190^{BCR\text{-}ABL}$, $b_2a_2P210^{BCR\text{-}ABL}$ or $b_3a_2P210^{BCR\text{-}ABL}$).
Van Denderen *et al.* (32-34) have shown that each of these BCR-ABL tumor-specific amino acid sequences is, as a fusion-point-peptide carrier-protein conjugate, suitable for immunization procedures. Accordingly, different (polyclonal) antibodies have been generated, each of them exclusively recognizing one of the chimeric BCR-ABL fusion-proteins.
From these initial studies it was concluded that *tumor-specific* BCR-ABL fusion-point sequences are *antigenically* exposed and can serve as a *new* means for tumor-*specific* diagnostic purposes (32-34).

To investigate whether a 'fusion-point peptide' based immunization strategy is a useful instrument to develop antibodies through which other characteristic tumor types can be specifically diagnosed, we immunized mice with an E2A-PBX1 fusion-point peptide (35). The E2A-PBX1 fusion-protein is exclusively found in t(1;19) positive leukemias in which *E2A* and *PBX1* genes are rearranged and fused (36).
Instead of using a fusion-point-peptide carrier-protein conjugate to enhance the peptide's immunogenicity, we immunized mice with a multiple antigenic E2A-PBX1 peptide, or MAP antigen. By using the MAP antigen as an immunogen, we minimized the chance to develop antibodies that would cross-react with the protein-carrier (37). The antiserum that was generated, termed BP 1/19, specifically recognizes the fusion-point of E2A-PBX1 proteins and, while a tumor-specific *microscopic* detection of Ph-chromosome positive tumor-cells had not been realized upon immuno-staining experiments with polyclonal anti-BCR-ABL fusion-point specific antisera, the results of BP 1/19 immunostaining experiments indicate that our anti-E2A-PBX1 fusion-point antiserum is particularly useful to distinguish, at the cellular level, t(1;19) positive leukemias from t(1;19) negative leukemias or normal cells (35, 38).

Regarding the limitations of polyclonal antibodies (e.g. limited supplies, variable specificity upon subsequent immunization and background problems in immunoassays), monoclonal anti-fusion-point specific antibodies are clearly favored. Essen-

tially by using conventional hybridoma technologies, we were able to generate two of such anti-fusion-point specific monoclonal antibodies (termed: ER-GO4 and ER-FP1, respectively) (39, 40). The results of different immuno analytical assays, such as peptide inhibition studies, (sepharose-) Western blotting experiments and protein tyrosine kinase assays, verified the specificity of our antibodies: i.e. ER-GO4 moAb specifically reacts with an E2A-PBX1 fusion-point epitope and, ER-FP1 moAb specifically reacts with the e1a2 junction in $e_1a_2P190^{BCR\text{-}ABL}$ proteins. Additionally, immunostaining experiments have demonstrated that moAb ER-GO4 specifically identifies COS cells transfected with cDNA encoding E2A-PBX1 proteins but does not detect *E2A* transfected COS cells nor does moAb ER-GO4 detect *PBX1* transfected COS cells. Likewise, COS cells transfected with cDNA encoding $e_1a_2P190^{BCR\text{-}ABL}$ proteins are specifically identified by using moAb ER-FP1, while COS cells transfected with cDNA encoding $b_2a_2P210^{BCR\text{-}ABL}$ or $b_3a_2P210^{BCR\text{-}ABL}$ proteins are not recognized by ER-FP1 antibodies. These immunofluorescence studies prove that tumor-specific fusion-point epitopes are *antigenically* exposed at the cellular level, on both E2A-PBX1 as well as e_1a_2 BCR-ABL proteins (39, 40).
The results from our experiments with both polyclonal and monoclonal antibodies indicate that, when fusion-point epitopes from chimeric proteins are antigenically exposed within a cell, they can: (1) act as tumor-specific targets, and (2) allow a tumor-specific, *microscopic* identification of leukemic cells. Unfortunately, both of our monoclonal antibodies do not, when used in immunofluorescence microscopy, discriminate between leukemic cells and normal cells. Regarding the performance of ER-GO4 moAbs and ER-FP1 moAbs in immunofluorescence experiments, we suggest that the level of expression of either E2A-PBX1 or $e_1a_2P190^{BCR\text{-}ABL}$ proteins is probably too low for specific recognition of *single* $t(1;19)^{E2A\text{-}PBX1}$ or e_1a_2-$t(9;22)^{BCR\text{-}ABL}$ positive leukemic cells by our monoclonal antibodies (39, 40).

Various immunological methodologies have been developed through which the presence of particular tumor-cells in a patient's sample are specifically demonstrated at first diagnosis (e.g. protein tyrosine kinase assay, Western-blotting and the BCR-ABL dipstick assay (41)). Unfortunately, most of these techniques are often less valuable to detect residual tumor-cells during therapy. One of the exceptions that enables the detection of small numbers of leukemic cells, is immunofluorescence microscopy. Depending on the immunophenotype of the leukemia, immunological, microscopic techniques are extremely sensitive; able to detect one aberrant cell among 10,000 normal cells (14). However, present microscopic techniques still rely on the recognition of *non*-tumor-specific differentiation-antigens and are, as a result, not capable to identify tumor cells specifically. It is evident that an immunological, *tumor-specific* microscopic diagnosis is favored.
Exemplified by the tumor-specific recognition of single $t(1;19)^{E2A\text{-}PBX1}$ tumor-cells by our BP 1/19 antiserum: an antibody-based, tumor-specific microscopic identification of leukemic cells is feasible. Yet, both of our anti-fusion-point specific monoclonal antibodies are not suitable for leukemia diagnosis. The tumor-specific, immunologi-

cal recognition seems hampered by particular problems that concern: (1) the intracellular concentration of the tumor-specific chimeric protein, (2) the affinity of the monoclonal antibodies and/or, (3) the peptide-based immunization strategy that is used to generate such antibodies.

New problems to be solved:

The relatively low intra-cellular concentration of both E2A-PBX1 as well as e_1a_2 BCR-ABL proteins limits the intended use of our monoclonal antibodies, i.e. the specific *microscopic* identification of either $t(1;19)^{E2A\text{-}PBX1}$ or e_1a_2 $t(9;22)^{BCR\text{-}ABL}$ positive leukemic cells by moAb ER-GO4 or moAb ER-FP1, respectively. Unless the intracellular concentration of either chimeric protein is sufficiently (and specifically) increased (e.g. by activating the promotor or enhancer regions of the chimeric gene in question), both antibodies are not suitable for microscopic diagnostic procedures. Besides the fact that the promotor and enhancer regions of these genes are not yet known, it would be impractical, given the low frequencies with which the t(1;19) and the t(9;22) occur in childhood ALL (6% and 5%, respectively), to submit all new leukemic samples to such hypothetical diagnostic procedures.
More realistic solutions to our current, antibody based, diagnostic microscopic problems are to be found when one attempts to: (I) increase the antibody's affinity and/or the antibody's avidity towards the tumor-specific epitope or, (II) change the procedure through which such antibodies are generated. In this paragraph we propose several solutions that would benefit a tumor-specific, microscopic diagnosis by immunological means:

I. Increasing the antibody's affinity and/or avidity:

*a. **monoclonal antibodies:*** it is important to realize that a continuous epitope of a chimeric fusion-protein is tumor-specific when it: 1) *exclusively* occurs on tumor-specific proteins, and 2) contains both parts of the chimeric protein flanking the fusion-point. Given the observation that the smallest part of a protein fragment

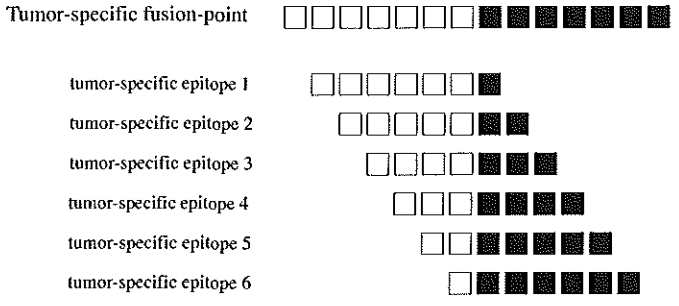

Figure 1 Schematic representation of the tumor-specific fusion-point of a hypothetical chimeric fusion-protein. Different continuous tumor-specific epitopes are available for antibody recognition.

which is able to bind to an immunoglobulin molecule, is estimated at 5-7 amino acids (31), it is obvious that at least 4-6 different tumor-specific epitopes are available for antibody recognition (see Figure 1). Since paratope-epitope interactions not only depend on the epitope's composition of the contiguous stretch of amino acid residues, but also depend on the epitope's conformation, it is imaginable that, within a particular tumor-specific protein, different tumor-specific fusion-point-epitopes are at hand to be specifically recognized by monoclonal antibodies.
Thus, although both moAb ER-FP1 as well as moAb ER-GO4 are limited in their diagnostic use, it might be possible to discriminate at the cellular level, tumor-cells from normal cells, by generating new monoclonal antibodies towards these different tumor-specific epitopes.

b. ***oligoclonal antibodies:*** when considering the polyvalent nature of our polyclonal antiserum BP 1/19, it can be concluded that the microscopic, tumor-specific recognition of $t(1;19)^{E2A\text{-}PBX1}$ tumor-cells by BP 1/19 antiserum is caused by a combination of differently reactive anti-fusion-point specific antibodies. Although it is well known that the concomitant presence of *non* specific immunoglobulins in antisera generally increases the background of various immunoanalytical assays, it is expected that when different anti-fusion-point specific (monoclonal) antibodies are properly mixed, they enhance the avidity of the oligoclonal antibody mixture towards the fusion-point epitope (42). As exemplified in Figure 2, it is estimated that background problems can be held to a minimum by using oligoclonal antibody mixtures, despite cross-reaction of certain anti-fusion-point antibodies with *non* tumor-specific cellular proteins.

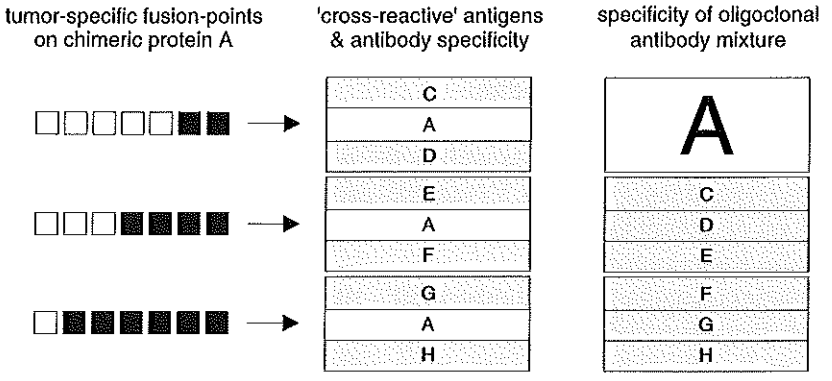

Figure 2 Schematic representation of the specificity of an oligoclonal antibody mixture directed towards the tumor-specific fusion-points of a hypothetical chimeric protein.
A single anti-fusion-point antibody may bind to a tumor-specific epitope that is not only specific for chimeric protein A but also for other antigens (C and D or, E and F or, G and H). The avidity of oligoclonal anti-fusion-point antibody mixture would be high towards the fusion-point epitope of the chimeric protein but low to any other antigen.

Thus, by generating an oligoclonal pool of different monoclonal antibodies, it should be possible to differentiate, by immunological means, between tumor-cells and normal cells, at the microscopic level.

Even though either of both of the suggestions depicted above, could result in the development of a new immunological tumor-specific, microscopic identification of characteristic leukemic cells, both of them involve the generation of new monoclonal antibodies. When considering to generate new anti-fusion-point specific antibodies by using conventional hybridoma procedures, one should anticipate to a vast amount of work and a small chance of success in finding a suitable antibody (e.g. moAb ER-GO4 is the only anti-E2A-PBX1 reacting antibody that resulted from ten different cell fusion experiments or, approximately 8,000 different hybridomas tested). Moreover, especially during our attempts to generate anti-BCR-ABL antibodies, we noted a discrepancy between the reactivity of so-called anti-*peptide* and anti-*protein* antibodies. While anti-BCR-ABL peptide antibodies were frequently detected during initial hybridoma screening procedures regarding their reactivity towards coated BCR-ABL peptides (ELISA), we found that most of the anti-BCR-ABL peptide antibodies did not recognize the cognate BCR-ABL proteins (Western blotting or protein tyrosine kinase assay; unpublished results). This discrepancy in reactivity between anti-peptide antibodies and anti-protein antibodies is well acknowledged and is largely attributed to the conformational differences between the peptides and their cognate proteins (31).
However, considering the need to direct the immune response towards the tumor-specific epitope in order to diminish the chance of raising antibodies towards *non* tumor-specific epitopes (see paragraph: 'tumor-specific proteins as antigens'), it seems unavoidable to use a peptide based strategy. Peptide immunization procedures appear to be, despite their limitations, the only suitable option when deciding to generate anti-fusion-point specific antibodies by using conventional hybridoma technologies.

II. Alternative (phage) antibodies:
Recent molecular genetic engineering of (human) antibodies seems to have bypassed both the hybridoma technology as well as the need to immunize laboratory animals (43). Large repertoires of antibody V (variable) genes, either harvested from lymphocyte populations or assembled *in vitro*, have been cloned for display on the surface of filamentous bacteriophages.
From libraries containing large numbers (10^7 - 10^{12}) of phages expressing single chain fragments that are encoded by variable regions from *IG* genes (scFv), rare antibody phages can be selected upon binding to an antigen that has been coated on a solid surface. Once specific phages have bound to the coated antigen, *non* binding phages are simply washed away. Specific antibody phages that have bound to the antigen are subsequently eluted from the solid surface by using conventional methods (43). As these phage particles are infectious they can be further grown in bacterial culture before subjecting these phages to further rounds of selection. Accordingly, phages can

be enriched to a 10^7-fold by four rounds of selection within two weeks at most (44). When considering the 'clonal' outgrowth of selected phages it can be stated that the phage display technology mimics the antigen-driven proliferation of B cells *in vitro*. The *in vitro* selection of antibody phages circumvents the immunization of laboratory animals according to time-consuming immunization schedules and, likewise, evades the need to test the antibody responses of these animals towards the injected immunogen. Exemplified by the poor antibody responses of mice injected with the multiple antigenic E2A-PBX1 peptides, i.e. approximately only one in ten immunized mice develops a sufficiently specific antibody response that allows the exclusive detection of $t(1;19)^{E2A\text{-}PBX1}$ positive cells (unpublished results), it is obvious that the phage technology limits the time and the number of laboratory animals otherwise needed to immunize and screen for proper antibodies. Moreover, when one realizes that generally only one in 10^5 splenocytes can be immortalized by using the conventional hybridoma technology (45), it is not difficult to imagine that, when deciding to immortalize the splenocytes from an immunized animal using conventional cell fusion techniques, one risks to lose the anti-fusion-point specific, antibody producing cell.

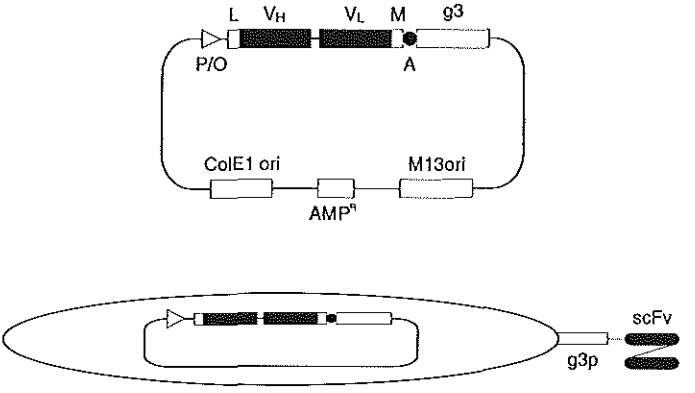

Figure 3 Phagemid pHEN1 (top) and a phage-antibody (bottom). P/O, lacZ promotor/operator; L, pelB leader; V_H + V_L, scFv encoding gene; M, Myc-tag; A, amber codon; g3, gene3; colE1 ori, E.coli origin of replication; AMP^R, ampicillin resistance gene; M13 ori, phage intergenic region; g3p, coat protein 3

Thus, when considering the conventional hybridoma technology; its low efficiency and the vast amount of work that involves testing the various supernatants of the hybridoma cell lines, it is obvious that the phage technology is preferred. There are various formats for antibody phage display. The format used in the following paragraphs is based on the phagemid vector pHEN1. The scFv fragments that are displayed on the surface of the phage particle are encoded by V_H gene and V_L gene fragments, inserted in line in pHEN1 (Figure 3) (46). The phage display technology is evolving fast and already has potential to generate *anti-fusion-protein specific* antibodies by a variety of means.

a. ***new monoclonal 'phage antibodies':*** our preliminary, unpublished studies indicate that it is possible to select phages that specifically recognize the multiple antigenic E2A-PBX1 peptide. These anti-*peptide* phages have been selected after *pre-absorbing* cross-reactive phages on a *non* tumor-specific mixture of multiple antigenic E2A peptides and multiple antigenic PBX1 peptides. The remaining antibody-phage repertoire (i.e. the phage library *without* cross-reactive anti-E2A and anti-PBX1 antibody phages) was used for binding to the multiple antigenic, *tumor-specific* E2A-PBX1 peptide. By repeating the pre-absorption and selection procedure 4-5 times, we were able to enrich single antibody phages. These phages are specifically reactive with the multiple antigenic E2A-PBX1 peptide and do not recognize multiple antigenic E2A or PBX1 peptides (unpublished results). Unfortunately, these phage antibodies did not bind to *(in vitro* translated) E2A-PBX1 proteins and their specificity seems restricted to conformational epitopes that are specifically exposed on the multiple antigenic E2A-PBX1 peptide.
It is likely that a peptide based selection procedure of antibody phages suffers from the same problems that were also found during screening procedures following conventional hybridoma techniques. The discrepancy of antibodies reacting with the peptide, but not with the cognate protein, makes a peptide based selection strategy less preferable to generate anti-fusion-protein phages.
Yet, the phage display technology does not necessarily depend on peptide based selection procedures: pre-absorbing antibody-phages from the complete repertoire on wild type, recombinant proteins, such as E2A and PBX1 proteins, diminishes the chance of rendering phage antibodies that are directed towards non tumor-specific epitopes. A subsequent selection from the remaining library of phages towards the recombinant tumor-specific protein (e.g. E2A-PBX1) should then be performed to select those phages that are reactive with tumor-specific epitopes.
The phage technology offers the opportunity to select antibodies that specifically recognize both *continuous* tumor-specific fusion-point epitopes as well as *discontinuous* tumor-specific epitopes. In contrast to the conventional hybridoma technology, the phage display technology has the potential of eliminating cross-reactive antibodies by pre-absorbing procedures. Such procedures may prove useful to identify any *discontinuous* tumor-specific epitope and provide an alternative for generating antibodies recognizing continuous tumor-specific epitopes.

b. ***mutated monoclonal 'phage antibodies':*** theoretical studies suggest that the greater the variability of the phage display library, the greater the chance of finding antibodies reactive to a given epitope and, likewise, the greater the possibility of selecting phage-antibodies with a high affinity towards the antigen (47). Yet, it is imaginable that high affinity anti-fusion-point specific antibody-phages are simply not present in the library used. Both *in vitro* (e.g. error prone polymerase reaction (48)) as well as *in vivo* (e.g. by using mutator strains of bacteria (49)) antibody V-gene mutations offer an opportunity to increase the low affinity of

selected antibody phages. Accordingly, new phages can be generated and selected for higher affinity towards a particular tumor-specific epitope.
Such antibody V-gene mutations are not only suited for increasing the specificity of low affinity antibody phages, but are also useful for increasing the affinity of existing monoclonal antibodies that have been generated by conventional hybridoma technologies. The V-genes from hybridoma cell lines, such as those producing ER-FP1 moAbs or ER-GO4 moAbs, are relatively easy cloned, mutated and inserted for display on filamentous bacteriophages. This newly generated library is liable to contain anti-fusion-point specific antibody-phages and it is expected that subsequent selections towards respective chimeric proteins, i.e. e_1a_2 BCR-ABL or E2A-PBX1, result in high affinity antibody-phages.
Thus, by mimicking the affinity maturation of B-cells *in vitro*, it should be possible to select new antibody phages with high affinity towards the fusion-point epitope of a chimeric protein. Such anti-fusion-point specific antibody-phages are particularly useful in microscopic and/or flow cytometric diagnostic assays. Given the fact that the specificity of an antibody largely depends on the analytical assay used (i.e. performance testing) (50), we propose that new antibody phages are initially selected on the basis of the antibody's staining pattern on fixed and permeabilized cells (e.g. COS cells transfected with cDNAs encoding e_1a_2 BCR-ABL proteins or E2A-PBX1 proteins). We expect that antibodies that have been selected according to these proposed procedures are more liable to be of practical use in future, tumor-specific diagnostic procedures.

c. ***bivalent and bispecific 'phage antibodies':*** molecular genetic engineering techniques are not only useful to enhance the affinity of a particular antibody(-phage) by mutating the antibody V-genes, but can also be used to mimic the bivalent, 'Y-shaped' structure of immunoglobulin molecules.
Protein interaction domains, derived from leucine zipper transcription factor encoding genes such as *FOS* or *JUN*, can be used to form *homodimeric* complexes. Once the cDNA of a leucine zipper domain is cloned it can be fused, together with cDNA that encodes the hinge region of an immunoglobulin molecule, to previously isolated scFv DNAs. The flexibility of the hinge area permits the two antigen binding sites to operate independently. It has been demonstrated that the subsequent transcription and translation of '*scFv-hinge-FOS*' genes, result in spontaneous formation of *bivalent* antibody fragments: i.e. (scFv-hinge-FOS:FOS-hinge-scFv) homodimers or, in short, $(scFv)_2$ (51). When considering to generate anti-fusion-point specific bivalent $(scFv)_2$ antibody fragments, a greater affinity and specificity towards the fusion-point is expected from bivalent $(scFv)_2$ antibodies, than that of single chain scFv fragments.
Peptide fragments from FOS or JUN transcription factors can also be used to form *heterodimeric* complexes. By reducing, reshuffling and reoxidizing homodimeric $(scFv\text{-hinge-FOS})_2$ and $(scFv\text{-hinge-JUN})_2$ fragments, it is possible to generate *bispecific* antibody fragments such as (scFv-hinge-FOS:JUN-hinge-scFv) (51).

These antibody fragments contain two differently reactive scFv molecules, dimerized as one single, *bispecific* antibody fragment. Given the observation that chimeric BCR-ABL proteins are specifically recognized by the combinatorial use of two differently reactive antibodies in the 'BCR-ABL dipstick assay', it is imaginable that, as long as the two separate scFv fragments of a bispecific antibody recognize distinctive epitopes flanking the BCR-ABL protein fusion-point and do not hamper each other's binding, anti-BCR-ABL bispecific antibody fragments can be generated. In this respect, anti-BCR-ABL bispecific antibodies are relatively easy made from existing antibody V-genes present in hybridoma cell lines such as ER-FP1 and 8E9. When considering to generate anti-BCR-ABL bispecific antibody fragments by combining the reactivity of ER-FP1 antibodies (i.e. recognizing e_1a_2 $P190^{BCR\text{-}ABL}$ proteins and possibly other proteins) with that of 8E9 antibodies (i.e. recognizing the carboxyl terminus of both ABL and BCR-ABL proteins), it is estimated that these bispecific antibody fragments have a greater affinity and specificity towards the e_1a_2 BCR-ABL fusion-protein than that of the monoclonal antibodies, ER-FP1 and 8E9, respectively. It should be realized, however, that much of the specificity of such bispecific antibodies depends on the flexibility of the hinge-region that is located between the scFv and FOS or JUN peptide fragments.

Conclusion:

Antibodies that specifically recognize cancer cells facilitate the microscopic diagnosis of characteristic tumor-types. Among the many different antigens that are usually identified in or on tumor-cells, chimeric proteins are one of the few cellular proteins that are *not* expressed by normal cell types. Chimeric proteins are the main repercussion of gene-fusion and it should be realized that fusion-genes are prominent features found in both hematological as well as *non*-hematological tumors (52).
It is evident that chimeric proteins are excellent tumor-specific markers, distinguishable from non-tumor-specific proteins by their presence of characteristic tumor-specific fusion-point epitopes. We have demonstrated that tumor-specific fusion-point epitopes of chimeric e_1a_2 BCR-ABL and E2A-PBX1 proteins are antigenically exposed, both at the peptide, the protein as well as at the cellular level. Our microscopic studies have verified that these tumor-specific epitopes are useful for tumor-specific diagnostic purposes by microscopic means. BP 1/19 antiserum is the first immunological reagent through which chimeric E2A-PBX1 proteins are specifically identified at the microscopic level in t(1;19) positive leukemic cells.
There are various techniques available, all capable of a tumor-specific diagnosis. Yet, it is evident that immunological, tumor-specific microscopic techniques are preferred: they are simple, quick, cheap and very sensitive. There is a clear future for the tumor-specific recognition of chimeric proteins. Tedious procedures that involve the 'old fashioned' hybridoma-techniques are no longer necessary. Phage display technolo-

gies offer many opportunities to detect both continuous as well as discontinuous tumor-specific epitopes. It is expected that new *monoclonal* or *oligoclonal* anti-tumor-specific (phage-)antibodies can be developed through which chimeric proteins, such as BCR-ABL and E2A-PBX1, are exclusively detected.

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Summary

Cancer is a heterogeneous disease regarding both its biology as well as its prognosis. A simple, universal treatment is, because of the heterogeneity of the disease, not possible. The kind of therapy chosen does not only depend on the organ affected by the primary tumor, but also depends on the outcome of so-called prognostic 'risk factors'. Risk factors predict the malignant potential of a certain type of tumor and some of these risk factors, e.g. the size and the metastatic spread of the tumor, are highly associated with the patient's chance of surviving cancer.
Only recently, researchers clearly demonstrated that most cancers have a genetic basis. Various genetic aberrations have been categorized already and nowadays, it is possible to distinguish between different cancer types more accurately.
This thesis describes our experimental work aimed at the development of new immunological diagnostic techniques. Accurate, but also simple diagnostic techniques are necessary to asses the prognosis of an individual patient more properly and to select (or develop) the most appropriate therapy.
To place our experimental work in perspective, this thesis starts with a general review of the concepts about cancer.

In *Chapter 1*, the classification of cancer is described. Traditionally, cancer diagnosis is based on both the cell type and tissue type from which a cancer originates. Its heterogeneity and its clonal character are mentioned briefly and the basic treatment protocols are summarized. Before ending this chapter, we emphasize that the effectiveness of anti-cancer treatment depends on a correct tumor-diagnosis.
Chapter 2 covers the theoretical basis of cancer. Malignant cells are characterized by uncontrolled cellular proliferation. To understand their malignant behaviour, regulation of non-neoplastic, normal cell proliferation is discussed first. The malignant behaviour of previously normal, healthy cells is then explained upon introducing typical chromosomal, molecular genetic and protein aberrations found in cancer cells.
In *Chapter 3*, the heterogeneity of cancer is exemplified by presenting the sub-classification of cancer. Leukemia is a life-threatening disease that is characterized by uncontrolled proliferation of blood-forming cells located in the bone marrow. Different types of leukemia are distinguishable and, depending on the expected duration of the (untreated) disease, they are traditionally classified as acute or chronic. A further diagnostic sub-classification is usually made according to morphological and (cyto-) chemical features of the cell type involved.
Yet, even within each morphological distinguishable subgroup, not all leukemias respond equally well to treatment. Apparently, the malignant behaviour of leukemic cells is only partly reflected by a morphological sub-classification of leukemia. A more refined treatment becomes feasible when the morphological classification is combined with data gathered upon analyzing the chromosomal and/or molecular genetic aberrations often found in leukemic cells.

Chromosomal and molecular genetic aberrations are at the basis of leukemogenesis. They are divided into those leading to aberrant expression of normal genes (e.g. *TAL1*) and those leading to the expression of aberrant chimeric genes, such as *BCR-ABL* and *E2A-PBX1*. Chimeric means: composed of parts with a different genetic background. The *BCR-ABL* gene results from the reciprocal exchange of chromosome fragments between chromosomes 9 and 22. This chromosomal translocation; the t(9;22)(q34;q11), fuses part of the *ABL* gene to part of the *BCR* gene on chromosome 22. Likewise, the t(1;19)(q23;p13) results in a chimeric E2A-PBX1 gene, in which part of the *PBX1* gene is fused to part of the *E2A* gene on chromosome 19. Both chimeric fusion-genes encode unique fusion-proteins that, as far as protein function is considered, do not resemble normal BCR, ABL, E2A or PBX1 proteins. In this respect, abnormal chimeric fusion-proteins are, in contrast to aberrantly expressed normal proteins such as TAL1, prototypes of a new kind of leukemogenesis. Both *BCR-ABL* as well as *E2A-PBX1* genes are involved in the oncogenic conversion of previously normal cells and both genes have typical prognostic and therapeutic implications for an individual leukemia patient.
As such, the chimeric *BCR-ABL* and *E2A-PBX1* genes are tumor-specific; both genes do not occur in normal healthy cells and both genes are only found in malignant cells with previously mentioned chromosomal translocations. Moreover, these chimeric genes provide excellent tumor-markers: e.g. the *BCR-ABL* gene is specifically detected by the fluorescent in situ hybridization technique (FISH) and, after reverse transcription of mRNA into cDNA, the chimeric BCR-ABL or E2A-PBX1 mRNA is specifically detected by the polymerase chain reaction (RT-PCR) technique.

During the past few years we have attempted to develop diagnostic reagents through which it should be possible to specifically identify chimeric fusion-proteins by using immunological methods.
In *Chapter 4* we describe the generation of a polyclonal antiserum, termed BP 1/19. This antiserum reacts specifically with E2A-PBX1 proteins and does not recognize E2A nor does it recognize PBX1 proteins. E2A-PBX1 proteins resemble the normal wild-type E2A and PBX1 proteins to a great deal. The only part of the protein which is present in E2A-PBX1 proteins but not in normal E2A or PBX1 proteins, is located at the fusion-point of the E2A-PBX1 protein. By immunizing mice with a multiple antigenic peptide with an amino acid sequence identical to that of the E2A-PBX1 fusion-point we were able to generate an antiserum that specifically identifies E2A-PBX1 proteins. We show that this antiserum distinguishes $t(1;19)^{E2A\text{-}PBX1}$ positive cell lines from t(1;19) negative cell lines, not only at the protein but also at the microscopic level. Incubating $t(1;19)^{E2A\text{-}PBX1}$ positive cells with diluted BP 1/19 antiserum results in a characteristic, granular nuclear fluorescence. These results verify that the fusion-point of E2A-PBX1 proteins is antigenically exposed and is, as such, suitable for tumor-specific diagnosis by immunological methods.
The diagnostic applicability, i.e. the specific diagnosis of $t(1;19)^{E2A\text{-}PBX1}$ positive pre-B

acute lymphoblastic leukemia (pre-B ALL), is demonstrated in *Chapter 5*. Here, we evaluated the specificity of BP 1/19 immunostaining on different cell lines in more detail. To this purpose, an extended array of cell lines was used. This array represents various hematopoietic cell types normally found in both peripheral blood as well as bone marrow samples. The specificity of the BP1/19 immunostaining technique proved correct as BP 1/19 only stained the nuclei of t(1;19) positive cell lines but not the nuclei of t(1;19) negative cell lines. The sensitivity of the BP 1/19 immunostaining technique was determined to one malignant $t(1;19)^{E2A\text{-}PBX1}$ positive cell among 10,000 normal cells through artificial mixing experiments. Its diagnostic value was ascertained when BP 1/19 specifically stained the leukemic cells isolated from patients suffering from t(1;19) positive pre-B ALL. Nuclear staining was not observed in t(1;19) negative pre-B ALL or common ALL patients, nor was it observed in the leukemic cells from a patient with a t(1;19) negative, t(9;22) positive chronic myeloid leukemia (CML) in blast crisis.

Yet, in tumor-diagnosis, monoclonal antibodies are clearly favored over polyclonal antibodies. In *Chapter 6* we describe a monoclonal antibody, termed ER-GO4. The ER-GO4 hybridoma cell line is the only 'anti-E2A-PBX1 fusion-point specific antibody' producing cell line that resulted from ten different conventional cell fusion experiments performed (or, approximately 8,000 hybridoma cell lines tested). Its specificity towards the E2A-PBX1 fusion-point was confirmed at both the peptide as well as the protein level. Unfortunately, in contrast to the BP 1/19 antiserum described above, ER-GO4 antibodies do not distinguish between $t(1;19)^{E2A\text{-}PBX1}$ positive cell lines and t(1;19) negative cell lines when they are used in cell-staining experiments. However, ERGO4 antibodies do recognize intracellular E2A-PBX1 proteins expressed in COS cells transfected with cDNA encoding E2A-PBX1 proteins. It is likely that the limited applicability of ER-GO4 antibodies in tumor-diagnosis is a matter of a relatively low E2A-PBX1 protein concentration in single $t(1;19)^{E2A\text{-}PBX1}$ positive cells.
Another fusion-point specific monoclonal antibody, termed ER-FP1, is described in *Chapter 8*. The ER-FP1 antibody is directed towards $e_1a_2P190^{BCR\text{-}ABL}$ proteins. The $e_1a_2P190^{BCR\text{-}ABL}$ proteins belong to the BCR-ABL proteins which are exclusively expressed in t(9;22)(q34;q11) positive leukemic cells. The specificity of ER-FP1 monoclonal antibodies was confirmed at both the peptide as well as the protein level: ER-FP1 specifically recognizes $e_1a_2P190^{BCR\text{-}ABL}$ but does not recognize the resembling b_2a_2-$P210^{BCR\text{-}ABL}$ or $b_3a_2P210^{BCR\text{-}ABL}$ proteins. However, like ER-GO4, ER-FP1 does not discriminate between leukemic and normal healthy cells at the microscopic level. To investigate whether the e1a2 fusion-point of e_1a_2 $P190^{BCR\text{-}ABL}$ proteins is antigenically exposed in intact, albeit fixed cells, we performed immunostaining experiments with ER-FP1 antibodies on transfected COS cells. These COS cells were transfected with cDNA either encoding $e_1a_2P190^{BCR\text{-}ABL}$, $b_2a_2P210^{BCR\text{-}ABL}$, or $b_3a_2P210^{BCR\text{-}ABL}$ proteins. Since ER-FP1 specifically detects e1a2 BCR-ABL transfected COS cells and not the b2a2 BCR-ABL nor the b3a2 BCR ABL transfected COS cells, we could verify that the fusion-point epitope of e_1a_2 $P190^{BCR\text{-}ABL}$ proteins remains intact after cell fixation.

Our experimental results clearly demonstrate that the e1a2 fusion-point is antigenically exposed and is as such, suitable for tumor-specific diagnosis by immunological means.

The need to develop new kinds of diagnostic techniques is exemplified by the poor prognosis of BCR-ABL positive acute lymphoblastic leukemias. Unlike other ALLs, BCR-ABL positive ALLs frequently relapse during or shortly after their first round of (induction) therapy. Obviously, it is important to specifically diagnose BCR-ABL positive leukemias before starting 'conventional' induction therapy. New (experimental) therapies such as more aggressive induction chemotherapy with or without bone marrow transplantation are expected to improve the otherwise insecure future of patients suffering from BCR-ABL positive ALLs.
In *Chapter 8* we describe the development of a new simple and rapid diagnostic technique: the 'BCR-ABL dipstick assay'. This assay is based on the successive detection of distinctly different antigenic determinants present on BCR-ABL proteins. The combination of differently reactive antibodies in the BCR-ABL dipstick assay allows for the detection of BCR-ABL proteins in a relatively easy, yet, specific fashion. Early therapy adjustment becomes feasible, as the BCR-ABL dipstick assay allows for a specific diagnosis of BCR-ABL positive leukemias within 24 hours.
The final chapter of this thesis, *Chapter 9*, summarizes the different categories of antibodies used in tumor-diagnosis. Most antibodies available recognize antigens that are also expressed by normal healthy cells (e.g. differentiation antigens). The diagnostic value of single antibodies is limited, although some of these antibodies are extremely helpful in tumordiagnosis when they are combined with other antibodies (e.g. 'multi-parameter' analysis). In contrast to differentiation antigens, chimeric antigens, such as the BCR-ABL and E2A-PBX1 proteins, are truly tumor-specific. Even though chimeric proteins strongly resemble proteins also present in normal, healthy cells, they can be discriminated from other proteins by one (or more) tumor-specific antigenic determinant(s). We have demonstrated that $t(1;19)^{E2A\text{-}PBX1}$ positive tumor-cells are, at the microscopic level, specifically identified by BP 1/19. The tumor-specific epitope of E2A-PBX1 proteins is, like the tumor-specific epitope of $e_1a_2P190^{BCR\text{-}ABL}$ proteins, antigenically exposed. It is expected that tumor-specific epitopes from different chimeric tumor-proteins can be recognized by using a similar strategy. In this respect, it is estimated that the phage display technology offers new opportunities to develop tumor-specific phage antibodies in a relatively easy fashion, realizing accurate diagnosis of malignant cells.

Samenvatting

Kanker is, vanuit een biologisch en prognostisch standpunt bezien, een heterogene ziekte. Een eenvoudige, algemeen geldende behandeling is door de heterogeniteit van de ziekte, niet mogelijk. De gekozen behandelingswijze hangt niet alleen af van het orgaan dat door de primaire tumor is aangedaan, maar hangt ook af van de uitkomst van zogenaamde prognostische 'risicofactoren'. Risicofactoren voorspellen het kwaadaardig karakter van een bepaald type tumor en sommige risicofactoren, bijv. de grootte en de uitzaaiing van de tumor, zijn in belangrijke mate gekoppeld aan de kans dat een patiënt de kanker overleeft.
Onlangs werd door onderzoekers duidelijk aangetoond dat de meeste kankers een genetische achtergrond hebben. Verschillende genetische afwijkingen zijn reeds in kaart zijn gebracht en het is tegenwoordig mogelijk om een beter onderscheid te maken tussen de verschillende soorten kanker.
Dit proefschrift beschrijft ons experimentele werk dat is gericht op de ontwikkeling van nieuwe immunologische, diagnostische technieken. Accurate, maar tevens eenvoudige diagnostische technieken zijn noodzakelijk om de prognose van een individuele kankerpatiënt beter in te schatten en om de meest geschikte therapie te kiezen (of te ontwikkelen).
Om ons experimentele werk in een breder perspectief te plaatsen, begint dit proefschrift met een algemene beschouwing van de denkbeelden omtrent kanker.

In *hoofdstuk 1* wordt de indeling van kanker beschreven. De diagnose kanker is van oudsher gebaseerd op zowel het celtype als het weefseltype van waaruit kanker ontstaat. De heterogeniteit en het klonale karakter van kanker worden in het kort besproken en de algemeen geldende therapieën worden samengevat. Aan het einde van dit hoofdstuk, benadrukken we dat de effectiviteit van kankerbehandeling afhangt van een correcte tumordiagnose.
Hoofdstuk 2 gaat over de theoretische achtergrond van kanker. Maligne cellen worden gekenmerkt door ongecontroleerde celproliferatie. Om het kwaadaardige gedrag van kankercellen te begrijpen wordt eerst de regulering van niet-neoplastische, normale celproliferatie besproken. Het kwaadaardig karakter van voorheen normale, gezonde cellen wordt vervolgens verklaard na een eerste introductie van, voor kankercellen, typische chromosomale, moleculair genetische en eiwit afwijkingen.
In *hoofdstuk 3* wordt de heterogeniteit van kanker verduidelijkt door de onderverdeling van leukemie naar voren te brengen. Leukemie is een levensbedreigende ziekte welke wordt gekenmerkt door ongecontroleerde deling van bloedvormende cellen die zich in het beenmerg bevinden. Verschillende typen leukemie zijn te onderscheiden en zij worden vanouds, afhankelijk van de te verwachten ziekteduur van de (niet behandelde) ziekte, onderverdeeld in acute en chronische leukemieën. Een verdere diagnostische onderverdeling wordt doorgaans gemaakt volgens morfologische en (cyto-) chemische kenmerken van het betrokken celtype.

Echter, zelfs binnen iedere morfologisch te onderscheiden subgroep, reageert niet iedere leukemie evengoed op de behandeling. Blijkbaar wordt het kwaadaardige gedrag van leukemiecellen slechts ten dele weergegeven door de morfologische onderverdeling van leukemie. Een beter afgestemde behandeling is te verwezenlijken indien de morfologische onderverdeling wordt gecombineerd met gegevens verkregen na analyse van de, in leukemiecellen vaak aangetroffen, chromosomale en/of moleculair genetische afwijkingen.
Chromosomale en moleculair genetische afwijkingen liggen ten grondslag aan het ontstaan van leukemie. Zij worden onderverdeeld in afwijkingen die leiden tot abnormale expressie van normale genen (bijv. *TAL1*) en afwijkingen die leiden tot de expressie van abnormale chimere genen zoals *BCR-ABL* en *E2A-PBX1*. Chimeer betekent: samengesteld uit delen van verschillend genetische herkomst. Het *BCR-ABL* gen komt voort uit de reciproque verwisseling van chromosoomfragmenten tussen de chromosomen 9 en 22. Deze chromosoom translocatie; de t(9;22)(q34;q11), koppelt een deel van het *ABL* gen aan een deel van het *BCR* gen op chromosoom 22. Op een vergelijkbare wijze resulteert de t(1;19)(q23;p13) in een chimeer *E2A-PBX1* gen, waarin een deel van het *PBX1* gen is gekoppeld aan een deel van het *E2A* gen op chromosoom 19. Beide chimere genen coderen voor unieke fusie eiwitten die, voor wat betreft eiwitfunctie, niet lijken op normale BCR, ABL, E2A of PBX1 eiwitten. In dit opzicht zijn abnormale chimere fusie eiwitten, in tegenstelling tot abnormaal geproduceerde normale eiwitten zoals TAL1, het voorbeeld van een nieuwe ontstaanswijze van leukemie. Zowel *BCR-ABL* als *E2A-PBX1* genen zijn betrokken bij de oncogene conversie van voorheen normale cellen en beide worden gekenmerkt door typische prognostische en therapeutische gevolgen voor een individuele leukemie patiënt.
De chimere *BCR-ABL* en *E2A-PBX1* genen zijn als zodanig tumor specifiek: beide genen komen niet in normale, gezonde cellen voor en beide genen worden alleen gevonden in kwaadaardige cellen met eerder genoemde chromosomale translocaties. Bovendien, chimere genen leveren uitzonderlijk goede tumormarkers op: het *BCR-ABL* gen wordt bijvoorbeeld specifiek gedetecteerd door de fluorescentie in situ hybridisatie techniek (FISH) en, na omzetting van mRNA in cDNA, wordt het chimere BCR-ABL of E2A-PBX1 mRNA specifiek door de polymerase ketting reactie (RT-PCR) aangetoond.

Gedurende de afgelopen jaren hebben we geprobeerd diagnostische reagentia te ontwikkelen waardoor het mogelijk moest worden om, m.b.v. immunologische detectiemethoden, chimere fusie-eiwitten specifiek aan te tonen.
In *hoofdstuk 4* beschrijven we de ontwikkeling van een polyklonaal antiserum, genaamd BP 1/19. Dit antiserum herkent specifiek E2A-PBX1 eiwitten en reageert niet met E2A noch met PBX1 eiwitten. E2A-PBX1 eiwitten lijken in sterke mate op de normale wildtype E2A en PBX1 eiwitten. Het enige deel van het eiwit dat aanwezig is in E2A-PBX1 maar niet in normale E2A eiwitten of PBX1 eiwitten, bevindt zich op het fusiepunt van het E2A-PBX1 eiwit. Door muizen te immuniseren met een meer-

voudig antigeen peptide waarvan de aminozuur volgorde overeenkomt met dat van het E2A-PBX1 fusiepunt waren wij in staat om het BP 1/19 antiserum te genereren dat specifiek E2A-PBX1 eiwitten herkent. We laten zien dat dit antiserum t(1;19)$^{E2A\text{-}PBX1}$ positieve cellen onderscheidt van t(1;19) negatieve cellijnen, niet alleen op eiwitniveau maar ook op cellulair niveau. Het incuberen van t(1;19)$^{E2A\text{-}PBX1}$ positieve cellen met verdund BP 1/19 resulteert in een karakteristieke, granulaire kernfluorescentie. Deze resultaten bevestigen dat het fusiepunt van E2A-PBX1 eiwitten op een antigene wijze tot expressie komt en als zodanig geschikt is voor specifieke tumordiagnostiek d.m.v. immunologische detectiemethoden.
De diagnostische toepassing van het BP 1/19 antiserum, d.w.z. de specifieke diagnose van t(1;19)$^{E2A\text{-}PBX1}$ positieve pre-B acute lymfatische leukemie (pre-B ALL), komt in *hoofdstuk 5* ter sprake. In dit hoofdstuk, wordt de specificiteit van de BP 1/19 immunologische celkleuring op verschillende cellijnen nader beschouwd. Een uitgebreide verzameling cellijnen werd hiervoor gebruikt. Deze verzameling is representatief voor de verscheidenheid aan hematopoëtische celtypen welke normaal in zowel perifere bloed- als beenmergmonsters voorkomen. De BP 1/19 immunologische celkleuring is specifiek, omdat BP 1/19 alleen de kernen van t(1;19) positieve cellijnen aankleurt maar niet de kernen van t(1;19) negatieve cellijnen. De sensitiviteit van de BP 1/19 aankleuringstechniek, zoals vastgesteld werd d.m.v. artificiële mengexperimenten, is één kwaadaardige t(1;19)$^{E2A\text{-}PBX1}$ positieve cel op 10.000 normale cellen.
De diagnostische waarde van BP 1/19 voor tumordiagnostiek werd duidelijk op het moment dat dit antiserum specifiek de leukemische cellen van patiënten lijdend aan t(1;19) positieve pre-B ALL aankleurde. De typische kernkleuring van BP 1/19 antilichamen werd niet waargenomen in cellen afkomstig van t(1;19) negatieve pre-B ALL of common ALL patiënten, noch werd de typische kernkleuring waargenomen in leukemiecellen van een patiënt lijdend aan een t(1;19) negatieve, t(9;22) positieve chronische myeloide leukemie (CML) in blastencrisis.

Echter, voor wat betreft tumordiagnostiek geldt dat monoklonale antilichamen duidelijk de voorkeur genieten boven polyklonale antilichamen. In *hoofdstuk 6* beschrijven we een monoklonaal antilichaam, genaamd ER-GO4. De ER-GO4 hybridoma cellijn is de enige 'anti-E2A-PBX1 fusiepunt specifieke antilichaam' producerende cellijn die voortkwam uit tien verschillende conventioneel uitgevoerde celfusie experimenten (m.a.w. ongeveer 8000 geteste hybridoma cellijnen). De specificiteit voor het E2A-PBX1 fusiepunt werd bevestigd op zowel het peptide als op het eiwitniveau. In tegenstelling tot het BP 1/19 antiserum maken ER-GO4 antilichamen, indien zij gebruikt worden in celkleuring experimenten, helaas geen onderscheid tussen t(1;19)$^{E2A\text{-}PBX1}$ positieve en t(1;19) negatieve cellijnen. Echter, ERGO4 antilichamen herkennen wel de intracellulaire E2A-PBX1 eiwitten in COS cellen getransfecteerd met cDNA coderend voor E2A-PBX1 eiwitten. Het is aannemelijk dat de gelimiteerde toepassing van ER-GO4 antilichamen in de tumordiagnostiek een kwestie is van een relatief lage E2A-PBX1 eiwitconcentratie binnen afzonderlijke t(1;19)$^{E2A\text{-}PBX1}$ positieve leukemiecellen.

Een ander fusiepunt specifiek monoklonaal antilichaam, genaamd ER-FP1, wordt besproken in *hoofdstuk 7*. Het ER-FP1 monoklonaal is gericht tegen $e_1a_2P190^{BCR-ABL}$ eiwitten. De $e_1a_2P190^{BCR-ABL}$ eiwitten behoren tot de BCR-ABL eiwitten die exclusief tot expressie komen in t(9;22)(q34;q11) positieve leukemie cellen. De specificiteit van ER-FP1 werd op zowel het peptide- als op het eiwitniveau bevestigd: ER-FP1 herkent specifiek $e_1a_2P190^{BCR-ABL}$, maar herkent niet de sterk gelijkende $b_2a_2P210^{BCR-ABL}$ of $b_3a_2P210^{BCR-ABL}$ eiwitten. Echter, evenals ER-GO4, maakt ER-FP1 op microscopisch niveau geen onderscheid tussen leukemie cellen en normale gezonde cellen. Om te onderzoeken of het e1a2 fusiepunt van $e_1a_2P190^{BCR-ABL}$ eiwitten op een antigene wijze tot expressie komt in intacte, doch gefixeerde cellen, hebben we immunologische celkleuring experimenten met ER-FP1 antilichamen op getransfecteerde COS cellen uitgevoerd. Deze COS cellen waren getransfecteerd met cDNA coderend voor òf $e_1a_2P190^{BCR-ABL}$, $b_2a_2P210^{BCR-ABL}$ òf $b_3a_2P210^{BCR-ABL}$ eiwitten. Omdat ER-FP1 specifiek e1a2 BCR-ABL getransfecteerde COS cellen herkent en niet de b2a2 BCR-ABL of de b3a2 BCR-ABL getransfecteerde COS cellen, konden we bevestigen dat het fusiepunt van $e_1a_2P190^{BCR-ABL}$ intact blijft na celfixatie. Onze experimentele resultaten laten duidelijk zien dat het e1a2 fusiepunt op een antigene wijze tot expressie komt en als zodanig geschikt is voor specifieke tumordiagnostiek d.m.v. immunologische methoden.

Het belang om nieuwe diagnostische technieken te ontwikkelen wordt verduidelijkt door de slechte prognose van BCR-ABL positieve acute lymfatische leukemieën. In tegenstelling tot andere acute lymfatische leukemieën, recidiveren BCR-ABL positieve leukemieën vaak al tijdens of kort na de eerste (inductie) therapieronde. Kennelijk is het belangrijk om BCR-ABL positieve leukemieën te herkennen voordat gestart wordt met de 'conventionele' inductie therapie. Van nieuwe (experimentele) therapieën zoals een veel agressievere inductie chemotherapie eventueel aangevuld met beenmergtransplantatie wordt verwacht dat deze de anders onzekere toekomst van patiënten lijdend aan BCR-ABL positieve ALLs verbeteren.
In *hoofdstuk 8* beschrijven we de ontwikkeling van een nieuwe, eenvoudige en snelle diagnostische techniek: de 'BCR-ABL dipstick analyse'. Deze methode is gebaseerd op de achtereenvolgende detectie van duidelijk verschillende antigene determinanten op BCR-ABL eiwitten. De combinatie van verschillend reagerende antilichamen in de BCR-ABL dipstick analyse maakt het mogelijk om BCR-ABL eiwitten op een relatief eenvoudige, doch specifieke wijze aan te tonen. Een vroege therapiebijstelling wordt mogelijk doordat de BCR-ABL dipstick analyse een specifieke diagnose van BCR-ABL positieve leukemieën binnen 24 uur mogelijk maakt .
Het laatste hoofdstuk van dit proefschrift, *hoofdstuk 9*, vat de verschillende groepen antilichamen die gebruikt worden in de immunologische tumordiagnostiek samen. De meeste antilichamen herkennen antigenen die ook bij normale, gezonde cellen tot expressie komen (bijv. differentiatie antigenen). De diagnostische waarde van dergelijke antilichamen is beperkt, ofschoon enkele antilichamen bijzonder geschikt zijn in de tumordiagnostiek indien zij in combinatie met andere antilichamen gebruikt wor-

den (i.e. 'multi-parameter' analyse). In tegenstelling tot differentiatie antigenen zijn chimere antigenen, zoals de BCR-ABL en E2A-PBX1 eiwitten, werkelijk tumor-specifiek. Ofschoon chimere eiwitten sterk lijken op eiwitten die ook in normale cellen voorkomen, kunnen zij van andere eiwitten onderscheiden worden door een (of meerdere) tumor-specifieke antigene determinant(en). We hebben aangetoond dat $t(1;19)^{E2A\text{-}PBX1}$ positieve tumorcellen specifiek herkend worden door BP 1/19. De tumor-specifieke epitoop van E2A-PBX1 eiwitten komt, evenals de tumor-specifieke epitoop van $e_1a_2P190^{BCR\text{-}ABL}$ eiwitten, op een antigene wijze tot expressie. Het is te verwachten dat tumor-specifieke epitopen van andere chimere tumor eiwitten op eenzelfde wijze specifiek zijn aan te tonen. In dit opzicht wordt verwacht dat de 'phage display' techniek nieuwe kansen biedt om tumor-specifieke 'faag' antilichamen op een relatief eenvoudige manier te ontwikkelen en zodoende accurate detectie van kwaadaardige cellen mogelijk maakt.

List of abbreviations

A	adenosine
ABL	gene homologous to the Abelson murine leukemia virus
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
AML1	acute myeloid leukemia gene 1
ANLL	acute non-lymphoblastic leukemia
APL	acute promyelocytic leukemia
ATP	adenosine triphosphate
B-ALL	B-cell acute lymphoblastic leukemia
*Bam*HI	restriction endonuclease isolated from Bacillus amyloliquefaciens H
B-cells	bone marrow derived lymphocytes
BCL	B-cell leukemia-lymphoma gene
BCR	breakpoint cluster region gene
(b-) HLH	(basic) helix-loop-helix DNA binding motif
BM	bone marrow
BP-1	anti b_2a_2 $P210^{BCR-ABL}$ fusion-point specific antiserum
BP 1/19	anti E2A-PBX1 fusion-point specific antiserum
BP-2	anti b_3a_2 $P210^{BCR-ABL}$ fusion-point specific antiserum
BP-ALL	anti e_1a_2 $P190^{BCR-ABL}$ fusion-point specific antiserum
BSA	bovine serum albumin
C	cytosine
cALL	common ALL
CD	cluster of differentiation (or, designation)
cDNA	complementary desoxyribonucleic acid
CML	chronic myeloid leukemia
CLL	chronic lymphocytic leukemia
Cy	cytoplasmic
der	derivative
DNA	deoxyribonucleic acid
E2A	gene encoding E2-box binding proteins
*Eco*RI	restriction endonuclease isolated from Escherichia coli
ER-FP1	anti e_1a_2 $P190^{BCR-ABL}$ fusion-point specific monoclonal antibody
ER-GO4	anti E2A-PBX1 fusion-point specific monoclonal antibody
FAB	French American British
FCS	fetal calf serum
FITC	fluorescein isothyocyanate
FISH	fluorescent *in situ* hybridization
G	guanosine
GRB2	gene encoding growth factor receptor binding protein 2
ETO	eighty-twenty-one gene
GATA	gene encoding proteins that specifically bind to (A/T)GATA(A/G)
*Hind*III	restriction endonuclease isolated from Haemophilus influenza Rd
HOX	homeobox gene
Ig	immunoglobulin

IGH	immunoglobulin heavy-chain gene
IGL	immunoglobulin light-chain gene
IL3	interleukin 3 gene
kb	kilo base
kDa	kilo Dalton
KLH	keyhole Limpet Hemocyanin
LCK	lymphocyte specific kinase gene
LYL	lymphoid leukemia gene
MAP	multiple antigenic peptide
m-BCR	minor breakpoint cluster region
M-BCR	major breakpoint cluster region
moAb	monoclonal antibody
mRNA	messenger ribonucleic acid
Mw	molecular weight or molecular mass
MYC	gene homologous to the avian myelocytomatosis virus
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBX1	pre-B cell leukemia gene
PCR	polymerase chain reaction
Ph	Philadelphia
PML	promyelocytic leukemia gene
pre-B ALL	acute lymphoblastic leukemia of pre-B cell origin
RARα	gene encoding retinoid acid receptor α
RAS	Rous Avian sarcoma gene
RBTN	rhombotin-like gene
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
Sm	surface membrane
SP	synthetic peptide
SV40	simian sarcoma virus 40
T	thymidine
TAN1	translocation associated *Notch* homologous gene
TAL1	T-cell acute leukemia gene 1
TEL	translocation *ETS* leukemia gene
T-cells	thymus derived lymphocytes
TCR	T-cell receptor gene
TRITC	tertramethyl rhodamine isothyocyanate
U	uridine
WT1	Wilms' tumor gene 1

Dankwoord

Het dankwoord is misschien nog wel het moeilijkst te schrijven gedeelte van een proefschrift.
Zeker tijdens wetenschappelijk onderzoek geldt dat er gezien de vaak teleurstellende resultaten van experimenteel werk, veel voor nodig is om gemotiveerd naar het werk te blijven gaan. Vele mensen zijn een belangrijke bron van steun geweest en het liefst zou ik de namen van al deze mensen in dit dankwoord willen vermelden.
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Ofschoon de onderzoeksprojecten op lab 853 zeer divers zijn, de mensen van lab 853 zijn in elkaars werk geïnteresseerd en zijn bereidwillig om elkaar met raad en daad bij te staan. Voor wat betreft het laatste, wil ik dan ook in het bijzonder dr Pieter Leenen en dr Maarten Egeler bedanken voor hun inzet om het 'breukpuntenwerk' een duw in de goede richting te geven.

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Minstens zo belangrijk voor het onderzoek was de inbreng van dr Marc van Dijk. Als 'E2A-PBX1-wetenschapper' was jij mijn hofleverancier van de verschillende plasmiden en van *in vitro* getransleerd E2A-PBX1. Bovendien bleek ook jij niet te beroerd om nieuwe testmaterialen te ontwikkelen. Zo is bijvoorbeeld het 'wildtype E2A-PBX1 eiwit' van grote waarde is gebleken voor de selectie van de ER-GO4 hybridoma cellijn.
Op het lab van dr Ton Logtenberg werden mijn eerste schreden gezet op de 'phage-display' technologie. Niet alleen het fagen-werk, doch ook de muzikale werkomgeving zorgden voor nieuwe impulsen welke snel hun navolging hebben gekregen in Rotterdam. Ofschoon de eerste proeven niet direct tot het gewenste resultaat hebben geleid (de tijd om het echt goed uit te zoeken ontbrak gewoonweg), ben ik ervan overtuigd dat anti-fusiepunt specifieke fagen zeker een toekomst hebben in de tumordiagnostiek.
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Paul Berendes.

Curriculum vitae

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1975-1983: Atheneum B, openbare scholengemeenschap Professor Casimir te Vlaardingen

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project: Monoklonale antistoffen gericht tegen breukpunt epitopen bij leukemie

1992-1996: assistent in opleiding o.l.v. prof. dr W. van Ewijk, afdeling Immunologie, Erasmus Universiteit Rotterdam.
project: Detectie van het tumor-specifieke fusiepunt antigen bij t(1;19) pre-B cel acute lymfatische leukemie

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