Homing on Track

Rare cell detection methods to study homing of leukemic and normal hematopoietic stem cells

Homing op Weg

Methoden ter detectie van laagfrequent voorkomende cellen voor het bestuderen van de homing van leukemische en normale hematopoietische stamcellen

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Homing on Track

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Chapter 1 Introduction

Chapter 1

Introduction

1.1 General introduction and scope of this thesis

Hematopoiesis is the process of blood cell formation. A relatively small number of pluripotent hematopoietic stem cells lies at the basis of the entire cascade of progressively more mature progenitor cells of the different hematopoietic lineages, culminating in the formation of mature, functional blood cells (Figure 1.1).
The regulation of hematopoiesis relies on complex interactions between hematopoietic cells and stromal cells, growth factors and their receptors, extracellular matrix molecules and cell-cell interactions through specialized cell adhesion molecules. The sites where hematopoietic stem cells can find just the right mixture of these regulatory interactions are referred to as niches [1].
During mammalian embryogenesis the hematopoietic system originates from mesodermally derived cells localized in the yolk-sac. At a later stage in the development of the fetus totipotent hematopoietic stem cells are predominantly found in the liver. Still later, hematopoiesis shifts to the spleen and the bone marrow. The spleen then gradually becomes a less important hematopoietic organ, so that at birth hematopoiesis in humans is almost exclusively situated in the bone marrow [2].

The rigorous temporal and spatial regulation of the development of the hematopoietic system implies that stem cells (or their descendant daughter cells, originating from self-renewing divisions) can undertake targeted migration from one site in the embryo to another. This phenomenon of targeted migration via the circulation to a specific tissue is referred to as "homing" [3]. The concept of homing comprises of a number of steps: a cell leaving the site where it was formed, entering the blood stream, leaving the circulation and entering the target organ's tissue. The term is also used in the context of clinical bone marrow transplantation, where hematopoietic stem cells are introduced into the blood circulation of a host and home to niches in the bone marrow to establish an entire new hematopoietic system.

Leukemic cells are hematopoietic cells that escape regulatory signals meant to control their proliferation and maturation. This results in uncontrolled proliferation and survival of immature, non-functional leukemic cells which take up space in the bone marrow, displacing the normal hematopoietic stem cells from their niches [4] and leading to fatal shortages of func-

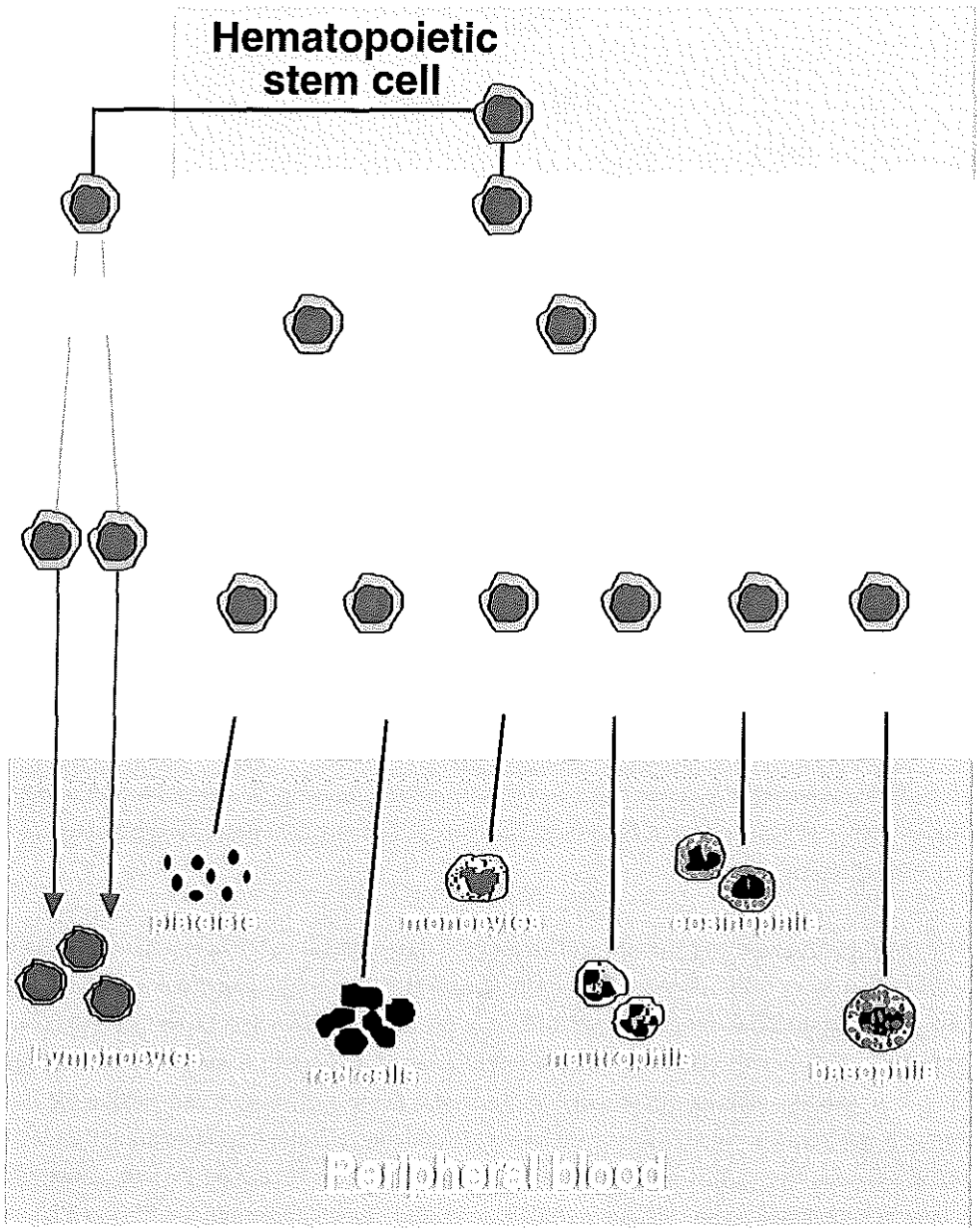

Figure 1.1 Schematic overview of hematopoiesis

tional blood cells [5]. Sometimes leukemic cells from patients or leukemic animals are easily grown *in vitro*, which has led to the establishment of clonal leukemic cell lines [6-12]. More often, however, leukemic cells require specific culture conditions to be grown *in vitro* : some require the addition of growth factors [13-21], some require a supporting stromal layer [22, 23]. In animal models leukemia can be transferred *in vivo* from one individual of an inbred

strain to another [24, 25], showing specific distribution patterns of the transferred leukemic cells [4, 26]. Some of these cell lines grow exclusively *in vivo* [24, 25]. Human leukemic cells have been grown *in vivo* in nude mice [27-30] and especially in SCID mice [31-33], showing distribution patterns similar to those in the donor [34, 35]. Similarly, genetic marking experiments have shown that human leukemic cells reinfused with an autologous bone marrow graft can contribute to a leukemia relapse [36-38]. Taken together, these data demonstrate that in many cases leukemic cells require the support of niches for their growth [39], and that leukemic cells, as well as normal hematopoietic stem cells, are capable of homing to such niches.
A thorough understanding of the homing of normal hematopoietic stem cells and of leukemic cells could have important implications. Active support of the homing of stem cells might improve the efficiency of bone marrow transplantation. This could allow transplantation of smaller graft sizes, which would be advantageous for several possible reasons. In allogeneic bone marrow transplantation it could lead to a reduced need for graft versus host disease prophylaxis. Whenever stem cells of the desired makeup are in short supply, as in many current gene therapy protocols [40-42] and in cord blood stem cell transplants [43-45], supported homing could improve the chance of a successful transplantation. In autologous bone marrow transplantation a reduction of the graft size could reduce the chance of transplanting leukemic stem cells with the autograft. In the case of autologous bone marrow transplantation it would also be advantageous to specifically disrupt the homing of leukemic stem cells; this would reduce the chance of a leukemia relapse caused by an autograft.
Concurrently, identification and characterization of stem cell niches will aid in the identification of the stem cell regulation mechanisms that operate *in vivo*. This thesis describes experiments aimed at the development and application of techniques that should serve such studies.

1.2 Homing and adhesion of cells

Homing of cells is a widely occurring process. For example, embryonic pattern formation is to a great extent the result of active migration of cells. Lymphocytes, granulocytes and monocytes travel widely throughout the body to provide immunologic surveillance. Endothelial cells and fibroblasts migrate during the wound healing process. Metastasis of solid tumor cells and dissemination of leukemic cells exhibit tissue specificity, and can therefore also be considered as homing phenomena.
Short range targeted cellular migration usually consists of active movement across a substratum. This type of migration can be targeted by chemotactic signals [46] or by specific interactions between adhesion molecules and their ligands.
Homing as defined within the scope of this thesis is long range migration: a sequence of passive transportation via the blood stream or the lymphatic system, followed by anchoring to the endothelium and entering specialized tissues. Here too, specific interactions between

Table 1.1 Adhesion at a glance: overview of the most important adhesion molecule families

Molecule		CD-classification	Expression (normal tissues)	Ligands
Selectins				
L-selectin		CD62L	Lymphocytes, myeloid cells	Glycam-1, CD34, MadCAM-1, CLA
E-selectin		CD62E	Activated endothelial cells	ESL-1, PSGL-1, CLA, sLe(x)
P-selectin		CD62P	Activated endothelial cells, platelets	PSGL-1, sLe(x)
Integrins	Subunits			
β_1-integrins			Expression widespread	
VLA-1	$\alpha_1\beta_1$	CD49a/CD29		Laminin, collagen
VLA-2	$\alpha_2\beta_1$	CD49b/CD29		Laminin, collagen
VLA-3	$\alpha_3\beta_1$	CD49c/CD29		Fibronectin, collagen
VLA-4	$\alpha_4\beta_1$	CD49d/CD29		Fibronectin, VCAM-1, integrin α_4, ICAM-2
VLA-5	$\alpha_5\beta_1$	CD49e/CD29		Fibronectin
VLA-6	$\alpha_6\beta_1$	CD49f/CD29		Fibronectin
	$\alpha_8\beta_1$	CD49f/CD29	Smooth muscle, mesenchymal cells, neural cells	Tenascin, fibronectin, vitronectin
	$\alpha_v\beta_1$	CD51/CD29		Vitronectin, fibrinogen
β_2-integrins				
LFA-1	$\alpha_L\beta_2$	CD11a/CD18	Lymphoid cells, monocytes, neutrophils	ICAM-1, ICAM-2
Mac-1	$\alpha_M\beta_2$	CD11b/CD18	monocytes, macrophages, NK cells	ICAM-1, ICAM-2
p150,95	$\alpha_X\beta_2$	CD11c/CD18	Monocytes, macrophages, granulocytes, NK cells	ICAM-1, ICAM-2
β_3-integrins				
VNR-α	$\alpha_v\beta_3$	CD51/CD61	Megakaryocytes, platelets, NK cells	Vitronectin, fibrinogen, vWf, fibronectin, osteopontin, PECAM-1
GPIIb/IIIa	$\alpha_{2b}\beta_3$	CD41/CD61	Platelets	Vitronectin
β_4-integrins				
	$\alpha_6\beta_4$	CD49f/CD104	T-cells, hemidesmosomes	Laminin
β_5-integrins				
	$\alpha_v\beta_5$	CD51/-	Epithelial cells	Vitronectin, fibronectin, osteopontin
β_6-integrins				
	$\alpha_v\beta_6$	CD51/-	Epithelial cells	Fibronectin
β_7-integrins				
LPAM-1	$\alpha_4\beta_7$	CD49a/-	Lymphocytes	MadCAM-1, VCAM-1, integrin α_4
Not yet classified adhesion molecules				
CLA			Lymphocytes homing to cutaneous endothelial cells	E-selectin
PSGL-1			Lymphocytes, myeloid cells	P-selectin
ESL-1			Myeloid cells	E-selectin
Lu-ECAM-1			Lung epithelial cells	?

Molecule	CD-classification	Expression (normal tissues)	Ligands
Immunoglobulin superfamily			
LFA-2	CD2	T-cells, NK cells	LFA-3
LFA-3	CD58	Expression widespread	LFA-2
ICAM-1	CD54	Expression widespread	LFA-1, hyaluronan
ICAM-2	CD102	Endothelial cells, lymphocytes, platelets	LFA-1, Mac-1
ICAM-3	CD 50	Leukocytes	LFA-1
VCAM-1	CD106	Monocytes, activated endothelial cells, constitutive on BM endothelial cells	VLA-4
NCAM-family	CD56	Neuroectodermal cells, T-cells, NK cells	NCAM
PECAM-1	CD31	Endothelial cells, platelets, monocytes, neutrophils, naive T-cells	PECAM-1, Integrin $\alpha_v\beta_3$
MUC-18		Smooth muscle cells, vascular endothelium	?
L1		Neural cells, Schwann cells, uro-epithelial cells, lymphoid cells	L1
P0		Neural cells	P0
CEA-family	CD66a-CD66e	Granulocytes, endothelial cells	CEA
Sialomucin, vascular addressin family			
CD34	CD34	Stem cells, hematopoietic precursor cells, endothelial cells	L-selectin
Glycam-1		endothelial cells	L-selectin
MadCAM-1		Mucosal HEV, endothelial cells	LPAM-1
Hyaluronate receptor family			
CD44-family	CD44	Expression widespread	Hyaluronan, laminin, fibronectin, collagen, serglycin (gp600)
Syndecan family		Epithelial cells	Amphoterin (p30), collagen, fibronectin, thrombospondin, bFGF
Aggrecan		Cartilage	Hyaluronic acid, link protein
Cadherin family			
E-cadherin		Epithelial cells	E-cadherin, other cadherins
N-cadherin			N-cadherin, other cadherins
P-Cadherin			P-cadherin, other cadherins
VE-cadherin		Endothelial cells	VE-cadherin, other cadherins

adhesion molecules and their ligands play a role to establish contact between the circulating cells and the
cells lining the circulatory compartment. Six major families of adhesion molecules have been identified and characterized, as outlined in Table 1.1. While some of these molecules seem to act simply as "dumb sticky receptors", others have recently been shown to exhibit sophisticated features such as intracellular signalling capabilities and conformationally controlled binding affinity [47]. Research is rapidly progressing in this field, and adhesion molecules, cell-cell interactions and homing are important subjects of interest for many research groups. The following paragraphs will summarize part of the current knowledge on the subject.

1.3 Homing and adhesion of leukocytes

Every organism must find ways to deal with the continuous threat of invasion by infectious agents. Mammals have developed a highly complex immune system to deal with this threat. The targeted migration of cells of the immune system to the sites where invasions of infectious agents are ongoing or where they are most likely to occur is referred to as leukocyte homing. Three main leukocyte homing pathways can be distinguished:

1. The continuous circulation of naive lymphocytes from the blood stream to peripheral lymph nodes and Peyer's patches and back into the blood flow via the lymphatic system.
2. Tissue specific migration of lymphocyte subsets to extralymphatic sites in mucosal epithelia and the skin.
3. Targeted infiltration of inflammation sites by lymphocytes, neutrophilic granulocytes and monocytes.

Homing in each of these three pathways follows a similar course of events: binding of adhesion molecules on the leukocyte to ligands expressed on endothelial cells leads to (A) rolling of the cells along the endothelium lining the vessel wall. First contact can induce activation events [48] which in turn lead to (B) the establishment of close contact between the leukocyte and the endothelium, forcing the leukocyte to an arrest. This stage is also referred to as tethering. The next step is referred to as (C) firm adhesion, which leads to flattening of the cells. Finally (D) the leukocytes transmigrate though the vessel wall and enter the surrounding tissue [46, 48-52].

Each of these four steps is mediated by adhesion molecules. Some adhesion molecules are specifically involved in one of the four elements of this adhesion cascade, while others take part in two or more steps. Each of these steps can be regulated by modulation of the expression and/or the binding characteristics of the adhesion molecules involved. Restricted expression and activation of adhesion molecules on the leukocytes' cell membranes and of ligands on endothelial cells leads to site specific homing patterns of defined cellular subsets.

1.3.1 Lymphatic tissue homing of naive lymphocytes

Lymphocytes are the effector cells of the specific immune system, one of the two major branches of the mammalian's defense system against infections. Non-antigen primed, naive lymphocytes with a CD45RA/CD62L^{hi}/CD44^{low}/CD49d^{low} phenotype [53-55] continuously travel throughout the body, entering lymph nodes from the blood stream, leaving them via the lymphatic drainage system and re-entering the circulation via the thoracic duct [54]. The extravasation of naive lymphocytes specifically takes place in high endothelial venules (HEV), the venules that are directly connected to the capillaries of the lymph nodes [56]. HEV's have a specialized endothelium consisting of large cuboidal cells [57] which express adhesion molecules as counterreceptors for lymphocyte homing receptors. The lymphocyte homing receptor L-selectin (CD62L) [58] is the adhesion molecule that establishes the first contact of naive lymphocytes with the HEV's endothelium and mediates rolling, most likely via Glycam-1 and/or CD34 expressed on the resting endothelial cells [59]. Tight adhesion is subsequently mediated by the interaction between A) lymphocytic Lymphocyte Functional Antigen-1 (LFA-1; CD11a/CD18) with primarily Intercellular Adhesion Molecule-2 (ICAM-2; CD102), which shows strong constitutive expression on resting endothelial cells, and to a lesser extent with constitutively expressed ICAM-1 (CD54) on the endothelial cells [60, 61] and in addition by B) interaction of Very Late Antigen-4 (VLA-4; CD49d/CD29) expressed on the lymphocytes and Vascular Cell Adhesion Molecule-1 (VCAM-1; CD106) expressed on the endothelial cells.

1.3.2 Homing of memory and effector lymphocytes to Peyer's patches and extra-lymphatic tissues

Memory and effector lymphocytes with a CD45RO/CD62L^{low}/CD44^{hi}/VLA-4^{hi} phenotype [54, 62] also continuously recirculate through the body. With respect to non-inflamed tissues, however, these cells predominantly home to Peyer's Patches (PP) and mucosal lymphatic tissues, and to tertiary lymphatic tissues, especially to the skin. In the HEV of PP and mucosal lymphoid tissues the first adhesion step is binding of L-selectin and/or integrin $\alpha_4\beta_7$ on memory lymphocytes to Mucosal Addressin Cell Adhesion Molecule-1 (MadCAM-1) expressed on endothelial cells [63-67]. Arrest and tight adhesion of memory and effector cells to mucosal HEV are mediated by the interaction between integrin $\alpha_4\beta_7$ expressed on the lymphocytes to MadCAM-1 expressed on the endothelial cells, and by the interaction of integrin $\alpha_4\beta_1$ on the lymphocytes and VCAM-1 on the endothelial cells.

A small proportion of memory lymphocytes recirculates through the skin [54]. These lymphocytes adhere to the flattened endothelial cells that line the capillaries and enter the tissue. Although homing interactions of skin-seeking lymphocytes and normal, non-activated cutaneous endothelial cells have not been studied in detail to date, it is likely that in the establishment of tight adhesion and extravasation of these cells interaction between LFA-1 and ICAM-1 is involved [62].

1.3.3 Inflammation induced homing of leukocytes

Inflammation leads to profound changes in endothelial cells. Cytokines and chemokines produced in inflammation sites induce upregulation of P-selectin (CD62P), E-selectin (CD62E), VCAM-1 and ICAM-1 on endothelial cells [68, 69]. Furthermore, endothelial cells present inflammatory substances by scaffolding them to glycosaminoglycans such as CD44 and syndecan [70]. Lymphocytes as well as neutrophilic granulocytes and monocytes show greatly enhanced homing to sites of inflammation. All three selectins as well as VLA-4 can mediate rolling as the first step of leukocyte homing to inflammatory sites. Rolling leads to (1) a significant slowdown of the circulating cells prolonging their presence at the inflammatory site and (2) increased proximity to the endothelial cells. The net effect of this prolonged presence and increased proximity is a sufficient exposure to inflammation related chematokines to induce activation events in the leukocytes which lead to upregulation and/or affinity increase of integrin adhesion molecules which can subsequently mediate tethering, firm adhesion and extravasation [71].

Lymphocytes can roll on P-selectin using P-Selectin Glycoprotein Ligand-1 (PSGL-1) [72] and, more specifically, skin-homing lymphocytes can roll on E-selectin using cutaneous lymphocyte antigen (CLA) [62, 73, 74]. VLA-4 may also play a role in rolling of lymphocytes on endothelial cells, through interaction with VCAM-1 [75]. Tethering of rolling lymphocytes is mediated through integrin VLA-4 on the lymphocytes and VCAM on the endothelial cells [62, 75] and also by interaction of integrin LFA-1 on the lymphocytes and ICAM-1 and ICAM-2 on the endothelial cells [61, 62, 75, 76].

Neutrophilic granulocytes show low frequency rolling in non-inflamed tissues, mediated by PSGL-1 expressed on the neutrophils and P-selectin expressed on endothelial cells [77]. In inflammation sites neutrophils show the strongest rolling response to P-selectin, followed by L-selectin and E-selectin [66, 78-80]. Firm adhesion ensues, mediated by interaction of VLA-4 with VCAM-1 and by interaction of LFA-1 and/or Mac-1 with ICAM-1 and ICAM-2 on the endothelial cells [81]. Finally, extravasation of granulocytes and monocytes can be blocked by antibodies to Platelet/Endothelial Cell Adhesion Molecule-1 (PECAM-1; CD31), indicating that PECAM-1 mediates diapedesis [82].

Future research on the homing of leukocytes will mainly focus on the identification of possible further ligands of the selectins, on intracellular signalling events mediated by adhesion molecules and on further unraveling the cellular interactions involved in firm adhesion and diapedesis.

1.4 Homing and metastasis of malignant cells

Cancer originates when a primary neoplasm arises as an aberration of a single cell which escapes normal cellular control mechanisms and exhibits dysregulated proliferation and sur-

vival. This leads to the formation of a malignant cell population of clonal descent. With time, such a clonal population often shows genetic instability, leading to the formation of malignant subclones. Clinical symptoms can occur through a multitude of causes, such as compression of normal tissues causing obstructions or pain, competition for nutrients and space with normal cells, and tissue destruction, thereby interfering with normal body functions. Without treatment cancer generally leads to fatal complications and causes death.

One fundamental problem in the treatment of cancer is metastasis: cancer cells can move away from the site where the primary neoplastic event occurred and establish themselves at secondary sites throughout the body. Metastasis is a complex process, in which five major steps are distinguished [83]: 1. Detachment of tumor cells from their site of formation (usually detachment from other tumor cells) 2. Entry of blood or lymphatic vessel 3. Dissemination by passive transport through the vessel 4. Adhesion, extravasation and active movement into tissue. 5. Establishment of a new malignant colony .

As of step 3, this sequence of elements of the metastatic process closely parallels the events that play a role in leukocyte homing, so that the term homing of malignant cells adequately summarizes these crucial steps in the development of cancer. Other parallels are tissue specificity and the use of adhesion molecules by the cells to adhere to endothelial cells and to enter the tissues.

Knowledge of the involvement of adhesion molecules in homing of cancer cells stems from (A) correlation studies between clinical course of disease and adhesion molecule expression on the malignant cells, from (B) *in vitro* adhesion studies using purified molecules or monolayers of endothelial cells, usually human umbilical vein endothelial cells (HUVECs) as ligands for primary cancer cells or cell lines, and from (C) *in vivo* studies in animal models, often immune compromised strains.

From these studies it can be concluded that adhesion often plays a dual role in metastatis: while on the one hand increased adhesive potential of a cell can reduce its chance of being released from its site and entering into the circulation, it may on the other hand increase its ability to invade a tissue [83]. For example, reduced expression of E-cadherin, an adhesion molecule that is involved in homophilic cell-cell adhesion in normal tissues, is associated with increased invasiveness of a variety of malignancies [84].

The dual role of adhesion in metastasis is especially salient in the case of the integrins, where expression of one and the same molecule can be associated with increased as well as decreased metastatic potential, depending on the cell type. For example, increased expression of VLA-2 has been associated with increased metastatic capability of rhabdomyosarcoma cells [85]. Conversely, reduced or absent expression of VLA-2 has been associated with increased invasiveness and disease progression in breast cancer and endometrial cancer [86-88]. This paradox may well be connected to the tissue-specific utilization of different extracellular matrix components to maintain tissue integrity.

Clearly, only a correlation between metastatic potential and *increased* expression or activity

of an adhesion molecule should point towards a role for an adhesion molecule in the *homing* phase of metastasis. The following section will present an overview of the available data on the role of adhesion molecules in this segment of the metastatic process, with a separate subsection devoted to homing of leukemic cells.

1.4.1 Adhesion molecules involved in homing of malignant cells derived from solid tumors

1.4.1.a Selectins

Increased metastatic potential and poor prognosis in human colorectal carcinoma is associated with increased expression of the carbohydrate ligands sialyl-Lewis(x) (sLe^x) and sialyl-Lewis(a) (sLe^a) [89], which can bind to E- and P-selectin [90]. Concurrently, expression of E-selectin on intratumoral vessels was correlated with rapid disease progression in a study of human melanoma [91]. Similarly, expression of sLe^x and sLe^a have been found to correlate with metastatic potential of cell lines in animal models [92]. Expression of sLe^x and sLe^a has been connected to E-selectin binding [93, 94], and finally, several colon and breast cancer cell lines and the leukemic cell line HL-60 were directly shown to bind to E-selectin [95-97]. In one instance this binding was shown to induce rolling of the cells along a monolayer of stimulated endothelial cells [97]. These results all indicate that especially E-selectin plays an important role in metastasis formation, most likely by mediating rolling behaviour of the cells on activated endothelia. Although P-selectin and L-selectin can also bind to carbohydrate ligands on cancer cells [98], there is little evidence that directly connects these molecules to metastatic adhesion. It does seem, however, that binding of malignant cells with P-selectin expressed on platelets may play a role in inducing activation of endothelial cells [99], which in turn could lead to increased binding due to the upregulation of E-selectin and also ICAM-1 and VCAM-1 on the endothelial cells. Such a mechanism has, however, not yet clearly been elucidated.

1.4.1.b Integrins

Apart from binding to specific cellular adhesion molecules on endothelial cells, a cell might also bind to extracellular matrix elements of the glycocalyx to anchor to and begin invading a tissue. This would imply a role for the integrins. The integrins are a large family of adhesion molecules, all consisting of an alpha and a beta subunit (cf Table 1.1). Apart from the $ß_2$ integrins and integrin $\alpha_4ß_7$ all of the integrins can serve as receptors of extracellular matrix components. Indeed many of the integrins have been connected with metastatic potential of malignant cells.

As mentioned above, VLA-2 has been associated with metastatic potential of rhabdomyosarcoma cells [85] and melanoma cell lines [100] in nude mice. VLA-3 expression was shown to correlate with disease progression in melanoma [101] and in head-and-neck squamous cell

carcinoma formation [102]. VLA4 expression has been associated with adhesion of sarcoma, melanoma and lung carcinoma cell lines to activated endothelial cells [95, 103] Mattila et al. [103] demonstrated that this adhesion was mediated by interaction of VLA-4 with VCAM-1. This correlates in part with the findings of Kawaguchi et al., [104] who demonstrated a correlation between VLA-4 expression levels and disease progression of osteosarcoma, but failed to detect expression of VCAM-1 in their samples. Expression of VLA-4 was also seen to be correlated with disease progression and clinical outcome in melanoma [91, 105]. Expression of VLA-5 has been associated with clinical disease staging of mammary tumors [106] and of melanoma [107], as well as with metastatic spread to the liver of murine mammary tumor cell lines [108].

Only few reports have implied the β_2 integrins in malignant cell homing. These reports are concerned with metastatic spread of lymphoma. Roos et al. [109] have shown that interaction between LFA-1 (integrin $\alpha_L\beta_2$, cf Table 1.1) expressed on lymphoma cells and ICAM-1 or ICAM-2 expressed on hepatocytes mediates hepatic spread of murine T-cell hybridomas. Zahalka et al. [110, 111] showed the involvement of LFA-1 in splenic invasion of murine T-cell lymphoma.

Integrin $\alpha_{IIb}\beta_3$ has been implied in murine melanoma-associated platelet aggregation and lung metastasis [112]. Integrin $\alpha_v\beta_3$ was found to correlate with disease progression in human melanoma [101, 113] and head-and-neck squamous cell neoplasms, along with integrin $\alpha_6\beta_4$ [102]. Integrin $\alpha_6\beta_4$ has also been connected to liver metastasis of a murine mammary carcinoma cell line [114], and with in vitro invasiveness of human prostatic carcinoma cells [115]. Finally, expression of integrin $\alpha_v\beta_5$ showed a correlation with disease progression in gastric carcinoma [116].

These results indicate that extracellular matrix adhesion via integrins plays a major role in the homing of malignant cells, and disintegrins [117-120] and extracellular matrix mimetics [121-123] are therefore currently under study as metastasis-blocking agents.

1.4.1.c Immunoglobulin Superfamily

Apart from the interaction between the integrin VLA-4 on tumor cells and the immunoglobulin superfamily (IgSF) member VCAM-1 expressed on endothelial cells only few of the other IgSF adhesion molecules seem involved in the homing of malignant cells.

ICAM-1 was shown to be directly involved in the binding of experimental lymphoma cell lines to hepatocytes [124] and with bone marrow invasiveness [125]. Recently, ICAM-1 on human glioma cells was shown to bind to integrin β_2 on activated bovine endothelial cells [126]. ICAM-1 is furthermore associated with disease progression in metastatic melanoma, but this is probably not a homing-related phenomenon [127-129]. The same may be true for MUC-18, an IgSF adhesion molecule that is also associated with disease progression in melanoma. The role of the homophilic adhesion inducing MUC-18 remains unclear, although the recent detection of MUC-18 on endothelial cells may indicate that it may be a homing receptor for melanoma cells [130, 131].

Finally, the tumor marker carcinoembryonic antigen (CEA) has been shown to mediate homotypic adhesion of tumor cells [132] and is associated with disease staging in cervical carcinomas [133] and in colon carcinoma [134, 135]. Jessup et al. [136] showed that CEA is associated with the formation of liver and lung metastases by colorectal carcinoma cell lines. The recent demonstration that CEA can be expressed on endothelial cells indicates that the role of CEA in the metastatic process may indeed be at the level of tumor cell - endothelial cell binding, thus ranging CEA among the putative malignant homing receptors [137].

1.4.1.d CD44 family

CD44 is a glycoprotein that serves as a receptor for hyaluronic acid [138, 139] and other extracellular matrix components such as fibronectin, collagen Type I, laminin [140] and serglycin (gp600) [141]. The gene for CD44 consists of a large number of exons that can be differentially spliced to form variants of the CD44 protein. Günthert et al. [142] showed that artificial expression of CD44v6 was sufficient to induce metastatic behavior in a rat pancreatic carcinoma cell line, and it was subsequently shown that this metastatic behavior could be blocked by a monoclonal antibody to the CD44v6 [143], indicating that the molecule is involved in homing of the malignant cells to distant sites. There is furthermore a wealth of clinical evidence connecting elevated or aberrant expression of CD44v6 and/or other splice variants with metastatic behavior and poor prognosis of many cancers, such as colorectal cancer [144-149], cervical cancer [150, 151], gastric cancer [152-154] and non-Hodgkin lymphoma [155, 156]. Although the data obtained thus far strongly indicate a possible role for CD44 as a homing receptor for malignant cells, no direct connection between CD44-conferred metastatic potential and interaction with a specific ligand has been demonstrated to date [157].

1.4.1.e Other adhesion molecule families

Of the not yet classified adhesion molecules only Lu-ECAM-1 [158], an adhesion molecule exclusively expressed on endothelial cells of pleural capillaries and sub-pleural venules in mice and man [159], has been shown to be involved in metastasis. Lu-ECAM-1 was shown to mediate the binding of murine B16-F10 melanoma cells to lung endothelium [160], and blocking of Lu-ECAM-1 using a monoclonal antibody led to a reduced formation of lung metastatic colonies by the B16-F10 cells [158, Zhu, 1992 #450]. None of the members of the other adhesion molecule families has been directly linked to homing of cancer cells.

1.4.2 Dissemination and homing of leukemic cells

Leukemic cells originate fom the bone marrow, their native extravascular compartment. Dissemination of leukemia implies that leukemic cells enter the circulation by crossing the barrier of the sinus endothelium, and similarly to metastasizing solid tumor cells they can invade organs such as spleen and liver by extravasation and subsequent ectopic growth. This sequence of events closely resembles the course of metastasis from solid tumors, as described

in Section 1.4.1. Dissemination of leukemia and metastasis of solid tumor cells are therefore basically the same [161]. The diffuse growth pattern of disseminated leukemia, the most conspicuous difference between solid tumors and leukemia, can be explained from a lack of homotypic adhesion between leukemic cells. In this way leukemic cells resemble their normal hematopoietic counterparts, since maintaining tissue integrity through homotypic adhesion is not normally a function of hematopoietic cells. Another difference between solid tumors and leukemia is that in general the life-threatening effects of leukemia, i.e. leukopenia and thrombocytopenia, arise through suppression of normal hematopoiesis in the bone marrow, not through the invasion of secondary tissue compartments.

Normal hematopoietic cells express a range of adhesion molecules through which they can interact with bone marrow stromal cells and extracellular matrix components [162, 163]. Several reports show altered expression of adhesion molecules on human leukemic cells as compared to their non-malignant counterparts [162, 164-166]. Also, some reports indicate altered expression of adhesion molecules on BM stromal cells from leukemia patients [167] and impaired adhesion of normal hematopoietic cells to stromal layers from leukemia patients [168, 169]. These studies mainly show loss of adhesive capacity in leukemia, which may be associated with increased egress of leukemic cells into the peripheral blood [170], similarly to increased release of solid tumor cells through loss of adhesion.

Prins and van Bekkum [26] observed significant differences in the homing and dissemination patterns of two *in vivo* animal leukemia models, the Brown Norway Myeloid Leukemia (BNML) and the acute lymphocytic leukemia L4415. This demonstrated the specificity of homing and dissemination of leukemia. While it is very likely that this specificity is at least to some extent attributable to differences in the expression patterns of adhesion molecules, relatively little is known about the exact role and the clinical significance of adhesion molecules in leukemia.

In two reports no correlation between disease progression or prognosis and expression of CD54 [171] or of VLA-2, VLA-4 or VLA-5 was observed, neither in acute lymphocytic leukemia (ALL) nor in acute myelocytic leukemia (AML) [172]. Notably, no correlation was found in these studies between adhesion molecule expression and extramedullary presentation (tumoral syndrome), which would have pointed towards a role for any of the examined molecules in extramedullary homing of leukemia. In contrast, such a correlation was found in B-cell chronic lymphocytic leukemia (B-CLL), where absence of β_1, β_2 and β_3 integrin expression was associated with favourable prognosis and reduced extramedullary involvement, clearly indicating a role for the integrins in extramedullary homing [173, 174]. Furthermore, LFA-3 (CD58) and CD44 expression independently defined a subclass of B-CLL with less favourable prognosis [173, 174]. Conversely, LFA-3 was found to be associated with favourable prognosis in ALL and AML [171]. The latter may be, however, less connected to the homing behaviour of these cells than to LFA-3 dependent susceptibility of these leukemic cells to lysis by cytotoxic T-cells or NK cells [175-177].

Additional analyses will be needed to clearly establish the exact role of adhesion molecules in different leukemia subtypes and to explain the seemingly controversial results. For example, the possibility that the adhesion characteristics of leukemic cells could have been changed by genetic aberrations leading to constitutively active or inactive conformations of adhesion molecules remains hitherto unexplored. So far however, the limited value of expression of adhesion molecules to differentiate leukemias according to disease progression or prognosis indicates that the adhesion defects observed in leukemic cells have little effect on their capacity to fill up the bone marrow cavities and cause suppression of hematopoiesis.
Within the context of homing it is of greater clinical significance that leukemic cells cotransplanted with an autologous bone marrow graft can contribute to a leukemia relapse [37, 38]. The frequently observed expression of VLA-4 on leukemic cells [166, 172] and the constitutive expression of VCAM-1 on BM sinus endothelium and on BM reticular cells [178] indicates that VLA-4/VCAM-1 interaction may play a key role in the homing of leukemic cells. No reports on in vivo leukemia models establishing such a role for any adhesion molecule are available as yet.

1.5 Homing in bone marrow transplantation

Barnes et al. (1956) [179] were the first to report on the treatment of murine leukemia by combining lethal irradiation with a bone marrow transplantation. This set off the development of bone marrow transplantation in humans as a treatment for human leukemia. Some forty years later, transplantation of bone marrow or hematopoietic stem cells from peripheral blood or cord blood is now the treatment of choice for many diseases, ranging from leukemia and lymphoma to radiation disease, a range of immunodeficiency syndromes, lysosomal storage diseases, and some solid tumors [180]. Recently, great effort is made to use hematopoietic stem cells as the cellular target in gene therapy protocols, extending the potential applicability of bone marrow transplantation even further. The success of every transplantation relies on the ability of hematopoietic stem cells to home to niches where they can lodge and establish hematopoiesis.
Historically the study of hematopoietic stem cell homing has developed in parallel with the study of the hematopoietic stem cell itself: the most important limitations were the purity of the stem cells suspensions that could be obtained and the assays that were available. A major step forward came with the development of the CFU-S assay [181], long thought to be an assay for pluripotent hematopoietic stem cells [182]. Much of the current knowledge of the *in vivo* homing of hematopoietic cells still stems from studies employing the CFU-S assay.
Bone marrow transplantation is usually preceded by a conditioning regimen, consisting of irradiation and/or cytostatic treatment. Shirota et al. [183, 184] demonstrated that both these components of conditioning regimens disrupt the endothelial barrier which separates the bone

marrow from the circulation. This provided the rationale for using *in vitro* adhesion assays that employ BM stromal cells as models for the processes that play a role in *in vivo* homing of hematopoietic stem cells.
Finally, analysis of adhesion molecule expression in transplanted cell populations and hematopoietic recovery parameters obtained from transplantation patients has yielded additional information on the role of adhesion molecules in bone marrow transplantation.
All these studies have indicated the involvement of several adhesion molecules, but a clear understanding of the entire sequence of adhesive interactions between hematopoietic stem cells and bone marrow stromal elements that constitutes *in vivo* homing is yet to be gained.

1.5.1 In vivo characterization of CFU-S homing

The first attempts to characterize the in vivo homing pattern of CFU-S consisted of retransplantation experiments into secondary recipients to determine the distribution of injected cells and the efficiency of CFU-S homing to the spleen and to the bone marrow. As reviewed in detail in Chapter 5, this resulted in a number of publications reporting different spleen seeding factors (f-factors) [185-187]. Visser and Eliason [187] used the same technique to determine an f-factor for CFU-S homing to the bone marrow of irradiated mice. Lord et al. then used the bone seeking radioactive isotope ^{89}Sr to kill any CFU-S that entered the bone marrow, and thus showed that the CFU-S take no more time than 24 hours to reach their final lodging, and that they do not redistribute later on [188]. Also, Lord et al. [189] demonstrated that CFU-S localize preferentially in the periphery of the femoral bone marrow cavity.
While providing information on the efficiency and kinetics of CFU-S homing to spleen and bone marrow, these studies have not led to a detailed account of the localization of stem cells in the bone marrow and of the composition of their niches, nor have they provided an insight into the mechanisms of stem cell homing and the molecules involved.

1.5.2 Adhesion molecules involved in homing of hematopoietic stem cells

1.5.2.a The lectin homing receptor

A direct approach towards identification of homing receptors was followed by the group of Tavassoli. Based on reports from Reisner et al. [190] and Samlowski et al. [191] that manipulating carbohydrates on the membranes of hematopoietic stem cells changed the seeding patterns into spleen and bone marrow they hypothesized that carbohydrate recognition would play a role in stem cell homing, and that the homing of stem cells would be mediated by lectin-like molecules. They then began to use monosaccharides coupled to bovine serum albumin (BSA) as probes of lectin activity. Galactosyl-BSA as well as mannosyl-BSA could be used to reversibly block adhesion of CFU-S and CFU-C and the formation of cobblestone areas by hematopoietic stem cells inoculated onto stromal layers [192, 193]. Radioactively labeled galactosyl-BSA preferentially accumulated in the bone marrow, indicating the presen-

ce of a galactosyl-binding receptor that could mediate internalization of galactosyl-containing glycoproteins [194]. The relevance of this interaction to *in vivo* homing was confirmed in a murine survival assay where infusion of galactosyl-BSA or mannosyl-BSA together with the bone marrow graft led to approximately 50% reduced survival of lethally irradiated recipients [195]. The artificial glycoproteins also bound to the primitive hematopoietic cell lines B6SUT and FDCP-1. Scatchard analysis indicated a binding affinity of these cells to gal-BSA of about $2.3x10^{-7}$ M, and 10^6 binding sites per cell. Mannosyl-BSA binding analysis demonstrated biphasic binding with affinities of about $2.5x10^{-8}$ M and $1.0x10^{-7}$ M respectively, and about $7.4x10^5$ and $3.7x10^5$ binding sites per cell [196]. This biphasic binding was later reinterpreted as being an artefact caused by impurity of the man-BSA, so that it was concluded that there was the same number of binding sites for man-BSA as for gal-BSA with an affinity of approximately $2x10^{-7}$ M [197]. From the fact that applying man-BSA and gal-BSA in blocking experiments led to roughly similar results and from the absence of an additive or synergistic effect [192, 193, 195] it was concluded that the homing receptor mediates its effect through interaction with a particular carbohydrate configuration containing both one or more galactose and mannose residues [197]. Subsequently a protein was isolated from these cell lines using affinity chromatography with immobilized galactosyl and mannosyl residues. The protein was shown to be a 110 kD heterodimer containing approximately 5% carbohydrate and consisting of subunits of 23 kD and 87 kD [198]. Further characterization of this protein as the putative homing receptor for hematopoietic stem cells is still ongoing [199].
For practical purposes, the homing lectin is provisionally classified here as an adhesion molecule, although this has not been formally demonstrated in any classical adhesion assay. In all the experiments described in this section the blocking neoglycoprotein was always present during the entire experiments. Therefore, it can not be ruled out that galactosyl-BSA and mannosyl-BSA interfere with for example the interaction between a growth factor and its receptor which is essential for proliferation. Such an effect would lead to similar results in the described experiments and could therefore be mistaken for a homing blockade.

1.5.2.b Integrins VLA-4 and VLA-5

There is ample evidence that hematopoietic stem and progenitor cells can adhere to fibronectin, which is abundantly present in the extracellular matrix of bone marrow stromal cells, and to the adhesion molecule VCAM-1 [200-205]. VCAM-1 is constitutively and inducibly expressed on bone marrow endothelial cells and on bone marrow stromal cells [178, 203]. Fibronectin-receptors expressed on hematopoietic progenitor cells are VLA-4 and VLA-5 [200-204]. VLA-4 can bind to both fibronectin and VCAM-1 [206].
Van der Sluijs et al. [207] found that 90% of late (day 28-35) cobblestone area forming cells (CAFC) adhered to fibronectin, whereas only 11% of day-10 CAFC's bound to fibronectin. Late CAFC's correspond to cells with marrow repopulating ability (MRA) and long-term repopulating ability (LTRA), whereas day-10 CAFC's correspond to day-12 CFU-S [208].

This corresponded to the findings of Williams et al. [200], who showed binding of 50% of day-12 CFU-S to extracellular matrix from a hematopoiesis-supporting stromal cell line and to fragments of fibronectin that contained the CS1 alternatively spliced domain of fibronectin, the fibronectin binding site of VLA-4, not VLA-5. This binding could be blocked by adding an antibody to integrin alpha-4. Furthermore, they showed long-term hematopoietic chimerism in mice that had been injected with the cells that bound to CS1-containing fibronectin fragments, demonstrating that murine long term repopulating hematopoietic stem cells can use VLA-4 to bind to fibronectin. Preincubation of bone marrow cells with rabbit antibodies to $ß_1$ integrin led to unspecified reductions of spleen colonies and myeloid colonies in femora, consistent with a role for integrin VLA-4 in the homing of hematopoietic stem cells.

Seemingly conclusive evidence for a role of VLA-4/VCAM-1 adhesion in *in vivo* homing of CFU-S was presented by Papayannopolou et al. [209]. They directly assayed for the effect of blocking VLA-4/VCAM-1 mediated adhesion in a series of direct retransplantation CFU-S assays at 3 hours after the primary transplantation. VLA-4 was blocked by incubating the cells with antibodies to VLA-4, whereas in another exprerimental group VCAM-1 was blocked by injecting antibodies to VCAM-1 into the recipient animals. Reduced numbers of CFU-S in the bone marrow were observed, compared to increased numbers of CFU-S in the spleens of the primary recipients. They concluded that the blockade of VLA-4 or VCAM-1 molecules led to a reduced capacity of the cells to lodge in homing sites in the bone marrow, thereby increasing the chance of the cells to home to the spleen. This confirms the notion that spleen homing of CFU-S is mediated via a mechanism different from bone marrow homing (see also Chapter 5). It was also shown that treatment of recipients with the antibodies to VLA-4 as well as antibodies to VCAM-1 induces mobilization of CFU-C in untreated recipients. Unfortunately, the experiments described in this report were flawed. In the *in vivo* blocking experiments a non-relevant isotype control was used as a control. This should have been an isotype-matched antibody to a non-adhesion related cell surface marker to control for F_c-related effects. Furthermore, as the authors show in this report, the injection of antibodies to VCAM-1 into normal recipient mice leads to massive mobilization of progenitor cells into the circulation on day 4 after the first injection. It can not be ruled out that mobilization has already taken place at four hours after injection of anti-VCAM in the primary recipients used in the homing assay. This could have led to competition of the mobilized cells with the stem cells in the bone marrow graft, thus causing the reduced numbers of CFU-S in the bone marrow. The conclusions brought forward in this paper do therefore not seem entirely warranted. However, if indeed the effects described in this paper are correct, and blocking integrin alpha-4 would cause an increase in spleen colony formation while blocking integrin ß1 would lead to a reduction of spleen colony numbers [200], then it seems likely that spleen homing of CFU-S is, at least in part [207], mediated by integrin VLA-5. Further experiments in this direction using direct homing assays which can also provide more information on the behaviour of earlier stem cell subsets are needed.

1.5.2.c Selectins

A number of studies with human material have recently implied the selectins in the homing of hematopoietic stem cells. Möhle et al. [163] showed strong expression of L-selectin on $CD34^+/HLA\text{-}DR^-$ and $CD34^+/HLA\text{-}DR^{dim}$ cells in leukapheresis products mobilized by G-CSF/cytotoxic treatment. These are considered to be more primitive stem cells than $CD34^+/HLA\text{-}DR^{bright}$ cells. A correlation between time-to-platelet-recovery in transplant patients and the number of L-selectin expressing CD34-positive cells from leukapheresis material was found by Dercksen et al. [210]. Both groups speculated on a role for L-selectin in the homing of transplanted hematopoietic stem cells. This would also be in line with the observed CD34-mediated adhesion of $CD34^+$ cells to cultured bone marrow endothelial cells [211]. Constitutive expression of CD34, a ligand for L-selectin, and low level constitutive expression of E-selectin, a possible ligand for PSGL-1 and SLe^x, was demonstrated in endothelial cells isolated from human bone marrow samples [212]. Adhesion to P-selectin and concurrent expression of PSGL-1 in CD34-positive bone marrow cells was demonstrated [213, 214], and so was expression of SLe^x [213, 215]. Staining of endothelial cells with antibodies to P-selectin has been observed in plastic-embedded sections of human [216], but not murine bone marrow samples [217]. Thus, the prerequisites for a role of all three selectins and their ligands seem fulfilled. However, no conclusive data on a direct functional involvement of the selectins in homing of hematopoietic stem cells has been presented so far.

1.5.2.d Growth factors and growth factor receptors

Several hematopoietic growth factors have been implicated in the interaction between stromal cells and hematopoietic stem cells [218-221]. Growth factors and their receptors generally bind with very high affinity which should be sufficient to directly mediate adhesion. It has been speculated that the mechanism involved would be sequestration of the growth factors to extracellular matrix components, where they would be accessible to the receptors expressed on hematopoietic stem cells [222, 223]. Recent data suggest, however, that growth factors enhance adhesion indirectly, through upregulation [220, 224, 225] and/or modulation of the adhesiveness [226-228] of adhesion molecules.

1.5.3 Out of the maze - direct *in vivo* homing studies

As described above, a plethora of possible adhesion mechanisms between hematopoietic stem cells and bone marrow stromal and endothelial have been identified, mostly in *in vitro* model systems. Homing research has now reached a stage where detailed *in vivo* studies are needed to elucidate which of the *in vitro* defined interactions are operational *in vivo*.

1.6 Technical aspects of *in vivo* homing studies

Several considerations are important when performing in vivo homing assays. The cell suspensions to be injected should be as pure as possible, so that reliable conclusions can be drawn about the cell type under study. Proliferation and differentiation may take place, possibly accompanied by migration of the cells within the stroma. Cell cycle kinetics must therefore be taken into account, depending on the exact experimental setting (Chapter 3).
When blocking antibodies are used in vivo, F_c mediated sequestration of the injected cells or complement mediated cytotoxicity of *in vivo* targeted cells can be a problem, so inter-species F_c/F_c-receptor crossreactivity must be considered [229]. Clearance of injected antibodies may be a problem, so antibody titers should be monitored [230].
Injected control cells should be labeled with a species- and isotype- matched antibody to a non-homing related epitope that is expressed at an approximately equal level to that of the target epitope.
Care has to be taken that adhesive sites and niches are not saturated by injection of vast numbers of cells. This would lead to non-specific effects and artefactual localization of the injected cells. Thus, the need for specificity coincides with the difficulty of obtaining large quantities of well-defined stem cells suspensions to a situation where limited numbers of stem cells are injected into the animals to be assayed. As a result, the frequencies of injected cells in the target organs are generally low. This places exacting demands on the choice of cellular markers and on the assays used for the detection of the injected cells.

1.6.1 Selection criteria for cellular markers

Several criteria must be considered when selecting cellular markers for the tracking of stem cells or leukemic cells in *in vivo* assays:
1. Toxicity. The label should exhibit minimal toxicity to the labeled cells.
2. Stability. Persistent presence of label in or on the cell is required. Label loss through internalization, "leaching" or metabolic activity should be minimal. Enzyme markers should exhibit stable and preferably high level and uniform expression.
3. Inertia. The label should have a minimal influence on the homing and migration of the cells, as well as a minimal influence on their proliferation and differentiation.
4. Immunogenicity. The label should be non-immunogenic. This is especially important in long-term experiments.
5. Detectability. High labeling indices should be achieved, to reduce the chance of detecting false positives. This is especially important when detecting low frequencies of labeled cells.
6. Versatility. The availability of a range of well-defined sensitive detection assays for labeled cells is advantageous by allowing the choice of an optimally suited assay for every experimental setting.

Basically, there are two ways of obtaining labeled cells: A) Genetic marking through introduction of marker genes into cell lines or the production of transgenic mice carrying a marker gene, or B) Transient marking through physical or chemical modification of the cells.

1.6.2 Genetic markers

Essentially any genetic polymorphism can be used as a marker for distinguishing cells of host or donor origin. This includes naturally occurring polymorphisms as well as artificially introduced marker genes. The most straightforward genetic marker is sex. Sex mismatch is widely used in transplantation research. The use of sex difference as a genetic marker imposes minimal restrictions on the choice of the animal model system. A disadvantage is the possibility of H-Y transplantation antigen-mediated immune reactions [231-234]. Sensitive detection of male cells using antibodies to H-Y antigens has proven impossible due to the limited specificity of antibodies to H-Y [235-239]. Therefore, detection of male cells in a female background relies on Southern blotting [240-242], in situ hybridisation [242-245] or PCR-based techniques [246]. Other naturally occurring polymorphisms have been employed as markers in transplantation biology, such as the glucose phosphate isomerase [247] which is analyzed by electrophoresis, and the Ly5.1/5.2 polymorphism [248], which can be analyzed using monoclonal antibodies. In situ hybridisation and in situ PCR are both relatively labour-intensive techniques. Moreover, in situ PCR is only beginning to become a routinely applicable technique. Southern analysis is relatively insensitive, and it is not suited for in situ detection of marked cells on a single cell level. Exact quantification of labeled cell frequencies is also difficult to achieve with this technique. The same is true for electrophoretic identification of glucose phosphate isomerase (GPI). Detection methods based on monoclonal antibodies are limited in sensitivity because of non-specific binding of antibodies.

The introduction of exogenous marker genes is a means of tailoring existing model systems to the *in vivo* study of cellular behaviour. These marker genes are cloned into a construct containing regulatory sequences to drive its expression, and subsequently the construct is introduced into the genome of the target cells. This can be achieved through a number of techniques, such as microinjection, calcium phosphate precipitation, lipofection, or electroporation. Alternatively, the gene can be cloned into a retroviral vector, which is used to infect the target cells. Depending on the exact application, this step is followed by selection of successfully marked cells, or the cells can be directly used in the model system. These techniques can also be used to modify embryonal stem cells from which transgenic animals can be produced. These can then serve as donors for genetically marked cells [249].

Most of the genes used in marking strategies are non-mammalian enzymes. Widely employed marker genes are chloramphenicol-acetyltransferase (CAT) [250], luciferase (LUC) [251], aminoglycoside phosphotransferase (Neo^R) [252], and ß-galactosidase (LacZ) [253-256]. Expression of these exogenous enzymes leads to intracellular levels of enzyme activity that can be used to select and detect marked cells. CAT assays are based on separation of radiola-

beled or fluorescently labeled acylated chloramphenicol from the unmodified substrate [257-259]. For luciferase there are very sensitive fluorencence assays [260, 261]. Both assays use cell lysates. The Neo^R gene confers neomycin resistance to modified cells. These cells can be detected by culturing under selective neomycin pressure. This is a single cell-based assay which is not suited for in situ detection of marked cells, and depends on the ability to grow the cells in vitro [37, 262]. The use of LacZ has the advantage that there is a wide variety of assays to choose from, both bulk assays on cell lysates as well as cell-based systems [263-267]. A disadvantage is the presence of several mammalian enzymes that exhibit endogenous ß-galactosidase activity.

1.6.3 Transient markers

Essentially every detectable non-hereditary change that can be conferred to a cell can serve as a transient marker. Such a marker is either lost when a cell dies, or becomes useless through dilution among daughter cells or through non-ideal behaviour such as leaching. These labels are generally used for in situ tracking and lineage analysis of cells. Over the years a wide range of transient markers has been used. All of the assays used for homing studies have had their limitations. For example, metabolic labeling of leukemia cells using tritiated thymidine [26] can only be applied to proliferating cells, and may thus not be very suitable for use in stem cell homing assays. The viable DNA-stain Ho33342 which has been used for the tracking of bone marrow derived prothymocytes [268, 269] could only be used in very short-term because of dye leakage. ^{51}Cr showed unstable and variable labeling of erythrocytes [270]. ^{111}In showed variable labeling, radiotoxicity and altered cellular function of lymphocytes [271, 272]. Covalent labeling of lymphocytes using FITC and TRITC showed reduced cellular life span [273, 274] and dye leakage [269].

In recent years two new classes of cell trackers have become available. The PKH-class of cell trackers consists of lipophilic molecules that become stably incorporated into the cell membrane. There are two fluorescent dyes of the PKH class and one of the trackers is a ^{125}I coupled cell tracker [275]. These trackers have so far shown little or no toxicity, stable membrane labeling, very high labeling indices [276], and little influence on cellular function [277, 278]. This makes the fluorescent forms very suitable for flow cytometric detection of labeled cells, especially for proliferation studies.

The second new class of cell trackers is a series of fluorochromes coupled to a chloromethyl moiety. These molecules can enter the cell and become covalently linked to glutathione molecules, leading to stable labeling [279-281]. These dyes are also suitable for flow cytometric detection, and seem especially promising for in situ detection of labeled cells in sections of paraffin embedded tissue specimens.

1.7 Introduction to the experimental work

The development of techniques for the in vivo detection of low numbers of marked cells is a prerequisite for reliable studies of the mechanisms that govern *in vivo* homing of normal hematopoietic stem cells and leukemic cells. Chapter 2 analyzes four fluorescent cell trackers and presents a survey of the applicability of each of these dyes. Chapter 3 shows that PKH26 can be used for *ex vivo* detection of very low frequencies of labeled cells. Homing of CFU-S into conditioned and non-conditioned recipients is compared and it is also shown that injected cells rapidly cycle upon injection into irradiated as well as non-irradiated recipient mice. Differences in the mechanism of spleen homing and bone marrow homing are observed and a new h-factor is introduced, to facilitate the analysis of homing kinetics and of the composition of an injected bone marrow fraction. In Chapter 4 the production of a LacZ marked rat leukemic cell line, LT12NL15, is described which is to be used in homing studies and in other systems where detection of very low numbers of leukemic cells is desirable. A new enzyme-kinetic assay for the presence of LacZ-marked cells is introduced in Chapter 5. An important element of this assay is a new method of suppressing background mammalian galactosidase activity, also presented here. Chapter 6 concerns the further characterization of the LT12NL15 cell line using the newly developed extremely sensitive sticky plate assay for the LacZ marked cells. Homing of the cells and growth kinetics are analyzed, followed by a comparison of the result from this "presence" assay with that of a "functional" culture assay after cytostatic treatment of LT12NL15 carrying rats. Implications of the detection of minimal residual disease (MRD) in leukemia patients are discussed. In Chapter 7 a new immunostaining method for the detection of LacZ expressing cells in paraffin sections of decalcified bone is presented. This assay further increases the choice of assays for LacZ marked cells and will aid in the analysis of the homing of LT12NL15 cells. Chapter 8 summarizes the results, indicates the issues that are still open, and discusses how the data presented in this thesis will prove their value in addressing them.

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Chapter 2

Evaluation of fluorescent marker molecules for homing studies of leukemic cells and normal hematopoietic cells

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Chapter 2

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Abstract

Staining protocols were optimized for the lipophilic fluorescent membrane stains PKH2 and PKH26 and for the intracellular fluorescent markers CM-BODIPY and CM-FDA, all four fluorescent cell trackers. This was followed by experiments to assess the influence of the markers on clonogenicity and growth rate of murine bone marrow cells and of cells from a rat leukemic cell line (LT12). It is shown that none of the trackers has a significant influence on these important kinetic parameters, and that all four can therefore be used for in vivo cell tracking experiments, depending on the exact purpose of the experiments.

Introduction

Fluorescent markers for homing studies of hematopoietic cells have to fulfill a number of prerequisites (Chapter 1.6.1). Based on reports in the literature on the use of PKH stains for *in vitro* cell kinetic studies of human epithelial cell lines [1, 2], leukemic cell lines [3] and peripheral blood monocytes [4] and for *in vivo* cell tracking studies of rabbit erythrocytes [5], sheep lymphocytes, [6], murine LAK cells and TIL's [7] and spleen cells [8] and of human peripheral blood leukocytes [9] we have tested and evaluated the membrane stains PKH2 and PKH26 for their applicability in our experiments. Both are non-polar molecules consisting of a double lipid-like tail coupled to a fluorescent moiety. Upon labeling, the lipid tail becomes incorporated in the cell membrane, leading to stable and persistent labeling of the cell for up to 60 days [5].

One of the objectives of the studies described in this thesis was microscopic detection of *in vivo* injected labeled cells to directly characterize stem cell and leukemic cell homing sites and niches. The disadvantage of lipid-like stains such as PKH2 and PKH26 is that they are washed away during the fixation and dehydration steps that are part of standard tissue processing procedures for microscopy. Therefore, two intracellular fluorescent markers, 5-chloromethylfluorescein diacetate (CM-FDA) and 8-chloromethyl-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene (CM-BODIPY) were also tested and evaluated for their usability in our in vivo studies. These molecules can be used as intracellular non-lipophilic labels

which should withstand dehydration and embedding. Furthermore, antibodies to allow visualization of both FDA and BODIPY labeled cells are commercially available[10].

CM-FDA is composed of an FDA molecule coupled to a reactive chloromethyl moiety. FDA is well known and widely used as a viability marker. It is non-polar and can therefore rapidly cross the cell membrane. Upon crossing the cell membrane, the diacetate moieties are hydrolyzed by intracellular esterase activity, leaving a fluorescein molecule. The chloromethyl moiety of CM-FDA will react with intracellular glutathione and free thiol groups that are present on cytoplasmic proteins, leading to an intracellular covalently coupled fluorescein molecule, which can easily be detected using standard fluorescent techniques. CM-BODIPY is a combination of the chloromethyl moiety with a fluorescent BODIPY molecule. This molecule can also cross the cell membrane and the reactivity of the chloromethyl moiety will lead to covalent intracellular entrapment of the fluorescent molecule.

Our evaluation consisted of experiments to determine optimal staining protocols of leukemic cells (LT12 and LT12NL15) and murine bone marrow cells for each marker. Subsequently, experiments were performed to monitor the effect of proliferation on the fluorescence of labeled cells and to assess the influence of the markers on clonogenicity and growth rate of hematopoietic and leukemic cells.

One of the characteristics of fluorescent cell trackers is that the fluorescent tracker molecules are equally divided among the daughter cells with every cell division. This is reflected in a gradual decrease of fluorescence intensity per cell in a proliferating cell population. Because of this phenomenon fluorescent cell trackers can be employed to study cell kinetic parameters, based on the expectation that when the average number of fluorescent molecules per cell decreases by 50% the fluorescence should also decrease by 50%. This assumption precludes disturbances such as dye leakage or varying "fluorescence efficiency" of the tracker molecu-

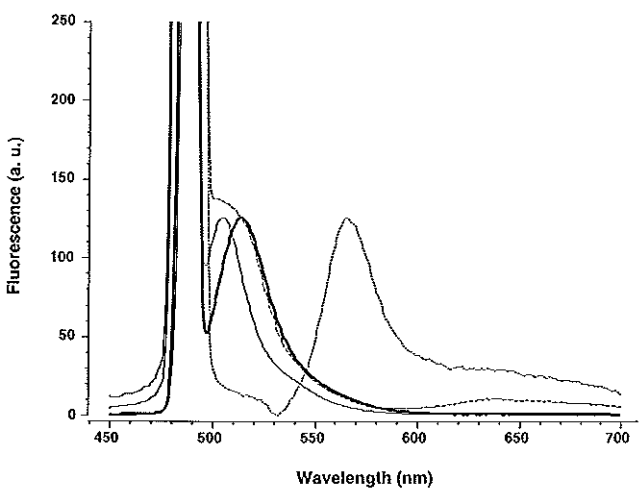

Figure 2.1 Fluorescence emission spectra of LT12NL15 cells labeled with fluorescent cell trackers. Excitation at 488 nm. Thick solid line: cells labeled with CM-FDA; thin solid line: cells labeled with PKH2; dashed line: cells labeled with CM-BODIPY; stippled line: cells labeled with PKH26.

les. To assess whether the '50% fluorescence reduction per cell division' holds true, the fluorescence intensity per cell of labeled and unlabeled leukemic cell cultures was measured by flow cytometry. This was combined with growth rate determination by frequently counting numbers of cells in the cultures.

Because bone marrow is a complex mixture of cell types, it cannot be ruled out that a uniform treatment of the cell suspension could have a different effect on the different stem cell subsets present. Therefore, the clonogenicity of fluorescently labeled normal hematopoietic stem cell subsets was measured using the Cobblestone Area Forming Cell assay (CAFC assay) as developed by Ploemacher et al. [11-13]. The major advantage of the CAFC-assay is that it can be used to assess all stem cell subsets in a bone marrow sample functionally and quantitatively. Originally developed and extensively validated in a murine system, it has recently been extended to the analysis of rat, rhesus monkey and human hematopoietic cells [14-16]. As a quantitative assay for stem cell subsets this assay can be used to determine the effects on hematopoietic stem cells of treatment with substances such as cytostatic drugs or fluorescent labels.

These experiments are of use to evaluate the applicability of these fluorescent cell trackers in the analysis of homing and proliferation of leukemic cells and normal hematopoietic cells.

Materials and methods

Animals

Bone marrow suspensions were prepared from femora and tibiae of B6/CBA mice bred under specific pathogen free conditions at the animal facility of the Erasmus University Rotterdam, the Netherlands. Suspensions were prepared by crunching femora and tibiae of donor mice in a mortar [17] containing 5 ml of Hanks' Balanced Salt Solution containing 3.5 mM HEPES, pH 6.8 (H+H). Single cell suspensions were prepared by passing the cells through 30 µm nylon gauze.

Density gradient centrifugation

Low density bone marrow cells were prepared by density gradient centrifugation. The cells were loaded on a composite gradient of Ficoll-400 (Pharmacia Biotech, Uppsala, Sweden) dissolved in 0.1 M sodium phosphate, pH 7.4. Densities were 1.078, 1.069, 1.051 g/cm^3, respectively. The gradients were centrifuged for 30 min at 23,500 g at 4° C. After centrifugation, the low density cells were harvested from the interphase between 1.069 and 1.078 g/cm^3 density, washed in a large volume of Alpha-MEM and used in the assays.

Leukemic cells

Leukemic cells were LT12 cells [18] and LT12NL15 cells, a LacZ expressing subsidiary of

the LT12 cell line [19]. Both are in vitro growing rat promyelocytic cell lines, derived from the in vivo growing Brown Norway Rat Myelocytic Leukemia (BNML) model for human acute myelocytic leukemia [20]. These cells were cultured in Alpha-MEM medium (Gibco, Breda, The Netherlands) containing 0.03% w/v glutamine, 100 IU/ml of penicillin, 100 µg/ml streptomycin and 10% v/v fetal calf serum (FCS). The cells were cultured at 37° C in an atmosphere of 5% CO_2 and >95% humidity.

PKH-staining

Cells were washed three times using H+H to remove all serum, and resuspended in a total volume of 250 µL diluent A or C (for PKH2 or PKH26, respectively) (Zynaxis Cell Science, Malvern, PA, USA) in an Eppendorf vial. Subsequently, 250 µL of PKH2 or PKH26 (Zynaxis) diluted in the appropriate diluent was added and mixed with the cells by gentle pipetting. After incubation for 1 to 60 minutes the staining reaction was stopped by addition 500 µL of H+H containing 10% v/v FCS. The cell suspensions were then underlayered with 200 µL FCS and centrifuged for 5 min at 400 g. The entire labeling procedure was performed at ambient temperature. After centrifugation the cells were washed once with H+H containing

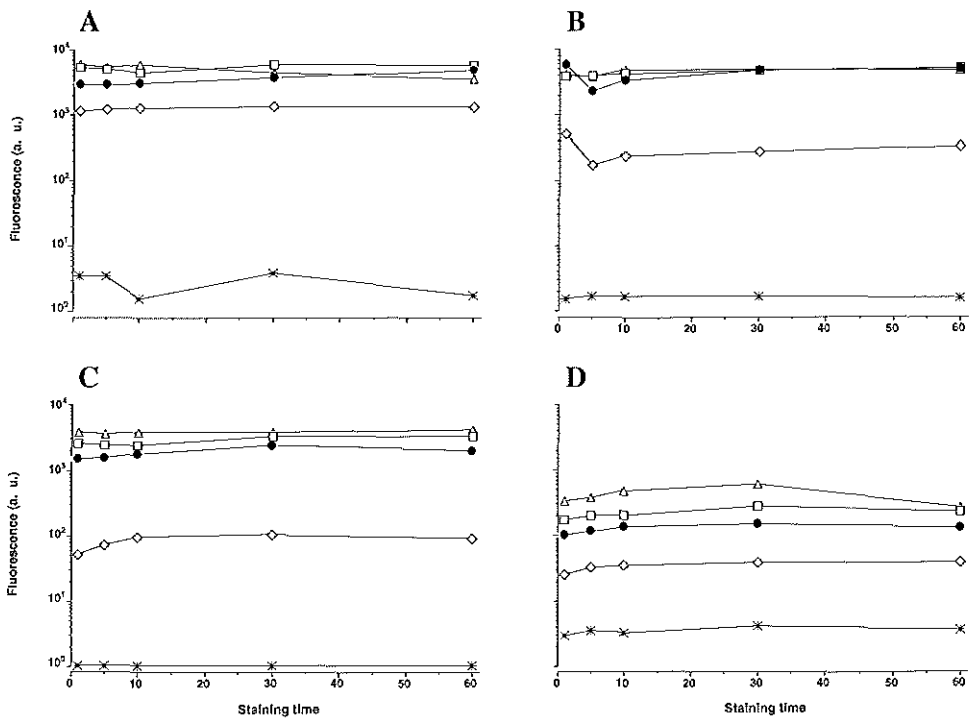

Figure 2.2A to 2.2D Optimization of staining time (min) and label concentration using LT12NL15 cells. Figure 2.2A: PKH2; Figure 2.2B: PKH26; Figure 2.2C: CM-FDA. Figure 2.2D: CM-BODIPY. Stars: control; diamonds: 1 µM; closed circles: 5 µM; squares: 10 µM; triangles: 25 µM.

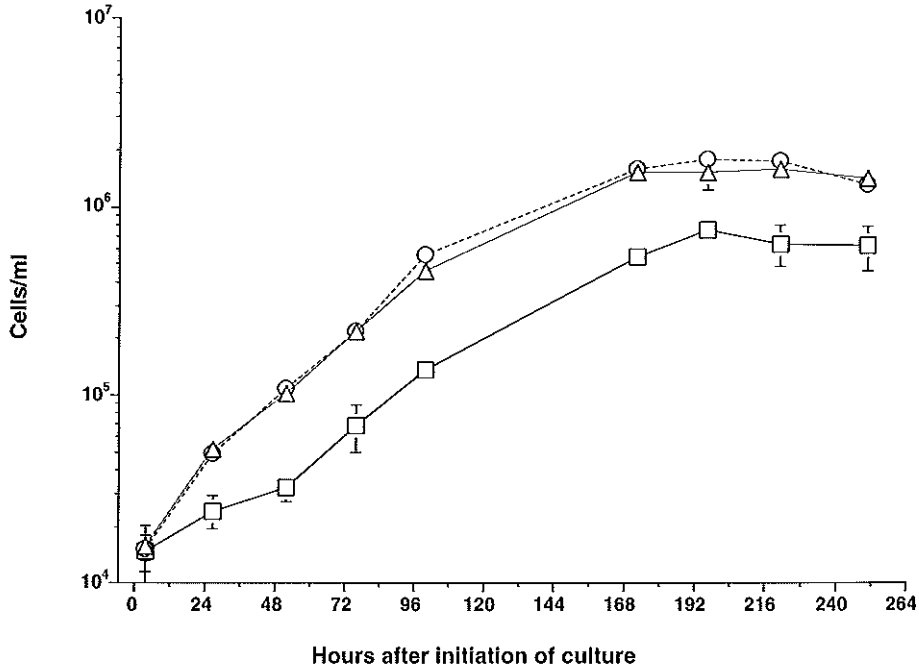

Figure 2.3 Growth curve of LT12 cells stained with PKH2. Triangles: unstained control cells; circles: mock-stained cells; squares: PKH2 stained cells. Error bars represent standard deviation of triplicate cell counts.

10% v/v FCS and used in the assays. Up to 10^7 cells were used in the staining procedure described. All labeling steps were performed in polypropylene vessels because the fluorescent cell trackers have a strong tendency to adhere to polystyrene liquid handling materials.

CM-FDA and CM-BODIPY staining

Stock solutions of 10^{-2} M in DMSO were prepared from both CM-FDA and CM-BODIPY (Molecular Probes, Eugene, OR) and stored at -20° C. For staining, aliquots of this stock solution were diluted to the appropriate concentration in H+H. Cells were washed serum-free as in the PKH-staining procedure, and subsequently resuspended in H+H. From this point the staining protocol followed the PKH-staining procedure as described above.

Fluorescence emission spectra of stained cells

Fluorescence emission spectra were measured of suspensions in PBS containing approximately equal numbers of LT12NL15 cells stained with the four fluorescent cell trackers. Excitation was at 488 nm, the most commonly used wavelength in fluorescence microscopy and flow cytometry for excitation of fluorescein and similar fluorochromes. Spectra were measured on a Perkin-Elmer LS-50 spectrofluorimeter (Perkin-Elmer Ltd., Beaconsfield,

Bucks., England). Excitation and emission slit width were set at 5 nm. For presentation, the emission curves were normalized to the green emission peak of CM-FDA.

CAFC-assay

Murine total bone marrow suspensions were fluorescently labeled and limiting dilution CAFC-assays were performed according to standard procedures [11-13] in 96-well plates on confluent layers of the murine bone marrow-derived stromal cell line NBM11F4G, which provides full support of all hematopoietic stem cell subsets (Ploemacher and Mayen, unpublished results). These stromal layers were grown in microwells pre-coated with 0.2% w/v gelatine to improve long-term attachment (1 h at room temperature or 24 hours at 4° C). The culture medium consisted of Alpha-MEM containing 3.5 mM HEPES, 10^{-7} M sodium selenite, 10^{-5} M hydrocortisone 21-hemisuccinate, $2x10^{-3}$ M glutamine, 10^{-4} M ß-mercaptoethanol, 100 IU/ml of penicillin, 100 µg/ml streptomycin. Before reaching confluency the medium was supplemented with 10% (v/v) FCS and 5% v/v horse serum (HS). After reaching confluency the cultures were maintained in medium supplemented with 20% v/v HS only. These cultures were grown in an atmosphere of 10% CO_2 and >95% humidity, at a temperature of 33° C. Cobblestone areas were counted weekly, followed by a change of half the culture medium. Wells had to contain cobblestone areas of at least five adjacent cells to qualify as positive. Limiting dilution analysis was performed assuming single-hit kinetics and using Maximum Likelihood statistics [21, 22].

Flow cytometry

The fluorescence of the labeled cells was measured on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). PKH2, CM-FDA and CM-BODIPY fluorescence were acquired in the *fluorescein* channel (FL1). PKH26 fluorescence was acquired in the *phycoerythrin* channel (FL2).

Results

Fluorescence spectra

Figure 2.1 shows the fluorescence emission spectra of cells labeled with the four cell trackers. PKH2 has a relatively small Stokes' shift of only 17 nm, leading to a distinct emission peak at 505 nm. CM-FDA shows a fluorescein-like emission peak at approximately 514 nm, slightly lower than that of fluorescein (530 nm). The fluorescencespectrum of CM-BODIPY does not exhibit a distinct peak as is observed with CM-FDA. The right hand slope of the CM-BODIPY-fluorescence is virtually congruent with that of CM-FDA, indicating that both labels should have comparable spectral measurement characteristics in a flow cytometer. PKH26 exhibits a very large Stokes' shift of 77 nm, with a distinct fluorescence peak at 565

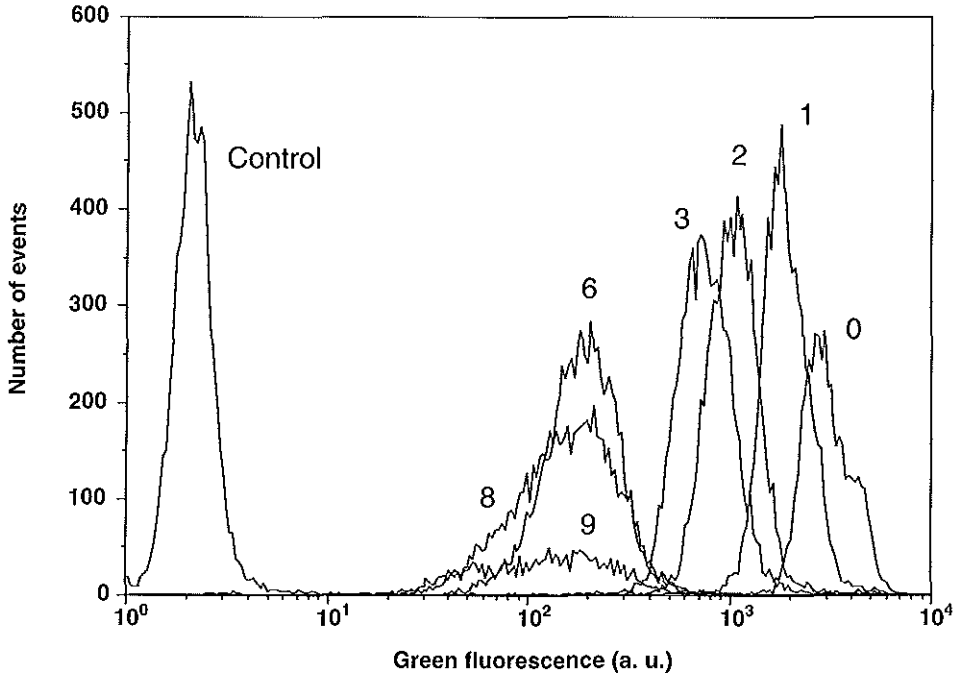

Figure 2.4 Fluorescence histograms of a culture of PKH2 stained LT12 cells, measured on consecutive days after initiation of the culture. Numbers with each graph indicate measuring day for each curve. Control indicates mock-stained control LT12 cells. Cells were counterstained with propidium iodide for live/dead discrimination. Graphs show live cells in samples of 10^4 events.

nm, and a broad slope towards higher wavelengths, leading to spectral overlap of PKH26 fluorescence with that of longer wavelength fluorochromes such as propidium iodide or Cy5.

Optimum staining protocols

Figures 2.2A to 2.2D show the effect of different staining times and tracker concentrations on the labeling intensity of the cells. Increasing the staining times beyond 1 minute did not lead to a substantial increase of the labeling intensity with any of the four fluorescent stains. Therefore, in all further labeling experiments a staining time of 1 minute was used. Figures 2.2A and 2.2B show that for both PKH2 as well as for PKH26 a concentration of 5 µM is sufficient to achieve maximum labeling intensity. Under these conditions there was no change of light scatter characteristics of the cells as measured by flow cytometry, as was observed at higher concentrations (not shown). Therefore, a standard labeling concentration of 5 µM was chosen with the PKH stains.

Figures 2.2C and 2.2D show similarly obtained graphs for CM-BODIPY and CM-FDA. At a concentration of 10 µM CM-FDA began to show saturated staining of approximately 3.5 logs above control. The same was observed with CM-BODIPY, although at a much lower labeling

intensity of two logs above control. For both stains, 10 μM was chosen as the optimal staining concentration.

Growth rate and fluorescence decay

Figure 2.3 shows a growth curve of PKH2 stained LT12 cells. As was normally observed, the curves consist of an exponential portion followed by a plateau caused by medium exhaustion. However, the culture of PKH2 stained cells showed a period of delayed growth during the first 48 hours after initiation of the culture. This was followed by a period of exponential growth with a curve parallel to that of control cells. Then the plateau phase set in at a lower level than with the control cells. PKH2 was the only dye with which such an initial growth delay was observed.

Figure 2.4 demonstrates that as the cells divide the fluorescent label of the cells is divided among the daughter cells, leading to a decrease of the average fluorescence in the population from day 0 to day 6. After day 6 the culture entered plateau phase, so that further dilution of the stain due to proliferation was only observed in a small subpopulation, leading to peak broadening on day 8 and a biphasic distribution on day 9.

Figure 2.5 shows a curve fit through the exponential portion of the growth curve of the PKH2-stained LT12 culture shown in Figure 2.4. This curve fit corresponds to a cellular fluorescence half life ($T_{1/2}$) of 37.6 hours. The population doubling time of PKH2 stained LT12 cells is 23.2 hours, equal to that of unstained cells.

Figure 2.6 shows the fluorescence peak position (mode) of LT12NL15 cells stained with different concentrations of CM-FDA for 1 minute. The cells were then cultured and measured at the indicated times after staining. After staining with 1 μM of CM-FDA the fluorescence showed a slow decrease with a T_d of 75.0 hours until 38 hours after staining. After 38 hours the fluorescence decay accelerated to a T_d of 14.3 hours. Cells stained with 5 and 10 μM CM-

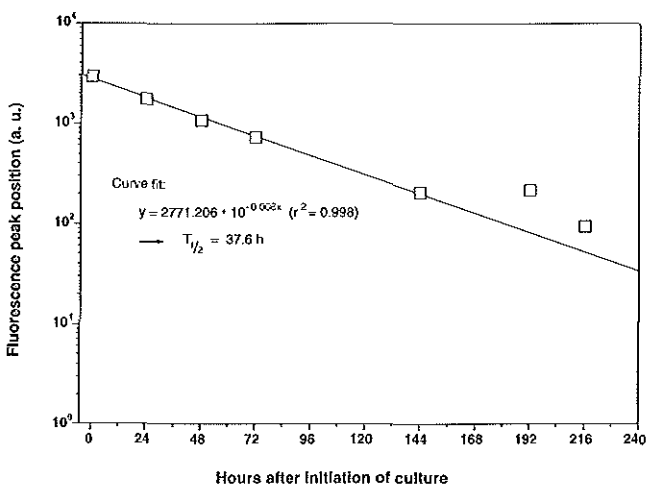

Figure 2.5 Analysis of fluorescence peak position as measured by flow cytometry of PKH2 stained LT12 cells, measured at different time intervals after initiation of culture (squares). Solid line indicates exponential curve fit through exponential part of the curve.

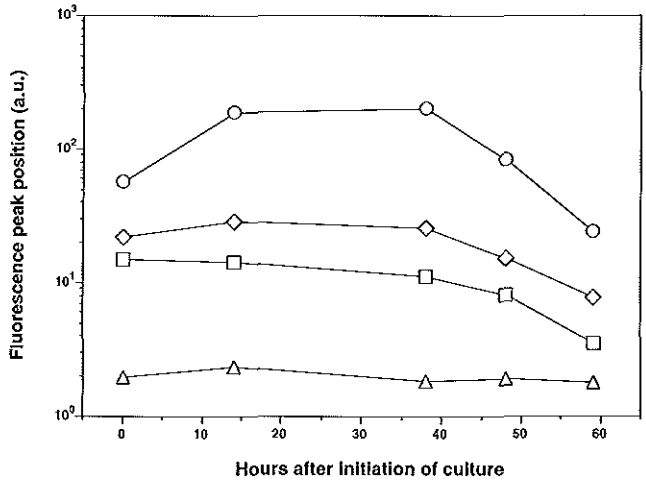

Figure 2.6 Analysis of fluorescence peak position as measured by flow cytometry of CM-FDA stained LT12NL15 cells, measured at different time intervals after initiation of culture. Triangles: unstained controls cells; squares: cells stained with 1 μM CM-FDA; diamonds: cells stained with 5 μM CM-FDA; circles: cells stained with 10 μM CM-FDA.

FDA showed an increase in fluorescence intensity until 38 hours after staining, followed by a steep decrease after 38 hours, with a T_d of 12.5 h and 6.8 h (5 and 10 μM , respectively).
Figure 2.7 shows the results of a similar experiment performed with CM-BODIPY as the intracellular stain. The fluorescence intensity was slightly higher than with CM-FDA. As expected, dye dilution was observed immediately after staining, because CM-BODIPY does not have to be hydrolyzed once loaded into the cells to become fluorescent. Direct proliferation analysis by cell counting indicated a population doubling time of 16.7 hours for both 5 μM and 10 μM labeled LT12NL15 cells. Analysis of the fluorescence decay, however, yielded population doubling times of 21.5 h, 30.0 h and 37.6 h (at 1, 5 and 10 μM, respectively).
Figure 2.8 shows the results of a CAFC-assay with murine bone marrow cells stained with the fluorescent cell trackers. The results indicate that clonogenicity as well as proliferation of all stem cell subtypes were not influenced by the staining with any of the four fluorescent dyes tested.

Discussion

As shown in Figure 2.1 PKH2 has an emission peak at 505 nm. This indicates that the standard fluorescein detection equipment used in these experiments is not optimally suited for detection of PKH2 stained cells, since it only detects the fluorescence from the right flank of the spectral curve of PKH2 fluorescence. This right flank, however, still yields enough of the emitted fluorescence to result in a labeling intensity of 2.5 log above background (Figure 2.2A). PKH26 fluorescence shows an emission peak at 565 nm, which renders it compatible with detection in the phycoerythrin FL2 channel of the FACScan flow cytometer. This peak is well separated from the fluorescein peak, indicating that PKH26 is well suited for double stai-

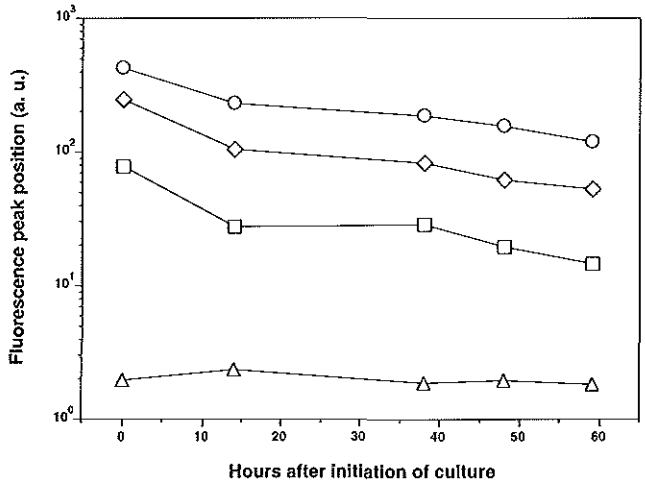

Figure 2.7 Analysis of fluorescence peak position as measured by flow cytometry of CM-BODIPY stained LT12NL15 cells, measured at different time intervals after initiation of culture. Triangles: unstained controls cells; squares: cells stained with 1 µM CM-BODIPY; diamonds: cells stained with 5 µM CM-BODIPY; circles: cells stained with 10 µM CM-BODIPY.

ning with fluorescein-isothiocyanate (FITC)-labeled antibodies. The considerable amount of fluorescence above 600 nm however, leads to problems in combining PKH26 with labels that exhibit long wavelength fluorescence, such as propidium iodide (PI). This is especially a problem because of the high labeling intensities that are achieved with PKH26. For live-dead discrimination it is advisable to use UV-excitable fluorochromes such as Hoechst 33258 or 4',6'-diamidino-2-phenylindole (DAPI) in conjunction with excitation by a UV light source, as described in Chapter 3 of this thesis. Spectral analysis of the fluorescence of CM-FDA and CM-BODIPY shows that these dyes are equally suited for detection with standard fluorescein detection equipment as used in fluorescence microscopy and flow cytometry. The slightly higher fluorescence of CM-BODIPY at higher wavelength means that electronic compensation of spectral overlap as used of flow cytometers should be slightly higher than with CM-FDA.

Figures 2.2A-2.2D show that with all four stains incubation times longer than 1 minute do not lead to substantially increased labeling intensities. One minute was therefore chosen as the standard staining time. Both PKH2 and PKH26 show saturating fluorescence intensities when stained at 5 µM. With the chloromethyl dyes saturation begins around 10 µM. Standard conditions were chosen accordingly.

Figure 2.3 shows that PKH2 staining of LT12 cells leads to a growth delay in the culture, caused by toxicity to the cells. The presence of the stain does not influence the growth rate of the culture during the exponential phase. This toxicity was not observed with any of the other cell trackers.

Figure 2.4 confirms that the fluorescence intensity of the cells decreases with their proliferation. The coefficients of variance (CV) of the curves remain roughly constant, at least during the exponential growth phase, which indicates that the fluorescent label is equally divided among the daughter cells. This fluorescence decrease does not, however,

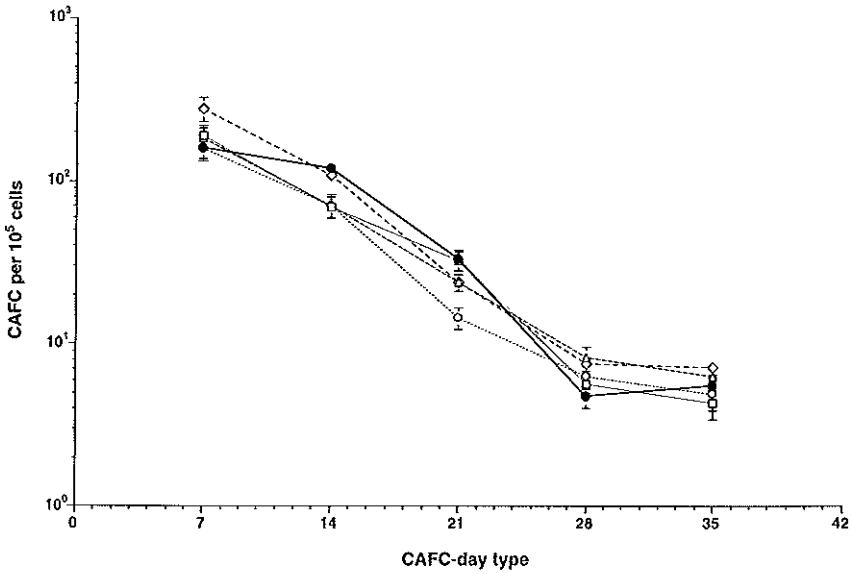

Figure 2.8 Cobblestone area forming cell assay of transiently labeled murine bone marrow cells. Closed circles: unstained control cells; open squares: PKH2 labeled bone marrow cells; open circles: PKH26 labeled bone marrow cells; open diamonds: CM-FDA labeled bone marrow cells; open triangles: CM-BODIPY labeled bone marrow cells. Error bars represent standard error of the mean (SEM).

correspond to a 50% reduction with every cell division. If this were true, then the cellular fluorescence half life should correspond to the population doubling time (T_d) of the cultures. As shown in Figure 2.5 the cellular fluorescence half life is considerably longer than the T_d. The most likely explanation is to assume that at high label concentrations inside the cell membrane there is considerable self quenching, which decreases when the tracker concentration decreases as a result of proliferation of the cells. A similar phenomenon is observed with CM-BODIPY. This can again be explained as a gradually declining self-quenching effect which is known to occur with high concentrations of CM-BODIPY (Ian Clements, Molecular Probes, personal communication). As expected, this effect is more pronounced with increasing CM-BODIPY concentration.

Cells stained with 1 µM CM-FDA initially show a slow fluorescence decline with a T_d of 75 hours, followed by a steep decline with a T_d of 14.3 hours from 38 h after intitiation of the cultures onwards. Staining with 5 and 10 µM CM-FDA leads to a fluorescence increase during the first 38 hours after initiation of the culture, followed by a rapid fluorescence decrease (T_d= 12.5h and 6.8 hours, respectively). This can be explained by assuming that the proliferation-induced dilution of the tracker is counteracted by an increase of fluorescence caused by ongoing hydrolysis of CM-FDA molecules by intracellular esterase activity. Also, decreasing self quenching at lower concentrations may play a role. The net effect is an increase or at least a slow decrease (1 µM) in fluorescence up to 38 hours after initiation of the cul-

Table 2.1 Summary of characteristics and specific applicability of fluorescent cell trackers. Undesired properties in italics.

	Colour	Localization	Brightness*	Distribution †	Toxicity	Stability	Decay ‡	Applicability
PKH2	Green, narrow spectrum, combines with PE or PI	Intra-membrane; stain not fixable	Stains brightly (3 log)	Narrow distribution	*Slight initial toxicity*	*Staining not always stable [3]*	Predictable decay	**Simple, short term flow applications, single laser**
PKH26	*Orange, wide spectrum, does not combine with PE/PI*	Intra-membrane; stain not fixable	Stains brightly (3 log)	Narrow distribution	Non-toxic	Stain stable	Predictable decay	**Flow cytometry, dual laser**
CM-FDA	Green, narrow spectrum, combines with PE or PI	Intracellular; stain fixable	Stains very brightly (>3log)	*Wide distribution*	Non-toxic	Stain stable	*Decay not predictable*	**Long-term tracking, histology (?)**
CM-BODIPY	Green, narrow spectrum, combines with PE or PI	Intracellular; stain fixable	*Staining not very bright (2 log)*	Very narrow distribution	Non-toxic	Stain stable	Predictable decay	**Short-term cell cycle studies, histology (?)**

* Brightness expressed as fluorescence intensity of stained cells compared to unstained control cells.

† Fluorescence distribution of a stained cell population, as measured by flow cytometry.

‡ Decay of fluorescence as caused by equal distribution of dye molecules with every cell division.

ture. After 38 hours the CM-FDA fluorescence decreases at a rate that is higher than could be caused by the proliferation of the cells, because the T_d is less than 23 hours. This implies that there is a secondary effect causing fluorescence decrease, presumably dye leakage from the cells. These data indicate that CM-FDA is not suited for short term proliferation studies of labeled cells, where the fluorescence decrease should follow an exponential curve. Also, the broader fluorescence distribution of CM-FDA makes it less suitable for such short term proliferation studies.

CAFC-analysis of fluorescent cell tracker labeled murine bone marrow cells showed that PKH26 staining has no effect on the clonogenicity or growth rate of any bone marrow stem cell subsets. This corresponded with CFU-S results obtained with PKH26 and CM-BODIPY labeled murine low density bone marrow cells (not shown). These results indicate that in principle all four trackers are suitable for studying the homing behaviour of all hematopoietic stem cell subsets.

The data presented in this Chapter suggest that none of the four tested fluorescent cell trackers has a significant influence on clonogenicity and growth rate of leukemic cells and hematopoietic stem cells. Therefore, the choice of the tracker to be used should depend mostly on the exact purpose of the labeling (See Table 2.1). Several reports in the literature have implied that PKH2 may be more toxic to cells and may stain less stable than PKH26 [3, 23]. Although our own data do not completely correspond to these findings (see also Figure 3.1), we chose to pursue our homing studies using PKH26. Because of the uniform fluorescence distribution and its more predictable behaviour, we chose to test CM-BODIPY for use as a marker for immunohistological detection of labeled cells in paraffin sections. So far, several attempts to develop a staining protocol using antibodies to CM-BODIPY have been unsuccessful.

In conclusion, this Chapter provides an insight in the parameters to be considered when selecting a fluorescent cell tracker for in vivo homing studies, leading to a rational choice based on experimental results.

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Chapter 3

Homing of fluorescently labeled murine hemopoietic stem cells

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Chapter 3

Homing of fluorescently labeled murine hemopoietic stem cells

Abstract

PKH-26 was used as a viable fluorescent membrane stain for murine hemopoietic stem cells. The presence of the dye on the cells was shown not to interfere with their ability to form day 8 and day 12 spleen colonies in lethally irradiated mice. In order to study their in vivo homing behaviour in detail, 10^4 labeled cells from a population enriched for CFU-S were injected intravenously into non-irradiated and into 3 hours previously lethally irradiated mice. At 17, 41 and 65 hours after injection animals were sacrificed and the numbers of labeled cells were quantified per organ, using the specially developed flow cytometric Fluorescence Hypercompensation procedure for the detection of rare events, which allows a detection sensitivity of 1 per 10^6. Spleen homing in irradiated and non-irradiated mice was virtually identical, whereas homing to non-irradiated bone marrow was 2.5 times higher than to irradiated bone marrow. This indicates a different homing mechanism for spleen and bone marrow. The results of this direct homing assay were placed in perspective with results of indirect homing studies from the literature, introducing a new "h-factor". From the CFU-S data putative specific enrichment factors for spleen-specific and bone marrow-specific homing were derived. Examination of the fluorescence intensity distribution among the labeled cell population indicated that virtually all cells started to proliferate rapidly after injection into both irradiated and non-irradiated animals. This indicates that specific signals from stromal elements in the stem cell niches are needed to keep the cells quiescent, and that the majority of the transplanted stem cells do not home to such niches. The potential use of PKH-26 for in vivo characterization of stem cell niches is discussed.

Introduction

A stem cell in the bone marrow is thought to be surrounded by a specific combination of stromal elements such as reticulum cells, stromal cells, fibroblasts and extracellular matrix elements. Such a special microenvironment is referred to as a niche [1]. As stem cells differentiate into more mature types of progenitor cells they may move from one type of niche to another [2-4]. In a niche the microenvironment is thought to regulate stem cell activity by providing specific combinations of cytokines and by establishing direct contact with the stem

cell [5-8]. Lack of contact leads to imbalanced maturation of stem cells [9]. For successful long term engraftment of bone marrow transplantation recipients it is necessary that at least some stem cells reach the specific niches. This process is referred to as homing.

Little is known about the mechanisms and the efficiency of stem cell homing. Indirect measurements of stem cell homing were performed by determining the seeding efficiencies (f-factor) of spleen colony-forming cells (CFC-S) in retransplantation experiments [10-13]. The f-factors determined with these experiments show considerable variation, ranging from 0.03 to 0.1 for the 24 hour-spleen seeding of normal bone marrow derived spleen-colony forming units (CFU-S). Some of this variation could be ascribed to differences in the mouse strains used [11, 14] and to recipient conditioning [15]. However, it has not been studied whether in interpreting these measurements the assumptions that were made about the effects of irradiation-induced spleen shrinkage on the number of stem cells in the spleen were justified, and whether the f-factor is indeed the same for the primary and the secondary transplantation. As it became clear that spleen colony forming units (CFU-S) themselves were only an indirect and not quantitative measure of pluripotent stem cells, the topic of spleen seeding efficiency has not been further studied in recent years. On the other hand CFU-S are a good measure of the cells providing radioprotection and short term repopulation of hemopoiesis in bone marrow transplantation. The study of CFC-S homing is therefore of relevance to the clinical application of sorted and expanded progenitor cell preparations used to shorten the aplastic phase after irradiation and high-dose chemotherapy of patients. Attempts to study the homing of purified progenitor cells in a direct way by using radioactive markers or Hoechst 33342 fluorescence were hampered by technical difficulties related to marker instability and diffusion, and to the difficulty of the reliable detection of low frequencies of grafted cells in tissue samples.

So far, most homing studies were performed using myeloablated recipients. Now that many laboratories are developing successful methods for gene transduction into hemopoietic stem cells, there is a growing interest in the homing and proliferation of stem cells transplanted into non-myeloablated recipients. A direct homing assay using fluorescently labeled cells could offer the possibility to detect low frequencies of labeled cells in the background of normal cells, present in the hemopoietic system of a non-myeloablated recipient.

We have now developed such a direct assay system to trace transplanted cells by employing PKH-26, a red fluorescent membrane dye [16, 17] to label purified murine CFU-S. To measure the numbers of labeled cells a new flow cytometric Fluorescence Hypercompensation procedure was used. This allows the detection of labeled cells in hemopoietic organs of recipients at a frequency of less than one per 10^6 unlabeled cells. Furthermore, the use of PKH-26 permits the study of the proliferation kinetics of the injected cells. With every division of a PKH-26 labeled cell, the dye is equally distributed among the two daughter cells, leading to a 50% decrease in fluorescence per cell. This principle allows calculation of the number of divisions that the cells have undergone at a certain point in time after staining [18-21].

Using this method we show that short-term homing and proliferation of CFC-S in irradiated recipients bears a striking resemblance with that in non-irradiated recipients, and that bone marrow homing is probably a specific phenomenon, whereas spleen homing is not. Furthermore, we show that disrupting the blood-bone marrow barrier by irradiation is not required to allow injected stem cells to enter the bone marrow [22, 23].

Materials and Methods

Animals

Donor mice were 7 week old male C57Bl/Ka, bred under specific pathogen free conditions at the animal facility of the ITRI-TNO institute in Rijswijk, The Netherlands. Recipient mice were from the same strain, 12-14 weeks old. Irradiated recipient mice received 8.75 Gy of ^{137}Cs gamma-rays 3 hours before tail-vein injection of appropriate cell numbers. The recipient mice for the homing assay received 10^4 labeled cells each. CFU-S assays were performed in groups of 7 and 10 irradiated mice for day 8 and day 12 spleen colony counts, respectively. These recipients received either $2\text{-}3x10^4$ stained or unstained total bone marrow cells, or 500-1000 labeled or unlabeled sorted cells. On day 8 and day 12 these mice were sacrificed and spleens were removed and fixed in Teleyesznicki's solution and macroscopically visible spleen colonies were counted.

CFU-S enrichment procedure

Bone marrow cells suspensions were prepared by crunching femora and tibiae of donor mice in a mortar [24] containing 5 ml of Hepes-buffered Hanks' Balanced Salt Solution (H+H). Single cell suspensions were prepared by passing the cells through 30 µm nylon gauze. Low density bone marrow cells were prepared and stained with wheat germ agglutinin (WGA) according to the method described by Visser and de Vries [25]. After centrifugation for 5 min at 400 g the bone marrow cells were resuspended in a 1.100 g/cm^3 metrizamide solution. Onto this layer, a metrizamide solution of 1.078 g/cm^3 containing 0.15 µg/ml of WGA-FITC was added. A top layer was added, consisting of a 1.016 g/cm^3 metrizamide solution. The pH of all metrizamide solutions was 6.9. $7x10^6$ Cells were applied per gradient tube. After centrifugation at ambient temperature for 10 min at 800 g, the low density cells were collected from the interface between the intermediate and the upper layer and pooled and washed once. WGA-FITC-bright blast cells were sorted on the RELACSII flow cytometer [26] using standard techniques (100 mW 488 nm argon ion laser light, p30/530 band pass filter). After sorting, the WGA-FITC was removed from the cells by adding a large excess of 0.2 M of N-acetyl-D-glucosamine and incubating for 15 min at 37° C. Subsequently the cells were washed and stained for monocytes and granulocytes using the 15.1.1-FITC monoclonal antibody [25, 27]. 15.1.1-Negative and low staining cells were sorted into H+H. From these cells aliquots

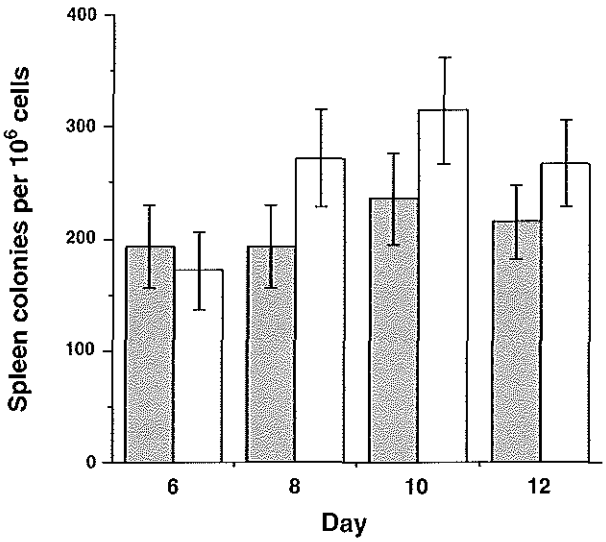

Figure 3.1 Histograms of numbers of spleen colonies found on different days in spleens of lethally irradiated recipient mice after injection of $2x10^4$ unstained (▨) and PKH-2 stained (□) total bone marrow cells. Day 6-10: n=7; day 12: n=10.

were taken to be used in the CFU-S assay. The remaining cells were transferred to an Eppendorf vial for PKH-26 staining.

PKH staining

Initially, both the green fluorescent (PKH-2) and the orange/red fluorescent (PKH-26) version of the PKH membrane dyes, were used in these studies . All experiments with sorted cells were performed with PKH-26. The sorted cells were spun down and resuspended in a total volume of 250 µL diluent A or C (for PKH-2 or PKH-26, respectively) (Zynaxis Cell Science, Malvern, PA, USA). Subsequently, 250 µL of 10^{-5} M PKH-2 or PKH-26 (Zynaxis) was added and mixed with the cells by gentle pipetting. After incubation for 1 min the staining reaction was stopped by adding 500 µL of H+H containing 10% fetal calf serum (FCS). The suspension was then underlayered with 200 µL FCS and centrifuged for 5 min at 400 g. The entire labeling procedure was performed at ambient temperature. After centrifugation the cells were washed once with H+H, counted and divided into aliquots for use in the CFU-S assay and in the PKH-homing assay. A small aliquot of the labeled cells was used for adjusting the flow cytometer.

Direct homing assay

Of the sorted and PKH-26 labeled cells 10^4 were injected intravenously into recipients that were either untreated or lethally irradiated. At time intervals of 17, 41, and 65 hours (day 1, 2 and 3) after injection two irradiated and one non-irradiated recipient were sacrificed for analysis. On several occasions organs from control mice that had not received cells were also analysed. For analysis, both femora, the thymus and a known portion of spleen and liver were isolated. Bone marrow suspensions were prepared from single femora by crunching in H+H.

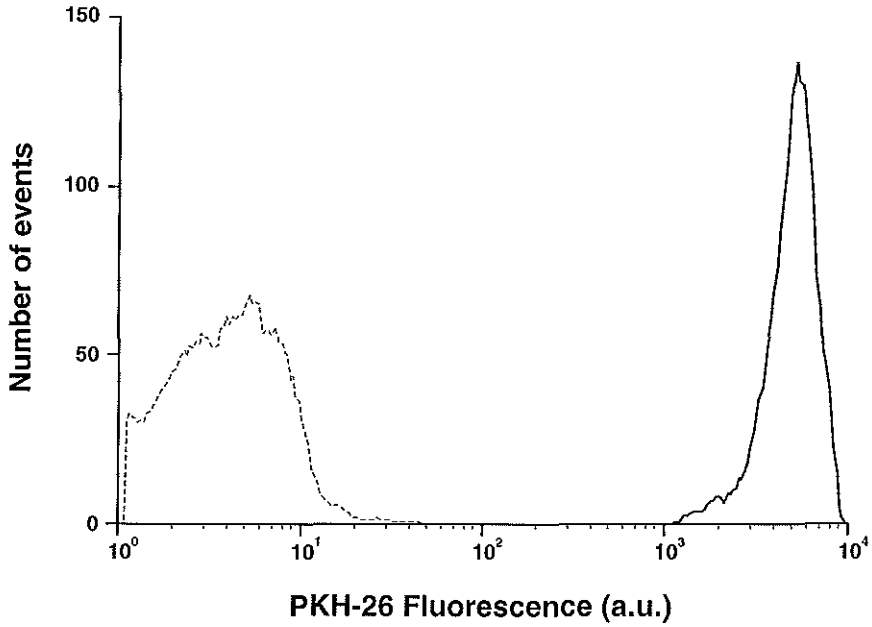

Figure 3.2 Overlay of orange fluorescence histograms, showing mock-stained control (dotted line) and PKH-26 stained cells (solid line) from murine bone marrow-derived lymphocytes, stained according to standard protocol. 10^4 Events per sample, data smoothed.

Spleen and liver samples and whole thymuses were put onto a moistened 30 µm nylon gauze and minced using a pair of surgical scissors. Subsequently, the gauze was flushed with 1-2 ml of H+H. Any remaining tissue fragments were then teased through the gauze using the plunger of a 2 ml syringe, and finally the gauze was flushed again with H+H. After passing through the sieve, the cell suspensions were transferred to 4 ml polystyrene FACS tubes. Special care was taken to reduce cell loss by repeatedly flushing mortar and pestle and the collection vial with small aliquots of H+H. Microscopic examination revealed that no labeled cells remained on the nylon gauze after filtering.

Flow cytometric analysis

Flow cytometric analysis of the samples was done on the RELACSII flow cytometer, using our newly developed Fluorescence Hypercompensation procedure. The argon ion laser was set to 488 nm. Green autofluorescence was measured through a 570 nm dichroic mirror and a p30/530 bandpass filter. The light reflected by the dichroic mirror (wavelength > 570 nm) was passed through a p10/577 bandpass filter to measure PKH-26 fluorescence. Perpendicular light scatter was measured from the light scattering on the dichroic mirror. First, the instrument was adjusted while running an aliquot of the freshly labeled sorted cells. The photomultiplier tube (PMT) for the PKH-26 was adjusted so that the labeled cells peaked

at 10^3 arbitrary fluorescence units on a four-decade logarithmic scale. Electronic compensation of the green autofluorescence signal according to the formula

$$FL(green)' = FL(green) - X \times FL(PKH\text{-}26)$$

was adjusted to the instrument's maximum value. In this way all the PKH-26 stained cells received a FL(green)' value of 5 or less than 5 arbitrary fluorescence units. In the dotplot display of orange PKH fluorescence versus green autofluorescence all the labeled cells fall on or just above the orange axis (values below 1 are changed to 1 by the electronics). The voltage on the PMT measuring the green autofluorescence was raised so that still 95% of all the PKH-26 stained cells were assigned a green fluorescence value of up to 5 arbitrary fluorescence units. This setting was used for all the measurements. PKH-26 positive cells were counted in a rectangular hardware sort gate (the PKH-26 gate), spanning the upper two decades of orange fluorescence (10^2-10^4 arbitrary units) and the lower half of the first decade of the hypercompensated green fluorescence (1-5 arbitrary units). Another gate, the control gate, was set directly adjacent to the PKH-26 gate, spanning 1.5 orange fluorescence decade from the center down, and the lower two decades of modified green fluorescence. Acquisition of reduced amounts of data was performed by measuring the samples with both gates on and counting electronically the numbers of cells within the gates. Using these rectangular hardware sort gates, approximately 98% of the total of events could be excluded from acquisition on disk and from software analysis. For reference purposes another 10^4 ungated events from each sample were separately acquired on disk. At least 33% of the volume of each sample was analyzed. Ungated event rates were up to 15.000 events per seconds. Red blood cells were eliminated by setting a threshold on forward light scatter (FLS).
In order to demonstrate that the events falling within the PKH-26 gate were from cells and not from electronic noise or other artifacts, a femoral sample was stained with Hoechst 33342 (1 µg/ml, incubation for 30 min at 37° C). Then during the analysis, the events falling within the PKH-26 gate were sorted onto a slide and examined and photographed using a Nikon Microphot FXA photomicroscope equipped with a 100x oil-immersion objective.

Results

In a first control experiment to see whether PKH staining would have an effect on the functionality of CFC-S, total bone marrow cells were stained with PKH-2 and their capacity to form spleen colonies was compared with that of control total bone marrow. As shown in Figure 3.1, no significant effect of PKH-2 staining on spleen colony formation on different days was observed with total bone marrow. This indicated that the PKH dyes would be suitable for tracking sorted cells in vivo. Based on later reports that PKH-26 performed better in terms of stability [20] and toxicity [28] than PKH-2 in some systems, it was decided to use

Table 3.1 Spleen colony formation after injection of total bone marrow cells and of PKH-26 stained sorted* cells.

Cells injected	Average per spleen	per 10^5 transplanted cells	Enrichment factor
	DAY 8 CFU-S		
$3x10^4$ total bone marrow cells	6.00 ± 2.45	20	**1**
10^3 cells unstained sorted* cells	17.71 ± 4.21	1771	**89**
500 PKH-26 stained sorted* cells	9.86 ± 3.14	1971	**99**
	DAY 12 CFU-S		
$3x10^4$ total bone marrow cells	7.33 ± 2.71	24	**1**
10^3 cells unstained sorted* cells	17.86 ± 4.23	1786	**89**
500 PKH-26 sorted* cells	11.71 ± 3.42	2343	**117**

Recipients received 8.75 Gy of ^{137}Cs gamma radiation 3 hours before injection of the cells.

*Sorted cells were low density, WGA-FITC positive and 1.5.1.1-FITC negative.

PKH-26 for the *in vivo* experiments with sorted cells. Similarly to PKH-2, PKH-26 does not affect spleen colony formation, as will be shown below.

Figure 3.2 shows the labeling intensity obtained after performing the staining protocol as described to stain murine lymphocytes with PKH-26. Staining is very uniform and very intense, with an orange fluorescence that is 1000-fold higher than that of control cells.

The employed enrichment protocol based on the method described by Visser and de Vries [25] yielded a cell population which is about 100x enriched in both CFU-Sd8 and CFU-Sd12 (Table 3.1). Assuming that most of the cells that yield a day 8 spleen colony are different cells from those that form day 12 colonies [29], and that the splenic f-factor is between 3% and 10% [12, 13, 30, 31], it can be calculated from Table 3.1 that this cell population consists of 34-100% pure CFC-S. For quality control we performed CFU-S assays with these sorted cells with and without PKH-26 staining. As can be seen in Table 3.1, the CFU-S enrichment factor (sorted cells vs. total bone marrow) of unstained sorted cells was 89-fold, both for

CFU-Sd8 and CFU-Sd12. Spleen colony numbers for stained sorted cells were similar, corresponding to 99-fold (CFU-Sd8) and 117-fold (CFU-Sd12) enrichment. These data demonstrate that PKH-26 staining of CFC-S does not lead to a significant difference in spleen colony formation.

Flow cytometric homing assay

The data reduction and hardware implementation of the Fluorescence Hypercompensation protocol permitted us to analyze the samples at a rate of up to 15.000 events per second. Analysis of femoral and splenic control samples from irradiated (Table 3.2) and non-irradiated control mice (Table 3.3) showed that false positive events were detected with a frequency of less than one per 10^6, with the additional remark that those false positives reached only just above the lower threshold level that was used (data not shown).

Figure 3.3 shows one of the sorted PKH-stained "events" from a spleen sample. It can be clearly recognized as a cell, because of the presence of the strong blue Hoechst 33342 fluorescence in the nucleus. On the membrane the typically patchy PKH-26 fluorescence can be seen. This result indicates that with the Fluorescence Hypercompensation analysis one does not detect strongly autofluorescent cells or random faulty events that are generated in the electronics circuitry, but indeed rare PKH-26 labeled cells.

Table 3.2 shows the numbers of labeled cells that were detected in irradiated recipients on day 1 by flow cytometry, using the Fluorescence Hypercompensation method. In the femora an average number of 54 (range, 29-72) labeled cells was detected. Considering that one femur contains 6.7% of the total murine bone marrow [32], this implies that an average of 806 donor cells were located in the bone marrow at the time of analysis. This corresponds to 8.06% of total injected cells. The spleens contain an average of 472 labeled cells at 17 hours, corresponding to 4.72% of the injected cells.

The livers also contained an average of 546 (range, 460-630) labeled cells. Since the liver of adult animals is no longer involved in hemopoiesis, but instead in the removal of dead and redundant cells, one might assume that the cells that were found were entrapped there for subsequent breakdown.

Very low numbers of cells (range, 3-11) were found in the thymus of these animals. The numbers are in the order of negative controls, so one can not draw firm conclusions from the results. Similar cell suspensions however, were shown to contain prothymocytes [33], and recent experiments by Hayes et al. [34] showed that prothymocytes are observed at very low levels in the thymus at 24 hours after injection of cells. It would therefore be possible that the few cells that were detected in the thymus are prothymocytes. Functional analysis of these cells is needed to establish this.

Adding up all the cells that were recovered from the recipient mice shows that still approximately 75% of the injected cells are not accounted for. In previous experiments (not shown) we also examined suspensions prepared from mesenteric lymph nodes, lungs, kidneys and

Table 3.2 Total numbers per organ of PKH-26 labeled sorted cells detected *ex vivo* using the Fluorescence Hypercompensation procedure, 17 hours after injection of 10^4 labeled sorted* cells.

	Experiment 1			Experiment 2		
	Fraction of total organ examined	**Number of PKH-26 cells detected**	**Calculated total number of cells per organ**	**Fraction of total organ examined**	**Number of PKH-26 cells detected**	**Calculated total number of cells per organ**
Irradiated recipient 1						
Femur1	0.65	36	**55**	0.56	40	**71**
Femur2	0.56	40	**72**	0.55	16	**29**
Total in bone marrow†			**1016**			**800**
Spleen	0.77	369	**480**	0.65	316	**485**
Liver	0.106	63	**595**	0.097	48	**497**
Thymus	0.94	5	**5**	0.68	6	**9**
Irradiated recipient 2						
Femur1	0.70	28	**40**	0.47	24	**52**
Femur2	0.70	31	**45**	0.40	27	**68**
Total in bone marrow†			**680**			**480**
Spleen	0.89	399	**450**			**N. D.**
Liver	0.092	58	**630**	0.100	46	**460**
Thymus	0.94	10	**11**	0.63	2	**3**
Irradiated control						
Femur1	0.76	0	**0**	0.71	0	**0**
Femur2	0.68	3	**4**	0.64	0	**0**
Total in bone marrow†			**"32"**			**0**
Spleen	0.63	0	**0**	0.63	0	**0**
Liver	0.064	0	**0**	0.100	0	**0**
Thymus			**N. D.**	0.58	0	**0**

Recipients received 8.75 Gy of ^{137}Cs gamma radiation 3 hours before injection of the labeled cells. Control animals received no labeled cells. N. D = not done.

*Sorted cells were low density, WGA-FITC positive and 1.5.1.1-FITC negative.

†Total bone marrow content calculation based on assumption that one femur contains 6.7% of total bone marrow [32].

Table 3.3 Total numbers per organ of PKH-26 labeled cells detected *ex vivo* using the Fluorescence Hypercompensation procedure, 17 hours after injection of 10^4 labeled sorted cells* into untreated recipient mice.

	Experiment 1			Experiment 2		
	Fraction of total organ examined	**Number of PKH-26 cells detected**	**Calculated total number of cells per organ**	**Fraction of total organ examined**	**Number of PKH-26 cells detected**	**Calculated total number of cells per organ**
Non-irradiated recipient						
Femur1	0.63	63	**101**	0.52	70	**136**
Femur2	0.71	69	**97**	0.45	87	**195**
Total in bone marrow†			**1586**			**2648**
Spleen	0.26	165	**636**	0.61	183	**299**
Liver	0.082	25	**305**	0.135	25	**185**
Thymus	0.57	1	**2**	0.61	3	**5**
Non-irradiated control‡						
Femur	0.97	5	**5**			
Spleen	0.76	0	**0**			
Liver			**N. D.**			
Thymus			**N. D.**			

*Sorted cells were low density, WGA-FITC positive and 1.5.1.1-FITC negative.

†Total bone marrow content calculation based on assumption that one femur contains 6.7% of total bone marrow [32].

‡Control measurements were performed in a separate experiment. Control animals received no labeled cells.

peripheral blood from recipient mice. Although our procedure was less refined at the time, we were never able to recover any significant numbers of labeled cells in samples of these organs, in contrast to samples of spleen, bone marrow and liver.

Table 3.3 shows the numbers of PKH-26 labeled cells in non-irradiated recipients. Numbers in spleen and thymus were comparable with those in the irradiated recipients, with an average number of 467 labeled cells in the two day-1 non-irradiated spleens. The femora of the non-irradiated recipients contained an average of 132 labeled cells, almost 2.5 times as many as the irradiated femora. Conversely, the non-irradiated livers contained an average number of 245 labeled cells, which is less than half the number found in the irradiated livers. Thus, the

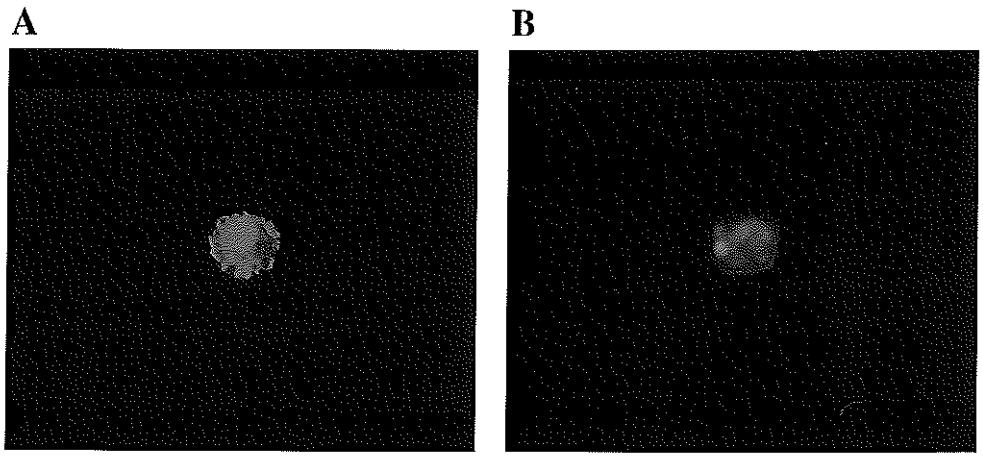

Figure 3.3 Photomicrograph of a cell from the PKH-26 gate, sorted onto a microscope slide. **(A)** Typically patchy orange PKH-26 fluorescence. **(B)** Hoechst 33342 fluorescence of the same cell.

irradiation has a striking inverse effect on the localization of stem cells to liver vs. bone marrow.

Proliferation kinetics

A disadvantage of the Fluorescence Hypercompensation method is that as the cells divide and their PKH-26 fluorescence decreases, the hypercompensation does no longer lead to reduction of Fl(green)' to 5 or below 5 fluorescence units of 95% of the labeled cells. This leads to a reduced recovery of stained cells after several divisions. As a consequence, it is impossible to use the results of the measurements of day 2 and 3 to determine the number of cells arising from the initial cells that homed in the organs. However, because all the cells that are hypercompensated are of donor origin, they can be used to monitor the short term proliferation kinetics of the cells qualitatively, and it is possible to see that there is no clearly recognizable subpopulation of cells that do not proliferate. Figure 3.4A/B shows how the labeled cells that were detected in the femora of irradiated and non-irradiated recipients behaved during the first three days after injection. Figures 3.4C/D and 3.4E/F show the labeled cells in spleen and liver, respectively. All histograms indicate that the fluorescence intensity of the PKH-26 labeled cells that were obtained from the organs on day 2 and 3 was significantly lower than that at day 1. This per cell decrease of fluorescence, caused by the equal distribution of dye among the offspring of each dividing cell indicates that virtually all the injected cells proliferated rapidly after injection, in irradiated as well as non-irradiated recipients. In the latter, however, the proliferation seems to proceed at a slightly lower rate at day 3 than in irradiated recipients.

Discussion

Comparison of direct and indirect measurements of the seeding efficiency

The CFU-S content of grafts correlates well with short-term repopulation of recipients [35-37] and the CFC-S may therefore be analogous to the cell type that is predominantly mobilized into the peripheral blood by treating patients with G-CSF and used for peripheral stem cell transplants. These are the cells that are responsible for short term repopulation and thereby rescue from radiation- or cytostatic drug-induced pancytopenia. Since this is the most important risk factor for patients undergoing marrow ablative treatment, it is very important

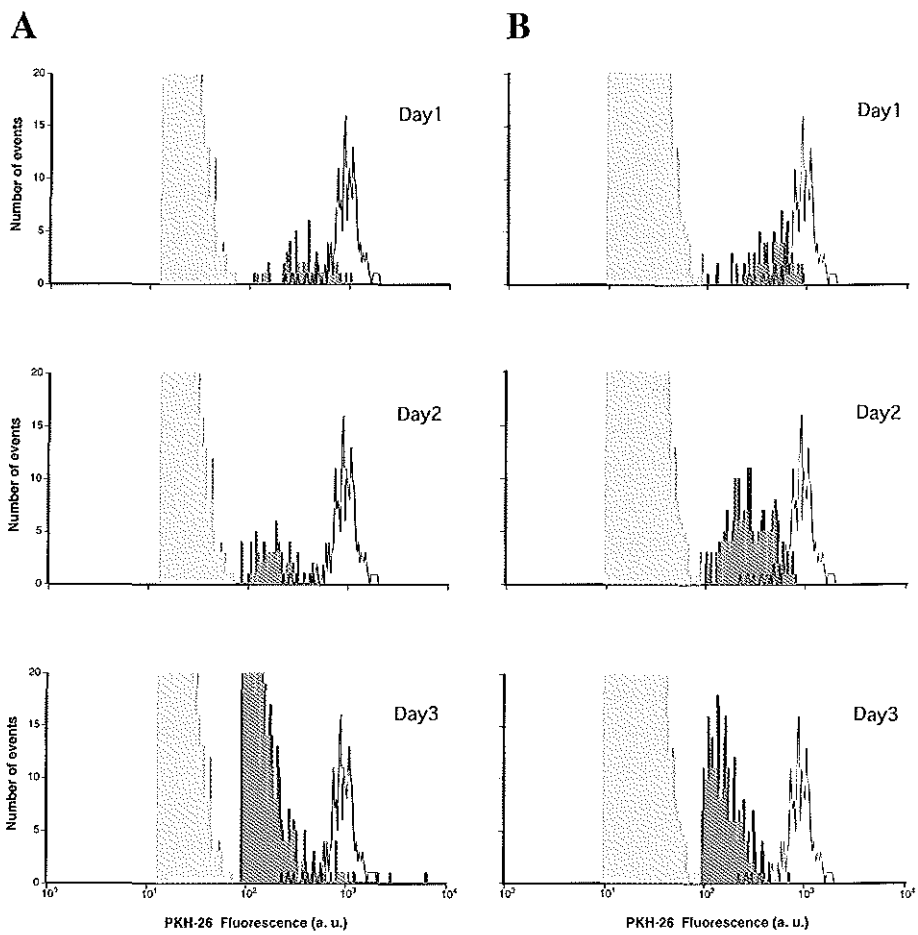

Figure 3.4 Histograms of orange fluorescence, showing fluorescence distribution of control samples (light grey solid bars), stained cells as measured at the time of injection (solid line) and of *ex vivo* detected PKH-26 stained sorted* cells (black bars). **(A)** Femora of irradiated and **(B)** non-irradiated recipients.

to study the possibilities of reducing this pancytopenic period by using short term repopulating stem cells for transplants. CFU-S transplants in mice can therefore be considered a relevant animal model for such studies.

We used a cell separation protocol in order to acquire sufficient numbers of a cell fraction that is highly enriched for CFU-S [38]. Working with this cell fraction, a homing assay was developed in which 10^4 labeled cells were injected into recipient mice. With the total bone marrow consisting of 5-6x10^8 cells, one might expect to find a maximal frequency of around 1 labeled cell per 5x10^4 bone marrow cells after injection of 10^4 labeled cells. Detection of such low frequencies of labeled cells is referred to as rare event analysis, and poses specific problems in flow cytometry [39-41]. Therefore, we have developed the Fluorescence Hypercompensation method, which bears some resemblance to the procedures described by Alberti [42, 43] for the discrimination of very dimly labeled cells from background for the detection of these low frequencies. The combination of strong PKH-26 fluorescence and the hardware gating and data reduction allows for sufficient background reduction and analysis speed to be able to detect labeled cell frequencies of 1 per 10^6.

Using this assay system, we describe for the first time a method in which the functional CFU-S assay is combined with a direct assay. In order to interpret the results it is necessary to compare them with the experiments in which CFU-S homing was determined using conventional methods. Until now, the spleen seeding factor (f-factor) was usually determined by retransplantation of recipient spleens. McCulloch and Till [44] showed that 24 hours after injection the number of CFU-S reaches a nadir, after which the number increases again. Lahiri et al. [45] showed that the decrease was caused by the shrinkage of the spleen due to the irradiation of the recipients. Lord et al. [12] showed that the increase cannot be caused by remigration of cells from the bone marrow, so it must originate from newly formed CFU-S. One of the attractive features of using PKH-26 as a label is that it can be used to study the proliferation kinetics of the cells. The results in Figure 3.4 show that most of the injected cells in bone marrow, spleen and liver have already undergone at least one round of cell division 17 hours after injection, indicating a rather short cycle time for at least most of the cells. This is in agreement with the data presented by Lahiri et al. [45], who showed that the number of CFU-S found 24 hours after injection is the resultant of homing, removal by spleen shrinking and new formation of CFU-S. It also means that the numbers of labeled cells in the organs at 24 hours do not directly reflect the homing of the cells to these organs. However, it does not affect the calculations made below, since all comparisons made are based on data derived from approximately the same time point (17 hrs and 24 hrs) and from mice that were irradiated 3 hours before injection of the grafts. According to Lahiri and van Putten [11], the number of CFU-S at 17 hours is only slightly higher than the number at 24 hours.

In retransplantation experiments it was always assumed that the splenic seeding efficiency in the primary transplantation (f_1-factor) equals that of the secondary transplantation (f_2-factor). Since only the f_2-factor could be determined in the serial transplantation experiments, the fol-

lowing assumption was made:

$$(\text{number of colonies})_1 = f_2 - \text{factor} \times \text{total number of CFC} - \text{S in the injected suspension} \quad (1)$$

where (# of colonies)$_1$ is the number of colonies found in the primary transplantation and where CFC-S is a cell capable of forming a spleen colony.

The f_2-factor for normal bone marrow-derived CFU-S was determined many times by several authors. Values between 0.03 and 0.10 were reported for normal bone marrow derived CFU-S [12, 13, 30, 31], and Visser and Eliason [13] reported a value of 0.0185 for CFU-S from an enriched bone marrow fraction.
Now that our direct assay has become available it is useful to take a more formal approach, in which for practical purposes an f_2-factor of 0.065 will be assumed, the average of the reported values in the abovementioned references. The number of primary colonies in a spleen is derived as follows:

$$(\text{number of colonies})_1 = f_1 - \text{factor} \times \text{total number of CFC} - \text{S in the injected suspension} \quad (2)$$

The relationship between the f_1-factor and the result from a direct homing assay can be described as follows:

$$f_1 - \text{factor} = \frac{\text{number of cells measured in spleen} \times \text{h-factor}}{\text{number of cells injected}} \quad \text{(h for homing)} \quad (3)$$

Here the new h-factor is introduced, which is composed of two elements:

$$h - \text{factor} = \text{"spleen selectivity"} \times \text{"splenic colony forming efficiency"} \quad (4)$$

Spleen selectivity should be envisaged as a form of CFC-S enrichment by specific homing or selective trapping in the spleen, and can be formally defined as:

$$\text{spleen selectivity} = \frac{\text{CFC} - \text{S purity of the labeled cells in the spleen}}{\text{CFC} - \text{S purity of total suspension}} \quad (5)$$

And the spleen plating efficiency is defined as:

$$\text{splenic colony forming efficiency} = \frac{\text{number of colonies formed in the spleen}}{\text{number of CFC} - \text{S in the spleen}} \quad (6)$$

In our measurements we observed that an average of 4.72% of the injected cells was present in the spleen 24 hours after injection, so equation 3 can be rewritten as:

$$f_1 - \text{factor} = 0.0472 \times h - \text{factor} \quad (7)$$

If the authors that performed the serial transplantation experiments made a justified assumption and indeed the f_1-factor = f_2-factor, then the average f-factor of 0.065 can be used to rewrite equation 7:

$$0.065 = 0.0472 \times \text{h-factor} \tag{8}$$

Thus, the h-factor amounts to 1.37. Extrapolation of the results from the CFU-S assay of the PKH-26 labeled cells leads to the expectation that from the 10^4 cells injected in the homing assay a total of 431 would have formed a spleen colony (Table 3.1, the sum of CFU-S day 8 and CFU-S day 12 per 10^4 injected cells). Magli et al. [29] showed that an average of 10% of the CFC-S in the spleen contributes to two colonies. Correcting for this, it can be concluded that 392 out of the average of 472 cells present in the spleen formed one or more colonies. This means that the minimum spleen colony forming efficiency in this experiment equals 83%, assuming that all the cells in the spleen were CFC-S. The maximum spleen colony forming efficiency is obviously 100%, which corresponds to a CFC-S purity of the labeled cells in the spleen of 83%. The upper and lower limits of the spleen colony forming efficiency are now set. Knowing the boundaries of the spleen colony forming efficiency it is also possible to determine the upper and lower limits for the spleen selectivity factor according to equation 4:

$$1.37 = \text{spleen selectivity} \times \text{splenic colony forming efficiency} \tag{9}$$

where $0.83 \leq$ spleen colony forming efficiency ≤ 1.

Solving equation 9 yields a spleen selectivity factor ranging between 1.37 and 1.65. Finally, one can determine a minimum CFC-S purity value for the injected cell fraction from equation 5. The CFC-S purity of the cells measured in the spleen can maximally be 1. In that case the spleen enrichment would amount to its maximum value of 1.65, and the initial minimum purity of the injected suspension would amount to 1/1.65=0.61, or 61% pure CFC-S. The maximum purity of the initial suspension would have to amount to 1/1.37=0.73, or 73%.
In Table 3.2 it was shown that, based on the assumption of equal distribution of all CFC-S throughout the bone marrow, 8.06% of the injected cells are located in the bone marrow of the irradiated recipient mice. With one femur containing 6.7% of the total bone marrow, and using the average of the femoral f-factors for unfractionated bone marrow cells as determined by several authors [11-13], a factor of 0.76, it follows that 16 x 0.76 = 12.16% of injected CFC-S were located in the bone marrow as a whole. This would indicate a femoral selectivity factor of 12.16/8.06 = 1.50 for the bone marrow, a similar value to that of the irradiated spleen.
It should be stressed that all these calculations are based on the assumption that the 24 hr f_1-factor and f_2-factor are the same and that the values used are averages taken from widely

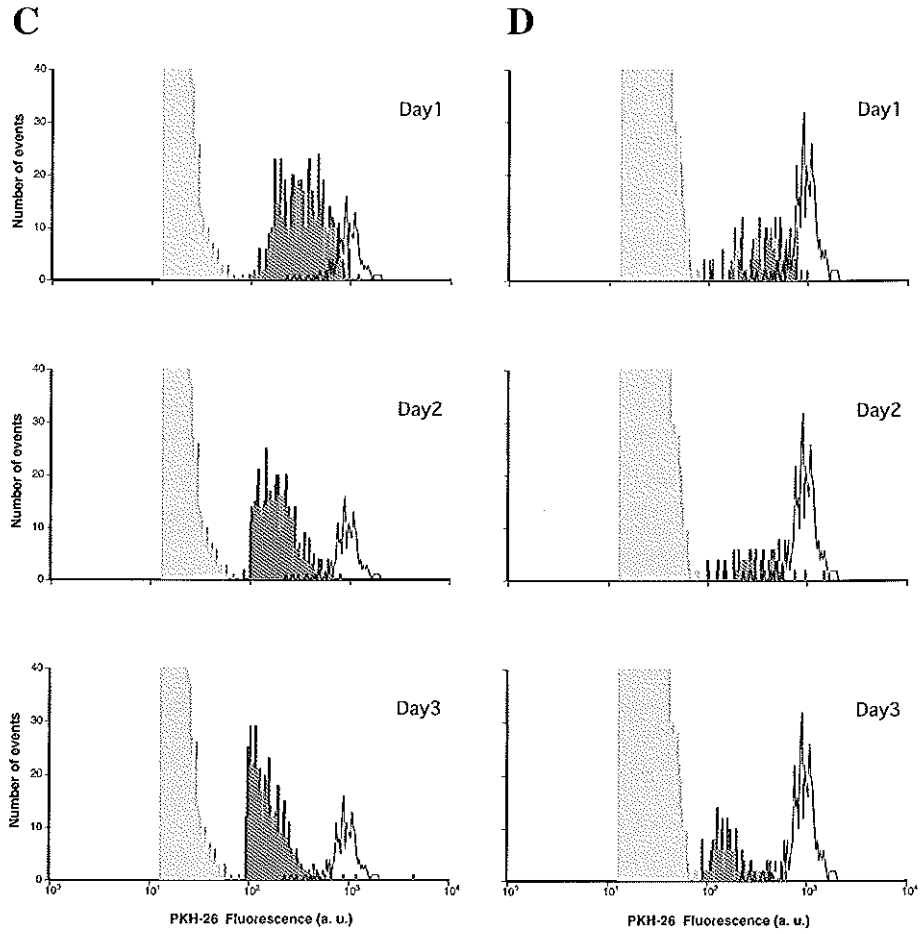

Figure 3.4 Histograms of orange fluorescence, showing fluorescence distribution of control samples (light grey solid bars), stained cells as measured at the time of injection (solid line) and of *ex vivo* detected PKH-26 stained sorted* cells (black bars). (**C**) Spleens of irradiated and (**D**) non-irradiated recipients. *Sorted cells were low density, WGA-FITC positive and 1.5.1.1-FITC negative.

varying values reported in the literature, especially those for the splenic f-factor. These values may not be very reliable, since not all the data used to obtain a representative value for the f_1-factor were based on spleen colonies counted on the same day after injection. In order to obtain more reliable values for the f_2-factors, combined retransplantations and direct homing assays should be performed.

The mechanism by which the spleen can specifically enrich CFC-S from the circulation can only be speculated on. It could be a specific mechanism, mediated by specific adhesion of the cells with the splenic endothelium. However, if this were the case, then one would expect different results in the non-irradiated spleens, where there is no radiation damage. The fact that

the numbers of labeled cells in non-irradiated recipients (467, average) and irradiated recipients (485, average) are so similar is more indicative of a non-specific process, such as for example trapping of the cells in the splenic microvasculature. The enrichment of the cells could be caused by for example size differences or by differences in the rigidity of the cell membrane, leading to preferential trapping of the CFC-S.

Homing in non-irradiated recipients

The situation in the spleen, where the numbers are very similar in irradiated vs. non-irradiated recipients, is in striking contrast with the difference between labeled cell numbers found in irradiated femora vs. non-irradiated femora. This suggests a different mechanism by which these organs can specifically select the CFC-S from the peripheral blood circulation, which is in line with the findings of Konno et al. [46] and of Aizawa and Tavassoli [47]. Further experiments are required to explain this observation. It may then be beneficial to use a less enriched CFC-S population, so that a possible difference in homing mechanism can be more clearly reflected in larger differences in organ-specific enrichment factors.

In the irradiated bone marrow the homing is decreased compared with the non-irradiated bone marrow. Aizawa et al. [48] showed that cells and substances that are to enter the bone marrow have to pass through the endothelial cells, not through the fenestrations between the endothelial cell. Clearly, this is an active process involving extensive cell-cell communication and interaction between adhesion molecules. In another study they showed that after irradiation the bone marrow sinus epithelium is very severely damaged [22]. This would lead to the expectation that homing in irradiated recipients should be severely compromised. That the observed difference between irradiated and non-irradiated bone marrow is only 2.5x could be explained by postulating a compensatory homing mechanism in irradiated bone marrow, in that due to the rupture of the sinus endothelial barrier cells are allowed to "roam freely" throughout the bone marrow cavities. Coincidental binding to stromal cells may then enable them to reach a stem cell niche. Histological examination of frozen sections of irradiated and non-irradiated femora taken during the homing of PKH-26 labeled cells will answer these questions.

Only 14-21% (irradiated recipients) to 25-31% (non-irradiated recipients) of the injected cells could be accounted for in our Fluorescence Hypercompensation analysis. This could be an artefact caused by differential cell loss during the sample preparation procedure. However, after staining at the concentration that we used, there have been no reports of increased cell loss. Stained red blood cells were less sensitive to hypotonic lysis than unlabeled cells [49, 50]. Therefore, differential cell loss does not seem very likely. The fact that normal spleen colony numbers are formed from the PKH labeled sorted cells indicates that PKH does not influence their *in vivo* functionality. Therefore the recovery of up to 31% of the injected cells in the organs that we examined seems to reflect the true *in vivo* situation. One explanation might be that there are unknown preferential homing sites, such as for example bone marrow

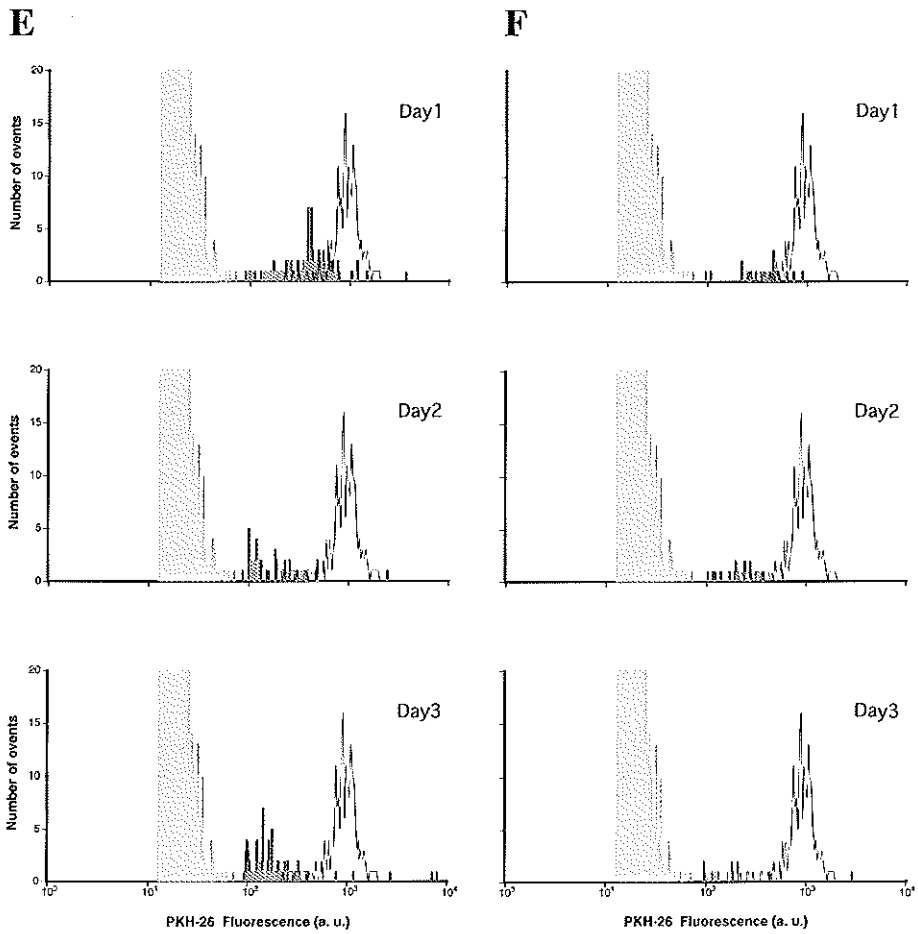

Figure 3.4 Histograms of orange fluorescence, showing fluorescence distribution of control samples (light grey solid bars), stained cells as measured at the time of injection (solid line) and of *ex vivo* detected PKH-26 stained sorted* cells (black bars). (**E**) Livers of irradiated and (**F**) non-irradiated recipients. *Sorted cells were low density, WGA-FITC positive and 1.5.1.1-FITC negative.

compartments other than femora. Another possible explanation is that already during the first 17 hours after injection a large proportion of the cells is sequestered and broken down. One would expect this to happen in the liver, although there are currently no data to support this explanation. Labeling of the sorted cells with the radioactive PKH-95 [51] will help to solve this problem.

One advantage of our homing assay system is that it can be applied to study donor engraftment of non-marrow ablated recipients. Recent developments in the field of gene therapy have led to a revived interest in the development of mild conditioning regimens for bone marrow transplantation. Several authors [52-56] have already shown that donor engraftment can

be achieved in non-conditioned recipient animals. Due to the relatively insensitive methods they used to assess donor repopulation (Southern blotting, isoenzyme heterogeneity, Y-probe in situ hybridization) they had to inject huge numbers of donor cells. Transplantation with genetically engineered bone marrow will require more elegant detection methods because the graft size will necessarily be small. The problem with the use of conventional functional assays is that the background of normal stem cell activity is not removed by the conditioning in these studies. Our direct assay can discriminate directly between donor and recipient cells. Surprisingly, the same rapid decrease of PKH-26 fluorescence caused by proliferation of the cells is observed after injection of CFU-S into non-irradiated recipients, as in irradiated recipients. Dye leakage from the cells as an alternative explanation for this fluorescence decrease is unlikely, since all our control experiments have shown only very little dye leakage, and there is an extensive body of literature presenting evidence that PKH-26 remains on the cell membrane *in vivo* for up to 40 days [20, 28, 49, 57-60]. One might expect that the hematological emergency caused by irradiation causes a lot of stimuli for the proliferation and maturation of all stem cells available. In the non-irradiated recipients, however, the hemopoietic system is completely intact and undisturbed, so there is no great demand for stem cell proliferation. Therefore, our observations support the notion that disruption of the stem cells from their niches drives them into cell cycle by removing the signals needed to keep them quiescent [9, 61].

In conclusion, we have developed a sensitive system for performing direct studies of the homing of hemopoietic progenitor and stem cells, which offers experimental options previously not available.

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Chapter 4

Retrovirus-mediated transfer and expression of marker genes in the BN rat acute myelocytic leukemia model for the study of minimal residual disease (MRD)

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Chapter 4

Retrovirus-mediated transfer and expression of marker genes in the BN rat acute myelocytic leukemia model for the study of minimal residual disease (MRD)

Abstract

To study minimal residual disease (MRD) in leukemia, we transferred the Escherichia coli genes encoding P-galactosidase (LacZ) and neomycin resistance (Neo^R) into the subline LT12 of the Brown Norway rat acute myelocytic leukemia (BNML), employing the retroviral BAG vector. In this way leukemic cells were genetically marked. Ten independent cell lines were characterized during in vitro growth as well as during two subsequent in vivo passages for expression of Neo^R for which the neomycin analogue G418 was used, and for LacZ expression for which the substrate 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal) was used. Out of 10 lines, four revealed permanent high expression of LacZ in all cells. In four other lines greatly varying LacZ expression between the individual cells from these lines was observed. In the remaining two lines LacZ expression was gradually lost. In contrast, Neo^R expression was gradually lost in eight out of the 10 lines, particularly rapidly during in vivo passaging. In the remaining two lines Neo^R expression was retained. The genetic modification did not alter the in vitro leukemogenicity of the cells. Long term in vivo expression of Neo^R and LacZ was followed in two selected lines up to 12 subsequent passages, i.e. one from the group of homogeneous high LacZ expression and one from the group of heterogeneous LacZ expression. In both lines LacZ expression was retained whereas Neo^R expression was rapidly lost after the third passage. The feasibility of using genetically marked leukemic cells for studies of minimal residual disease (MRD) was explored by injecting rats with leukemic cells, treating them with chemotherapy at full blown leukemia development to reduce the tumor load, mimicking the induction of a state of MRD and studying LacZ expression at relapse. LacZ expression was evident in 100% of the cells whereas Neo^R expression was lost in a considerable fraction. These results indicate that the viral vector BAG can be used to mark leukemia cells genetically although a selection of clones with the desired stability of long-term expression is required.

Introduction

The acute myelocytic leukemia in the Brown Norway rat (BNML) has served as a model for comparative studies with human acute myelocytic leukemia for a number of years and has

contributed considerably in understanding of the biological characteristics of minimal residual disease (MRD) in acute leukemia [1-4]. Various methods for the detection and quantification of MRD have been developed based on bioassays or flow cytometric techniques [5, 6]. However, these techniques are either time-consuming and/or expensive or they do not make it possible to study MRD in situ, which is a drawback for further studies on MRD. Therefore, the development of a specific genetic marker that would allow the detection of residual cells in situ would be particularly helpful.

Currently, retrovirus-mediated gene transfer permits the introduction of indicator genes into mammalian cells or embryos for the study of cell lineages or embryonic development [7-12]. The advantages of retroviral vectors include their unequalled high transfer efficiency, their expression in most cell types, accurate and stable integration of a single vector copy into chromosomal DNA, and a wide choice of different vectors with different host ranges.

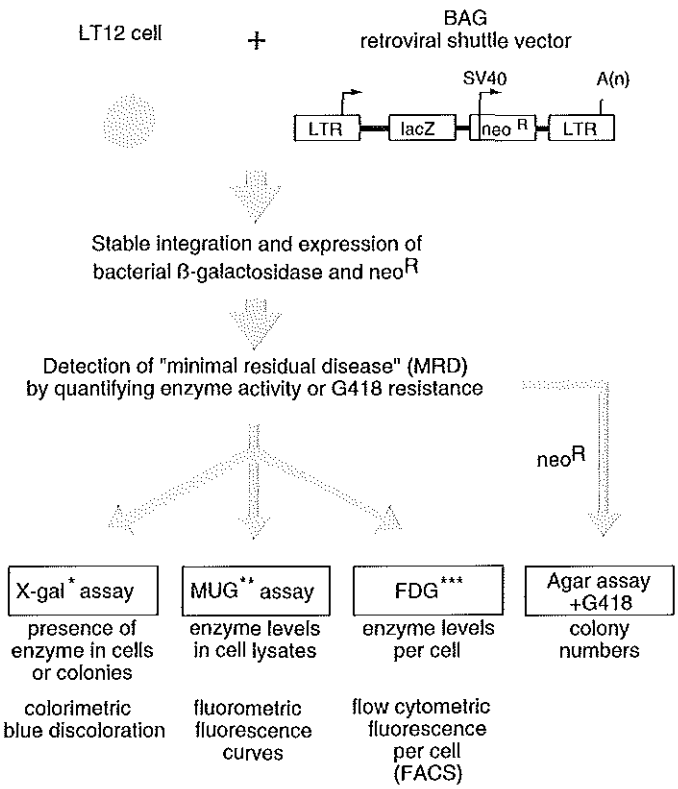

Figure 4.1 Schematic representation of the approach to genetically mark leukemic cells from the BN myelocytic leukemia model and the various detection methods for ß-galactosidase activity to detect and quantity leukemic cells during the state of minimal residual disease. X-gal, 5-bromo-4-chloro-3-indolyl-ß-D-galactoside; MUG, methylumbelliferyl-ß-D-galactoside; FDG, fluorescein di-ß-D-galactoside.

Retroviral vectors carrying the Escherichia coli LacZ gene, coding for ß-galactosidase and/or the Neo^R gene, conferring neomycin resistance, have been used effectively for genetic tagging of cells and the subsequent tracing of their progeny [13, 14], for the detection of micrometastases formation in experimental tumor models [15, 16], and to study gene expression in models for gene therapy purposes [17, 18]. Currently, attempts are about to be made to genetically mark human leukemia cells in marrow transplant patients to answer the question of the origin of a leukemia relapse after autologous bone marrow transplantation [19, 20].

Various assays can be employed to detect and quantify leukemic cells expressing the LacZ and/or the Neo^R gene. Our approach to explore these methods for the detection of MRD is illustrated in Figure 4.1 and includes, firstly, the colorimetric X-gal staining procedure where cells or colonies typically stain blue after conversion of a colorless substrate, i.e. 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal) by ß-galactosidase activity in the cells [9]. Secondly, the fluorimetric assay for measuring ß-galactosidase activity in cell lysates as described by Leaback and Walker [21], which is based on the conversion of 4-methyl-umbelliferyl-ß-D-galactoside (4-MUG) to the fluorescent product 4-methylumbelliferone (4-MU). Thirdly, the flow cytometric method described by Nolan et al. [22] in which expression of LacZ can be measured on a cellular basis after the intracellular conversion of the substrate fluorescein-di-ß-D-galactoside by ß-galactosidase. The expression of the Neo^R gene is measured by comparing the number of leukemic colonies grown in semi-solid agar cultures in the

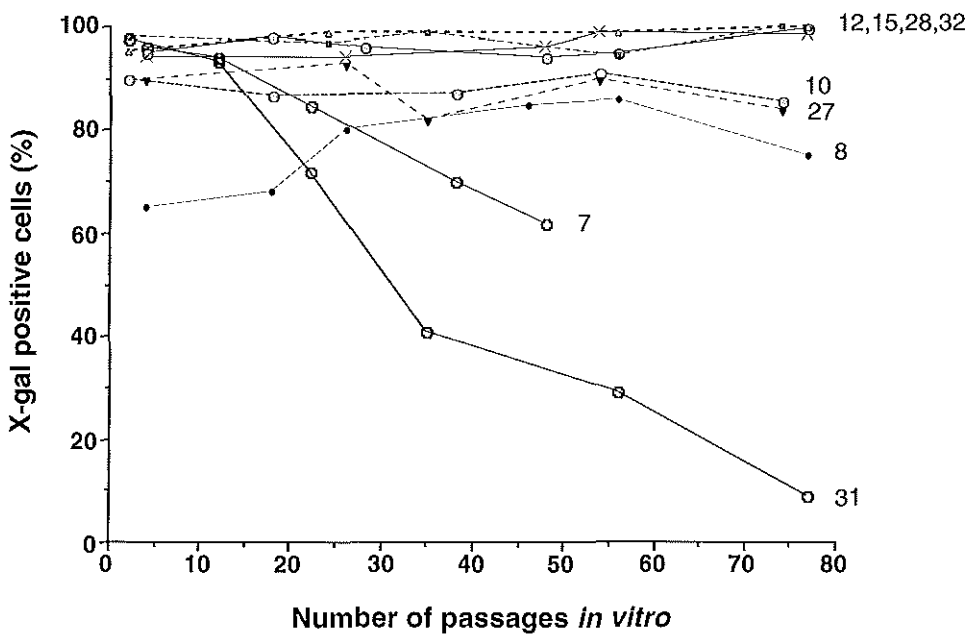

Figure 4.2 The expression of LacZ in various LT12NL cell lines during continuous in vitro culturing without G418 selection pressure, measured up to passage 77. The percentage of cells expressing LacZ is measured by staining the cells with X-gal in suspension (see Materials and methods). The line numbers are indicated in the figure.

presence or absence of the neomycin analogue G418. In this paper we report on the use of the X-gal staining method in liquid assays as well as in combination with agar cultures in absence or presence of G418 for the detection of LacZ and Neo^R expression in leukemic cells.
The retroviral vector BAG which carries LacZ as well as Neo^R [12] was used to genetically modify the in vitro growing subline LT12 [3] (originally described as IPC81 [23]) of BNML. Here we report how via characterization of the different LT12NL cell lines with respect to their in vitro and in vivo pattern of expression of both LacZ and Neo^R lines were selected to be used in MRD studies in the BNML model. In this respect the experiments in leukemic rats that were first brought into a state of MRD, using remission induction chemotherapy and in which the expression of the LacZ and Neo^R marker genes was studied at leukemia relapse, were of particular relevance.

Materials and methods

Experimental animals

The experiments were performed in the barrier-derived inbred BN rat strain BNBi/Rij produced in the Rijswijk breeding facility and maintained under specific pathogen-free conditions. Male rats between 16 and 20 weeks of age (circa 260 g body weight) were used.

Cells and in vitro culture

The LT12 cell line is an in vitro growing subline derived from the in vivo growing BN acute myelocytic leukemia cell line [24]. Cells were maintained and passaged in alpha minimal essential medium (α-MEM) (Flow Laboratories, Irving, UK) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine (Gibco, Paisley, UK) and antibiotics (penicillin 100 μg/ml and streptomycin 50 μg/ml). In colony assays, cells were cultured in a 0.3% agar-based medium containing 20% FCS with or without the neomycin analogue G418 at 200 μg/ml (Gibco).

Retroviral vector and infections

The BAG virus harboring the LacZ and Neo^R genes was developed by Price et al. [12] and obtained from the American Type Culture Collection (ATCC) depository (Psi-2-BAG, no. CRL 9560). Supernatant from the retrovirus-producing cell line was used to infect LT12 cells in a liquid culture system. Briefly, exponentially growing LT12 cells were incubated in the presence of 1 ml BAG viral supernatant in serial 10-fold dilutions in medium in the presence of 4 μg/ml polybrene (Sigma, St Louis, MI). After 36 hours, the infected cells were placed under G418 selection pressure in an agar culture system containing G418 (200 μg/ml, optimal for suppressing colony formation of parent LT12 cells) for 10 days. Individual G418-resistant leukemic colonies were isolated from the agar culture and expanded in liquid culture medium

containing G418 (600 µg/ml, optimal for suppressing growth of parent LT12 cells). LT12 cell lines resistant to G418 and expressing LacZ are referred to as LT12NL cells. Supernatants from cell cultures as well as serum from rats collected during the terminal stages after in vivo transfer of the leukemia cell lines were tested for the presence of helper virus. All tests were negative.

X-Gal staining

This histochemical procedure described by Sanes et al. [9] is used for demonstrating ß-galactosidase in cells. In brief, cells were centrifuged (400g, 10 min) resuspended in phosphate buffered saline (PBS), and 10 µL cell suspension (10^5/ml) was added into the wells of a 96-well, flat-bottomed, microtest tissue culture plate. The cells were not fixed prior to staining. To each well, 50 µL of an enzyme substrate solution was added i.e. a mixture containing 1 mg/ml X-gal (Molecular Probes, Eugene, OR), 5mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM $MgCl_2$, in PBS.

Cultured colonies were stained by pipetting 500 µl of the substrate solution on top of the agar layer in 35 mm culture dishes after 7-8 days of culture. After a 4 to 14h incubation period at 37° C the substrate was converted by the ß-galactosidase (LacZ) present in expressing cells and as a result the cells or colonies stained blue, which was scored visually under an inverted

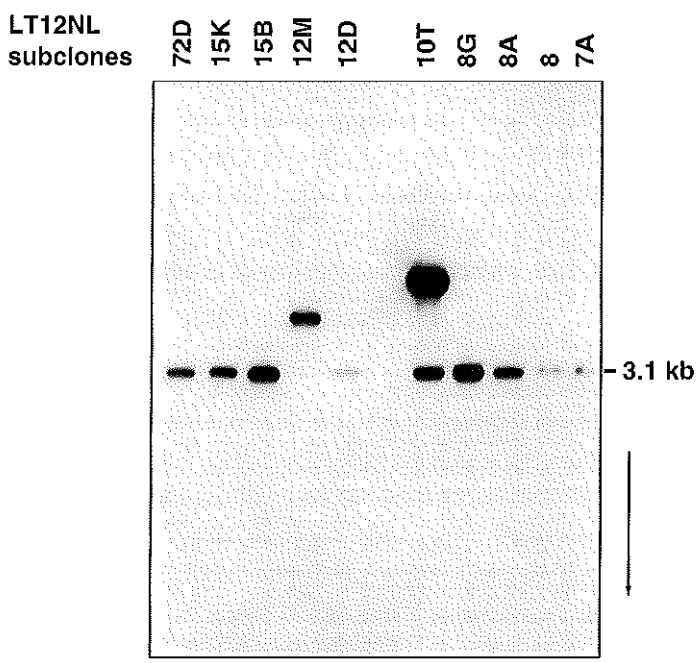

Figure 4.3 Southern blot analysis of BamH1 digested genomic DNA of different LT12NL subclones hybridized with the LacZ probe.

microscope. Cells in liquid suspension or in the cultured colonies not expressing the LacZ gene remained colorless.

4.3.5 Injection of rats and in vivo passaging

BN rats were injected intravenously with 10^6 LT12NL or LT12 cells. When the leukemic symptoms fully developed, i.e. increase in spleen and liver size and the first signs of paralysis of the hind legs, usually during the third week, the rats were killed and femoral bone marrow, spleen and peripheral blood were collected. Cell suspensions were made and used for analysis and in vivo passage into new recipient rats. For the latter purpose, cells from leukemic spleens were used.

Chemotherapy of leukemic rats

To induce a state of MRD, leukemic animals were treated with cyclophosphamide (CP, Janssen Chimica, Beerse, Belgium) on day 11 after intravenous (i.v.) transfer of 10^6 LT12NL cells, CP was administered intraperitoneally (i.p.) at a dose of 100 mg/kg bodyweight. In previously reported studies [4] this treatment was found to reduce the leukemic cell load by 5 to 6 logs.

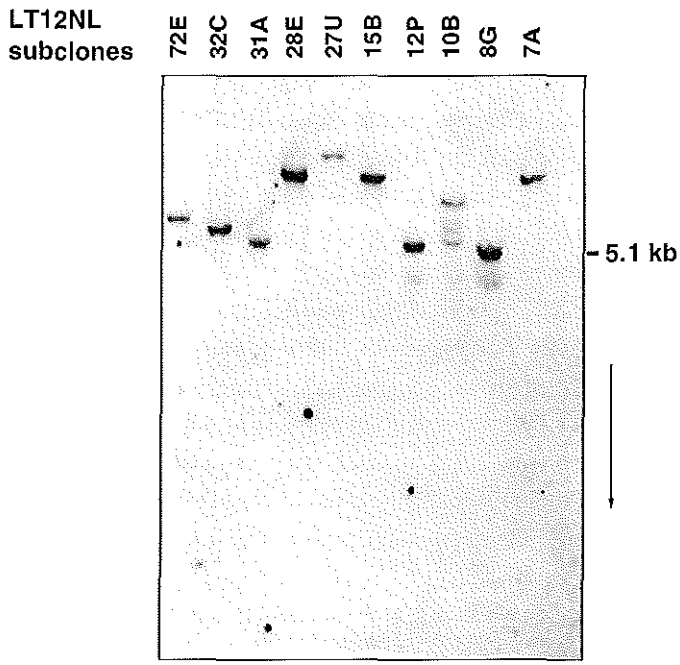

Figure 4.4 Southern blot analysis of EcoR1 digested genomic DNA of different subclones hybridized with the LacZ probe.

Determination of expression of Neo^R and LacZ

Cells obtained from in vitro cultures or from the tissues of leukemic rats were plated using the agar colony assay at 300 cells per dish in the absence of G418 or at 300, 3000, and 9000 cells per dish in the presence of G418. From the ratio of colony numbers in cultures with G418 and those without G418 the percentage of cells expressing Neo^R was calculated. After counting, all culture dishes were stained with X-gal to determine the percentage of colonies with LacZ expression. This procedure allowed the determination of the long term expression of LacZ and Neo^R of LT12NL cells during continuous in vitro culture or in vivo passaging.

Southern blot analysis

From the in vitro growing LT12NL lines, which were used for in vivo experiments, a number of subclones were expanded and genomic DNA was extracted for Southern blot analysis and hybridized with radioactively labeled LacZ or Neo^R probes.

Results

Gene transfer to LT12 cells

LT12 cells infected with the BAG virus were incubated in an agar culture selection system containing 200 µg/ml G418. Under these conditions parent LT12 cells cannot grow in contrast to G418-resistant cells. The latter develop into colonies, indicating that they harbor a successfully integrated and expressed Neo^R gene. Eighty-two individual colonies were picked from the agar plates. These independently derived infected LT12NL cell lines were expanded in culture medium with 600 µg/ml G418. The efficiency of infection and expression of Neo^R of the rat LT12 cells with the ecotropic BAG virus was found to be about 10^{-3}. Infection of NIH/3T3 cells indicated that the supernatant used for infection had a viral titer of $7x10^4$ colony-forming units (CFU)/ml.

The expression of the LacZ gene in the 82 neomycin resistant LT12NL cell lines was determined by X-gal staining. Twenty-one clones had no detectable levels of LacZ gene expression indicated by the fact that all cells remained white after X-gal staining. For 47 lines cultured it was directly obvious that they showed a heterogeneous pattern of expression, i.e. the color of the cells varied from white to dark blue even after prolonged X-gal staining. Fourteen showed a dark blue discoloration indicating a high expression of the LacZ gene. Ten lines of these were selected arbitrarily on the basis of the most uniformly and most dark blue staining for detailed investigation.

In anticipation of MRD detection studies, it was established that into a culture well with a surface of 32 mm^2 circa $5x10^5$ cells can be entered still allowing observation of the individual cells. Preliminary data from mixing experiments indicated that small numbers of 'LacZ-positive' darkly blue staining cells could easily be detected in a background of large numbers

Table 4.1 Comparison of long-term in vitro and in vivo expression of the LacZ and Neo^R genes in various LT12NL cell lines.

Line	In Vitro		In Vivo	
Number	LacZ (%)	Neo^R (%)	LacZ (%)	Neo^R (%)
7[a]	55	100	18	100
8	23	54	48	80
10	97	10	97	16
12	99	100	42	100
15	100	77	100	72
27	100	4	100	0.1
28	99	100	97	42
31	100	28	100	2
32	99	93	100	89
72	100	76	100	85

in vitro, after 75 passsages in vitro; in vivo: after two passages in vivo; LacZ, cells were cultured in agar and stained with X-gal; the percentage of colonies containing blue cells reflects the percentage of cells expressing LacZ; Neo^R, cells were cultured with G418 (200 µg/ml) or in its absence; the colony number ratio is used to calculate the percentage of cells expressing Neo^R. [a]Data at passage 50 in vitro; this line was lost thereafter.

of negative cells with a lower limit of one positive cell per $5x10^5$ negative cells (data not shown). When normal rat bone marrow cells were stained with X-gal, in the order of one blue cell per $5x10^4$ was detected, probably reflecting lysosomal ß-galactosidase activity. This background activity can be reduced to virtually zero by optimizing staining conditions, e.g. pH, temperature, and specific blocking of the lysosomal activity, which brings the lower limit of detection of residual leukemic cells down to one per 10^6-10^7 cells (unpublished results).

Characterization of in vitro growth

The cell population doubling times of four different genetically marked LT12NL lines were measured and compared with those of the parent LT12 cell line (four LT12 control cultures in total). Starting from 10^5 cells/ml, cell numbers were counted daily during nine consecutive days in liquid culture without G418. Exponential as well as Gompertzian growth curves were fitted to the data points by a non-linear least-squares minimization routine [24]. The growth curves of LT12 and LT12NL cell lines (not shown) appeared to be comparable and the small difference in the mean cell population doubling times between LT12 cells (T_d = 14.6h) and LT12NL cells (T_d = 16.6 h) during exponential phase was not statistically significant, suggesting that the in vitro growth kinetics of LT12 cells were not affected by the insertion of LacZ and Neo^R genes.

Table 4.2 Survival times and organ weights at death of leukemic rats after intravenous injection of 10^6 cells of the different LT12NL lines.

Line number	Survival time (days)	Spleen weight (g)	Liver weight (g)
7	19.5±0.5	2.3±0.1	14.4±1.1
8	23.4±0.3	3.9±0.4	17.9±0.7
12	19.5±0.5	2.5±0.1	12.5±2.8
15	22.8±0.5	4.1±0.5	10.6±3.6
27	19.8±0.3	2.2±0.1	13.4±0.3
28	26.3±0.5	1.1±0.1	7.8±0.6
31	24.3±1.3	1.5±0.2	9.0±1.1
32	22.3±0.6	2.3±0.7	8.9±2.0
72	20.3±0.3	4.6±0.1	23.4±1.1
LT12 control	19.8±0.5	2.5±0.2	14.1±0.8
Nonleukemic control	N. A.	0.5±0.05	10.0±0.5

Values are means of 3-4 animals per group ± SE. N.A.: does not apply.

LacZ gene expression was monitored in ten LT12NL cell lines at various time points during more than 75 in vitro passages (3-4 days per passage without G418 selection pressure). We used two method for this: (a) by determining the percentage of blue cells after X-gal staining in suspension, and (b) by first plating the cells in soft agar and allowing colonies to develop during a 10-day culture period and subsequently staining these with X-gal and determining the percentage of colonies staining blue. Two distinct patterns of X-gal staining were observed using the direct X-gal staining in liquid suspension. Firstly, in six LT12NL cell lines all cells stained uniformly blue (Figure 4.2, lines 7, 8, 12, 15, 28 and 32), suggestive of uniform expression levels of LacZ in all cells. However, in two of these a gradual decrease in the percentage of blue staining cells was seen (Figure 4.2, lines 7 and 8), which implies that LacZ expression was gradually lost in these two lines. Secondly, heterogeneous X-gal staining was seen in four lines (Figure 4.2, lines 10, 27, 3 1, and 72), i. e. in the same cell suspension the entire range from very light (almost white) to dark blue cells was observed. This means that in the cells from these lines LacZ expression levels vary from (very) low to high which is reflected in the intensity of the blue discoloration. For identifying the responsible factors further research is required which is, however, beyond the scope of this study.

When the cells were first cultured in agar and then stained with X-gal colonies from lines 12, 15, 28, and 32, all stained blue. Also all cells within the colonies were blue. Colonies cultured from lines 7 and 8 stained either white or blue, whereas all cells within the blue colonies were blue. The white colonies most likely were derived from cells in which LacZ expression was shut off. Colonies cultured from the lines 10, 27, 31 and 72, however, always stained blue

whereas within the colonies dark blue as well as light blue cells (nearly white) were seen. Only rarely colonies from these cell lines containing only white cells were observed.
In addition to measuring LacZ expression with X-gal staining of liquid cultures, a two-step assay was also used in which cells from the various cell lines were first cultured in the colony assay and subsequently stained with X-gal. In Table 4.1 (column 2) the expression of LacZ is shown measured after 75 passages in vitro. Not necessarily all cells within the colony needed to be blue to score colonies as positive. Therefore the percentages of X-gal-positive cells shown in Figure 4.1 (liquid X-gal staining data) could be lower as is observed for lines 10, 27, 31, and 72 when compared with the percentages shown in Table 4.1, column 2. In these lines the expression of LacZ apparently varies during cell proliferation and may at certain stages be below the level of sensitivity of the X-gal staining method in liquid. Lines 7 and 8 were found to exhibit loss of LacZ expression indicated by the presence of uniformly 'white' colonies (all cells in the colonies lost LacZ expression) as well as uniformly 'blue' colonies (persistence of LacZ expression). During continuous in vitro passage of the cell lines the percentages of X-gal positive cells in lines 7 and 8 progressively declined (compare with Figure 4.2). All colonies derived from lines 12, 15, 28, and 32 were uniformly 'blue'.
The expression of the Neo^R gene in the LT12NL cell lines was assessed at various intervals during long-term in vitro passaging (77 passages, without G418 selection) by comparing the colony formation efficacy of LT12NL cells in the agar culture in the presence or absence of G418. From the ten cell lines tested, three cell lines (lines 7, 12, and 28) retained their Neo^R expression (>95% G418-resistant colonies), while the remaining seven LT12NL cell lines (lines 8, 10,15, 27,31, 32, and 72) gradually lost neomycin resistance (Table 4.1, column 3). In conclusion, two cell lines (lines 12 and 28) maintained high expression levels of both genes (>95% of X-gal positive colonies and > 95% of G418-resistant colonies), following prolonged in vitro passaging.

Southern blot analysis

Subclones were cultured from the ten selected LT12NL lines and expanded for genomic DNA extraction to allow Southern blot analysis. To detect the total BAG construct XbaI was used, BamHI for the LacZ gene and BamHI x XhoI for the Neo^R gene. EcoRI was used for determining the number of integration sites. Hybridization was done with radioactively labeled LacZ or Neo^R probes. Most subclones from lines for which X-gal staining indicated loss or heterogeneity in LacZ expression or for which loss of Neo^R expression was seen, were found with changes in the hybridization pattern with the LacZ and Neo^R probes. An example is shown in Figure 4.3 where hybridization with the LacZ probe after BamHI digestion yields a hybridizing band for subclone 12M (X-gal staining 0%) which is not at the expected location. In contrast, the hybridizing band from subclone 12D (20% stains with X-gal) is found at the expected location. Another example is clone 10T which shows a second strong hybridizing band indicating that two or possibly three retrovirus integrations took place. In agreement

with this is the observation that hybridization of 10T with the Neo^R probe showed a more intense band of the normal size (not shown).
Loss of LacZ expression is not always indicated by changes in the hybrization pattern, which is also shown in Figure 4.3 where the hybridizing bands from subclones 15B (100% LacZ) and 15K (0% LacZ), 8G (100% LacZ) and 8A (0% LacZ) are found at the expected location. Obviously, only major structural changes can be detected with Southern blot analysis and not the minor changes, e.g. point mutations and methylations etc. The number of virus integrations was determined by Southern blot analysis of EcoR1 digested genomic DNAs of the different subclones from each of the lines studied

Characterization of in vivo growth

Cells from the ten LT12NL cell lines characterized in vitro were also injected into rats after about 30 passages in vitro without G418 to study the growth characteristics in vivo and to measure the in vivo expression of LacZ and Neo^R. Following the intravenous injection of 10^6 leukemic cells in rats the survival times and the spleen and liver weights at death were recorded. Most animals developed hind-leg paralysis, a phenomenon which has previously been

Table 4.3 Long term expression of the LacZ and Neo^R genes during repeated in vivo passage.

	LT12NL line 15		LT12NL line 72	
Passage number	LacZ (%)	Neo^R	LacZ (%)	Neo^R
1	100	75	99	97
2	97	21	97	23
3	100	20	100	37
4	nd	nd	97	2.3
5	100	nd	nd	nd
6	100	2-4	100	0.3
7	nd	nd	nd	nd
8	100	0.06	100	0.2
9	nd	nd	nd	nd
10	100	0.004	100	0.05
11	nd	nd		
12	100	<0.001		

LacZ, cells were cultured in agar and stained with X-gal; the percentage of blue colonies equals the percentage of cells expressing LacZ ; Neo^R, cells were cultured with and without G418; the colony number ratio indicates the percentage of cells expressing Neo^R; nd, not determined.

described for the BNML model [25, 26]. Differences were observed in the survival times with the mean values ranging from 19.5 to 26.3 days, as well as in the degree of organ involvement for the different cell lines (Table 4.2). In particular, organ weights varied greatly, i.e. the spleen weights varied from 1.1 to 4.6 g and the liver weights from 7.8 to 23.4 g compared to 0.5 g and 10.0 g, respectively, for non-leukemic control rats. From the bone marrow, spleen, and peripheral blood cells, suspensions were prepared for determining the percentage of cells expressing LacZ and Neo^R. After two in vivo passages, five of the 10 investigated LT12NL lines (lines 7, 8, 10, 12, and 28) showed loss in expression of LacZ, while Neo^R expression was lost in eight out of 10 lines (all lines except 7 and 12) (Table 4.1 and 5). For eight out of 10 lines the in vitro expression pattern of the marker genes correlated with the observed pattern in vivo (Table 4.1, columns 4 and 5). For the remaining two lines the in vitro and in vivo expression data did not correlate, i.e. line 12 lost expression of LacZ in vivo but not in vitro and line 28 lost the expression of Neo^R in vivo but not in vitro.

Long-term in vivo expression of the marker genes was studied by repeated in vivo passaging using LT12NL line 15, representing the cell lines with 100% of the cells staining blue, and LT12NL line 72, representing the cell lines characterized by heterogeneous expression using the liquid X-gal assay but 100% of the colonies cultured staining blue. In one series, leukemic spleen cells were directly transferred into recipient rats, while in another series the neomycin-resistant fraction from the leukemic spleen was first rescued during a short period (4-6 days) of in vitro culture in the presence of G418.

In Table 4.3 the results of the leukemic transfer without G418 are shown. The expression of LacZ persisted in both lines up to 12 repeated in vivo passages, whereas Neo^R expression was rapidly and totally lost. In a separate transplantation series the cells were cultured for a short time in vitro in the presence of G418 in between the in vivo passages. The G418 resistant cells were injected into new recipients rats. Already during the next in vivo passage the cells lost the expression of Neo^R, i.e. 6-10% of the cells from line 15 and 18-30% of the cells from line 72, respectively (Table 4.4).

LacZ and Neo^R expression in relapsing leukemia

Three animals injected with 10^6 leukemic cells from LT12NL line 72 were treated with a cyclophosphamide (CP) dose of 100 mg/kg i.p. at day 11 after i.v. leukemic cell transfer. Treated animals were killed when they were moribund which was at day 37, 38, and 41, respectively (Table 4.5). Compared to untreated leukemic control animals this corresponds to increases in the survival time of 17, 18, and 21 days, respectively. It is known for the BNML leukemia that a tenfold lower cell dose results in an increase in survival time of 4 days [3, 4]. A similar relationship has been found for LT12NL line 72. Hence, from the increase in survival time it can be calculated that the tumor load ($5x10^8$ leukemic cells on day 11) was reduced by the CP treatment to $5x10^3$ cells. At death the animals harbor a total of 10^{10} leukemic cells. This implies that during the regrowth process on average 21 cell doublings were made.

Table 4.4 Long term expression of the LacZ and Neo^R genes in vivo during repeated passaging with an intermittent neomycin resistance rescue.

	LT12NL line 15		LT12NL line 72	
Passage number	LacZ (%)	Neo^R	LacZ (%)	Neo^R
1	100	90	100	100
2	nd	nd	nd	nd
3	100	95	100	67
4	nd	93	100	70
5	nd	nd	nd	nd
6	100	97	nd	nd
7	100	94	100	73
8	100	95	nd	nd
9			99	88

After each in vivo passage leukemic spleen cells were cultured in the presence of G418 (600 µg/ml) until enough cells were available for injection into new recipient rats; LacZ, cells were cultured in agar and stained with X-gal; the percentage of blue colonies equals the percentage of cells expressing LacZ; Neo^R cells were cultured with and without G418; the colony number ratio indicates the percentage of cells expressing Neo^R; nd, not determined.

Cell suspensions from femoral bone marrow, spleen, and peripheral blood were cultured in soft agar in the presence or absence of G418. Expression of Neo^R was deduced from the ratio of colonies formed in the presence of G418 and the number of colonies formed in control cultures without G418. All colonies that were cultured became blue when stained with X-gal, indicating that LacZ expression was retained (Table 4.5). The expression of Neo^R varied between 64 and 95%. This indicates that the LacZ expression was not affected by the chemotherapy applied. Compared to the rapidly decreasing expression of Neo^R during continuous in vivo passaging of line 72 (Table 4.3), the LacZ expression remained remarkably high in the leukemic animals in which the leukemia relapsed after MRD-induction treatment.

Discussion

In this study it was shown that leukemic cells could be genetically marked using retrovirus mediated gene transfer of the LacZ and Neo^R genes. In eight of the ten cell lines investigated the expression of LacZ in vitro was stable after up to 75-77 in vitro passages, corresponding to at least 300 cell doublings. In the remaining two cell lines expression of LacZ appeared to be unstable after prolonged culturing. In four lines a high and stable expression of LacZ was

inferred, on the basis of homogeneous blue staining in the X-gal assay, implying that these lines were candidates to be used for MRD studies. In the other four cell lines a variable degree of X-gal staining was found. Whether the proliferative state of the cells (i.e. cell cycle phase, growth-arrest or log phase growth) influences the expression of the LacZ gene remains to be investigated. Under certain conditions or during certain stages of the cell cycle, the ß-galactosidase concentration in the cells may be below the level of sensitivity of the X-gal staining. To use the lines with heterogeneous expression of LacZ for MRD studies a two-step assay would be required. Normal progenitor cells do not form colonies in agar cultures, unless stimulated with hematopoietic growth factors. When bone marrow samples containing LT12NL cells show colony formation this would already be indicative for the presence of leukemic cells and suffice to measure the leukemic cell frequency. LacZ staining does in that case not really add to detection of residual disease.

In principle, also the Neo^R gene is a candidate to be used as a marker gene to determine clonal residual leukemic cells by the colony assay in agar medium containing G418 [27]. However, in most of the LT12NL cell lines in our study Neo^R expression was rapidly lost, in vitro as well as in vivo. In two out of 10 lines the Neo^R gene was expressed in 100% of the cells. A selection pressure can be used to achieve stable expression of an introduced gene in retrovirus-mediated gene transfer. For practical application it is, however, unrealistic to propose a selection pressure in vivo, especially in a situation of MRD because this would conflict with 'undisturbed' leukemia regrowth and the measured leukemic cell frequencies would be serious underestimations.

The in vivo studies on leukemia development in rats indicated that the transferred genes did not alter the leukemic properties of LT12 cells. The expression of LacZ was found to be stable in seven out of 10 LT12NL cell lines for at least two in vivo passages. One of the lines stable in vitro lost LacZ expression in vivo (line 12). Southern analysis indicated changes in the hybridization pattern for line 12 but this was not further investigated. For the two lines that were repeatedly passaged it was found that for up to 10 to 12 in vivo passages, corresponding to 120-130 cell doublings, LacZ expression was retained. Neo^R, however, was rapidly lost in these two lines. When the LT12NL cell lines were repeatedly transferred in vivo and resistant cells were rescued in between each passage by culturing them in the presence of G418 for a short period, the percentage of resistant cells declined during the next in vivo passage. The stability of the expression of a retroviral gene may depend upon several factors, which include the nature of the infected cells, the reporter gene used, methylation, the chromosomal integration site of the provirus, the relative position of the genes in a multigene vector, and the presence or absence of selection pressure [28, 29]. Both genetic and epigenetic events are described to lead, at times, to a high-frequency loss or shutdown of viral gene expression in vitro [30]. In the LT12NL cell lines, LacZ expression was stable in seven out of 10 cell lines in the absence of a G418 selection pressure. In some cases the LacZ gene expression might be competitively inhibited by the expression of the Neo^R gene under the neomy-

Table 4.5 Expression of the LacZ and Neo^R genes at death in relapsing leukemic rats following remission induction with cyclophosphamide.

Animal	Survival Time	Bone Marrow		Spleen		Blood	
	(days)	LacZ (%)	Neo^R (%)	LacZ (%)	Neo^R (%)	LacZ (%)	Neo^R (%)
1	37	100	82	nd	nd	100	71
2	38	100	77	100	80	100	95
3	41	100	78	100	64	nd	nd

Animals were treated with cyclophosphamide (100 mg/kg i.p.) on day 11 after i.v. transfer of 10^6 LT12NL (line 72) cells. Cell suspensions of the organs were plated in agar with or without G418 (for determining Neo^R expression), cultured for 7 days, and stained for LacZ expression with X-gal. nd, not determined. Note: untreated leukemic control rats survived 20 days.

cin selection pressure, by acting as a transcriptional silencer in a way as described by Artelt et al. [31]. Furthermore, evidence is emerging that the expression of two genes introduced into cells within a single retroviral vector may lead to transcriptional silencing [32]. In one of the lines that we studied (line 7) Neo^R expression was retained with a concurrent loss of LacZ expression. However, for the second cell line that retained Neo^R expression (line 28) LacZ was continuously expressed as well. The observed susceptibility of the Neo^R gene to shutdown of expression, implies that the BAG construct was inadequate to mark the leukemic cells with the Neo^R gene.

For MRD studies a relevant observation was the fact that leukemic cells that survived the remission-induction treatment with chemotherapy fully retained expression of LacZ . During this in vivo regrowth process cells increased from approximately $5x10^3$ after the MRD induction to 10^{10} cells at relapse. This observation showed that expression of LacZ was not influenced by the cytostatic drug treatment and that relapsing leukemic cells could be identified and quantitated using LacZ. For the detection of MRD the X-gal staining method has a potentially comparable sensitivity as reported earlier for the L-CFU-S bioassay [4] and for monoclonal antibody labeling and flow cytometry in the BNML leukemia model [5, 6]. Even when compared to methods based on polymerase chain reaction technology, considered to belong to the most powerful currently available [33] and for which levels of detection of one per 10^6 are reported, X-gal staining performs very well. Because the X-gal assay has a high specificity and sensitivity and because it allows rapid analysis of large numbers of samples it will be a valuable assay, for instance to determine homing characteristics of leukemic cells in animal model studies. In earlier studies we have provided evidence that leukemia cells are inhomogeneously distributed during the state of MRD, resulting in focal leukemia relapses [6]. This implies that studies of leukemic cells in situ, i.e. in their natural microenvironment, are required. X-gal staining of cryosections of bone marrow as well as other organs with leukemia involvement will be the most promising application for in situ detection of MRD. These stu-

dies, now in progress, may lead to a better insight into the distribution of MRD and will be of use for studying the interaction between leukemic cells and hemopoietic stroma, and may provide valuable information for designing new strategies to eradicate residual disease. Translated to the clinical treatment of acute leukemia with autologous bone marrow transplantation this implies that genetic labeling and re-infusion of human AML cells before initiation of chemotherapy may be helpful to resolve questions, e.g. about the origin of an eventual leukemia relapse.

In conclusion, our results indicate that leukemia cells can be genetically marked with the LacZ and Neo^R genes. In selected cell lines the expression of LacZ was found to be stable during long observation times both in vitro and in vivo so that they can be used for the study of MRD in leukemia.

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Chapter 5

Differential suppression of background mammalian lysosomal ß-galactosidase increases the detection sensitivity of LacZ-marked leukemic cells

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Chapter 5

Differential suppression of background mammalian lysosomal ß-galactosidase increases the detection sensitivity of LacZ-marked leukemic cells

Abstract

A method is described for the detection of E. coli ß-galactosidase (LacZ) expressing leukemic cells in ex vivo bone marrow samples. 4-Methylumbelliferyl-ß-D-galactopyranoside (MUG) is used as a substrate in a kinetic assay. D-Galactose is used to suppress endogenous lysosomal ß-galactosidase activity, yielding a sixfold increase in sensitivity. With this assay, the detection limit is 1 leukemic per 10^4 normal bone marrow cells.

Introduction

Although in recent years treatment protocols for acute myelocytic leukemia have been substantially improved, many patients still develop a leukemic relapse, caused by the regrowth of leukemic cells that survive the treatment. More knowledge of the localization and the growth pattern of these very low numbers of residual surviving cells may help in improving the treatment protocols.

The Brown Norway Rat Acute Myelocytic Leukemia model (BNML)[1] and its in vitro growing subline LT12 have been extensively characterized and accepted as a relevant animal model for human acute myelocytic leukemia [1]. Recently, a subline LT12NL15 was developed from the LT12 cell line, by using the BAG vector [2] to introduce the E. coli ß-galactosidase (LacZ) gene into its genome. LT12NL15 cells exhibit stable, high level expression of E. coli ß-galactosidase in every individual cell, in vitro as well as in vivo [3]. These genetically marked cells are used to study the in vivo homing behavior and growth patterns of leukemia cells in the BNML model. In these studies, it is essential to have ways of detecting very low numbers of the genetically marked cells in tissue samples containing very large numbers of cells.

[1]Abbreviations used: BNML, Brown Norway Rat Acute Myelocytic Leukemia model; *LacZ*, *E. coli* *ß*-galactosidase gene; BM, bone marrow; PBS, phosphate buffered saline; CBS, citrate buffered saline; TBS, Tris buffered saline; Tris, tris-hydroxymethylaminomethane; MUG, 4-methylumbelliferyl-*ß-D*-galactopyranoside; FIR, Fluorescence Intensity Increase Rate; 4-MU, 4-methylumbelliferon; X-gal, 5-bromo-4-chloro-3-phenylindolyl-*ß*-D-galactopyranoside; AMPGD, 3-(4-methoxyspiro[1,2-dioxetane-3,2'-tricyclo [3.3.1.1^{3,7}]decan-4-yl)phenyl-*ß*-D-galactopyranoside.

Most reports describing assays for the detection of LacZ expression in transfected cells have focused on the sensitive detection and sorting of single LacZ expressing cells using flow cytometry [4, 5] or on the detection of ß-galactosidase in lysates [6, 7]. The detection of LacZ-marked cells in vivo is so far almost exclusively based on the histochemical detection of ß-galactosidase expression using X-gal as a substrate [8]. These assays rely on visual inspection of the samples, which is rather time consuming and limits their applicability.
We report here the development of a kinetic ß-galactosidase assay using 4-methylumbelliferyl-ß-D-galactopyranoside (MUG) as a substrate, with which bone marrow samples can be assayed for the presence of LacZ-marked leukemic cells. When detecting E. coli ß-galactosidase activity in mammalian tissue samples, the detection sensitivity is limited by the presence of considerable amounts of endogenous lysosomal ß-galactosidase. Several authors, each describing the characterization of a specific ß-galactosidase enzyme, have reported compounds that suppress the respective enzyme [9]. We have compared a number of these compounds for their ability to selectively suppress rat lysosomal ß-galactosidase vs. E. coli ß-galactosidase. None of these compounds showed such an effect. However, we did find that product inhibition by D-galactose can cause a sixfold differential suppression of lysosomal ß-galactosidase vs. E. coli ß-galactosidase. Using our optimized assay we can detect 400 LT12NL15 cells in the presence of $5x10^6$ normal bone marrow cells, the standard sample size used in the assay. This implies a lower detection limit of one leukemic cell per 10^4 normal cells.

Materials and methods

Animals

Brown Norway rats from the BN/BiRij strain were bred at the Medical Biological Laboratory TNO, Rijswijk, The Netherlands. LT12 and LT12NL15 cells were cultured in α-MEM supplemented with 10% fetal calf serum (Gibco, Breda, The Netherlands). LT12NL15 cells were used as a source of E. coli ß-galactosidase. 4-Methylumbelliferyl-ß-*D*-galactopyranoside and 4-methylumbelliferon (4-MU) were purchased from Molecular Probes (Eugene, OR). D-galactose, dithionitrobenzene, D-galactonic acid, D-galactal and Triton-X-100 were purchased from Sigma (St. Louis, MO). Fluorescence was measured with a Perkin Elmer LS50 fluorimeter (Perkin Elmer, Beaconsfield, UK). Excitation was set to 365 nm, bandwidth 15 nm; emission was set to 440 nm, bandwidth 5 nm.

4-MU Fluorescence vs. pH

4-MU was dissolved in DMSO at a concentration of 1 mg/ml. Of this solution 20 µL was mixed with 2 ml of either citrate buffered saline (CB; 100 mM sodium citrate, 0.9 % NaCl), phosphate buffered saline (PB; 100 mM sodium phosphate, 0.9 % NaCl) or Tris buffered

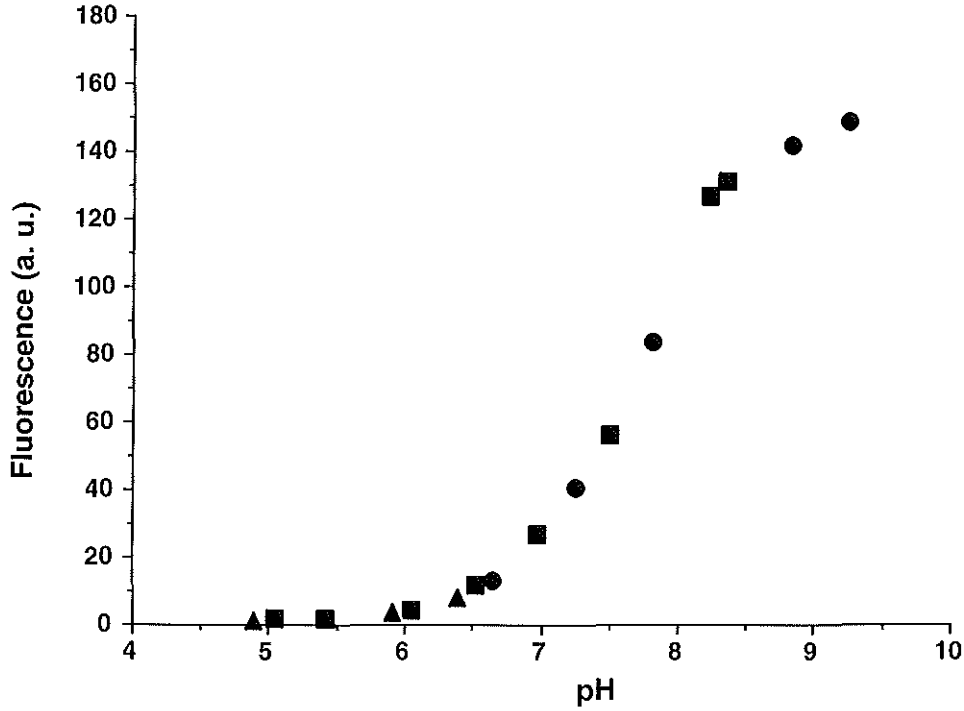

Figure 5.1 Effect of pH on fluorescence efficiency of 10 ng/ml 4-methylumbelliferon. Triangles: citrate buffered saline; squares: phosphate buffered saline; circles: Tris buffered saline.

saline (TB; 100 mM Tris-HCl, 0.9 % NaCl) covering a pH range of 4.9 to 9.3. The fluorescence intensity of the 4-MU was measured as a function of the pH.

Bone marrow cells

Rats were killed using carbon dioxide and femora were taken out and kept on ice. Bone marrow suspensions were prepared by flushing the femora with ice cold PBS (PBS; 12.7 mM sodium phosphate, 0.82 % NaCl, pH 7.4). To remove red blood cells the samples were mixed with an equal volume of ice cold erythrocyte lysis buffer and incubated for 10 minutes on ice. Subsequently, the cells were washed three times with PBS. Composition of the erythrocyte lysis buffer: 155 mM NH_4Cl, 11.9 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.4. After removal of red blood cells bone marrow samples were washed in PBS.

Lysates

Lysates were prepared by resuspending the cells in 0.1% Triton-X-100 in water, in a volume corresponding to $5x10^7$ cells/ml. This was followed by vigorous stirring for 1 minute. Finally, the lysates were centrifuged for 15 minutes at 1500 g to remove particulate matter.

MUG-assay

MUG was dissolved in DMSO at a concentration of 50 mM. Of this MUG solution 20 µl was pipetted into a quartz cuvette, and reaction buffer and cell lysate were added to a final volume of 2 ml. Reaction buffer was 100 mM Tris-HCl of varying pH, containing 2 mM $MgCl_2$ and containing variable concentrations of D-galactose. After thorough mixing, the increase of fluorescence intensity was recorded for 60 seconds. The fluorescence intensity increase rate (FIR) was calculated by linear regression analysis of the fluorescence intensity vs. time curve and taken as a measure of ß-galactosidase activity, expressed as arbitrary units (a. u.).

Inhibitors

Dithionitrobenzene, galactal, D-galactonic acid, and D-galactose were explored for their potential use as selective inhibitors of lysosomal ß-galactosidase activity. Concentrations were used that were stated in the literature to be effective in suppressing the respective ß-galactosidase [9]. In addition, product inhibition by D-galactose was tested for its differential effect on E. coli ß-galactosidase vs. lysosomal ß-galactosidase. The compounds were tested in

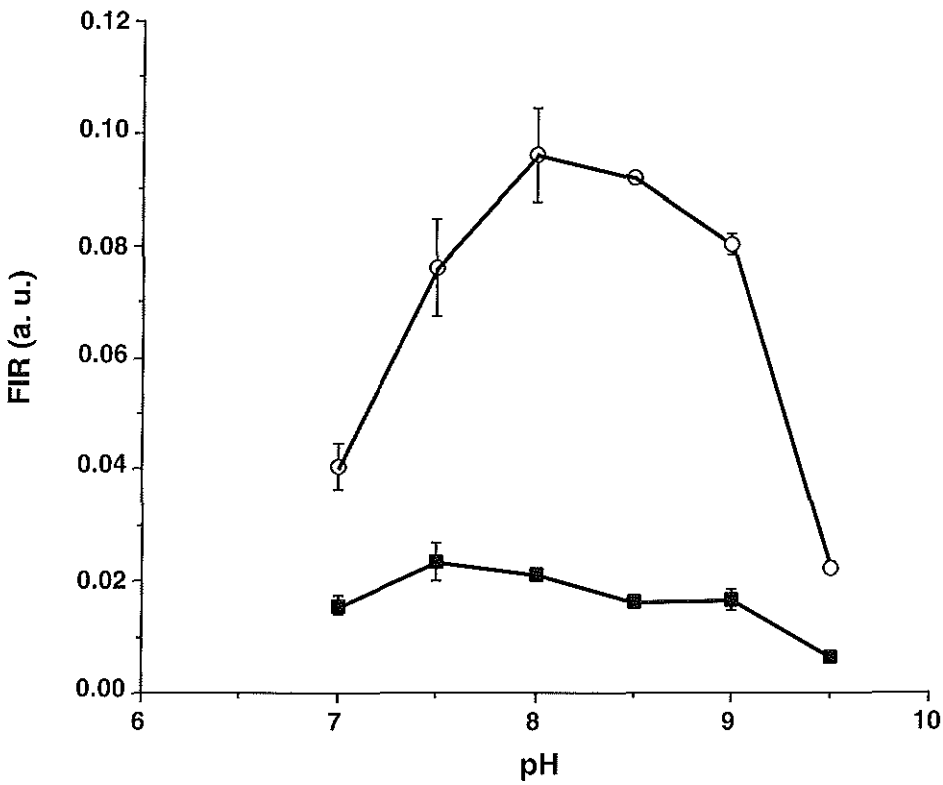

Figure 5.2 Effect of pH on fluorescence increase rate (FIR) of lysates from $1x10^4$ LT12NL15 cells. Squares: with the addition of 100 mM D-galactose; circles: no galactose added. Error bars represent standard deviation of triplicate measurements.

our kinetic MUG assay, using PBS as reaction buffer and lysates from $5x10^6$ normal bone marrow cells and from 10^5 LacZ marked cells as enzyme sources.

Results

Determination of optimum pH

In endpoint MUG assays the reaction is stopped by shifting the pH of the reaction mixture to pH 12. This denatures the enzyme and increases the fluorescence efficiency of 4-MU, the fluorescent reaction product of MUG. A real time kinetic assay must be performed at physiological pH values. The optimum pH of E. coli ß-galactosidase is at pH 7.0. The optimum fluorescence efficiency of 4-MU is at pH values above pH 9.4. (Figure 5.1). Therefore, in a kinetic MUG assay the observed increase of fluorescence intensity is a resultant of decreasing enzyme activity at basic pH values, and of increasing fluorescence efficiency of 4-MU. The combination of these effects led to an optimum pH for performing the assay at pH 8.0. (Figure 5.2, open circles).

Suppression of background activity

Lysates from $5x10^6$ bone marrow cells from healthy control rats show considerable background activity due to the presence of an endogenous ß-galactosidase in the lysosomes. Fiering et al. [4] reported on the use of chloroquine for the suppression of endogenous ß-galactosidase activity. Chloroquine is accumulated in intact lysosomes, which raises the intralysosomal pH and thereby reduces lysosomal ß-galactosidase activity. Obviously, this works only in

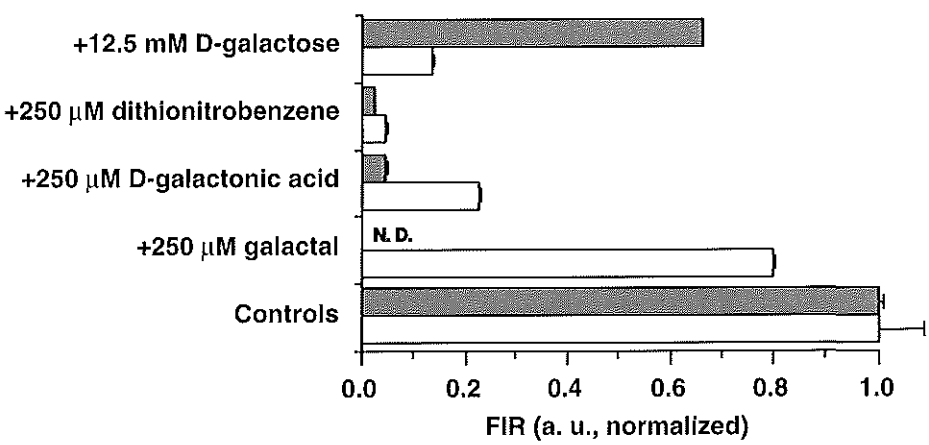

Figure 5.3 Exploring the suppressive effect of galactose derivatives on rat bone marrow (light grey) and E. coli (dark grey) ß-galactosidase activity. Measurements were done in duplicate. Bars express percentage of respective control. Error bars represent standard deviation of triplicate measurements.

intact cells. In order to find a way to selectively suppress background ß-galactosidase activity in lysates, we tested a number of substances that were reported as inhibitors of specific ß-galactosidases [9]. Of the substances tested (Figure 5.3), galactal showed only a minor suppression of lysosomal ß-galactosidase and was therefore not tested with E. coli ß-galactosidase. Dithionitrobenzene and *D*-galactonic acid had their strongest suppressive effect on the E. coli ß-galactosidase. Only D-galactose was seen to selectively suppress endogenous ß-galactosidase activity. In a dose ranging experiment it was shown that a concentration of 100 mM D-galactose led to an almost saturated suppression of background activity (Figure 5.4). This concentration was used in all further experiments.

Combined effect of D-galactose and pH

To investigate whether the use of D-galactose might have an influence on the optimum pH of the kinetic MUG assay, the pH was varied in the presence and in the absence of 100 mM D-galactose in the measurement of background activity (Figure 5.5) and in the measurement of E. coli ß-galactosidase activity from LT12NL15 cells (Figure 5.2). In the presence of 100 mM D-galactose the activity of lysosomal ß-galactosidase was suppressed to a very low level, independent of the pH. The suppression factor at pH 8.0 was 31.6.

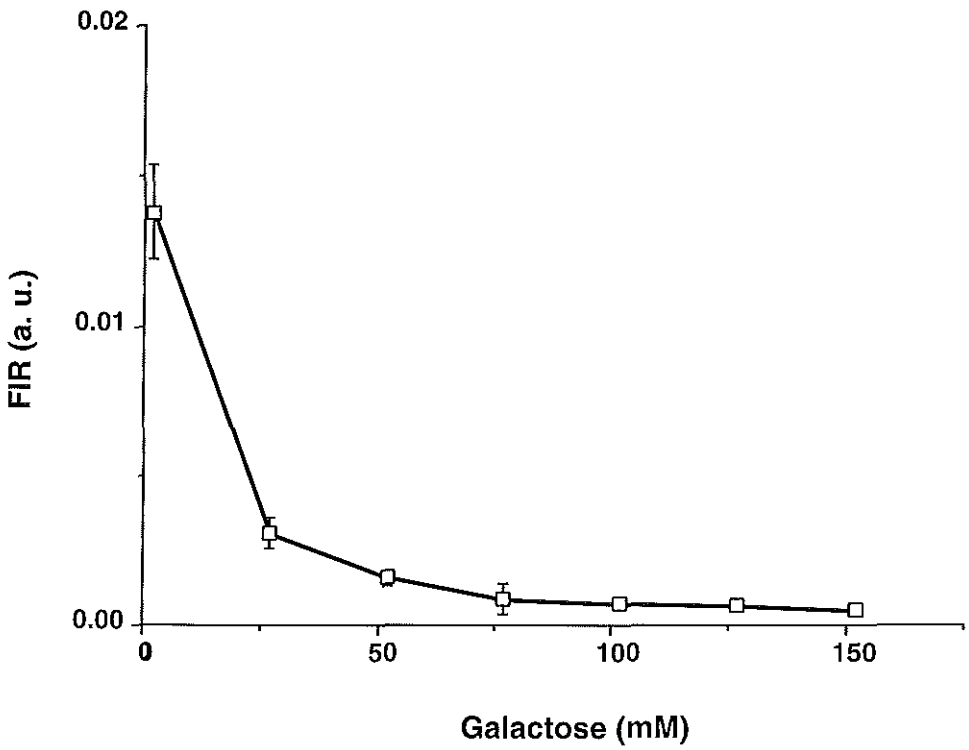

Figure 5.4 Dose-effect relationship between D-galactose and fluorescence intensity increase rate (FIR) of lysates from $5x10^6$ normal bone marrow cells. Error bars represent standard deviation of triplicate measurements.

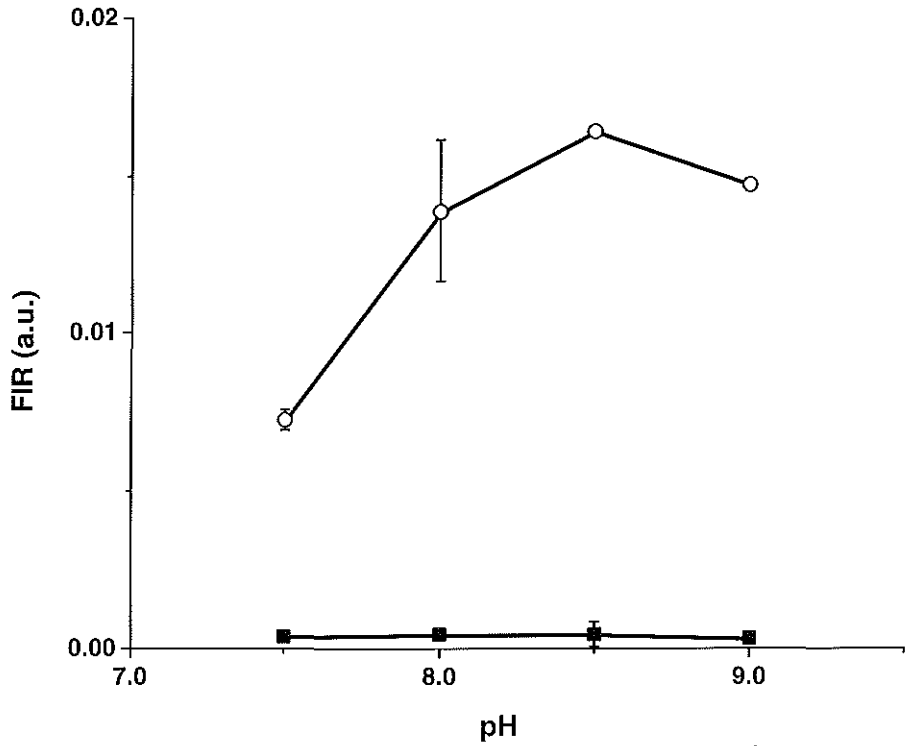

Figure 5.5 Influence of the pH on fluorescence intensity increase rate (FIR) of lysates from $5x10^6$ normal bone marrow cells. Squares: with the addition of 100 mM D-galactose; circles: no galactose added. Measurements were done in duplicate.

The optimum pH of E. coli ß-galactosidase was still at pH 7.5-8.0, and was similarly to that of lysosomal ß-galactosidase, less pH sensitive in the presence of D-galactose. At pH 8.0 we measured a 4.7-fold suppression of E. coli ß-galactosidase activity. Thus, the use of 100 mM D-galactose led to a 6.7-fold *differential* suppression of lysosomal ß-galactosidase activity vs. E. coli ß-galactosidase activity.

Determining the detection level

After determining the optimum assay conditions we determined the lower detection level of LT12NL15 cells in lysates from $5x10^6$ normal bone marrow cells (Figure 5.6). When the lysate from 400 LT12NL15 cells was added, the FIR was raised to twice the background level, which is generally considered to be the lowest significant level in an assay. Above 400 LT12NL15 cells, the FIR was seen to increase linearly along with the number of cells added. Therefore, the lower detection limit of the assay was at 400 LT12NL15 cells in a background of $5x10^6$ bone marrow cells.

Discussion

The therapeutic regimens that are tested in the BNML model have a strong, but variable influence on the cellular composition of the bone marrow. Some of these treatments, such as total body irradiation, are known to induce strong autofluorescence in bone marrow cells. Therefore, we chose to develop a kinetic assay, in which the initial background fluorescence in the samples does not influence the measurements. We used MUG as a substrate, because fluorimetric MUG assays are generally more sensitive than colorimetric assays using *o*-nitrophenyl-ß-*D*-galactopyranoside (ONPG) as a substrate. In order to optimize our assay, we first had to assess the effect of pH, because the pH influences three major components of the assay system: 4-MU fluorescence efficiency (Figure 5.1), lysosomal ß-galactosidase activity and E. coli ß-galactosidase activity. Figure 5.2 shows that the optimum pH for measuring E. coli ß-galactosidase activity alone is at pH 8.0, as a result of the combined effect of decreasing enzyme activity above its optimum pH of 7 and the increasing fluorescence efficiency of 4-MU. Surprisingly, lysosomal ß-galactosidase activity is measured most efficiently at pH 8.5. This is probably due to the fact that so far above its activity optimum (pH 4.5), the activi-

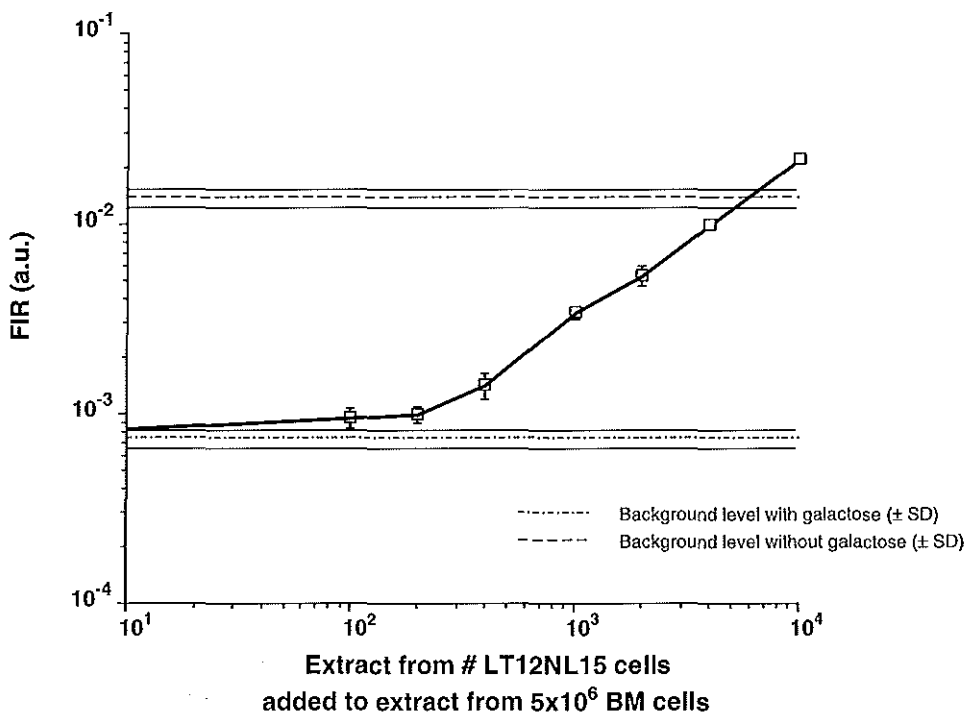

Figure 5.6 Determining the lower detection limit of LT12NL15 cells in a background of $5x10^6$ normal bone marrow cells. Error bars represent standard deviation of triplicate measurements.

ty of the lysosomal enzyme only slightly decreases with a rise in pH, whereas the fluorescence vs. pH curve of 4-MU increases rather steeply at pH 8-9.

The sensitivity of a detection method is determined by the level of background activity. Suppression of background is therefore crucial to achieve improved detection sensitivity. Young et al. [10] described the use of heat treatment for the suppression of endogenous ß-galactosidase activity in eukaryotic cell lines. Lysates of LT12NL15 cells, our LacZ expressing cell line, showed strong reduction of activity after 60' incubation at 50° C. This heat lability could be caused by the truncation of the first eight carboxy-terminal amino acids from the ß-galactosidase produced by the marker gene [11] in comparison to purified bacterial enzyme. The endogenous ß-galactosidase activity in bone marrow lysate was only slightly reduced by the heat treament. Thus, in our system heat treatment did not result in background reduction (results not shown). Therefore, several substances that were reported in the literature as inhibitors of ß-galactosidase activity were tested for their applicability as selective suppressors of lysosomal ß-galactosidase activity. Apart from D-galactose, all the compounds that were tested were more effective in suppressing LacZ activity or did not have any considerable effect at all. Only D-galactose was seen to inhibit lysosomal ß-galactosidase activity more than LacZ activity. After selecting 100 mM D-galactose as the standard concentration to work with (Figure 5.4), we verified that the optimum pH for performing the assay in the presence of D-galactose was still at pH 8.0. At this pH, the addition of 100 mM D-galactose led to a 6.7-fold differential suppression of lysosomal ß-galactosidase activity.

After optimizing the assay conditions, we determined the detection limit of our assay by mixing lysates of varying numbers of LT12NL15 cells with lysates of a fixed number of $5x10^6$ normal bone marrow cells. At 400 LT12NL15 cells, the FIR is twice that of background, a level that is generally considered as the lowest detectable. This means that fewer than 500 leukemic cells per $5x10^6$ normal bone marrow cells can be detected with this assay. This may not seem very sensitive when compared to, for example, the luminometric AMPGD assay described by Jain and Magrath [6], which can detect the ß-galactosidase activity of a single LacZ expressing cell. However, this assay, as indeed most assays described, was only optimized for measuring E. coli ß-galactosidase expression in cell lines, not for large tissue samples containing a large amount of endogenous lysosomal background activity. When compared to other assays for the detection of rare leukemic cells, our MUG assay shows comparable or better sensitivity than other assays such as antibody staining and agar culture assays [1], and is less time consuming.

The use of D-galactose for the differential suppression of background activity may be generally useful in all LacZ assays to increase detection sensitivity, not only in rat cells, but also in human and murine systems, where it has a comparable effect on background activity (data not shown). In histochemical staining assays using X-gal as a substrate for ß-galactosidase, D-galactose can prevent background containing cells from developing a "threshold" amount of staining, making such assays free of any background problems, so that every stained cell can

be positively identified as a cell expressing the LacZ gene (data not shown).

In conclusion, we have optimized a kinetic fluorimetric assay for the detection of LacZ-marked leukemic cells in the presence of large numbers of normal bone marrow cells. The assay was optimized by carefully defining the optimum pH of the assay and by using D-galactose, which causes a 6.7-fold differential suppression of mammalian lysosomal ß galactosidase activity. This led to a detection sensitivity of 1 leukemic cell per 10^4 normal bone marrow cells.

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Chapter 6

Monitoring of leukemia growth using a highly sensitive assay for the detection of LacZ marked leukemic cells

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Chapter 6

Monitoring of leukemia growth using a highly sensitive assay for the detection of LacZ marked leukemic cells

Abstract

A very sensitive assay for the detection of LacZ marked cells of an in vitro growing subline of the Brown Norway Rat Myelocytic Leukemia (BNML) model was developed. By combining cytochemical X-gal staining with D-galactose mediated suppression of endogenous background ß-galactosidase activity, a detection sensitivity of 1 leukemic cell per 10^8 normal bone marrow cells could be achieved. A detailed analysis of the in vivo growth pattern and kinetics of this cell line is presented. Also, it is shown that after cyclophosphamide treatment of leukemic rats no leukemic colonies are formed in an agar-colony assay, whereas the leukemic cells remain detectable in the bone marrow for a considerable time period. Eventually, however, all leukemic cells disappear from the marrow. These findings are discussed in the light of prolonged detection of rare leukemic cells in patients in continuing remission.

Introduction

Cytostatic drug treatment of leukemia patients leads in many cases to the induction of a complete remission, during which no leukemic cells can be detected by microscopic evaluation of blood or bone marrow smears. A significant proportion of these patients are then in a state of minimal residual disease (MRD), which implies that during their remission period these patients have low numbers of surviving leukemic cells. Many patients will eventually develop a leukemia relapse due to the proliferation of the residual leukemic cells. Considerable effort is invested into the detection and quantification of MRD, because this can be considered as one of the keys to further improvement of leukemia therapy, especially for those cases where bone marrow transplantation is not an option.

Immunologically- and polymerase chain reaction (PCR)-based detection systems have been developed that are able to routinely detect leukemic cells with a sensitivity of 1 per 10^5 normal marrow or blood cells [1-5]. The relevance of such sensitive detection is however not clear. There does not seem to be conclusive evidence that positive detection of the presence of leukemic cells or, more accurately, the presence of DNA sequences specific for leukemic cells, is always indicative of an imminent leukemia relapse. So far, clinicians are not inclined to start cytostatic treatment based on positive MRD test results.

Martens et al. [6] have performed MRD studies in the BNML model, an animal model for human acute myelocytic leukemia (AML). They showed that MRD detection can give unreliable results due to focal regrowth of leukemic cells, which leads to serious sampling errors. It has also been shown that the kinetics of MRD differs from that of overt leukemia. Extrapolation of the growth curves of leukemic cells from animals suffering late relapses after cytostatic drug treatment showed that the cells causing the eventual relapse had been in a non-dividing state for prolonged periods of time [7, 8].

In order to pursue these studies in greater detail and to elucidate the role of localization of MRD, LT12 cells, an in vitro growing subline of the in vivo growing BNML model, were genetically modified using a retroviral vector [9]. The resulting subline, LT12NL15, expresses high levels of E. coli ß-galactosidase (LacZ) in every individual cell, which serves as a genetic enzyme marker for the detection of these leukemic cells. In this paper the sticky plate assay is described, which is based on a cytochemical staining procedure using 5-bromo-4-chloro-3 indolyl-ß-D-galactopyranoside (X-gal) as a substrate, in combination with D-galactose mediated suppression of endogenous ß-galactosidase activity in normal cells [10]. With this assay MRD detection at a sensitivity of 1 per 10^8 can be achieved. Using this assay, it was shown that after leukemia treatment (100 mg/kg cyclophosphamide) which resulted in cure from the disease, leukemic cells remain detectable in the bone marrow of the animals for prolonged periods, but eventually disappear. In parallel, a functional agar culture assay

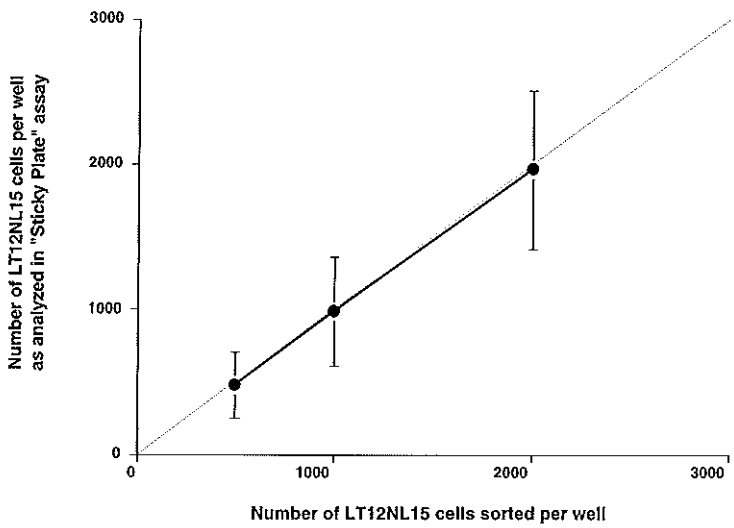

Figure 6.1 Validation of sticky plate assay results by analysis of artificial mixtures of LT12NL15 cells and normal rat bone marrow cells. Exact numbers of LT12NL15 cells were sorted into tubes containing the normal bone marrow cells. Closed circles: numbers measured in sticky plate assay. Dotted line: expected numbers. Wells were optically sampled using an eyepiece grid. Error bars represent SEM (n=10 wells).

showed that as early as 24 h after cytostatic drug treatment the LT12NL15 cells whose presence was easily detected with the sticky plate assay were no longer able to form colonies in vitro.

Materials and methods

Animals

Brown Norway (BN) rats from the BN/BiRij strain were bred at the Medical Biological Laboratory TNO, Rijswijk, The Netherlands. LT12 and LT12NL15 cells were cultured in Alpha Modification of Eagle's Medium (a-MEM) supplemented with 10% fetal calf serum (Gibco, Breda, The Netherlands).

Cytostatic drug treatment

Rats received 100 mg/kg cyclophosphamide intraperitoneally by injection of a solution of 20 mg/ml cyclophosphamide (Sigma Chemie, Bornem, Belgium) in Phosphate Buffered Saline (PBS).

Cell suspensions

In the experiments where peripheral blood was assayed for the presence of leukemic cells the animals were anesthetised using ether. All the peripheral blood was drawn through the aorta, using heparin to prevent clotting. Femora, spleens, lungs, livers and thymuses were then taken out and kept on ice. Bone marrow suspensions were prepared by flushing the femora with ice cold Hanks' Buffered Salts Solution (HBSS; Gibco) using a 22 gauge injection needle. After flushing the cells were passed through a 30 µm nylon gauze.

Spleen, lung and thymus cell suspensions were made by mincing the spleens or known fractions of spleens with a pair of scissors, whereupon the cells were passed through a 30 µm nylon gauze. From spleen, lung and bone marrow cell suspensions the erythrocytes were removed by mixing the samples with an equal volume of ice cold erythrocyte lysis buffer and incubating for 10 min on ice. Subsequently, the cells were washed three times with PBS. Composition of the erythrocyte lysis buffer: 155 mM NH_4Cl, 11.9 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.4. After removal of red blood cells bone marrow samples were washed twice in HBSS. Cell loss was assessed by counting nucleated cells before and after the lysis procedure.

Liver cell suspensions were prepared using the same method of mincing and filtering. To remove hepatocytes from the suspensions the cells were loaded on LSM (Lymphocyte Separation Medium; Organon Teknika, Boxtel, The Netherlands) and centrifuged for 15 min at 1500 g. Non-hepatocytes were collected from the Buffer/LSM interface and washed twice in HBSS. This procedure results in separation of hepatocytes and debris from other cells con-

tained in the liver, largely cells from the blood flowing through the liver and leukemic cells [11]. This separation step was applied because hepatocytes and debris interfere with microscopic examination in the sticky plate assay.

Cell sorting

Using the custom-built RELACS II cell sorter [12] known numbers of LT12NL15 cells were sorted to prepare artificial mixtures of LT12NL15 cells with normal bone marrow cells. Sorting and re-analysis experiments showed that pre-set cell numbers were sorted with an accuracy of 98% (not shown).

Agar culture assay

An agar culture assay of LT12NL15 cells was performed by plating series of 10^6-10^5-10^4 cells in 1 ml cultures using 3 cm polystyrene petri dishes. The semi-solid culture medium was based on Dulbecco's Modification of Eagle's Medium (DMEM; Gibco) and contained 20% Fetal Calf Serum (FCS), 0.03% glutamine, 1 mM sodium pyruvate, 50 µM ß-mercaptoet-

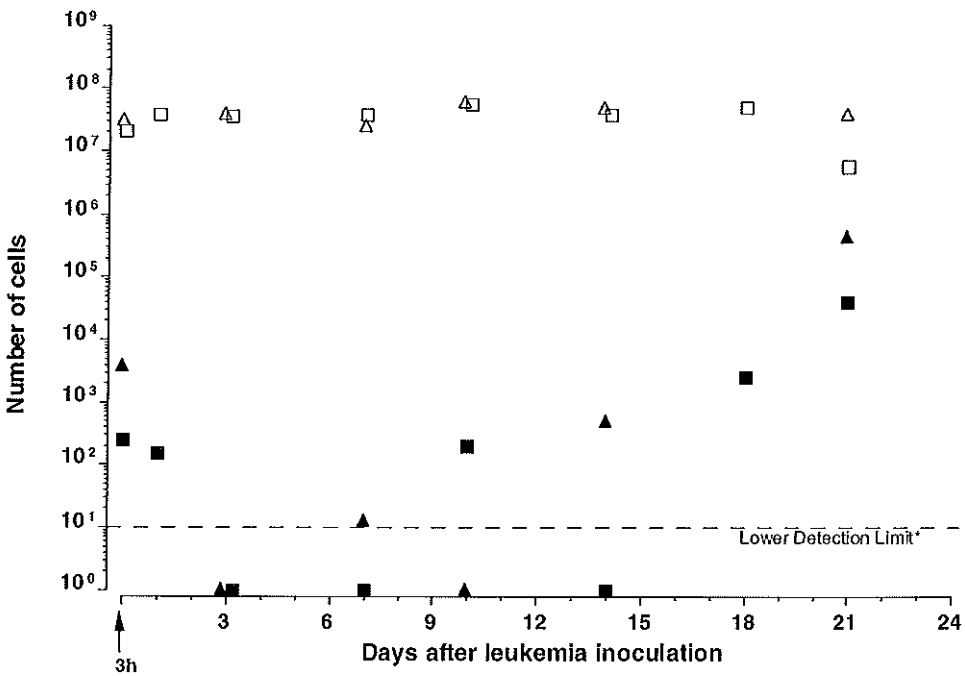

Figure 6.2 Analysis of LT12NL15 in vivo growth pattern in BN rats: total nucleated cell numbers and total numbers of LT12NL15 cells in total peripheral blood as determined by sticky plate assay. Triangles and squares represent results from different rats; closed symbols: total number of LT12NL15 cells per organ; open symbols: total numbers of nucleated cells in the blood as obtained after lysis of erythrocytes. * Lower detection limit is calculated as the number of total LT12NL15 cells per organ corresponding to a count of 1 blue cell in the largest sample analyzed.

hanol, 100 IU/ml of penicillin, 100 µg/ml streptomycin and 6 mg/ml agar (Bacto-Agar, Difco Laboratories, Detroit, MI). Cultures were kept for 7-10 days in a humidified atmosphere at 37° C and 10% CO_2. No growth factors were added. Under these conditions only leukemic colonies develop in the cultures. This was verified by adding to each culture dish 1 ml of a standard X-gal staining solution (Molecular Probes, Eugene, OR, USA), consisting of 50 µg/ml X-gal, 5 mM $K_4[Fe(CN_6)]$, 5 mM $K_3[Fe(CN_6)]$ and 10 mM $MgCl_2$ dissolved in PBS [13]. After staining overnight at 37° C the blue colonies were counted. No white colonies were observed.

Sticky plate assay

Into each well of flat bottom polystyrene 96-well plates 100 µL of poly-L-lysine 70.000-150.000 molecular weight (Sigma Chemie) dissolved in distilled water was pipetted. These wells were incubated for at least 30 min at 4° C. Subsequently, the wells were washed three times with PBS. Then $5x10^5$ cells in a maximum volume of 100 µL were pipetted into the

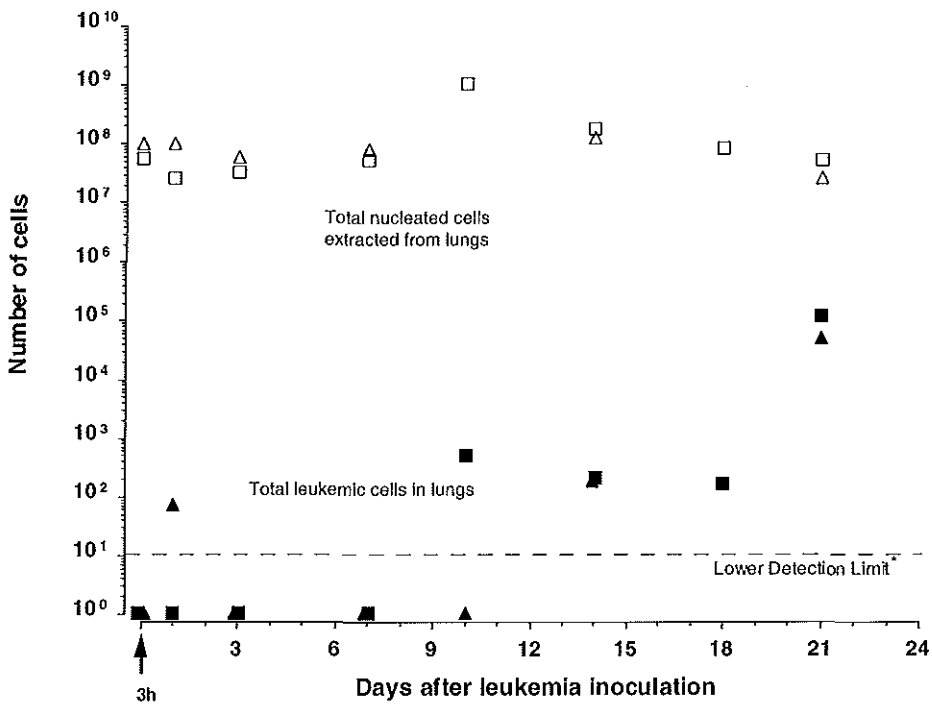

Figure 6.3 Analysis of LT12NL15 in vivo growth pattern in BN rats: total nucleated cell numbers and total numbers of LT12NL15 cells in the lungs as determined by sticky plate assay. Triangles and squares represent results from different rats; closed symbols: total number of LT12NL15 cells per organ; open symbols: total numbers of nucleated cells per organ. * Lower detection limit is calculated as the number of total LT12NL15 cells per organ corresponding to a count of 1 blue cell in the largest sample analyzed.

wells. At this cell number the bottoms of the wells become evenly covered with cells. The plates were then centrifuged for 5 min at 400 g, 4° C. After centrifugation the plates were placed on ice and 100 µL of a solution containing PBS and 2% paraformaldehyde and 0.2% glutaraldehyde was carefully added to the wells. After a 5-min fixation period the plates were carefully inverted and the supernatant was removed by gentle flicking with the plate. The wells were then washed three times with PBS. The plates had to be handled very carefully to prevent cell loss. Cell loss can be seen as areas in the plate were there is no uniform coverage with cells. Quantitative assessment of cell loss during the procedure (counting of cells in the washing fluids) showed a cell loss factor of 0.5-5 % (not shown). After washing the wells were filled with 100 µL of an X-gal solution as described above, with the addition of 100 mg/ml D-galactose. This serves as a specific suppressor of background staining [10]. The plates were then placed overnight at 37° C. After overnight staining 100 µL of the fixative solution was added and the plates were sealed with sellotape and kept at 4° C until microscopic evaluation of the numbers of blue stained cells in the wells. Microscopic examination was

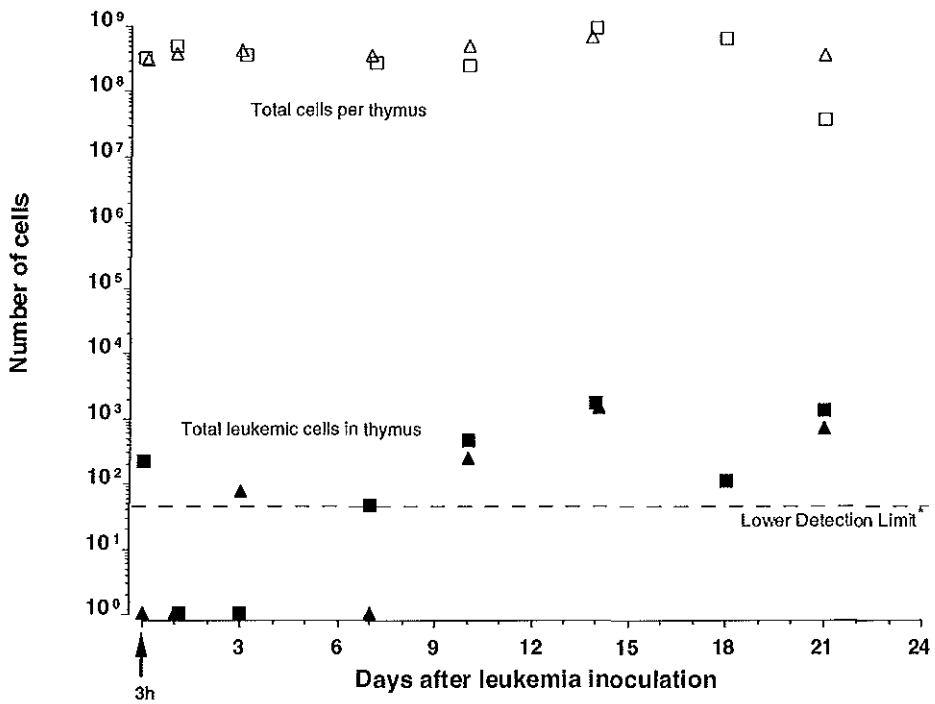

Figure 6.4 Analysis of LT12NL15 in vivo growth pattern in BN rats: total nucleated cell numbers and total numbers of LT12NL15 cells per thymus as determined by sticky plate assay. Triangles and squares represent results from different rats; closed symbols: total number of LT12NL15 cells per organ; open symbols: total numbers of nucleated cells per organ. * Lower detection limit is calculated as the number of total LT12NL15 cells per organ corresponding to a count of 1 blue cell in the largest sample analyzed.

done on an Olympus inverted microscope, using 20x magnification. Wells containing up to 200 LT12NL15 cells were counted completely. Wells containing higher numbers of blue cells were evaluated using an eyepiece grid to take optical samples. The number of cells per well was then calculated using a correction factor for the fraction of the well's surface that was optically sampled. In the Figures total numbers of LT12NL15 cells per organ are presented, which were calculated by extrapolating the number of blue cells in the analyzed sample to the number of blue cells in the total organ.

Experimental designs

First it was shown that the sticky plate assay yields reliable counts of LT12NL15 cells in artificial mixtures with normal bone marrow cells. Subsequently, the assay was used for a detailed analysis of the growth pattern of LT12NL15. Finally, the results of an agar culture assay were compared to the results of the sticky plate assay to analyze the kinetics of LT12NL15 leukemia after remission-induction therapy.

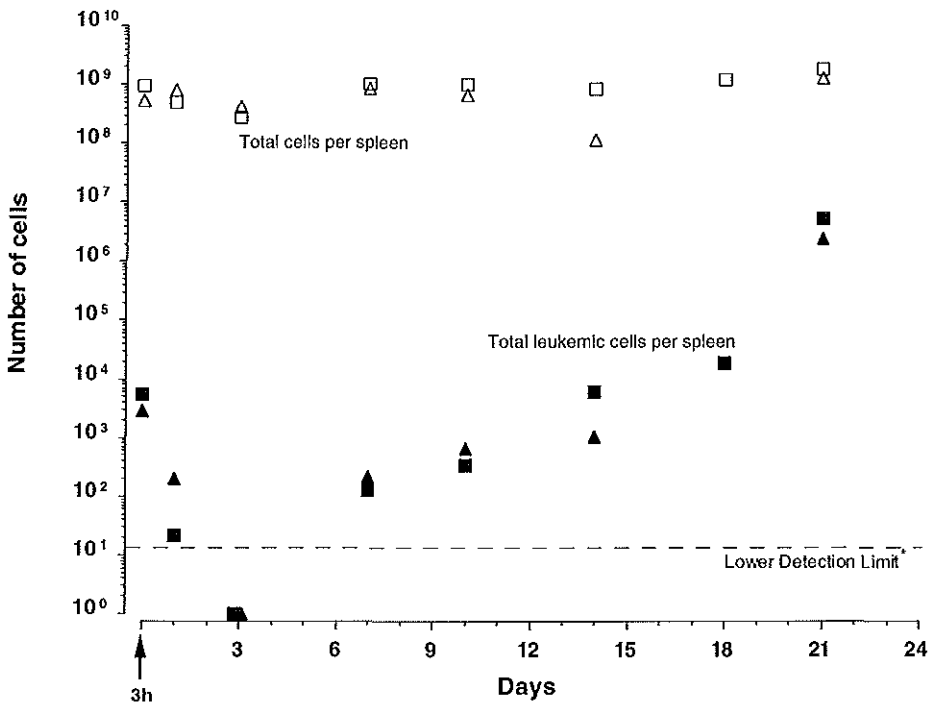

Figure 6.5A Analysis of LT12NL15 in vivo growth pattern in BN rats: total nucleated cell numbers and total numbers of LT12NL15 cells per spleen as determined by sticky plate assay. Triangles and squares represent results from different rats; closed symbols: total number of LT12NL15 cells per organ; open symbols: total numbers of nucleated cells per organ. * Lower detection limit is calculated as the number of total LT12NL15 cells per organ corresponding to a count of 1 blue cell in the largest sample analyzed.

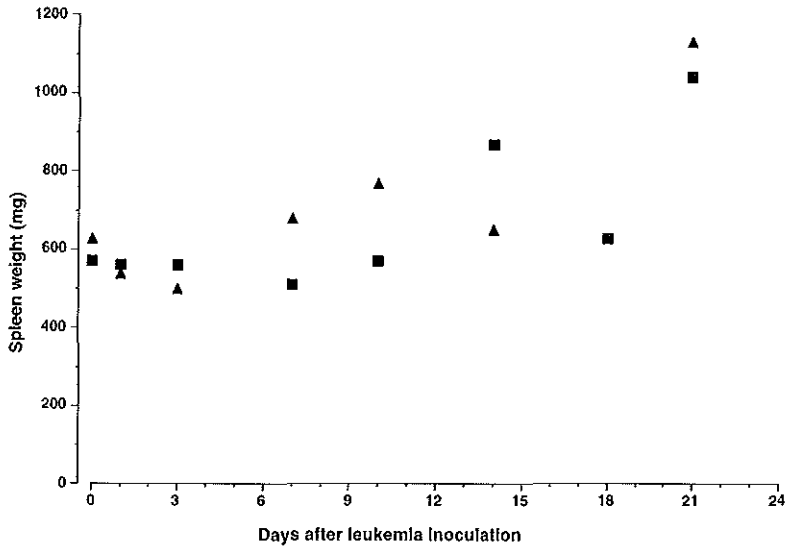

Figure 6.5B Spleen weights during the development of LT12NL15 myelocytic leukemia in BN rats. Triangles and squares represent results from different rats, corresponding to Figure 6.5A

Results

Sticky plate assays of exactly known numbers of LT12NL15 cells (flow sorting) mixed with $5x10^5$ normal bone marrow cells showed a linear relationship between the number of cells expected per well and the number of cells observed (Figure 6.1). The observed numbers are slightly lower than expected, reflecting cell loss of 0.5-5% during the wash steps (data not shown).

The sticky plate assay was used to study the homing behaviour and the in vivo growth pattern of LT12NL15 cells. Rats were injected with 10^6 LT12NL15 cells and on consecutive days animals were sacrificed and peripheral blood, bone marrow, spleen, lungs, liver and thymus were examined for the presence of LT12NL15 cells using the sticky plate assay. Organ weights (liver, spleen) and total numbers of nucleated cells per organ were also determined.

Figure 6.2 shows that 3 h after injection the leukemic cells are well above the detection limit in the peripheral blood of the animals. Then the number rapidly declines to below the detection level on day 3 after injection. As time progresses, the leukemic cell numbers in the peripheral blood remain very low, and show considerable variation. Only towards the terminal stage, which is accompanied by hind leg paralysis around day 21, the numbers in the peripheral blood increase sharply. A nearly identical pattern is observed in the lungs of these animals (Figure 6.3). In the thymus (Figure 6.4) the leukemic cell numbers remain very low throughout the entire development of the leukemia in the animals.

In the spleens (Figure 6.5A) again, a steep decrease of leukemic cell numbers from 3 h after

injection to day 3 after injection is observed, followed by an exponential increase between day 7 and day 18 (Population doubling time (T_d) = 39.3±1.7 h, R^2 = 0.90), culminating in a sharp increase between day 18 and 21. The slow exponential increase in leukemic cell numbers is accompanied by a gradual increase in spleen weight, as shown in Figure 6.5B.

Figure 6.6A shows that in the liver there is again a decrease of the number of LT12NL15 cells between day 0 and day 3, but the number does not decrease below the detection level. From day 3 onward, the number of leukemic cells in the liver increases exponentially with time (T_d = 24.5±1 h, R^2= 0.93). On day 21 the total liver LT12NL15 cell number amounts to only 10^6 cells, which is too low to lead to a significant increase in liver weight (Figure 6.6B).

In the bone marrow (Figure 6.7), as in all other compartments, a decrease in leukemic cell numbers is observed during the initial phase. A nadir is already reached 24 h after injection. From day 3 after injection onwards, the leukemia growth in the femora can be described by fitting the data points to a growth curve that consists of an exponential curve contiguous to a Gompertz curve, as used by Schultz et al. [14] to describe the growth of BNML cells in vivo.

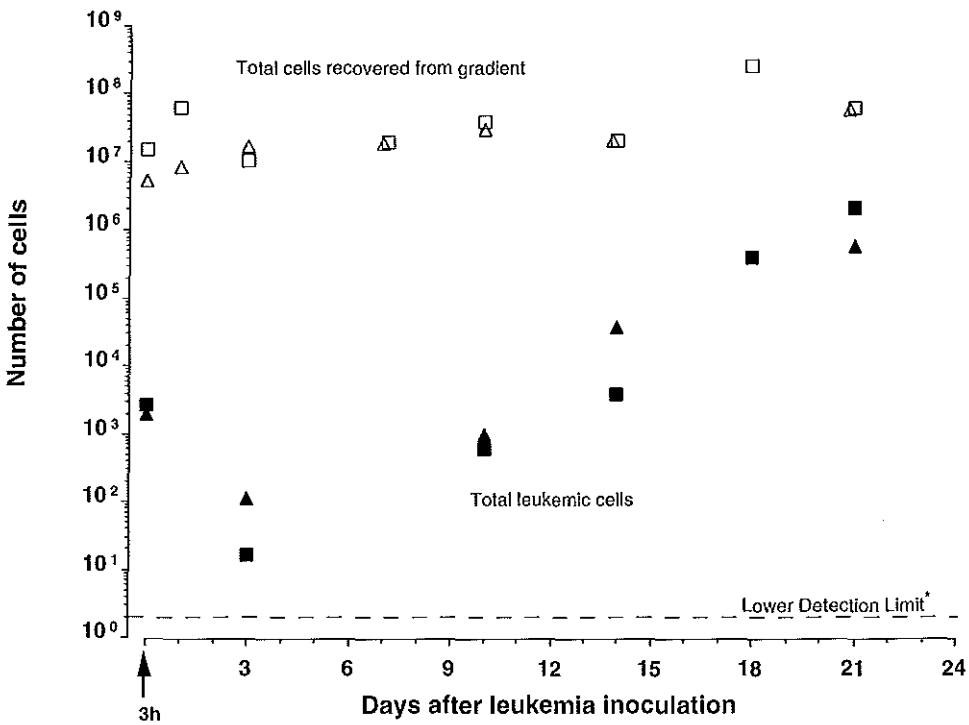

Figure 6.6A Analysis of LT12NL15 in vivo growth pattern in BN rats: total nucleated cell numbers and total numbers of LT12NL15 cells per liver as determined by sticky plate assay. Triangles and squares represent results from different rats. Closed symbols: total number of LT12NL15 cells per organ. Open symbols: total numbers of nucleated cells per organ. * Lower detection limit is calculated as the number of total LT12NL15 cells per organ corresponding to a count of 1 blue cell in the largest sample analyzed.

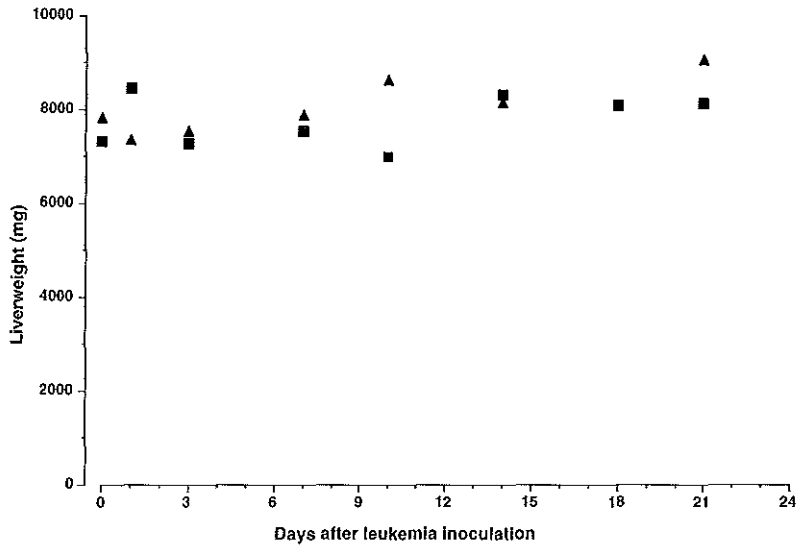

Figure 6.6B Liver weights during the development of LT12NL15 myelocytic leukemia in BN rats. Triangles and squares represent results from different rats, corresponding to Figure 6.6A

The population doubling time during the exponential phase is 16.2±0.2 h (R^2= 0.99), and the time of exponential to Gompertzian growth transition (T_g) is at 10.4 days (Figure 6.7). This corresponds with the in vitro doubling time of LT12NL15 cells (15.8±0.2 h, data not shown). Taking the starting point of exponential growth at day 3, extrapolation of the exponential part of the growth curve to the time of injection shows that an average of 145 cells effectively homed to the femoral bone marrow. Assuming that one femur contains 2.59% [15] of the total bone marrow, this means that only around $5.6x10^3$ cells out of 10^6 injected homed effectively and started to grow, corresponding to 1 per 179 cells.

In another experiment the sticky plate assay was used to monitor the leukemic cell population of rats that received remission-induction therapy of 100 mg/kg cyclophosphamide (i.p.) on day 11 after the injection of $6x10^5$ LT12NL15 cells. These results were compared to the results of the agar culture assay. As shown in Figure 6.8, the the curves for both assays are closely parallel until day 11. This indicates that both assays correlate quite well. The colony assay yields lower absolute numbers of cells in the femur, which indicates that the plating efficiency of the agar culture assay is only 1-10%. As of day 12 an extreme difference in the two curves is observed: in the agar culture assay no more clonogenic cells were detected, whereas the sticky plate assay curve showed that readily detectable numbers of leukemic cells persisted in the animals for a prolonged time period of 16 days, after which the numbers disappeared below the detection level. Two animals survived for more than six months after treatment without any sign of relapse, indicating that the treatment resulted in cure from leukemia.

Discussion

X-gal staining of LT12NL15 cells consistently results in intense blue staining in more than 99% of the cells. Furthermore, X-gal staining of agar cultures of LT12NL15 shows 100% blue colonies [9]. This indicates that the cells of this LT12 subclone constitutively express high levels of E. coli ß-galactosidase in every individual cell. To develop a highly sensitive MRD-assay using the genetic marker of these cells, the sticky plate assay was developed. The addition of 100 mM galactose in the X-gal staining solution leads to selective suppression of this endogenous ß-galactosidase, as shown by Hendrikx et al. [10]. This suppression is so effective that in two sticky plate assays of each 10^8 normal rat bone marrow cells no blue cells were observed. By inference, this leads to a theoretical lower detection threshold of 1 leukemic cell per 10^8 normal cells. Without galactose suppression a background of blue staining normal cells is observed at a frequency of 1 per 10^4.

Figure 6.1 shows that there is a good correlation between expected *vs.* observed LT12NL15

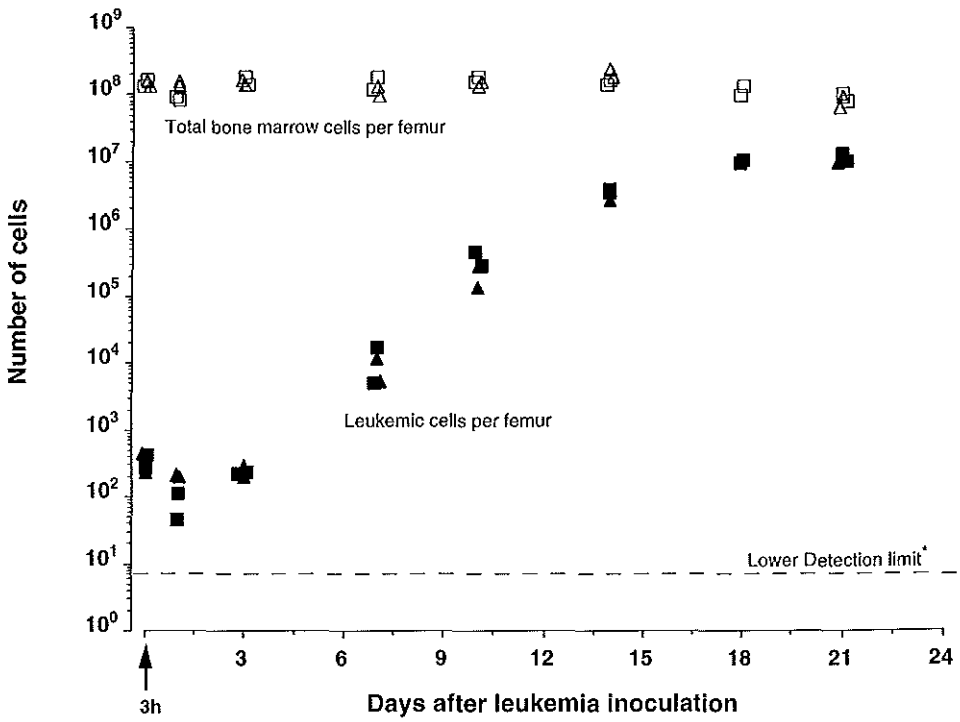

Figure 6.7 Analysis of LT12NL15 in vivo growth pattern in BN rats: total nucleated cell numbers and total numbers of LT12NL15 cells per femur as determined by sticky plate assay. Both femora from each rat were analyzed separately. Triangles and squares represent results from different rats; closed symbols: total number of LT12NL15 cells per organ; open symbols: total numbers of nucleated cells per organ. * Lower detection limit is calculated as the number of total LT12NL15 cells per organ corresponding to a count of 1 blue cell in the largest sample analyzed.

cells, even when optical sampling is used to extend the dynamic range of the assay to frequencies higher than 1 leukemic per $2.5x10^3$ normal cells. Therefore, the sticky plate assay in combination with LacZ-marked cells offers sufficient sensitivity and dynamic range to make monitoring of leukemia in vivo feasible from the time of injection of the cells to the terminal stage.

The sticky plate assay was first used to characterize the growth kinetics of LT12NL15 cells in vivo. Previous experiments have shown that the in vivo growth pattern of LT12 sublines show considerable variation, so that each subline has to be characterized separately [9].

As seen in Figures 3 and 4, only very low numbers of LT12NL15 cells are detected in the lungs and in the peripheral blood during the early stage of the disease. The sharp increase in leukemic cell numbers during the terminal phase does not originate from the increased number of leukemic cells in the blood, since the number of leukemic cells in the lungs is greater than the number found in the total obtained peripheral blood (ca 5 ml). The blood-free wet lung weight of rats is approximately 0.8 g [16], and the lung weights of the animals in our experiments averaged 1.6±0.2 g (s.d.), so the average blood content was approximately 0.8 ml. The leukemic cells in the lung are probably located within the lung tissue, or, alternatively, the relatively large LT12NL15 cells are trapped in the lung capillaries. The slow increase during the first phase of the disease indicates that lungs do not support growth of the leukemic cells. The very low numbers of leukemic cells in the thymus throughout the course of the disease may also indicate non-specific trapping of cells.

The fact that the leukemic cell number in the femora remains stable or declines during the first 3 days after injection implies that during this phase the leukemic cell population dynamics is the resultant of the decline of cells that do not home effectively and of the exponential growth of cells that do reach a site in the bone marrow that supports their growth. From day 3 onwards all the non-homed cells have disappeared, and exponential growth is observed. This is followed by a third phase where the growth fraction of the leukemic cell population decreases, possibly because the bone marrow cavity is completely filled with cells. Through ongoing proliferation cells are forced to leave the bone marrow and they invade other organs. In this phase the femoral growth curve follows a Gompertz growth curve, the result of the release of cells into the circulation, the decrease of the growth fraction of the population and of cell loss through cell death [14]. Finally a plateau in the number of leukemic cells in the femur is reached.

The population doubling time in the spleen is 39.3 h (Figure 6.5A), which is much longer than the in vitro doubling time or the doubling time in bone marrow or liver. This indicates that either the spleen presents a much less favorable environment for the growth of LT12NL15 cells, or that cells emigrate from the spleen to the peripheral blood and hence to other organs. The steep increase in splenic LT12NL15 numbers indicates that migration between compartments does play a role in the spleen, but it is contradictory to the possibility of emigration. Therefore, it seems plausible to conclude that the spleen does not provide a

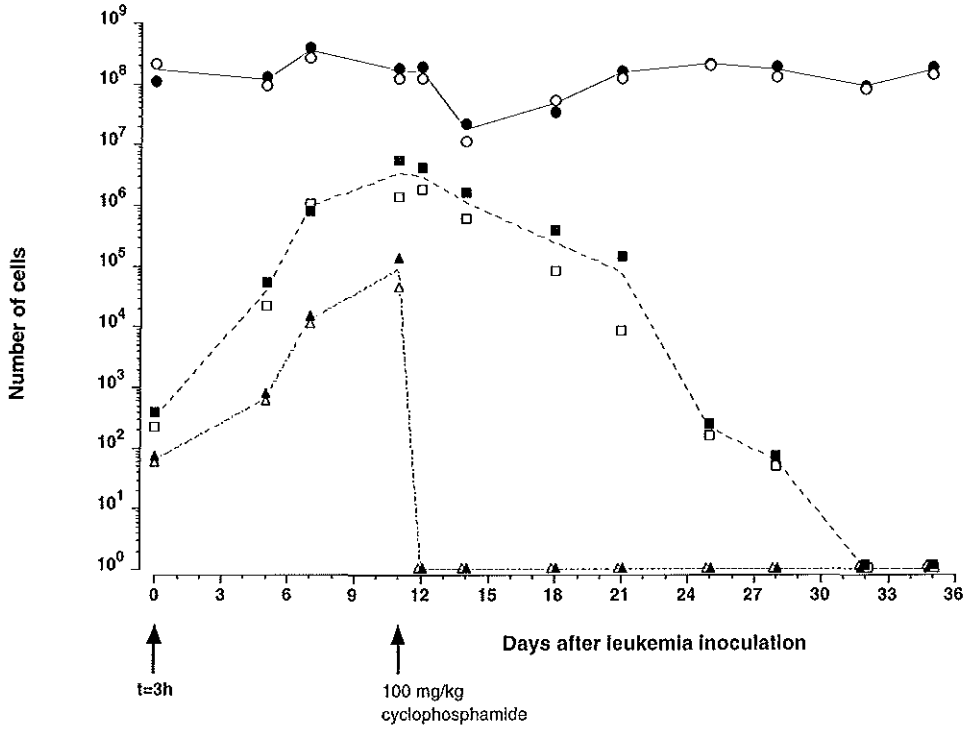

Figure 6.8 Total BN rat femoral content of total nuclear cell numbers and of LT12NL15 cells numbers as determined by sticky plate assay and by agar culture assay in BN rats, before and after cyclophosphamide treatment. Circles: total nucleated cell content; squares: sticky plate assay results; triangles: agar colony assay results. Open and closed symbols represent results from different animals. Lines connect average values.

favorable environment to sustain the growth of LT12NL15 cells. This is in agreement with the prolonged period of decline of leukemic cells which is observed during the initial phase. In the liver exponential growth takes place, commencing between day 3 and day 10. Again the growth rate is lower than in the bone marrow, but at the terminal phase no saturation is observed. This shows that the liver can support the growth of LT12NL15 cells, though not as well as the bone marrow, and that inter-organ migration plays only a minor role in the leukemic population dynamics in the liver.

Only one per 179 cells homed effectively to the bone marrow and started to grow. This is in the same order as the ED_{50} value for LT12NL15 (253±61 cells). This means that LT12NL15 cells show much less effective homing than BNML cells, which have an ED_{50} value of 25 cells [17]. Extrapolation of the exponential curve fit to day 0 shows that even though considerable numbers of LT12NL15 cells are found in the spleen soon after injection, only a very low number of leukemic cells actually start to grow in the spleen. The steep increase during the terminal phase probably reflects the sieving function of the spleen, comparable to the

lung-trapping hypothesis. A similar phenomenon can be observed in the liver. Therefore, it seems unlikely that there are specific homing receptors for LT12NL15 cells in the spleen or in the liver. Altogether these data indicate that homing of LT12NL15 cells is very ineffective, and may therefore be mediated by non-specific trapping in the capillaries of the organs.

Comparison of sticky plate analysis and agar culturing shows that after cyclophosphamide treatment no clonogenic leukemic cells could be detected anymore in the agar culturing assay. Together with the survival of rats for more than six months without showing signs of relapse, this indicates that the treatment was sufficiently effective to eradicate all leukemic cells. The sticky plate results show that leukemic cells do remain detectable for a prolonged period of 16 days after treatment before decreasing to below the detection level. This means that MRD detection based on the mere presence of cells of leukemic origin is not necessarily relevant. Only a functional assay, i.e. a clonogenic assay, or detection of increasing numbers of leukemic cells in a "non-functional" assay is indicative of a relapse. Also, our results show that when a cure is achieved in this model, the number of leukemic cells steadily decreases to below the detection level. This suggests that the prolonged presence of cells of leukemic origin which is often observed in leukemia patients [18] may be caused by low level proliferative activity of very small numbers of leukemic stem cells [19]. It could be speculated that these cells are localized in body compartments which sustain only slow proliferation rates of these cells. When during this slow proliferation a leukemic stem cell would self-renew and produce a new leukemic stem cell that is released into the circulation and homes to a compartment where leukemia growth is better supported, then this leukemic stem cell could start a chain reaction of rapid proliferation, which would lead to higher production of new leukemic stem cells which in turn would proliferate, finally resulting in a leukemia relapse. This model could serve as a possible explanation for the occurrence of late relapses in leukemia, which are often preceded by prolonged periods during which low numbers of leukemic cells can be detected in bone marrow and peripheral blood of patients [18].

Our results indicate that the sticky plate assay is a very sensitive assay for the detection of LacZ marked cells. This assay may be used to study the occurrence of late relapses as observed after high-dose anti-leukemia treatment. Also, it will be applied to the study of the behavior of leukemic cells in a model of autologous bone marrow transplantation where the graft contains low numbers of leukemic cells, a situation which often occurs in the clinic [20]. Because of its exquisite sensitivity, this assay may also prove useful for detecting and determining the fate of very low numbers of LacZ labeled cells in gene therapy protocols.

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Chapter 7

LacZ staining of paraffin tissue sections

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Chapter 7

LacZ staining in paraffin embedded tissue sections

Abstract

Femora and tibiae of rats carrying leukemia from a LacZ marked acute promyelocytic leukemia-derived leukemic cell line (LT12NL15) were decalcified using EDTA and routinely embedded in paraffin. Sections were used to develop for the first time an immunostaining method for LacZ, employing catalysed reporter deposition (CARD) based on the deposition of biotinylated tyramine. This method is used to study homing and adhesion of leukemic cells.

Introduction

In acute myelocytic leukemia (AML) infiltration of the bone marrow with leukemic cells is accompanied by severe suppression of normal hematopoiesis, eventually leading to life-threatening bleeding problems, recurrent infections and anemia. To investigate whether this suppression of normal hematopoiesis is caused by spatial competition between normal hematopoietic stem cells and leukemic cells, Prins and Van Bekkum [1] injected tritiated thymidine-labeled leukemic cells from the in vivo growing Brown Norway acute Myelocytic Leukemia (BNML) cell line, a well characterized model of human AML (reviewed by Martens et al., 1990), into rats. It was subsequently observed that these cells were preferentially localized in the subendosteal region of the femoral bone marrow, in contrast to L4415 cells, a rat model for human acute lymphocytic leukemia [1, 3]. This led to the assumption that AML cells specifically compete for space in the bone marrow compartment that is normally occupied by immature hemopoietic progenitor cells [4]. To extend the study of the growth pattern and localization of acute myelocytic leukemia cells and to elucidate the role of adhesion molecules in this process, a more versatile and permanent method to mark leukemic cells was required.

Genetic marking using the *Escherichia coli* ß-galactosidase gene (LacZ) is a widely used method for the identification and localization of transplanted cells in vivo [5]. Retroviral gene transfer was used to introduce the LacZ gene into LT12 cells, an in vitro growing derivative of the BNML cell line [6]. This resulted in the development of a genetically marked leukemic cell line, LT12NL15, which exhibits stable expression of large amounts of *E. coli* ß-galactosidase in the cytoplasm of every cell [7, 8]. These cells were used to set up a sensitive system to

study homing and growth of leukemic cells [8]. Here we report the development of an immunohistochemical staining method for LacZ in paraffin sections of formalin-fixed, decalcified tibiae and femora of rats carrying LT12NL15 leukemia. This method allows detailed visualization of genetically marked leukemic cells within the undisturbed spatial context of the bone marrow and the enclosing bone. This procedure may also be of value to other studies where detection of LacZ labeled cells in paraffin sections can offer increased specificity, greater convenience and superior tissue preservation compared to frozen sections.

Materials and Methods

Cells

LT12 is an in vitro as well as in vivo growing rat acute promyelocytic leukemia cell line, derived from the in vivo growing BNML cell line [9]. LT12 cells were genetically marked using a retroviral vector containing the LacZ gene, resulting in the subline LT12NL15. Briefly, LT12 cells were cocultured with the BAG retrovirus [10] in the presence of polybrene. After 36 hours of coculture the cells were placed under G418 selection pressure in an agar culture system. Ten days later resistant colonies were picked and expanded. After extensive in vitro and in vivo passaging of the resulting series of sublines the LT12NL15 subline was selected for use in further experiments [7]. LT12NL15 cells were cultured in Alpha Modification of Eagle's Medium (α-MEM) supplemented with 10% fetal calf serum (Gibco, Breda, The Netherlands).

Animals

SPF-Quality Brown Norway (BN) rats from the BN/RijHsd strain were purchased from Harlan CPB, Inc., Zeist, The Netherlands. Leukemia was induced by injecting LT12NL15 cells via the lateral tail vein of ether-anesthetized rats.

Immunological reagents

The primary antiserum was a rabbit antiserum to *E. coli* ß-galactosidase (5-Prime 3-Prime, Inc., Boulder, Co.). The secondary antibody was donkey anti rabbit-HRP conjugated, absorbed for rat, human and mouse (DAR-HRP; Amersham, Arlington Heights, Ill.). Biotinylated tyramine and streptavidin-HRP conjugate were as supplied in the Tyramide Signal Amplification kit (DuPont/New England Nuclear, Boston, MA).

Staining

Rats were ether-anesthetized and bled through the aorta. Subsequently, femora and tibiae were removed, cleaned of muscle tissue and fixed in 4% buffered paraformaldehyde for one to five weeks. Decalcification was performed for 3 weeks at room temperature under conti-

Table 7.1 Selection of epitope unmasking technique for LacZ staining on paraffin sections

Retrieval solution	Time	Effect on section	Staining result: Mouse MoAb (Sigma[1])	Rat Moab (Savelkouls[2])	Rabbit polyclonal (5Prime-3Prime[3])
Control	-	-	no staining	no staining	no staining
Microwave treatment in 0.01 M citric acid, pH 6.0	4x5 min.	severe damage to sections	no staining	no staining	N.D.
Microwave treatment in 0.01 M citric acid pH 6.0	2x5 min.	severe damage to sections	no staining	no staining	N.D.
Microwave treatment in 0.01 M NaHCO3, pH 6.0	2x5 min.	severe damage to sections	no staining	no staining	N.D.
Microwave treatment in 6M urea	2x5 min.	severe damage to sections	no staining	no staining	N.D.
DAKO Target Retrieval Solution, 96 °C	30 min.	bone removed	very weak staining?	N.D.	N.D.
Pronase 0.1%, 37 °C	10 min.	-	no staining	N.D.	
	15 min.	-	very weak staining?	N.D.	N.D.
	20 min.	-	very weak staining?	no staining	N.D.
Trypsin 0.1%, 37°	5 min.	-	no staining	no staining	very weak staining
	15 min.	-	no staining	no staining	N.D.
	20 min.	-	N.D.	N.D.	weak staining
	30 min.	-	very weak staining?	no staining	N.D.
	45 min.	-	very weak staining?	N.D.	clear staining

[1] Clone GAL-13, ascites, Sigma, Inc., Bornem, Belgium.

[2] Clone GL113, ascites. See Chatelain et al., J. Immunol. 148:1182-1187 (1994). Courtesy of Dr. HF Savelkoul, Dept. Immunology, Erasmus University Rotterdam, The Netherlands.

[3] Lot FA 143 A, IgG concentration 4.17 mg/ml. 5Prime-3Prime, Inc., Boulder, CO.

nuous stirring in 10% EDTA in distilled water, using sodium hydroxide to raise the pH to 8.0 in order to increase solubility of the EDTA. After decalcification the bones were dehydrated and embedded in paraffin according to standard procedures. Sections were prepared on SuperFrost microscope slides (Menzel, Braunschweig, Germany) coated with 3-aminopropyl-triethoxysilan (Sigma Chemicals, Bornem, Belgium) to improve adhesion of the sections to the glass surface. Before staining, the sections were deparaffinized and rehydrated to distilled water using a standard graded xylene-ethanol series. Extensive testing of multiple staining parameters led to the following reliable staining protocol:

1. Incubation in 0.1% trypsin dissolved in PBS, 45 minutes at 37°C.
2. Rinse three times in phosphate buffered saline solution (PBS).
3. Blocking of endogenous peroxidase activity in peroxidase blocking solution (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD), 1:10 diluted in distilled water, 5 minutes at room temperature.
4. Rinse in PBS (3x).
5. Blocking of non-specific binding sites: 1 hour incubation in blocking buffer at room temperature. Blocking buffer consisted of PBS containing 5% fetal calf serum, 5% horse serum, 5% goat serum and 10% rat serum and 0.2% Triton-X-100.
6. Rinse in PBS (3x).
7. Overnight incubation at 4° C with the primary antiserum. The antiserum was diluted 1:100 in blocking buffer containing 0.0002% NaN_3.
8. Rinse in PBS (3x).
9. Incubation in peroxidase conjugate: DAR-HRP, 1:400 diluted in blocking buffer. One hour at room temperature.
10. Rinse in PBS (3x).
11. Incubation in biotinylated tyramine, 1:150 diluted in diluent supplied with amplification kit. Ten minutes at room temperature.
12. Rinse in PBS (3x).
13. Incubation with streptavidin-HRP, 1:100 in blocking buffer. Thirty Minutes at room temperature.
14. Rinse in PBS (3x).
15. Develop HRP staining by incubating in 0.75% Diaminobenzidine (DAB; Fluka, Buchs, Switzerland) dissolved in PBS, containing 0.03% H_2O_2. Fifteen minutes at room temperature.
16. Rinse briefly in distilled water
17. Counterstain in hematoxylin
18. Rinse briefly in distilled water
19. Dehydrate through an ethanol/xylene series and mount in Entellan (Merck, Darmstadt, Germany).

Table 7.2 Optimization LacZ staining in paraffin sections

	Procedure step										
1° AB Dilution	Pronase (min)	Trypsin (min)	1° AB 1h 37° C	1° AB 1h RT	1° AB O/N 4° C	End. perox. blocking	NaN_3	Goat serum	Horse serum	Rat serum	Tyramide amplification
1:10		45'			x	+	+	+	+	+	+
1:100	-	-	x			-	-	+	+	-	-
1:100	-	-			x	-	-	+	+	-	-
1:100	10'				x	-	-	+	+	-	-
1:100	15'				x	-	-	+	+	-	-
1:100	20'				x	-	-	+	+	-	-
1:100		5'			x	-	-	+	+	-	-
1:100		15'			x	-	-	+	+	-	-
1:100		20'			x	-	-	+	+	-	-
1:100		30'			x	-	-	+	+	-	-
1:100		45'			x	-	-	+	+	-	-
1:100		45'			x	+	-	+	+	-	-
1:200		45'			x	+	-	+	+	-	-
1:400		45'			x	+	-	+	+	-	-
1:800		45'			x	+	-	+	+	-	-
1:1600		45'			x	+	-	+	+	-	-
1:100		45'		x		-	-	-	+	-	-
1:100		45'		x		+	+	-	+	-	-
1:100		45'		x		+	+	+	-	-	-
1:100		45'		x		+	+	+	+	+	-
1:100		45'		x		+	+	+	+	+	+
1:100		45'			x	+	-	-	+	-	-
1:100		45'			x	+	-	+	+	-	-
1:100		45'			x	+	-	-	+	+	-
1:100		45'			x	+	+	+	+	-	-
1:100		45'			x	+	-	+	+	+	-
1:100		45'			x	+	+	+	+	+	-
1:100		45'			x	+	+	+	+	+	+

[1] Clone GAL-13, ascites. Sigma, Inc., Bornem, Belgium.

[2] Clone BG-01, culture supernatant. See Slavíková et al., Folia Biologica 38:350-357 (1992). Courtesy of Dr. PA Dráber.

[3] Clone GL113, ascites. See Chatelain et al., J. Immunol. 148:1182-1187 (1994).
Courtesy of Dr. HF Savelkoul, Dept. Immunology, Erasmus University Rotterdam, The Netherlands.

[4] Lot FA 143 A, IgG concentration 4.17 mg/ml. 5Prime-3Prime, Inc., Boulder, CO.

[5] Biogenesis, Ltd., Poole, England. Courtesy of Dr. A. Tunnicliffe.

Antibody (source)										
Mouse Moab (Sigma) [1]		Mouse Moab (Draber) [2]		Rat Moab (Savelkouls) [3]		Rabbit polyclonal (5Prime-3Prime) [4]		Rabbit polyclonal (Biogenesis) [5]	
Controls [6]	Leukemic [7]	Controls	Leukemic	Controls	Leukemic	Controls	Leukemic	Controls	Leukemic
		no DAB product	no spec. staining [8]						
BG [9]	no spec. staining								
BG	no spec. staining					BG + non-spec. [10]	no spec. staining		
BG + non-spec.	no spec. staining			BG + non-spec.	no spec. staining				
BG + non-spec.	no spec. staining			BG + non-spec.	no spec. staining				
BG + non-spec.	no spec. staining			BG + non-spec.	no spec. staining	BG + non-spec.	weak specific staining		
BG + non-spec.	no spec. staining			BG + non-spec.	no spec. staining				
BG + non-spec.	no spec. staining			BG + non-spec.	no spec. staining				
BG + non-spec.	no spec. staining			BG + non-spec.	no spec. staining				
BG + non-spec.	no spec. staining								
BG + non-spec.	no spec. staining					BG + non-spec.	specific staining, variable [11]		
BG + non-spec.	no spec. staining					BG + non-spec.	specific staining, variable		
						BG + non-spec.	specific staining, variable		
						BG	weak specific staining, variable		
						BG	no spec. staining		
						BG	no spec. staining		
						BG + non-spec.	weak specific staining, variable		
						non-spec.	weak specific staining, variable		
						non-spec.	weak specific staining, variable		
						no DAB product	weak specific staining, variable		
				BG + non-spec.	no spec. staining	no DAB product	weak specific staining	non-spec.	weak specific staining
						BG + non-spec.	specific staining, variable		
						BG + non-spec.	specific staining, variable		
						BG + non-spec.	specific staining, variable		
						non-spec.	specific staining, variable		
						BG	specific staining, variable		
						no DAB product	specific staining, variable		
		no DAB product	no spec. staining			no DAB product	specific staining, reproducible	non-spec.	specific staining

[6] Summarizes results of sections from control rats and of sections from leukemic rats stained without primary antibody

[7] Results from sections of leukemic rats.

[8] Staining not clearly distinguishable from controls.

[9] DAB reaction product present in sections stained without I antibody.

[10] Signifies non-specific reaction of primary antibody on sections from control rats.

[11] Staining not consistently positive among sections.

Results

Extensive testing resulted in a reliable method to stain LacZ-marked cells in paraffin sections of decalcified bones. A 45-minute trypsinization step gave optimal staining results while preserving the bone matrix integrity in the sections. Endogenous ß-peroxidase was quenched using a special peroxidase blocking solution. Background and non-specific staining were reduced through the use of fetal calf serum, horse serum and rat serum, as described in materials and methods. To improve the reliability of the staining among different sections a biotine-tyramide amplifications step was introduced. This led to a sensitive and reliable staining protocol.

Figure 7.1A shows a photomicrograph of a tibia of a rat 12 days after injection of 10^6 LT12NL15 cells. Quantitative analysis of the bone marrow using the sticky plate assay [8] showed that the frequency of leukemic cells in the bone marrow of this rat was approximately 1 per 10^2 normal marrow cells (data not shown). The LacZ expressing leukemic cells can easily be recognized by their DAB staining. This Figure shows a representative example of the typically observed growth pattern of LT12NL15 cells which is characterized by focal growth in a loose formation, seemingly unconnected to any structural element of the bone marrow. Figure 7.1B shows an adjacent control section that was stained without the primary antibody. Note the absence of any DAB reaction product. Other controls were sections of control rats, stained with and without primary antibody. No DAB reaction product was seen in these sections (not shown). Figure 7.1C shows a higher magnification image of LacZ stained LT12NL15 cells. The DAB reaction product is localized in the cytoplasm of the leukemic cells.

Discussion

The LacZ gene which encodes for ß-galactosidase in *Escherichia coli* is widely used as a genetic marker of cells, as a reporter gene in genetic modification experiments and as a reporter sequence in the production of fusion proteins. This frequent application is owed to the wide range of assays available to detect its activity [11] and the robustness of the enzyme [12].

In mammalian tissues sometimes problems are encountered with background activity from various endogenous ß-galactosidases [13]. This problem can to a great extent be alleviated by the application of D-galactose in staining solutions, which acts as a differential suppressor of background activity [14], or by using immunohistochemical procedures [15].

Because of the robustness of the enzyme X-gal staining of frozen sections has been the method of choice for most localization studies. For the analysis of X-gal localization in bone marrow this is however not a very attractive option, since only specialized and time consu-

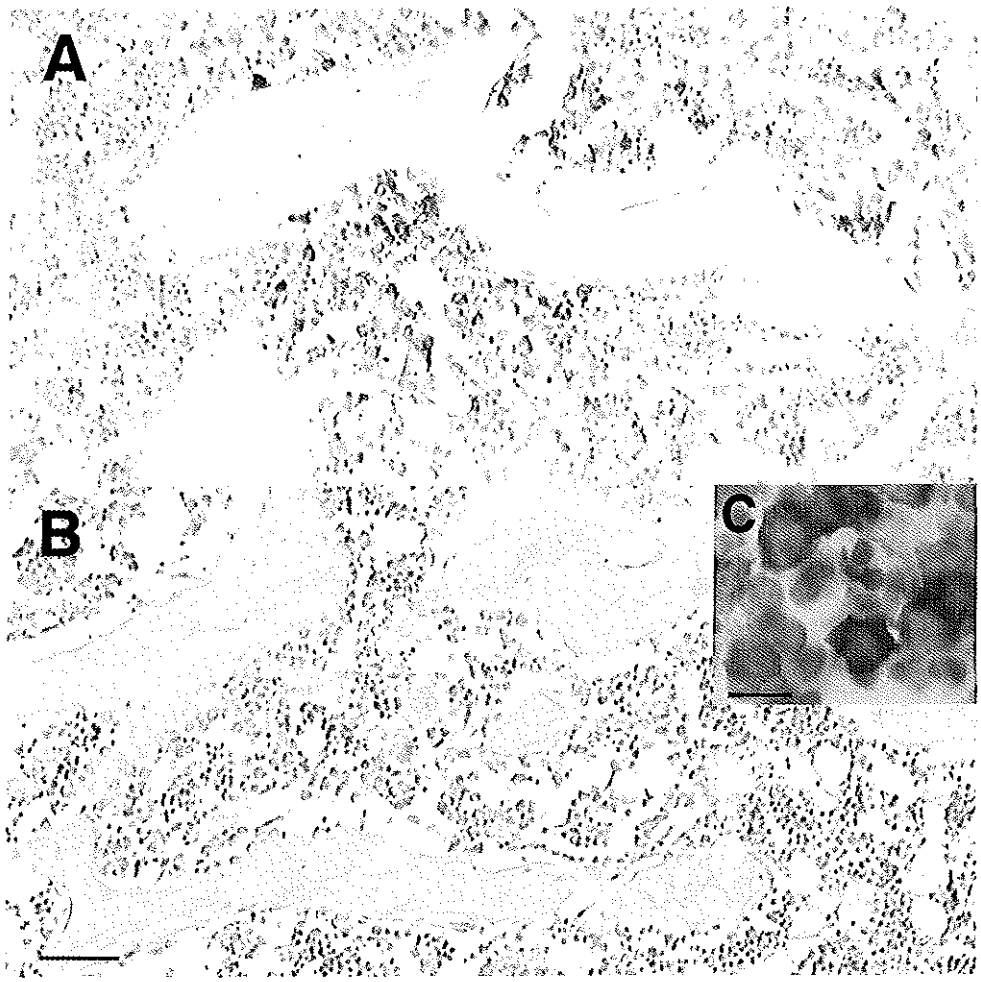

Figure 7.1A Low magnification photomicrograph of rat bone marrow in tibia of a rat, five days after injection of 10^6 LT21NL15 cells. Brown DAB reaction product shows LacZ-positive LT12NL15 cells. **Figure 7.1B** adjacent section, stained without primary antibody. Bar represents 50 µm. **Figure 7.1C** high magnification photomicrograph of LacZ stained LT12NL15 cells. Bar represents 10 µm.

ming methods for the preparation of frozen sections from undecalcified bone have been described [16, 17]. Another option is the cold glycol methacrylate embedding method as described by Lin et al. [18], but this requires the use of heavy duty microtomes for sectioning hard tissues which are not readily available in every laboratory. We therefore decided to develop a protocol for staining routine paraffin embedded sections of decalcified bones. Enzyme histochemistry is then no longer an option, since the enzyme becomes inactivated in the process of decalcification and embedding. Several monoclonal and polyclonal antibodies to *E. coli* ß-galactosidase were tested, in conjunction with a range of epitope-unmasking techni-

ques. Using the 5-Prime-3Prime polyclonal rabbit antiserum [15, 19] it was shown that trypsinization could render the paraffin sections stainable with a simple procedure, although with variable result. Incorporation into the protocol of a biotinylated tyramide-intensification step (CARD) [20-22] led to a reliable and reproducible staining procedure. As demonstrated in Figure 7.1, this method shows unequivocally the focal growth of LT12NL15 leukemic cells in the bone marrow.

While this method allows for LacZ staining of bone marrow paraffin sections, its good tissue preservation, ease of use, and the absence of background ß-galactosidase staining provides the perspective of a much wider applicability. Furthermore, this method will bring the use of LacZ marking within the scope of clinical studies where bone marrow autografts are genetically marked to study the kinetic and spatial behaviour of transplanted LacZ marked hemopoietic stem cells and of co-transplanted residual tumour cells [23, 24].

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Chapter 8

General Discussion

General Discussion

Homing: time to rethink

In the bone marrow everything seems to stick to almost everything else. A plethora of in vitro adhesion studies has shown a vast number of possible adhesion mechanisms between hematopoietic stem cells and bone marrow stromal cells, extracellular matrix components and bone marrow endothelial cells. This indicates that there may be widespread adhesive redundancy to safeguard the proper localization and targeting of hematopoietic cells. It can not be ruled out, however, that of all the possible adhesion mechanisms identified in different in vitro models only few are actually operative in vivo, and only under specific circumstances. The importance of homing and adhesion and possible therapeutic implications of new knowledge on the subject warrant further investigation. In view of the need to identify the truly relevant interactions greater emphasis should be placed on in vivo assays to clarify a number of issues and thereby show the way for future research.

Homing of hematopoietic stem cells

Endothelium or stromal cells?

One of the issues that remain to be resolved is the role of the bone marrow endothelium versus stromal cells in mediating the first steps of stem cell homing. Many studies have focused on the adhesion between hematopoietic stem cells and stromal cells. While it is quite likely that the stromal cells and extracellular matrix elements are instrumental in the maintenance and regulation of hematopoiesis, it is very well possible that the first crucial event of the homing process is the establishment of contact between the hematopoietic cell and endothelial cells. This must certainly be so in the case of bone marrow transplantation into non-conditioned hosts, which is being explored by several laboratories and which may become an important element in human gene therapy protocols [1, 2]. On the other hand it is not certain whether in the case of bone marrow transplantation into conditioned hosts the bone marrow sinus endothelium still forms an intact barrier against entry into the bone marrow. Shirota et al. [3, 4] indeed showed that the endothelium is severely damaged after conditioning for bone marrow transplantation.

The use of fluorescently marked stem cells or LacZ-marked stem cells derived from transgenic animals will be of value in resolving this matter by providing a means of identification of transplanted cells for quantification and localization purposes. In this thesis a method is described for the detection of LacZ labeled cells in paraffin sections of decalcified bones that

contain the bone marrow, the target organ for hematopoietic stem cell homing (Chapter 7). The method described also offers a starting point to the development of a staining method for cells labeled with intracellular markers such as CM-BODIPY and CM-FITC. The combination of this staining method with labeling for endothelial and stromal markers will shed light on the role of endothelial cells vs. stromal cells in stem cell homing. This will in turn direct the selection of relevant in vitro model systems for further studies.

What is a niche ?

Almost twenty years after the term was introduced [5] the stem cell niche is still a viable, but abstract concept. During this period it has been demonstrated that hematopoiesis is indeed regulated through the interaction of hematopoietic stem cells with external factors: cytokines, adhesion molecules and extracellular matrix molecules. However, a clear image of the stem cell niche in vivo has not yet emerged. Combined staining of bone marrow stromal elements and of genetically or transiently marked purified stem cells that have homed into the bone marrow of recipients will finally let the niche materialize.

Which are the relevant adhesion molecules ?

In vivo homing studies should serve in separating the sheep from the goats by identifying which adhesion molecules are specifically relevant in vivo under defined conditions. First of all this will help elucidating whether the observed distinction in adhesion molecule expression patterns of stem cells derived from bone marrow, cord blood and mobilized peripheral blood stem cells [6-8] are causally related to differences in the homing capacity of these cells and hence to the as yet unexplained differences in repopulation kinetics of stem cell grafts from these different sources [9-11].

Moreover, these in vivo studies will help to focus experiments aiming at improved homing efficiency of stem cells on those adhesion molecules. Stem cell research has invariably been hampered by the inability to obtain pure, well-defined stem cell suspensions. This has always led to the "hematopoietic Heisenberg uncertainty principle" [12]: if you can localize a cell in vivo, you don't know if it is a stem cell; conversely, once you have shown in a functional assay that a cell is a stem cell, you don't know where it was in vivo. Basic stem cell knowledge is now rapidly approaching the point where this paradox may be solved, because it will probably soon be possible to obtain pure stem cell suspensions. This will simplify experiments and will make the interpretation of the obtained results more straightforward. The low frequency of hematopoietic stem cells will nevertheless continue to make it difficult to obtain large numbers of stem cells, and therefore in vivo homing experiments will always be rare event detection assays. This thesis presents a flow cytometric method for the detection of rare fluorescently labeled cells (Chapter 3) and a method for the detection of very low frequencies of LacZ labeled cells (Chapter 6), using a special background-suppression method (Chapter 5). These methods will greatly facilitate the problem of rare event detection in in vivo homing

studies taking a quantitative approach.
The staining technique described in Chapter 7 should be of use in studies employing a qualitative approach by allowing detailed colocalization of transplanted cells and the adhesion molecules they interact with. Once the relevant molecules have been identified the described techniques will continue to be useful for monitoring the in vivo effectiveness of methods to manipulate the homing of hematopoietic stem cells.

Homing of leukemic cells

How often do reinfused leukemic cells cause relapse after autologous stem cell transplantation?

The most important aspect of homing of leukemic cells is the fact that leukemic cells reinfused with an autologous bone marrow graft can cause a leukemia relapse. Numerical simulation studies in leukemia models suggested that the contribution of reinfused leukemic cells to causing relapse would be negligible [13], but recent clinical evidence has shown that the contribution of genetically marked reinfused leukemic cells to leukemia relapses is considerable [14]. This finding shows that homing of leukemic cells is a clinically relevant phenomenon. Furthermore it stresses the importance of finding allogeneic stem cell donors for leukemia patients. These results have also shown conclusively that purging of autologous stem cell grafts is necessary. Genetic marking of autografts has opened for the first time the possiblity of monitoring the success of purging protocols. In order to fully analyze the frequency with which reinfused leukemic cells contribute to leukemia relapses a large number of marking studies have been initiated, most of them using the neomycin resistance gene. However, the use of the E. coli LacZ instead of the Neo^R gene for genetic marking of autografts in combination with the highly sensitive sticky plate assay (Chapter 6) and with detection in paraffin sections of bone marrow (Chapter 7) provides a much more versatile assay system, yielding information that would be difficult to obtain with Neo^R marking. Application of LacZ would make descriptive studies of homing and localization of leukemic cells as well as normal hematopoietic stem cells in patients feasible, and it could also be useful for MRD detection and relapse prediction. In short: every autologous stem cell transplantation should be combined with LacZ marking.

Which adhesion molecules are relevant?

Further exploitation of the LacZ-marked leukemic cell line LT12NL15 (Chapter 4) will provide information on the adhesion molecules used by this cell line to home to the bone marrow. Preliminary blocking experiments in which LT12NL15 cells were preincubated with function-blocking antibodies to VLA-4 or ICAM-1 prior to injection into rats showed reduced numbers of leukemic cells in the bone marrow of the recipients at different time intervals

after injection, and an increased survival time in the case of VLA-4 blocked leukemic cells. Controls were animals that received non-pretreated leukemic cells (data not shown). These results indicate that VLA-4 and possibly ICAM-1 play a role here. In this model it will also be possible to compare homing and adhesion in conditioned and non-conditioned hosts. This may help in elucidating the role of the endothelium in homing per se, an issue outlined in section 8.2.1. LacZ marking of a panel of other leukemic cell lines might be used to compare the functions of different adhesive interactions among leukemia subtypes.
Combinations of antibody blocking and genetic marking with two or more different genetic marker genes could be applied in clinical transplantation protocols to obtain conclusive confirmation in the human system of the role of specific adhesion molecules in stem cell and leukemic cell homing.

Homing: the long run

In perspective, one may expect that a detailed insight in the homing of hematopoietic stem cells and of leukemic cells may eventually lead to new therapeutic approaches. Improved homing, for example by using activating antibodies, could potentially be beneficial in certain situations where small graft size or low engraftment efficiency is a problem. These two problems coincide in gene therapy approaches aiming at transplantation of modified stem cells into non-conditioned recipients. Here supported homing could be particularly useful. In autologous stem cell transplantation it might be possible to use stimulated homing to reduce the graft size, thereby reducing the chance of cotransplanting leukemic cells or circulating solid tumor cells. The discovery of differences in the adhesion molecules employed by normal stem cells and leukemic cells could open a window of opportunity for developing strategies to specifically block leukemic cell homing in autologous stem cell transplantation protocols. In allogeneic bone marrow transplantation a smaller graft size may allow a reduction of graft versus host disease prophylaxis. A third application could be in improving the engraftment efficiency of human stem cells or leukemic cells in chimeric SCID mouse models, e.g. for diagnostic purposes.
Another therapeutic approach one might think of could be in exploiting differences in homing efficiency between normal hematopoietic stem cells and leukemic cells without transplantation. Comparison of ED_{50}-values of BNML cells and LT12NL15 cells shows that there is considerable variation in the homing capability of leukemic cell lines, indicating that such an approach could be useful in certain cases of homing-impaired leukemia. Mobilization protocols using cytokine combinations and/or adhesion molecule antibodies might be used to mobilize both leukemic and normal cells from the bone marrow compartment. If the normal stem cells would have a homing advantage above leukemic cells they could possibly reclaim the niches that were formerly taken up by the leukemic cells, while a comparatively large

proportion of the leukemic cells would be sequestered, most likely in the spleen and the liver. Such an approach could potentially lead to a selective reduction of the leukemic cell load with less severe side effects than cytostatic treatment.

In summary, the rare cell detection techniques described in this thesis offer the means to relate in vitro adhesion assays to sensitive in vivo assays, and will thereby contribute to enhance developments in stem cell transplantation, gene therapy and leukemia treatment.

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Summary

Summary

Mature functional blood cells are derived from pluripotent hematopoietic stem cells through a complex differentation process. As the cells are shuttled through this differentiation programme, they gradually exhibit a series of structural changes which are used to classify cells according to their differentiation stages (see Fig. 1.1). This process of blood cell formation is referred to as hematopoiesis. In adult mammals, such as man, hematopoiesis is predominantly located in the bone marrow. Hematopoiesis is controlled through a range of interactions between hematopoietic cells and their microenvironment. Growth factors and their receptors, extracellular matrix molecules, and specialized adhesion molecules all play important roles in mediating these interactions. Those places in the bone marrow where hematopoietic stem cells can find exactly the right combination of regulating interactions are called "niches".

During embryogenesis hematopoietic stem cells enter the fetal blood circulation to move through several steps of targeted migration from the yolk sac to the fetal liver and hence to liver and bone marrow. This targeted migration is called homing. A similar phenomenon is observed in stem cell transplantation: stem cells are injected into the circulation of the host and from there they find their way to niches in the bone marrow, where they can establish an entire new hematopoietic system. This is also referred to as homing.

When a serious misfortune happens to a hematopoietic cell, this can lead to its leukemic transformation. Such a cell evades the normal regulatory stimuli and will start to proliferate without control. The hematopoietic stem cells are then displaced from their niches in the bone marrow, and eventually life-threatening shortages of functional blood cells occur. Although they fail to respond in the proper manner to the regulatory stimuli from the niches, leukemic cells are often dependent on the niches for their growth and survival, just as normal hematopoietic stem cells. In addition, leukemic cells can be transplanted, which proves that they are also capable of homing.

This thesis deals with the development of techniques to study the homing of hematopoietic stem cells and leukemic cells. These techniques can be used to obtain a better understanding of the exact mechanisms and molecules involved. This may lead to the development of methods to influence homing, which may be of use in stem cell transplantation, gene therapy and leukemia treatment.

In **Chapter 1** an overview is given of adhesion and homing in a number of well characterized physiological systems: the recirculation of naive lymphocytes via lymphatic tissues, homing of memory and effector lymphocyte subsets to extralymphatic sites in mucosal epithelia and the skin, and infiltration of inflammation sites by lymphocytes, neutrophilic granulocytes and monocytes.

This is followed by an analysis of a non-physiological variant of homing: metastasis of tumor

cells. Along the lines of the most important adhesion molecule families those reports in the literature are discussed where increased expression or activity of adhesion molecules correlates with increased metastatic activity of tumor cells, indicating a possible role for the respective molecule in the homing of the tumor cells.

Subsequently, an overview is presented of the available literature on homing and adhesion of hematopoietic stem cells. A vast array of adhesive interactions between stem cells and stromal elements has been described. However, most of these reports are based on in vitro systems, and not all these interactions may be operative in vivo. It is stated here that in vivo experiments are required to identify those interactions that are actually relevant in vivo.

Finally a basic outline is given of marking cells for use in in vivo experiments, based on the distinction between genetic, permanent markers and transient cellular markers.

In **Chapter 2** four transient markers of two different classes are outlined: 1) PKH2 and PKH26, fluorescent lipophilic molecules that are incorporated into the cell membrane, and 2) CM-FDA and CM-BODIPY, fluorochrome molecules coupled to a chloromethyl moiety. These molecules can traverse the cell membrane, and once inside the cell the chloromethyl group can react with protein thiol groups or glutathion. In this way the markers are covalently bound inside the cell. All these markers are redistributed among the daughter cells of every cell division, which leads to label dilution as the cells proliferate. An overview of the labeling characteristics of the different molecules is given, and standard labeling protocols are described. Cells from the in vitro growing cell line LT12NL15, which was derived from the Brown Norway Myelocytic Leukemia model (BNML), were labeled with the markers and experiments which show their influence on cellular kinetics are presented. Only with PKH2 some initial toxicity was observed, while none of the other markers changed this cell line's growth curve. A shift of the mean fluorescence intensity is demonstrated with PKH2 labeled LT12NL15 cells, and it is also shown that the half-life of this fluorescence decay does not correspond to the doubling time of the cell culture. CM-FDA and CM-BODIPY also exhibited unexpected fluorescence decay patterns. As the most likely explanation self quenching of the emitted fluorescence is assumed. Finally it is shown that none of the four markers has any effect on the formation of cobblestone areas from murine bone marrow in a CAFC assay, which indicates that these markers can be safely used for marking pluripotent hematopoietic stem cells in homing experiments.

In **Chapter 3** the use of PKH26 as a marker to stain a population of murine stem cells that consists of CFU-S is described. After staining these cells were injected into irradiated or non-irradiated mice, and at different time intervals the numbers of labeled cells were quantified, and their fluorescence was measured, serving as an indicator of any proliferative activity of the cells. It is clearly seen here that the key problem in performing realistic in vivo homing assays is the low frequency of the labeled cells. To solve this problem a flow cytometric method is presented, which allows the detection of labeled cells up to 3 days after injection, to a frequency of approximately 1 per 10^6. It is observed that spleen homing is identical in

irradiated and non-irradiated animals, whereas the homing to the bone marrow is 2.5 times reduced in irradiated animals, supporting the notion that homing to spleen and bone marrow are mediated through different mechanisms. In the general discussion of this Chapter a formal analysis is performed, which shows that through this new method an extra parameter, the h-factor, can be measured in this transplantation system, so that the homing selectivity to bone marrow and spleen can be determined. This may be used to gain insight into the organ specificity of the homing of the stem cell subset that is used in an experiment. From the rapid reduction of the cellular fluorescence it is concluded that the cells rapidly proliferate upon injection into the animals. It is suggested that this may be caused by a failure of the majority of the cells to reach a niche where they receive specific "quiescence" signals.

In **Chapter 4** it is described how a large number of LacZ and Neo^R expressing cell lines is produced from LT12 cells, a leukemic cell line derived from the Brown Norway rat. LacZ and Neo^R are marker genes whose expression leads to detectable levels of ß-galactosidase activity (LacZ) or to neomycin resistance (Neo^R). Extensive analysis of the expression stability in these sublines of both marker genes in vivo as well as in vivo is presented. Most noticeable is the frequent loss of expression of the Neo^R gene, especially after in vivo passaging. LacZ generally showed more stable expression, although here, too, a variety of expression patterns was observed. One subline, LT12NL15 was selected for further experiments. This cell line exhibits homogeneous, stable and high expression of LacZ in every individual cell.

A kinetic assay was developed, described in **Chapter 5**, using a fluorescent ß-galactosidase substrate. This assay allows quantification of the ß-galactosidase activity in cell lysates, which is a measure for the number of LT12NL15 cells in the starting material. Through the use of 100 mM D-galactose in the assay mixture the background activity of endogenous ß-galactosidases was reduced by a factor of 10, which eventually allowed a maximum detection sensitivity of 1 per 10^4 bone marrow cells.

This same method for the suppression of background activity from the rat cells was used to develop the much more sensitive "sticky plate assay", described in **Chapter 6**. In this assay the cells are fixed to flat bottom microwells, followed by overnight staining in an X-gal staining solution. LacZ positive cells acquire an intense blue staining, and can be easily detected when the wells are scanned through an inverted microscope. The detection limit of this method is approximately 1 per 10^8. Using this method detailed growth curves of LT12NL15 cells in bone marrow, spleen, thymus, liver and peripheral blood were obtained. From these curves it was concluded that the number of LT12NL15 cells in the bone marrow reaches a nadir as many as 24 hours after injection. Sticky plate and agar culturing results were compared in animals that were treated with cyclophosphamide at 11 days after injection of the LT12NL15 cells. Twenty-four hours after the treatment leukemic cells could no longer be detected using the agar culture assay. Control animals that were treated similarly survived for more than 6 months after the treatment and were therefore considered cured. However, with the sticky plate assay LacZ-marked leukemic cells were still detectable in the bone marrow

for more than 14 days, indicating that their numbers decreased only very slowly. It is concluded that highly sensitive detection methods such as the sticky plate assay and PCR assays, which cannot distinguish between live and dead cells, can easily produce false positive results. Furthermore the results showed that eventually the number of leukemic cells decreased to below the detection limit. Based on this it is speculated that the sometimes observed long-term presence of very low numbers of leukemic cells in the bone marrow of leukemia patients indicate that there is production of leukemic cells at a very low rate in these patients, possibly by leukemic stem cells that reside in sub-optimal niches. If one of these leukemic stem cells or one of its offspring would reach a more favourable niche, then that might be the event that triggers the occurrence of a leukemia relapse.

In **Chapter 7** an immunohistochemical staining method is presented for the detection of LacZ in paraffin sections of decalcified rat bones. LacZ is normally detected in frozen tissue sections. However, it is quite difficult to prepare frozen sections from femora and tibiae of rats and mice. Paraffin sections of decalcified bones can be routinely prepared, and allow detailed histological analysis. Until now LacZ could not be detected in paraffin sections due to loss of enzyme activity caused by the embedding procedure. The method described in this Chapter is based on the use of polyclonal antibodies to LacZ. To achieve optimal reliability of the procedure, an amplification step was added, based on in situ deposition of biotinylated tyramine radicals. In sections prepared with this method it was observed that the in vivo growth pattern of the cell line LT12NL15 is not associated with any distinct structure in the bone marrow. This leukemia initially forms loosely associated clusters of cells in the bone marrow, and even during the later stages the growth pattern does not become entirely diffuse throughout the bone marrow.

Finally, **Chapter 8**, the General Discussion, places the contents of this thesis in perspective by identifying the most relevant issues within the context of homing and adhesion, and by indicating the role of the described methods in resolving them.

Samenvatting

Samenvatting

Rijpe, functionele bloedcellen ontstaan via een complex differentiatieproces uit pluripotente hematopoietische stamcellen. Terwijl de cellen dit differentiatietraject afleggen ondergaan ze structurele veranderingen die het mogelijk maken om een groot aantal differentiatiestadia te onderscheiden (zie Fig. 1.1). Dit proces heet hematopoiese. De hematopoiese is bij volwassen zoogdieren, waaronder de mens, voornamelijk in het beenmerg gelokaliseerd. Regulatie van de hematopoiese vindt plaats door een reeks van interacties tussen hematopoietische cellen en hun micro-omgeving, waarbij belangrijke rollen zijn weggelegd voor groeifactoren en hun receptoren, extracellulaire matrix moleculen en speciale adhesiemoleculen. Die plekken in het beenmerg waar de stamcellen precies de juiste combinatie van regulerende interacties vinden worden "niches" genoemd.

Tijdens de embryogenese ondernemen de stamcellen via de foetale bloedcirculatie een aantal gerichte migratiestappen van de dooierzak naar de foetale lever en vandaar naar milt en beenmerg. Deze gerichte migratie wordt aangeduid met de term "homing". Iets vergelijkbaars vindt plaats bij beenmergtransplantatie: stamcellen worden in de bloedbaan van de ontvanger geïnjecteerd en vinden zelf hun weg naar de niches in het beenmerg van waaruit ze een compleet hematopoietisch systeem kunnen opbouwen. Ook dit wordt homing genoemd.

Door een maligne transformatie van een hematopoietische cel kan er leukemie ontstaan. De cel onttrekt zich dan aan de normale regulerende stimuli en gaat ongebreideld groeien. Daarbij worden de normale hematopoietische cellen uit hun niches in het beenmerg verdrongen en ontstaat uiteindelijk een levensbedreigend tekort aan functionele bloedcellen. Hoewel ze niet op de juiste manier reageren op de regulerende stimuli in de niches zijn leukemische cellen net als normale hematopoietische stamcellen in de meeste gevallen voor hun groei en overleving afhankelijk van niches. Bovendien blijkt uit het gegeven dat leukemische cellen transplanteerbaar zijn dat ze ook in staat zijn tot homing.

Dit proefschrift beschrijft de ontwikkeling van technieken om de homing van hematopoietische stamcellen en leukemie-cellen te bestuderen. Deze technieken kunnen gebruikt worden om een nader begrip te verkrijgen van de precieze mechanismen en moleculen die hierbij een rol spelen. Dit kan mogelijk leiden tot de ontwikkeling van methoden ter beïnvloeding van homing die bruikbaar kunnen zijn bij stamceltransplantatie, gentherapie en de behandeling van leukemie.

In **Hoofdstuk 1** wordt aan de hand van de wetenschappelijke literatuur een overzicht gegeven van adhesie en homing in een aantal goed gekarakteriseerde fysiologische systemen: de recirculatie van naïeve lymphocyten tussen het bloed en perifere lymfeklieren en Peyerse plaques, de homing van memory- en effector lymphocyten subsets naar extralymfatische lokalisaties

in slijmvies-epithelia en de huid, en de homing van lymphocyten, neutrofiele granulocyten en monocyten naar ontstekingshaarden.
Vervolgens wordt aandacht besteed aan een niet-fysiologische vorm van homing: de metastase van kankercellen. Aan de hand van de belangrijkste families van adhesiemoleculen worden gegevens besproken waar een correlatie tussen een verhoogde kans op metastasevorming en verhoogde expressie of activiteit van een adhesiemolecuul kan duiden op een rol voor het betreffende molecuul bij de homing van de tumorcellen.
Als volgende stap wordt een overzicht gegeven van wat er bekend is van homing en adhesie van hematopoietische stamcellen. Er blijkt een enorm aantal verschillende adhesieve interacties tussen stamcellen en stromale elementen mogelijk te zijn. De meeste van de beschreven studies zijn echter uitsluitend gebaseerd op in vitro systemen, en het is niet zeker dat in vivo ook inderdaad deze interacties voorkomen. Hier wordt dan ook gesteld dat in vivo experimenten nodig zijn om die interacties te identificeren die daadwerkelijk in vivo relevant zijn.
Tenslotte wordt een basaal overzicht geschetst van de mogelijkheden die er zijn om cellen zodanig te markeren dat ze kunnen worden gebruikt in in vivo homing experimenten. Hierbij wordt onderscheid gemaakt tussen genetische, permanente markers en transiënte markers.
In **Hoofdstuk 2** worden vier transiënte markers besproken uit twee verschillende klassen: 1) PKH2 en PKH26, fluorescerende lipofiele moleculen die in de celmembraan kunnen worden ingebouwd, en 2) de fluorescerende chloromethyl-markers CM-FDA en CM-BODIPY, fluorochroommoleculen die zijn gekoppeld aan een chloromethylgroep. Deze moleculen kunnen gemakkelijk de celmembraan passeren, en eenmaal in de cel kan de chloromethylgroep reageren met thiolgroepen van eiwitten of van glutathion. Op die manier worden deze markermoleculen covalent vastgelegd in de cel.
Voor alle vier deze markers geldt dat ze worden verdeeld tussen de dochtercellen van elke celdeling, wat leidt tot uitverdunning van het label naarmate de cellen prolifereren. In het hoofdstuk wordt een overzicht gegeven van de kleuringseigenschappen van de verschillende moleculen aan de hand waarvan standaard kleuringsprotocollen worden vastgesteld. Vervolgens worden experimenten met gelabelde cellen van de leukemische cellijn LT12NL15 besproken om de invloed van de verschillende labels op de celkinetiek te bestuderen. Slechts bij PKH2 was sprake van enige toxiciteit, de andere labels hadden geen invloed op de groeikinetiek van deze cellijn. Met PKH2 gelabelde LT12NL15 cellen wordt vervolgens getoond dat de gemiddelde fluorescentie per cel verschuift naarmate de cellen prolifereren, maar dat de halfwaardetijd van die vermindering niet overeenstemt met de verdubbelingstijd van de celcultuur. Bij CM-FDA en CM-BODIPY werden eveneens afwijkende fluorescentie-afnamepatronen waargenomen. Als meest waarschijnlijke verklaring wordt self-quenching van de geëmitteerde fluorescentie aangenomen. Tenslotte wordt aangetoond dat geen van deze markers de kinetiek van de vorming van cobblestone areas in een CAFC-assay beïnvloedt, wat erop wijst dat deze markers bruikbaar zijn voor het markeren van pluripotente stamcellen voor homingsexperimenten.

In **Hoofdstuk 3** wordt het gebruik van PKH26 beschreven als marker voor het kleuren van een populatie gesorteerde muizestamcellen die voornamelijk uit CFU-S bestaat. Na kleuring werden deze cellen ingespoten in bestraalde of onbestraalde muizen en op verschillende tijden werd gekeken hoeveel van de geïnjecteerde cellen konden worden teruggevonden, en hoe de fluorescentie veranderd was als indicatie voor de eventuele delingsactiviteit van de cellen. Daarbij werd duidelijk dat de lage frequentie van gelabelde cellen het grote probleem vormt in een realistische in vivo homing-assay. Om dit probleem aan te pakken wordt een flow cytometrische methode gepresenteerd, waarbij de gelabelde cellen tot drie dagen na injectie kunnen worden gedetecteerd tot een frequentie van ca. 1 per 10^6. Uit de resultaten blijkt dat de homing van de cellen naar de milt vergelijkbaar is in bestraalde en onbestraalde dieren, terwijl de homing naar het beenmerg met ca. een factor 2.5 gereduceerd. Dit ondersteunt het idee dat homing naar milt en naar beenmerg via een ander mechanisme verlopen. In de discussie bij dit hoofdstuk wordt aan de hand van een formele analyse aangetoond dat door het toepassen van deze methode een voorheen niet meetbare parameter in het systeem nu wel meetbaar is, waardoor nu ook de selectiviteit van de homing van de cellen uit een niet 100% zuivere stamcelsubset naar beenmerg en milt kan worden bepaald. Dit kan een indruk geven van de mate waarin de homing orgaanspecifiek is voor de betreffende stamcelsubset. Uit de waargenomen snelle afname van de fluorescentie van de gelabelde cellen wordt afgeleid dat de cellen meteen na injectie snel prolifereren. Er wordt verondersteld dat dit zou kunnen worden veroorzaakt doordat een groot deel van de geïnjecteerde cellen geen niches bereikt waar ze signalen zouden kunnen ontvangen die proliferatie voorkomen.

Hoofdstuk 4 beschrijft hoe van de LT12 cellijn, een van het Brown Norway rat acute Myeloïde Leukemie model (BNML) afgeleide leukemische cellijn, door middel van een retrovirale vector een groot aantal LacZ en Neo^R gemarkeerde sublijnen werden verkregen. De analyse van de stabiliteit van de expressie van de beide markergenen zowel in vitro als in vivo wordt beschreven. Daarbij is het opvallend dat vooral na in vivo passage vaak de expressie van het Neo^R gen werd afgeschakeld. LacZ bleek in de meeste lijnen stabieler tot expressie te komen, ofschoon ook hier een scala aan verschillende expressiepatronen werd waargenomen. Uiteindelijk werd de cellijn LT12NL15 geselecteerd voor verdere experimenten. Deze cellijn heeft een homogene, stabiele en hoge expressie van LacZ in elke individuele cel. Voor deze LT12NL15 cellen werd een kinetische assay ontwikkeld, gebruikmakend van een fluorescerend substraat voor ß-galactosidase. Dit wordt beschreven in **Hoofdstuk 5**. Hiermee kan de hoeveelheid ß-galactosidase activiteit in een cellysaat worden bepaald, en daarmee ook het aantal LT12NL15 cellen in het uitgangsmateriaal. Door het gebruik van 100 mM D-galactose in de meetoplossing kon de achtergrondactiviteit afkomstig van endogene ß-galactosidases uit de rattecellen met een factor 10 worden verlaagd, en werd uiteindelijk een maximale detectiegevoeligheid van 1 per 10^4 bereikt.

Deze zelfde methode om de achtergrondactiviteit van endogene ß-galactosidases van de rattecellen te onderdrukken werd gebruikt om de veel gevoeliger "sticky plate assay" te ontwikke-

len, beschreven in **Hoofdstuk 6**. Hierbij worden de te analyseren cellen door middel van een fixatief aan de bodem van een microtiterplaat vastgeplakt. Vervolgens wordt overnacht gekleurd met een X-galoplossing. De LacZ-positieve cellen worden hierbij intens blauw gekleurd, en kunnen gemakkelijk worden herkend bij inspectie door een omkeermicroscoop. De detectielimiet van deze methode ligt rond de 1 per 10^8. Met behulp van deze methode werden gedetailleerde in vivo groeicurves van de LT12NL15 cellen in beenmerg, milt, thymus, lever en perifeer bloed verkregen. Hieruit bleek dat pas 24 uur na inspuiten het aantal LT12NL15 cellen in het beenmerg van de rat het laagste punt bereikt. Sticky plate en agarkweek-analyse resultaten werden vergeleken in dieren die 11 dagen na het inspuiten van de LT12NL15 cellen werden behandeld met het cytostaticum cyclophosphamide. Vierentwintig uur na behandeling bleken er in de agarkweken geen LT12NL15 cellen meer aantoonbaar. Bovendien overleefden controledieren de behandeling langer dan zes maanden, en werden derhalve als genezen beschouwd. In de sticky plate assay bleek daarentegen dat leukemiecellen nog gedurende meer dan 14 dagen in het beenmerg detecteerbaar waren, en dus relatief langzaam in aantal afnamen. Dit leidde tot de conclusie dat zeer gevoelige detectiemethoden, zoals sticky plate en PCR assays, die niet kunnen onderscheiden of een maligne cel nog leeft of niet, vrij gemakkelijk vals positieve resultaten kunnen opleveren. Anderzijds lieten de resultaten zien dat uiteindelijk de leukemiecellen wel gestaag in aantal afnamen, tot beneden het detectieniveau. Aan de hand hiervan wordt gespeculeerd dat de soms na behandeling langdurig detecteerbare cellen in leukemie patienten erop wijzen dat er ergens in het beenmerg nog een trage productie van leukemiecellen plaatsvindt, mogelijk door leukemische stamcellen in ongunstige niches. Wanneer zo'n maligne stamcel of een van zijn nakomelingen in een gunstiger niche zou terechtkomen zou dat mogelijk het moment kunnen zijn dat het aantal leukemische cellen gestaag gaat toenemen en uitmondt in een leukemierecidief.
In **Hoofdstuk 7** wordt de ontwikkeling van een histochemische kleuring voor LacZ op paraffinecoupes van ontkalkte rattebotjes beschreven. Voor het detecteren van LacZ gemarkeerde cellen gebruikt men in het algemeen vriescoupes. Van femora en tibiae, die meestal worden gebruikt voor het bestuderen van beenmerg van proefdieren, kunnen slechts met grote moeite vriescoupes gemaakt worden. Paraffinecoupes van ontkalkte botten zijn routinematig te vervaardigen en geven een gedetailleerd histologisch beeld van het beenmerg. Tot nu toe was het niet mogelijk in paraffinecoupes LacZ aan te tonen, omdat door het inbedden de enzymactiviteit verloren ging. In de in dit hoofdstuk beschreven methode wordt gebruik gemaakt van polyclonale antilichamen om LacZ aan te kleuren. Om de hele procedure betrouwbaarder te maken werd bovendien een versterkingsstap toegevoegd, gebaseerd op in situ depositie van gebiotinyleerde tyramine-radicalen. Aan de hand van coupes die met deze methode waren gekleurd werd het duidelijk dat het in vivo groeipatroon van de cellijn LT12NL15 niet geassocieerd is met een duidelijk herkenbare structuur in het beenmerg. Deze leukemie vormt in eerste instantie losse groepjes cellen in het beenmerg en verspreidt zich ook in een laat stadium niet volledig diffuus door het hele beenmerg.

Tenslotte wordt in **Hoofdstuk 8**, de algemene discussie, dit proefschrift in een breder perspectief geplaatst door na te gaan wat precies binnen het kader van de homing van hematopoietische stamcellen en leukemiecellen de meest prangende vragen zijn, en hoe de beschreven methoden gebruikt kunnen worden bij het beantwoorden daarvan.

Curriculum Vitae

The author of this thesis was born on 30 July, 1962 in Arcen, The Netherlands. In July 1980 he graduated from Grammar school at the St. Thomas College in Venlo, The Netherlands.
In September 1980 he went to the Catholic University of Nijmegen. As a graduate student he participated in several research projects. At the department of Genetics: development of immuno-EM in the study the role of lampbrush chromosomes in spermatogenesis of Drosophila hydei. At the department of Botany: comparative field studies of vegetation regeneration of ski-slopes and basic classification studies of Rhododendron-shrubs. At the departments of Hematology and Nephrology of the St. Radboud University Hospital, Nijmegen, The Netherlands: production and characterization of antibodies to CALLA (CD10), and purification and characterization of CALLA (CD10) protein. During extracurricular courses he acquired the "First degree" certificate for teaching biology and the Radiation protection "Level 3" certificate. Graduation as a biologist followed in May 1988.
From November 1988 to March 1989 he worked as a research assistant at the Central Haematology Laboratory, St. Radboud University Hospital, Nijmegen, The Netherlands. This was followed by his appointment as a research assistant to the Radiobiological Institute, Rijswijk, The Netherlands from April 1989 to April 1991. Here he joined the research groups of Prof. Dr. J. W. M. Visser and Prof. Dr. A. Hagenbeek. He was responsible for the daily operation of RELACS II flow cytometer and the sorting of murine hematopoietic stem cells. Here, too, the work began on the development of protocols for application of fluorescent cell-tracking dyes and the development of a LacZ marked leukemic cell line, and of protocols for LacZ detection and for suppression of mammalian ß-galactosidase activity. From April 1991 to January 1996 he was appointed as a PhD Student on a grant from the Dutch Cancer Society at the TNO Institute of Applied Radiobiology and Immunology, Rijswijk, The Netherlands. In January 1993 he moved with the research group of Prof. Dr. Hagenbeek to form the Department of Developmental Therapeutics at the Institute of Hematology, Erasmus University Rotterdam, The Netherlands.
As of July 1996 Jan Hendrikx is building forth on the research described in this thesis as a postdoc researcher with the Stem Cell Laboratory of the Lindsley F. Kimball Research Institute at the New York Blood Center, New York, NY, USA.

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