Novel Approaches in the Detection and Characterisation of Circulating and Micrometastatic Tumour Cells in Epithelial Malignancies

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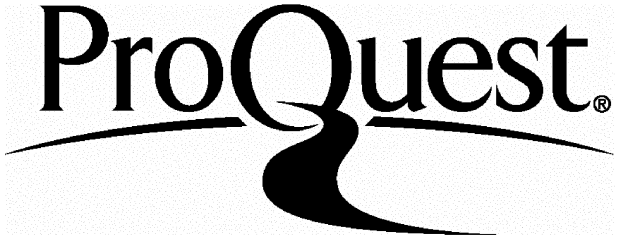

This thesis is dedicated in memory of my late

Grandmother – Sophia Panteli

"ΝΑ ΣΕ ΔΩ ΓΙΑΤΡΟ"

("I wish to see you a doctor")

Abstract

The detection of disseminated tumour cells has introduced a new opportunity to evaluate the diverse biological characteristics of the primary tumour that might favour the early dissemination of its cells, since conventional risk factors do not provide specific or sensitive enough information. An immunocytochemical (ICC) assay established during the study, was shown to be capable of detecting one cytokeratin-positive (CK^{+ve}) cell in 2 x 10^6 mononuclear cells (MNCs), by attachment to microscope slides coated with Cell-Tak® Cell and Tissue Adhesive. The clinical data indicate that 57% of samples examined from patients undergoing therapy for carcinoma of the breast (BrCa), showed detectable CK^{+ve} cells in peripheral blood (PB) or bone marrow (BM) aspirate samples. The incidence of tumour cell contaminated PB samples was higher in patients with metastatic disease than patients without overt metastatic disease ($p<0.0001$). In contrast, all control samples consistently tested negative for circulating CK^{+ve} cells.

Detection of micrometastases may also help to determine prognosis and allow development of new therapeutic approaches. In a study of the impact of chemotherapy on the presence of tumour cells in the PB, 24/33 high-risk non-metastatic BrCa patients had detectable tumour cells pre-chemotherapy (steady state). A reduction, but not complete eradication, in the number of circulating CK^{+ve} cells was observed in all patients during subsequent courses of chemotherapy ($p<0.0001$). This demonstrates that chemotherapy is not effective in eliminating all PB/BM tumour cells even in chemo-responsive patients.

In addition, the phenotype of these cells may determine whether overt metastases will develop. A highly sensitive assay combining tumour cell enrichment with immunolabelling and fluorescence *in situ* hybridization (FISH), was developed to characterise cytogenetically aberrant tumour cells in haematopoietic samples. Using DNA probes to the Her-2/*neu* (c-*erb*B-2) oncogene and chromosomes 7, 8, and 17 hybridized to metaphase chromosomes and immunolabelled interphase cells, oncogene overamplification and chromosomal alterations have been established. Additional studies have confirmed an association between the level of Her-2/*neu* gene amplification, clinical status and response to chemotherapy. Genotypic analyses suggest that Her-2/*neu* overexpression may enhance metastatic potential possibly by promoting steps in the invasion and metastasis process and that "shed" Her-2/*neu*$^{+ve}$/CK^{+ve} PB tumour cells might "home" to the BM, resulting in aggressive tumour behaviour and a poor prognosis.

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Abbreviations

#	chemotherapy course number
χ^2	Chi-square test
β-hCG	β-subunit of human chorionic gonadotropin
Ab	antibody
AC	adriamycin, cyclophosphamide
Ag	antigen
AMCA	amino-methyl-coumarin-acetic acid
AP	alkaline phosphatase
APAAP	alkaline phosphatase anti-alkaline-phosphatase
BM	bone marrow
BN	0.1M $NaHCO_3$, 0.05% NP-40, pH 8.0
BPH	benign prostatic hypertrophy
BrCa	breast cancer
BSA	bovine serum albumin
c.v.	coefficient variation
Ca	cancer
CCD	cooled coupled device
CD	cluster designation
cDNA	complementary DNA
CEA	carcinoembryonic antigen
CGH	comparative genomic hybridization
CI	confidence interval
CIN	cervical intraepithelial neoplasia
CK	cytokeratin
CK^-	cytokeratin-negative
CK^+ or CK^{+ve}	cytokeratin-positive
CMF	cyclophosphamide, methotrexate, 5-fluorouracil
CR	complete response
CT	computed tomography
Cy	cyclophosphamide
Cy3	cyanine 3
DAPI	4,6-diamidino-2-phenylindole
DB^+	double positive: (cytokeratin/Her-2/*neu*)$^+$
DFS	disease-free survival
DM	double minute
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
Dox.	doxorubicin
D-PBS	Dulbecco's-phosphate-buffered saline
EDTA	ethylene-diaminetetra-acetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
EMA	epithelial membrane antigen
EpCAM	epithelial cell adhesion molecule (17-1A)
ER	oestrogen receptor
FBS	foetal bovine serum
FISH	fluorescence *in situ* hybridization

FITC	fluorescein isothiocyanate
g	gravity
G-CSF	granulocyte-colony stimulating factor
Gy	Gray
hbFGF	human basic fibroblast growth factor
HBSS	Hank's balanced salt solution
HDC	high-dose chemotherapy
HDST	high-dose sequential therapy
HEA-125	human epithelial antigen-125
HEPES	N2-hydroxyethylpiperazine-N2-ethanesulphonic acid
hK2	human kallikrein-2
HLA	human leukocyte antigen
HMFG	human milk fat globule
HPV	human papillomavirus
HTC®	heavy polytetrafluoroethylene (Teflon®)-coated
ICAM-1	intercellular adhesion molecule-1
ICC	immunocytochemical
IF	immunofluorescence
Ig	immunoglobulin
ISHAGE	The International Society for Haematotherapy and Graft Engineering
kD(a)	kilodalton
Le^Y	Lewis Y
LFA-1	lymphocyte function-associated antigen-1
LOH	loss of heterozygosity
M	molar
MACS	high-gradient magnetic cell sorting
MAPK	mitogen-activated protein kinase
MEM	minimum essential medium
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
MNCs	mononuclear cells
MoAb	monoclonal antibody
MRD	minimal residual disease
MRI	magnetic resonance imaging
mRNA	messenger RNA
MUC	mucin
n or No.	number
NC	no change
NDF	neu differentiation factor
NK	not known
NP-40	Nonidet P-40
NSCLC	non-small-cell lung cancer
OS	overall survival
p	probability
PB	peripheral blood
PBS	phosphate-buffered saline
PBSC	peripheral blood stem cells
PBSCT	peripheral blood stem cell transplant
PCR	polymerase chain reaction
PN	0.1M NaH_2PO_4, 0.1M Na_2HPO_4, 0.1% NP-40, pH 8.0
PNM	5% non-fat dry milk, 0.02% NaN_3 in PN

POX	immunoperoxidase
PR	progesterone receptor
PSA	prostate-specific antigen
PSM	prostate-specific membrane antigen
pT	pathologically confirmed tumour
RB	retinoblastoma
rhmAb	recombinant humanised monoclonal antibody treatment
RNA	ribonucleic acid
RNase	ribonuclease
RP	radical prostatectomy
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute hydrogen-carbonate-buffered media
RT	room temperature
RT-PCR	reverse transcriptase-polymerase chain reaction
S.D.	standard deviation
SEM	standard error of mean
SSC	sodium salted citrate
TAG-12	tumour associated glycoprotein-12
TC	thiotepa, cyclophosphamide
TE	10mM Tris-HCl, 1mM EDTA, pH 8.0
TEC	tumour enrichment column
TNM	tumour-lymph node-metastasis classification
UICC	Union Internationale Contre le Cancer
uPA	urokinase plasminogen activator
VI	vascular invasion

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understanding whilst working on this project. Finally, a special mention must go to my wife, Demetra, not only for all her support, but especially her continual encouragement and belief in me, particularly during times of stress associated with the process of writing-up a PhD!

Chapter 1 Main Introduction

Malignant tumours of epithelial tissues are the most common form of cancer and are responsible for the majority of cancer-related deaths in Western industrialised countries. Because of progress in the surgical treatment of these tumours, mortality is linked increasingly to early metastasis, which is often occult at the time of diagnosis (Pantel and Riethmüller, 1996). For certain cancers e.g. breast/colorectal with no evidence of systemic metastases when the primary tumour is resected, traditional pathological staging parameters of the tumour (e.g., tumour size and grade and lymph node status) are determined; with this information and a statistical assessment of the risk of disease recurrence, the decision is made as to whether to give systemic adjuvant therapy to prevent metastatic relapse. Undetected micrometastases can contribute to the failure of primary treatment. Therefore, the identification of occult metastases in patients with early stage cancer could have a substantial clinical impact on the prognosis and optimal therapy for patients with cancer, provided effective treatment is available. For this reason, the improved direct identification of occult metastatic disease is particularly important. At later stages of the disease, it may be useful to determine the presence of and change in the number of residual malignant cells to determine appropriate choice and duration of treatment.

Occult dissemination of tumour cells in patients with operable cancer may be considered a determinant of subsequent metastasis formation, yet is usually missed by conventional tumour staging. Several groups have therefore designed immunocytochemical (ICC) (Table 1.1) and molecular assays to identify such micrometastases versus single cells.

Marker	Antibody	Disease Stages	Detection Rate	Tissue Prep^{n}.	Stain Tech.	Reference
Mucin	MBr1	I - III	20/121 (17%)	biopsy	IF	Salvadori *et al*, 1990
Mucin	LICR-LON-M8	I - III	4/50 (8%)	biopsy	POX	Courtemanche, 1991
CK, EMA	KL1	I - III	1/93 (1%)	biopsy	POX	Mathieu *et al*, 1990
EMA	E29	I - III	89/350 (25%)	smears	POX	Gogas *et al*, 1997
Mucin	LICR-LON-M8	I, II	12/25 (48%)	smears	POX	Kirk *et al*, 1990
EMA, CK	E29, K8/18/19	I - III	38/100 (38%)	smears	AP	Harbeck *et al*, 1994
TAG-12	2E11	I - III	315/727 (43%)	smears	AP	Diel *et al*, 1996
CK, TAG-12	AE1, C26, TI6	I - III	18/49 (37%)	smears	IF	Cote *et al*, 1991
CK	AE1, AE3	IV	27/71 (38%)	smears	AP	Singletary *et al*, 1991
CK	CK2	I - III	84/349 (24%)	cytospin	AP	Schlimok *et al*, 1992

Table 1.1: Immunocytochemical studies on the detection of micrometastatic bone marrow invoivement in breast cancer patients.

CK = cytokeratin; EMA = epithelial membrane antigen; TAG-12 = tumour-associated glycoprotein-12; IF = immunofluorescence; POX = immunoperoxidase; AP = alkaline phosphatase.

Among these organs, the bone marrow (BM) appears to be a clinically relevant indicator site for disseminated cancer cells in many epithelial malignancies, and has the advantage that it is easily accessed. The medullary space appears to be a site of intensive cell exchange between circulating blood and mesenchymal interstitium. The development of antibodies to epithelial differentiation antigens, such as cytokeratins, an essential constituent of the epithelial cytoskeleton, and tumour-associated cell membrane glycoproteins has opened a diagnostic window with the possibility to detect disseminated tumour cells as early as at primary diagnosis (Mansi *et al*, 1987; Schlimok *et al*, 1987). Along with emerging data in support of the prognostic relevance of this finding (Braun and Pantel, 1998) there is an urgent need for appropriate therapeutic approaches directed against micrometastatic cells. It is known from clinical practice that both locoregional and distant tumour recurrences occur in patients treated with curative intent, for example complete tumour resection (R_0) in patients without distant metastasis (M_0) – even if systemic cytotoxic chemotherapy is applied, which supports the existence of pre-existing chemotherapy resistant tumour cells.

Research into the molecular basis of tumour metastasis has identified numerous proteins that might influence this process (Figure 1.1). Conditions that allow growth of epithelial cells at metastatic sites are largely unknown but include the appropriate microenvironment for tumour cell growth (e.g., hormonal milieu, oxygenation, nutrients, or growth factors) and an environment for the formation of new blood vessels (angiogenesis). The factors determining the length of period from the dissemination of tumour cells until the appearance of clinically manifest metastases are also unclear. For instance, in analyses of single cells, most disseminated tumour cells in BM do not appear to be

proliferating at the time of primary surgery (Pantel *et al*, 1993a). For this reason, it may be important to use adjuvant therapies that are aimed at both proliferating and non-proliferating cells.

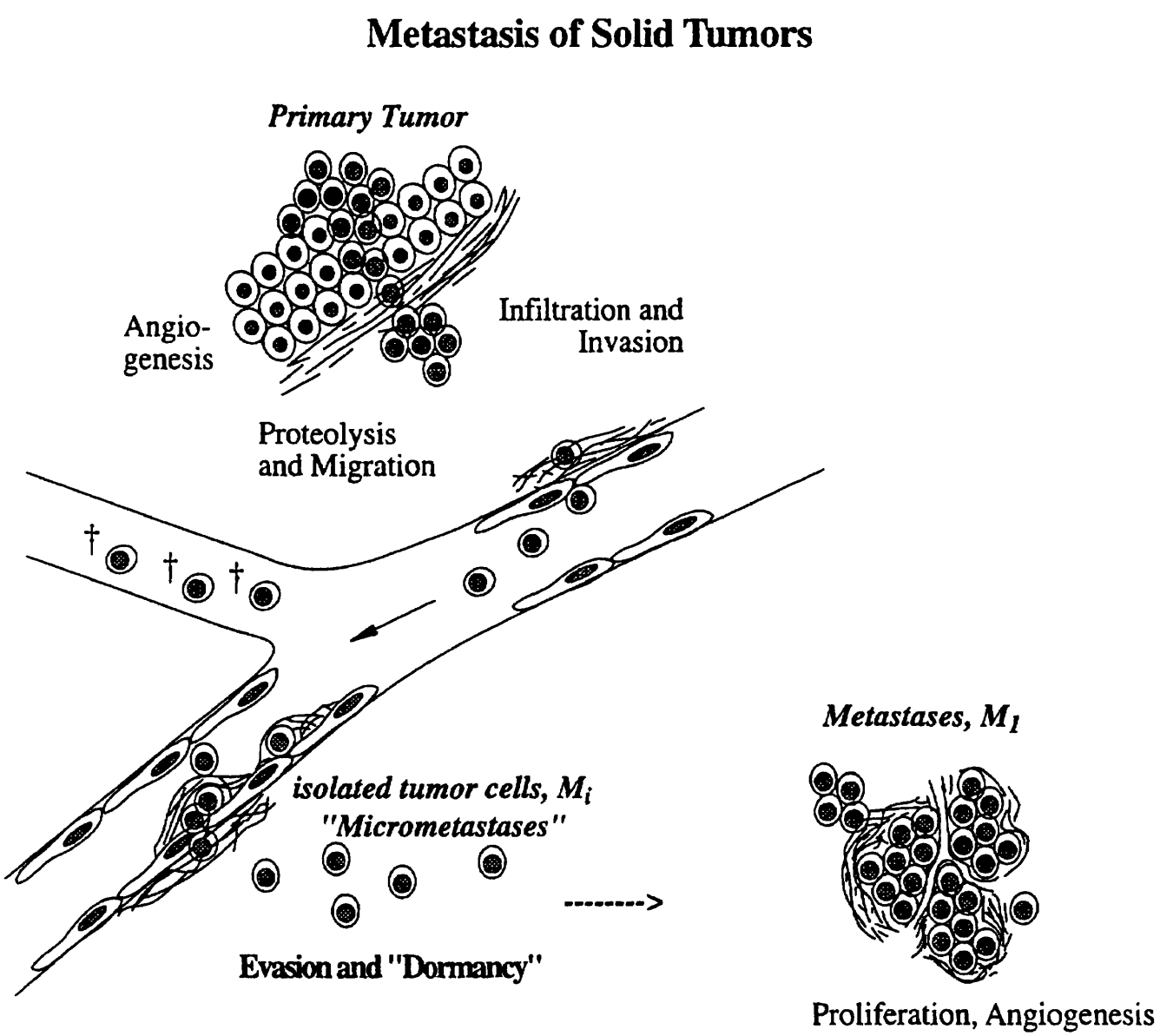


Figure 1.1: Diagram of tumour cell metastasis.

Cells from the primary tumour leave the tumour as a result of proteolysis, infiltrate and invade the circulatory system, and migrate to a new site where they adhere to the walls of the capillary and invade a new organ. At this site, micrometastases (i.e., isolated tumour cells or small clusters of tumour cells) that are undetectable by conventional tumour staging procedures are thought to be able to survive for several years. The cells can evade chemotherapy because they are in a dormant state. It has been is proposed that this stage be called "M_i". Later the cells can become proliferative, stimulate angiogenesis, and begin to form a metastatic tumour (stage M_1, tumour-lymph node-metastasis [TNM] classification system). Figure taken from Pantel *et al*, 1999.

1.1 Historical Background to the Development of Tumour Cell Detection

Although Ashworth first reported a case of cancer as early as 1869, in which cells similar to those in the patient's subcutaneous tumours were detected in the blood (examined under the microscope morphologically after the addition of magenta solution), the routine search for malignant cells in circulating blood was destined to fail because of the rarity of the event. Using a special staining-block technique, Engell (1955) was the first to detect circulating tumour cells in samples from groups of patients with various epithelial malignancies. The technique involved the haemolysis of cellular elements of heparinised blood using a 1% saponin solution. This was followed by the addition of citrated plasma which coagulated over the cells, and the coagulum treated according to ordinary histological technique, mounted in paraffin, cut into serial sections, and stained with Hansen's haematoxylin and 1% aqueous eosin. However, both specificity and sensitivity (1%) were low because of contamination with normal cells obtained from the peripheral and venous blood samples draining the tumour area at operation, and the lack of accurate quantitation of cells. Indeed, circulating marrow elements, especially megakaryocytes, were often confused with tumour cells, in the original studies reporting a very high rate of positivity (as high as 96%) among cancer patients.

In the initial studies, six to eight puncture sites (two each from sternum, sacrum, anterior and posterior iliac crests) were chosen from around the skeleton. Nowadays, almost all groups only take samples from two sites – either the two anterior or posterior iliac crests (Redding *et al*, 1983). Ingle *et al* (1978) studied 116 patients with primary and advanced breast cancer (BrCa) and correlated the results of BM examination, (biopsy [stained with

haematoxylin and eosin after processing] and aspirate smear [stained with a modified Romanovsky's stain]), with those of radiographic examination and bone scintigraphy. Positive bone biopsies were found in 55% of patients with positive radiograms, in 56% of patients with positive bone scans and overall in 40% of those with metastatic disease. In a comparable correlative study using haematoxylin stained bone biopsies, Landys (1982) found, 43%, 35% and 28.5%, respectively, in similar patient subgroups. While these initial results were obtained using bone biopsies, the detection rate was around 4% in patients with normal radiograms and scans. The advantage of the biopsy method clearly lies in the well-defined area of the sample and the possibility to investigate the surroundings of the tumour cells. Disadvantages are the low yield of tumour cells and the trauma to the patient during the biopsy procedure.

The detection of tumour cells in BM underwent a renaissance as a result of the introduction of immunocytological techniques. Between 1980 and 1982 Sloane, Dearnaley and co-workers from the Royal Marsden Hospital and the Ludwig Institute for Cancer Research in London presented a new method using tissue sections and BM aspirates. They were able to achieve detection rates for tumour residues in the region of 20-30% using a polyclonal anti-EMA antibody (epithelial membrane antigen) (Sloane *et al*, 1980a, 1980b; Dearnaley *et al*, 1981, 1983; Sloane and Ormerod, 1981). The description of these methods encouraged numerous groups in Europe and the USA to investigate tumour cell detection as a prognostic factor in various types of tumours.

1.2 Diagnostic Approaches of Micrometastasis Detection

1.2.1 Immunocytochemistry

To date, data on BM screening for cancer micrometastasis are almost exclusively based on ICC analyses. Bone marrow is an easily accessible site for aspiration, and is physiologically devoid of epithelial cells. Therefore, extrinsic epithelial cells can be discriminated from haematopoietic BM cells using monoclonal antibodies (MoAbs) directed against epithelial differentiation markers. Greatly divergent detection rates obtained from similar study populations, for example in BrCa as summarised in Table 1.1, however, made a systematic analysis of critical variables rather advisable. A detailed analysis of these reports showed that the ICC staining techniques and Abs used to detect disseminated cancer cells differed considerably, particularly with regard to the number of cells analysed, as well as different follow-up times and, in some cases, small numbers of patients (Pantel *et al*, 1994; Sloane, 1995; Braun *et al*, 1998a).

Since the specificity of the ICC assay for single tumour cells is one of the major concerns, and because haematopoietic BM cells produce endogenous peroxidase, alkaline phosphatase-based staining techniques are now preferred over immunoperoxidase methods applied for the detection of disseminated tumour cells in blood and BM (Mansi *et al*, 1988, 1991). Most investigators elected to use MoAbs rather than polyclonal anti-sera for tumour cell detection. Most of these MoAbs applied for epithelial tumour cell detection are directed to either cytokeratins (CKs) as major constituents of the epithelial cytoskeleton, or membrane-bound mucins, such as epithelial membrane antigen (EMA), human milk fat globules (HMFG), human epithelial antigen-125 (HEA-125) or tumour-associated glycoprotein-12 (TAG-12).

Analysis of a large series of non-carcinoma control patients, (Schlimok *et al*, 1990; Pantel *et al*, 1996; Braun *et al*, 1998a) however, revealed that the specificity of MoAbs to CK was superior to that of MoAbs against mucins (Table 1.2).

Antibody	Antigen	Fraction of BM Samples With Immuno-reactive Cells (%)
CK2[a]	CK18	6/215 (3%)
A45-B/B3[b]	pan-CK	2/165 (1%)
E29[c]	epithelial membrane antigen, EMA	20/75 (27%)[d]
2E11[b]	tumour-associated glycoprotein-12, TAG-12	66/105 (63%)[d]
HMFG1[c]	human milk fat globule, HMFG	32/75 (43%)[d]

Table 1.2: Immunocytochemical staining of bone marrow from non-carcinoma control patients.

[a]Per BM sample 4 x 10^5 mononucleated cells were stained (Schlimok *et al*, 1987; Pantel *et al*, 1994).

[b]Per BM sample 2 x 10^6 mononucleated cells were stained (Braun *et al*, 1998a).

[c]Per BM sample 1.5 x 10^5 mononucleated cells were stained (Schlimok *et al*, 1987).

[d]$p<0.001$ compared to CK2 or A45-B/B3 immunostaining (χ^2 test).

The epithelial nature of CK-positive (CK^+) cells in BM was also supported by double labelling analyses. To resolve concerns that single CK^+ cells are rare haematopoietic cells with aberrant CK expression, it was demonstrated that neither the leukocyte common antigen CD45 nor the mesenchymal intermediate filament protein vimentin were co-expressed by CK^+ cells (Schlimok *et al*, 1987; Pantel *et al*, 1994). In prostate cancer (prostate Ca) patients, disseminated CK^+ tumour cells exhibited co-expression of the prostate-specific antigen (PSA) in about 40% of cases, an incidence being consistent with the rate of PSA co-expression in primary carcinomas and the LNCap prostate Ca cell line (Gallee *et al*, 1986). Taken together, these findings suggested that these disseminated CK^+ cells were descendants of the epithelial cancer.

To estimate the sensitivity of the ICC assay, previous methodological studies have used surrogate model systems of BM samples seeded with cancer cells from cell lines, demonstrating that the technique can detect 2-4 cells at a concentration of 10 per 10^6 and, by extrapolation, a 95% chance of detecting one cancer cell at a concentration of 2 per 10^6 (Molino *et al*, 1991; Osborne *et al*, 1991).

Recent studies implied that a certain minimal amount of tumour cells might be required to initiate subsequent overt metastasis (Cote *et al*, 1991; Jauch *et al*, 1996). Determination of such a minimal residual tumour load required an exact quantification of the residual tumour burden which so far has not been performed in most earlier studies due to the use of cell smears (Cote *et al*, 1991; Diel *et al*, 1992, 1996; Harbeck *et al*, 1994). This technique does not permit a reproducible quantitative transfer of cells to the slide surface. More reproducible results were obtained by cytocentrifugation, allowing the transfer of a well-defined cell number to slides (Schwarz, 1991; Pantel *et al*, 1994).

Using an anti-CK Ab on cytopreparations of mononuclear cells (MNCs) isolated from BM aspirates, disseminated cancer cells were found in approximately 25% to 60% of patients with non-metastatic carcinomas (stage M_0, [see Section 1.3 – Clinical Relevance of Bone Marrow Micrometastasis]). However, most of the patients had fewer than 10 tumour cells per 8 x 10^5 mononuclear BM cells. As fewer cells are generally examined in histochemical analyses of BM biopsy sections, the prognostic value of this approach was regarded as very limited.

Thus far, little is known about the prognostic relevance of the residual tumour cell burden and the optimal time of BM screening. With a quantitative transfer of cells to the slides using a cytocentrifuge, Jauch *et al* (1996) were able to show for the first time that the residual tumour cell count correlates with the relapse rate in gastric carcinoma. In another study by the same group, the prognostic value of follow-up examinations was also confirmed (Heiss *et al*, 1995). In fact, the repeated identification over a 2-year period of tumour cells in the BM of patients with operable gastric carcinoma had even greater prognostic value than the identification of disseminated cells when the primary tumour was resected.

In a very recent report (Cote *et al*, 1999), immunohistochemical techniques were used to identify spread of cancer cells in the PB and lymph nodes. For both parameters an independent prognostic influence was delineated. This suggested that both lymphatic and haematogenous spread of rare single isolated cancer cells are independent, but clinically highly relevant, prognostic factors.

The time-consuming microscopic screening of large numbers of cytologic samples can now be facilitated through an automated analysis of stained

preparations using computerised image-analysis systems. As an alternative, Abs coupled to small magnetic beads have been used to enrich disseminated cancer cells from BM and blood samples (Naume *et al*, 1997, 1998; Makarovskiy *et al*, 1997; Martin *et al*, 1998; Ghossein *et al*, 1999a). The enriched tumour cell fraction can then be further characterised, using immunocytochemistry, cell culture, or nucleic acid-based techniques.

1.2.2 Nucleic Acid-Based Techniques for the Detection of Disseminated Cancer Cells

Molecular detection procedures have been used extensively to identify residual tumour cells in BM; for example, follicular lymphoma cells have been detected by specific genetic changes (bcl-2 translocation and immunoglobulin gene rearrangements) (Gribben *et al*, 1991; Zwicky *et al*, 1996). More recently, a number of molecular techniques have been used to detect disseminated cancer cells in various tissue samples (von Knebel Doeberitz and Lacroix, 1999). In principle, either DNA- or RNA-based markers can be used (Table 1.3), both with specific advantages, but also critical limitations (detailed below).

Tissue	Tumour Organ	Messenger RNA/DNA Marker(s)* (References)
Bone Marrow	Breast	CK19, CEA (Gerhard *et al*, 1994; Fields *et al*, 1996)
	Colorectum	Ki-ras mutations, CEA, CK19, CK20 (Gerhard *et al*, 1994; Gunn *et al*, 1996; Soeth *et al*, 1997; Dietmaier *et al*, 1999)
	Stomach	CEA, CK20 (Gerhard *et al*, 1994; Soeth *et al*, 1997)
	Pancreas	CEA (Gerhard *et al*, 1994)
	Prostate	PSA, CK19, hK2 (Corey and Corey, 1998)
	Head and neck	E48 antigen (Brakenhoff *et al*, 1999)
Lymph nodes	Breast	CK19, MUC1, β-hCG (Hoon *et al*, 1996; Noguchi *et al*, 1996; Schoenfeld *et al*, 1996)
	Lung	p53, Ki-ras mutations (Ahrendt *et al*, 1997)
	Cervix	HPV16, E6/E7 (Czegledy *et al*, 1995)
	Colorectum	p53 and Ki-ras mutations, CEA, CK19, CK20 (Hayashi *et al*, 1995; Gunn *et al*, 1996; Liefers *et al*, 1998)
	Pancreas	Ki-ras mutations (Tamagawa *et al*, 1997)
	Prostate	PSA, PSM, hK2 (Corey and Corey, 1998)
	Head and neck	p53 mutations (Brennan et al, 1995)
Blood	Breast	CK19, EGF receptor, β-hCG (Hoon *et al*, 1996; Hildebrandt et al, 1997; Mapara *et al*, 1997)
	Lung	Microsatellite alterations (Chen *et al*, 1996)
	Colorectum	CK20, Ki-ras mutations (Burchill *et al*, 1995; Hardingham *et al*, 1995; Soeth *et al*, 1997)
	Stomach	CK20 (Soeth *et al*, 1995)
	Pancreas	Ki-ras mutations (Tada *et al*, 1993)
	Liver	α-Fetoprotein (Matsumura *et al*, 1994)
	Prostate	PSA, PSM, hK2 (Corey and Corey, 1998)
	Head and neck	Microsatellite alterations (Nawroz *et al*, 1996)
Liver	Pancreas	Ki-ras mutations (Inoue *et al*, 1995)
Tumour resection margins	Head and neck	p53 mutations (Brennan *et al*, 1995)

Table 1.3: Detection of disseminated epithelial tumour cells by molecular methods.

*CK = cytokeratin; CEA = carcinoembryonic antigen; PSA = prostate-specific antigen; MUC = mucin; HPV = human papillomavirus; EGF = epidermal growth factor; PSM = prostate-specific membrane antigen; β-hCG = β subunit of human chorionic gonadotropin; and hK2 = human kallikrein-2.

Specific mutations of defined DNA sequences, for example proto-oncogenes (*k-ras*) and tumour suppressor genes (*p53*) (Hayashi *et al*, 1994, 1995; Sanchez-Cespedes *et al*, 1999); changes of the methylation status of defined genes (e.g., *p16*) (Belinsky *et al*, 1998); microsatellite instability (Mao *et al*, 1996); or even sequences of carcinogenic viruses (Kobayashi *et al*, 1998) have been used as markers. Because these alterations are not present in normal human tissues, they permit the identification of tumour-specific changes. One limitation of this approach is, however, the fact that DNA may be released from dying tumour cells. Because DNA sequences are stable in human tissues, detection of the mutant or altered sequences does not point to the presence of residual cancer cells in the sample under investigation. Instead, these assays point, with a high level of sensitivity, to the presence of tumour cells, somewhere in the organism, that might have released DNA fragments containing tumour-specific alterations (Chen *et al*, 1996; Nawroz *et al*, 1996; Goessl *et al*, 1998; Hibi *et al*, 1998; Sanchez-Cespedes *et al*, 1998). DNA-based assays consequently seemed to be most suitable to monitor the tumour burden (Fujiwara *et al*, 1999) or to detect cancers early (Chen *et al*, 1999). In some reports DNA-based markers have been used to detect residual tumour cells in lymph nodes (Sanchez-Cespedes *et al*, 1999) or even at the resection margins (Brennan *et al*, 1995), in both cases being clearly associated with a worse prognostic outcome based on ~~respective~~ RETROSPECTIVE studies.

Another approach to detecting disseminated cancer cells in clinical samples is the reverse transcriptase-polymerase chain reaction (RT-PCR)–based amplification of specific mRNA molecules, which are only expressed in the tumour cells and not in the normal adjacent tissues or other clinical samples under investigation (Raj *et al*, 1998; Ghossein *et al*, 1999b). One advantage of

using mRNA as opposed to DNA as a target for the detection assays is the reduced viability of RNA molecules in clinical samples once released from dying cells. Therefore, the detection of specific RNA transcripts points to the presence of residual tumour cells in the sample. For some cancers, specific transcripts that are selectively expressed in the tumour cells but not in normal non-neoplastic cells have been described. Among those are mRNAs transcribed from specific fusion genes in various soft tissue sarcomas (Willeke *et al*, 1998a, 1998b). Likewise, transcripts encoded by human papillomaviruses have been used to detect residual tumour cells of anogenital cancers, in particular cervical carcinoma (Czegledy *et al*, 1995). For most cancers such *true* tumour-specific mRNA molecules have not yet been characterised. Given that CKs are highly expressed in epithelial tumours, they have frequently been targets, particularly CK19 and CK20 (Burchill *et al*, 1995; Fields *et al*, 1996; Gunn *et al*, 1996; Noguchi *et al*, 1996; Schoenfeld *et al*, 1996; Soeth *et al*, 1997), although many epithelial markers have been evaluated (Table 1.3). Other transcripts used as markers include CEA (Gerhard *et al*, 1994; Liefers *et al*, 1998), epidermal growth factor receptor (Hildebrandt *et al*, 1997; Mapara *et al*, 1997), mucin-1 (Noguchi *et al*, 1996), human chorionic gonadotropin-β (Hoon *et al*, 1996), and α-fetoprotein (Matsumura *et al*, 1994). In prostate Ca, prostate-specific marker transcripts are available, including PSA, prostate-specific membrane antigen, and human kallikrein-2 (reviewed by Corey and Corey, 1998). Although these mRNA-based assays can detect one cancer cell in about 10ml of blood equal to 10^7 normal non-transformed cells, the sensitivity and specificity of each assay was largely dependant on various technical factors. The precise amplification conditions including temperature, the choice of amplification primers, the use of appropriate "nested" PCR-primers, and the

defined conditions of amplification reagents were all of particular importance. However, it is now becoming clear that many of these targets may not have the requisite specificity to distinguish epithelial tumour cells from haematopoietic cells. Several studies (Traweek *et al*, 1993; Krismann *et al*, 1995; Smith *et al*, 1995; Gunn *et al*, 1996; de Graaf *et al*, 1997; Henke *et al*, 1997; Zippelius *et al*, 1997; Bostick *et al*, 1998a; Ko *et al*, 1998; Ruud *et al*, 1999) have shown that these transcripts are consistently identified in normal BM, blood, and lymph node tissue. There are several possible reasons proposed for this lack of specificity, including the presence of pseudogenes and low-level transcription of epithelium-specific mRNA by haematopoietic cells. Besides the choice of the appropriate marker transcript, the specificity of the RT-PCR assay was largely dependant on the method of sample preparation and on the assay conditions (Neumaier *et al*, 1995; Traystman *et al*, 1997; Corey and Corey, 1998). For example, false-positive findings of RT-PCR assays for CK20 were avoided by analysis of MNCs instead of whole blood preparations, because normal granulocytes expressed CK20 (Soeth *et al*, 1997; Jung *et al*, 1998). Another limiting factor was the deficient expression of the marker gene (e.g., PSA) in micrometastatic tumour cells (Ferrari *et al*, 1997; Zippelius *et al*, 1997). To overcome this problem, a multimarker RT-PCR assay was established, as was done for melanoma-associated antigens (Hoon *et al*, 1995; Sarantou *et al*, 1997).

A range of reports from several clinical laboratories suggest that the detection of disseminated cancer cells in blood or BM using RNA amplification assays has a strong prognostic impact on the survival in cancer of the gastrointestinal tract (marker: CK20 [Soeth *et al*, 1997]), prostate (marker: PSA [Ghossein *et al*, 1997]), and in melanoma (marker: GAGE, tyrosinase [Mellado

et al, 1996, 1999; Ghossein *et al*, 1998; Keilholz, 1998; Cheung *et al*, 1999]). However, there are also reports in which such a prognostic impact could not be confirmed (Millon *et al*, 1999; Oefelein *et al*, 1999). Additional prospective trials with more patients and, in particular, standardised amplification protocols are needed before the clinical utility of these assays ~~has been~~ CAN BE defined (Jung *et al*, 1997).

The perioperative analysis by molecular methods of blood samples taken from patients who underwent surgery for colorectal cancer clearly points to a transient intraoperative dissemination of tumour cells (Denis *et al*, 1997; Weitz *et al*, 1998). Whether these cells might reach and survive in secondary organs and form overt metastases is still unknown.

Interest in using molecular markers to identify single disseminated cancer cells may increase and improve the diagnostic analysis of tumour-draining lymph nodes. Various publications have shown that a detailed immunohistopathologic work-up of tumour-draining lymph nodes is more sensitive and thereby more effective in identifying spread tumour cells, compared with the routine histopathologic analysis. Moreover, these single tumour cells in the draining lymph nodes were shown to have a strong adverse prognostic impact on the survival of patients with oesophageal and lung cancer (Izbicki *et al*, 1997; Kubuschok *et al*, 1999). In contrast to immunocytochemistry, molecular markers provided the opportunity to analyse the whole lymph node in a one-step, biochemical reaction. Preliminary studies on a limited number of patients with colorectal cancer, for example, indicated that using either DNA-based (Sanchez-Cespedes *et al*, 1999) or RT-PCR–based assays for CEA or CK20 mRNAs significantly increased the detection

rate of lymph node micrometastasis (Weitz *et al*, 1999), and that this finding was again linked to a worse clinical outcome (Liefers *et al*, 1998).

The increased sensitivity of the assay may be achieved by a loss of specificity, unless the selected marker genes are expressed exclusively in tumour cells. In addition, RT-PCR needs to be quantitative if RT-PCR determination of tumour cell numbers (burden) is to become an additional component of the detection of occult metastasis.

1.2.3 New Techniques for Detection of Disseminated Tumour Cells

The ICC detection of micrometastases has been developed during the last 10 years, and its clinical relevance has been validated in a number of studies. This method is currently the standard for the early detection of occult tumour cells disseminated from solid tumours. Microscopic analysis of many cytologic samples is, however, time consuming and requires considerable expertise. A new method of cytocentrifugation that permits the analysis of larger sample volumes (Schwarz, 1991) should address the first problem. The microscopic screening of large numbers of cytologic samples could be automated by the use of an image-analysis system (scanner). Systems of this type are currently being developed that have high sensitivity and specificity and are being used for screening occult metastases in patients enrolled in clinical trials, including lung cancer (Cote *et al*, 1997; Makarewicz *et al*, 1997). One way to increase the sensitivity of tumour cell detection in BM and blood is to selectively enrich for tumour cells (see Chapter 6).

Immunocytochemical methods relying on MoAbs against various epithelium-specific cytoskeletal and membrane antigens have been used to detect individual disseminated carcinoma cells in mesenchymal organs.

Methodological studies based on surrogate model systems consisting of BM samples to which cancer cells from cell lines have been added have demonstrated that immunocytologic techniques are superior to conventional histopathologic examinations. When immunocytochemistry and flow cytometry studies were compared, the results of the published studies were found to be heterogeneous, depending on the method used to detect tumour cells (Molino *et al*, 1991; Wingren *et al*, 1995; Vredenburgh *et al*, 1996; Gross *et al*, 1995). Molino *et al* (1991) and Vredenburgh *et al* (1996) claimed that immunocytochemistry was superior to flow cytometry. In contrast, Gross *et al* (1995) developed a flow cytometric assay with comparable sensitivity to immunocytochemistry; but the time involved to analyse one sample was 40 hours. Although flow cytometry is an conventional method for detecting occult metastases in patients with lymphoma and leukaemia (Jennings and Foon, 1997; Ciudad *et al*, 1998), no study using patient samples has compared the sensitivity of flow cytometry with immunocytochemistry for the detection of micrometastases in patients with epithelial tumours. Some discrepancies have been ascribed to the characteristics of the model cell lines used as surrogates for micrometastases (Simpson *et al*, 1995). For example, if CK Abs were used to detect epithelial tumour cells, the loss of CKs would render the cells undetectable. In BrCa cells, studied using multi-parameter DNA flow cytometry, the loss of CKs has been shown to be a function of the cellular factors present and the preparation procedure used (Wingren *et al*, 1995).

In light of earlier studies (Leon *et al*, 1977; Shapiro *et al*, 1983; Stroun *et al*; 1987, 1989) indicating that cancer patients may have large amounts of circulating DNA in serum or plasma, blood samples from patients with head and neck tumours or lung cancer have been analysed for microsatellite alterations

(Chen *et al*, 1996; Nawroz *et al*, 1996). With the rapid advancement of new technologies that allow the profiling of individual tumours (Kononen *et al*, 1998), the development of methods for patient-specific tumour cell detection may be possible. In high-density DNA microarrays, probes for the messenger RNA products of up to 10,000 different genes are present on a single 'chip', usually a glass slide. The chips are used to determine which of these genes are expressed (that is, which are transcribed into mRNA) in a selected cell type (Lockhart and Winzeler, 2000). This technology has already been used to classify cancers, such as leukaemia, according to their gene-expression profile (Berns, 2000; Lockhart and Winzeler, 2000). One drawback to microarray analysis is that it cannot identify post-transcriptional changes in protein expression or activity. Mutant ras, for example, can contribute to cancer but would not be identified by microarray analysis of human tumours, as only its activity – not its expression level – is altered by mutation. Another interesting application of DNA-based markers is the analysis of the p53 (also known as TP53) gene in cells of the resection margins, which are called tumour free by conventional histopathologic examination. This type of analysis has been shown to provide clinically important data for patients with squamous cell carcinomas of the head and neck (Brennan *et al*, 1995).

1.3 Clinical Relevance of Bone Marrow Micrometastasis

Several reports have indicated that the presence of immunocytochemically identifiable disseminated cancer cells in BM is associated with an unfavourable prognosis (see Table 1.4).

Tumour Origin	Marker Proteins	Detection Rate (%)	Prognostic Value	Reference
Breast	EMA	89/350 (25)	DFS, OS	Mansi *et al*, 1999
	TAG-12, EMA, CK	38/100 (38)	DFS, OS*	Harbeck *et al*, 1994
	CK	18/49 (37)	DFS*	Cote *et al*, 1991
	TAG-12	315/727 (43)	DFS, OS*	Diel *et al*, 1996
	CK	199/552 (36)	DFS, OS	Braun *et al*, 2000b
Colon/Rectum	CK18	28/88 (32)	DFS*	Lindemann *et al*,1992
Stomach	CK18	34/97 (35)	DFS	Schlimok *et al*, 1991
	CK18	47/78 (60)	DFS	Heiss *et al*, 1997
	CK18	95/180 (53)	DFS*	Jauch *et al*, 1996
Pancreas	CK	24/49 (48.9)	OS	Roder *et al*, 1999
Oesophagus	CK	37/90 (41)	DFS, OS	Thorban *et al*, 1996
Bronchus	CK	17/43 (40)	DFS	Cote *et al*, 1995
Lung	CK18	83/139 (60)	DFS*	Pantel *et al*, 1996
	CK18	15/39 (39)	DFS	Ohgami *et al*, 1997

Table 1.4: Immunocytochemical studies of the prognostic relevance of disseminated tumour cells in bone marrow.

*Prognostic value as independent parameter confirmed through multivariate analysis.

CK = cytokeratin; DFS = disease-free survival; EMA = epithelial membrane antigen; OS = overall survival; TAG-12 = tumour-associated glycoprotein-12.

However, controversial reports challenging the clinical relevance of disseminated cancer cells have been published over the past decade, also (Funke and Schraut, 1998). To exemplify that the phenomenon of tumour cell dissemination – which appeared to be common for tumour entities as diverse as the ones listed above – may have a prognostic impact independently from the manifestation of bone or BM metastasis, breast, colorectal and ovarian cancer were selected for detailed discussion.

1.3.1 Breast Carcinoma

Although less than 10% of women with primary BrCa present with Stage IV disease, the 5 year survival rate for BrCa is around 65% and 30-40% of women will develop metastatic disease. The main sites of metastases are bone, soft tissue, lung and liver. At first relapse, BM metastases are detectable in 23% of patients by conventional diagnostic techniques. As the disease progresses, the rate of BM involvement increases, up to 80% in *post-mortem* studies of patients with metastatic BrCa (Kamby *et al*, 1987).

Involvement of regional lymph nodes is the most powerful predictor for systemic relapse. Some studies have sought to correlate any relationship between conventional risk factors such as lymph node status with detection of epithelial antigen-positive cells. The results are not consistent. Some studies found significant correlations between BM positivity and nodal status (Berger *et al*, 1988; Diel *et al*, 1996; Funke *et al*, 1996), while others failed to confirm such an association (Harbeck *et al*, 1994) or found a trend towards correlation between the parameters (Schlimok *et al*, 1987; Cote *et al*, 1988). A more detailed analysis of these reports show that the ICC techniques used to stain the cancer cells differ considerably, particularly with regard to the number of

cells analysed and the Abs used to detect disseminated cancer cells. For example, a recent report from Mansi *et al* (1999) claimed that the detection of disseminated cancer cells in BM had no significant independent prognostic value; however, the EMA Ab used in the study has limited specificity, because it crossreacts with haematopoietic cells (Braun *et al*, 1998a). Furthermore, use of the Ab TAG-12, which is also directed against membrane-bound mucins, is also known for its crossreactivity with BM cells (Delsol *et al*, 1984; Heyderman and Macartney, 1985; Taylor-Papadimitriou *et al*, 1985), and the monospecific CK2 Ab directed against CK18 which is less sensitive than a broad-spectrum Ab (Braun *et al*, 1998a). Applying the broad-spectrum anti-CK MoAb A45-B/B3 for tumour cell detection, Pantel *et al* (1998) found a significant association of BM micrometastasis with diagnosis of inflammatory BrCa ($p = 0.006$), distant metastasis ($p<0.0001$), and extensive lymph node metastasis (≥ 10 nodes involved; $p = 0.009$). An interesting report derived from Fox *et al* (1997) showed that an assessment of tumour angiogenesis and vascular invasion gives a reliable indication of the probability of the presence of EMA-positive cells in BM from BrCa patients, and that both processes contribute to metastasis formation.

In order to assess the significance of isolated tumour cells in BM, clinical follow-up studies were initiated. Some of the most recent reports on the ICC evaluation of BM from BrCa patients are summarised in Table 1.4. A follow-up examination of 727 primary BrCa patients without manifest distant metastasis after a median follow-up time of 36 months (3-108 months) reported that the presence of TAG-12-positive cells identified patients with reduced metastasis-free ($p<0.001$) and overall survival ($p<0.001$) (Diel *et al*, 1996). The detection of TAG-12-positive cells was described as an independent prognostic indicator for

both metastasis-free and overall survival, being superior to axillary lymph node status, tumour stage, and tumour grade. Harbeck *et al* (1994) confirmed these results which examined BM aspirates from 100 patients with primary BrCa. Isolated tumour cells were detected in 38% of the patients using a cocktail of MoAbs to EMA, TAG-12 and CK. After a median follow-up of 34 months (7-64 months) multivariate analysis using the Cox proportional-hazard model revealed that BM positivity was a strong, significant prognostic indicator for relapse-free and overall survival.

In addition to the presence or absence of occult tumour cells, it now appears that the number of tumour cells detected may be clinically relevant. Preliminary work by Cote *et al* (1991) indicates that there is an increased risk of relapse in patients with mammary carcinoma who have more disseminated cells. They used a cocktail of MoAbs to cell-surface antigens (C26 and T16) and CKs (AE1), and demonstrated that the tumour burden in BM was an important risk factor. In their analysis of BM cell smears, the number of isolated tumour cells per sample (0 or <10 vs. ≥10 cells) was the only independent predictor of early recurrence ($p<0.003$). Thus, the number of occult disseminated tumour cells detected may reflect the tumour cell burden, and this number may be an important clinical variable.

1.3.2 Colorectal Carcinoma

In contrast to BrCa with its propensity for bone metastasis, it may surprise that disseminated tumour cells are detected in BM from patients with tumour types that rarely form skeletal metastasis, such as colorectal and ovarian cancer (see below). Clinically manifest metastasis are described in 1-4% of cases with colorectal cancer (Bonnheim *et al*, 1986), although this rate

increases to 6-12% in *post-mortem* studies (Welch and Donaldson, 1979; Weiss *et al*, 1986). While the tendency for dissemination during early stage disease (for example, M_0) was identical to that observed in BrCa patients (Table 1.5), BM positivity was significantly reduced in M_1 colorectal cancer patients as compared to M_1 BrCa patients (Pantel *et al*, 1993b).

Tumour Entity	No. of Patients	No. of Patients With CK18-positive Tumour Cells[a]
Breast Cancer	**135**	**49 (36.3%)**
M_0	116	35 (30.2%)
M_1	19	14 (73.7%)[b]
Colorectal Cancer	**277**	**85 (30.7)**
M_0	195	53 (27.2%)
M_1	82	32 (39.0%)[c,d]
Controls[e]	**215**	**6 (2.8%)**

Table 1.5: Frequency of CK18-positive tumour cells in bone marrow of patients with breast and colorectal cancer.

[a]Per BM sample 4 x 10^5 mononucleated cells were stained with Ab CK2 (Pantel *et al*, 1993b).

[b]$p<0.001$ compared to M_0 BrCa patients (χ^2 test).

[c]$p = 0.005$ compared to M_1 BrCa patients (χ^2 test).

[d]$p = 0.05$ compared to M_0 colorectal cancer patients (χ^2 test).

[e]Non-carcinoma control patients.

The appearance of this difference at such an advanced tumour stage was explained by a specific growth and/or survival advantage for the disseminated mammary carcinoma cells in BM, and with the presence of microenvironmental factors that support the growth of mammary tumour cells (Kjonniksen *et al*, 1992; Dong *et al*, 1994; Fodstad and Kjonniksen, 1994) but not the growth of colorectal tumour cells. This hypothesis also explained why clinically manifest skeletal metastasis from colorectal carcinoma is rare, despite the presence of disseminated colorectal tumour cells in BM (Welch and Donaldson, 1979; Weiss *et al*, 1986; Bonnheim *et al*, 1986). Besides the interaction of tumour cells with the surrounding organ milieu, the path that cells must follow in the circulatory system also plays a role in tumour dissemination. Colon carcinoma cells, for example, must first pass through the capillary bed of the liver, which traps many of the cells and enhances the chances of metastasis of colon carcinoma to the liver (Weiss *et al*, 1986).

In a study on 88 patients with colorectal cancer, Lindemann *et al* (1992) found 28 (32%) of cases with disseminated tumour cells in BM. After a median observation time of 35 (12-58) months, patients with a positive BM finding showed a significantly shorter disease-free survival than those without such a finding ($p = 0.008$); multivariate analysis confirmed BM positivity as the strongest independent determinant of relapse (relative risk 2.98; $p = 0.004$). Since BM was not the preferred site of tumour relapse, the detection of disseminated tumour cells was interpreted as the evidence for the disseminative capacity of an individual tumour (Lindemann *et al*, 1992). This interpretation was supported by the observation that patients with CK^+ cells in BM more frequently succumbed to distant metastasis, predominantly in the liver.

1.3.3 Ovarian Carcinoma

Epithelial ovarian cancer – the major cause of death from malignancies of the gynaecological tract in the U.S. (Cannistra, 1993) as well as in Europe (Black *et al*, 1997) – is characterised by lethal effects of local progression due to transcoelomic spread and disease outside the abdominal peritoneal cavity is rare. *Post-mortem* studies, however, indicate a considerable frequency of occult haematogenous metastasis at distant sites, such as liver, lungs, bone, and BM (Dauplat *et al*, 1987; Abdul-Karim *et al*, 1990). These observations already suggested that haematogenous dissemination of malignant cells is more frequent, as recently shown (Cain *et al*, 1990; Ross *et al*, 1995b), than can be expected from the clinically observed pattern of relapses.

In a large prospective study on ovarian cancer patients, Braun *et al* (1999) were able to detect disseminated tumour cells in 28 (30%) of 95 patients, indicating the capacity of the individual tumour for haematogenous dissemination. Haematogenous dissemination was identified only in cases with tumours that extended beyond the ovarian parenchyma (stages Ic-IV). Although no correlation was found between BM positivity and established risk parameters, including tumour grading, residual tumour, increased serum levels of the CA-125 tumour marker, and retroperitoneal lymph node involvement, evaluation of the clinical follow-up after a median observation time of 18 months revealed a significant correlation with early distant metastasis formation, predominantly in liver and lungs (Braun *et al*, 1999). In multivariate Cox's regression analysis, BM positivity turned out to be the only independent predictor for a distant metastatic relapse (Braun *et al*, 1999).

These data challenged the dogma that haematogenous spread of tumour cells is only a prerequisite of advanced tumour stages when tumour cells gain

access to blood vessels by continuous shedding. Haematogenous tumour cell dissemination was found in a considerable number of patients as early as at the time of first diagnosis of the tumour, and BM appeared to represent a relevant reservoir for viable tumour cells. In view of the increasingly improved control of local tumour growth by aggressive chemotherapy or surgery/radiotherapy, the detection of disseminated and potentially therapy-resistant tumour residues in BM emerged as a clinically relevant prognostic factor.

However, some studies have failed to confirm the prognostic relevance of occult tumour cell detection and it is possible that technical factors may have accounted for discrepancies (Funke and Schraut, 1998). For early stage BrCa, occult tumour cell detection rates of 4%-45% in BM have been reported (Osborne and Rosen, 1994). The choice of Abs and immunologic methods and the technical skills involved in performing the procedures and interpreting the results do introduce variation, and there is a risk of false-positive identification of non-epithelial cells, as previously referred to (Fadlon *et al*, 1996; Borgen *et al*, 1998). For example, Fadlon *et al* (1996) speculated that PSA-positive cells in the blood could be monocytes that have pinocytosed free PSA in serum or phagocytosed PSA-positive tumour cells. Thus, it will be important to define the critical variables in the methods and to introduce at least some level of standardisation to allow more reliable and reproducible results (Chaiwun *et al*, 1992; Pantel *et al*, 1994; Borgen *et al*, 1998). The International Society for Haematotherapy and Graft Engineering (ISHAGE) working group on tumour cell detection has initiated the first steps toward this goal (Borgen *et al*, 1999). Finally, differences in study design may account for some discrepancies; some studies have not been designed to allow evaluation of the association of occult tumour detection with clinical outcome.

1.4 Tumour Cell Dissemination Through Lymph Nodes

The single most important prognostic factor in patients with early stage BrCa is the presence of lymph node metastases. However, routine histologic examination of the lymph nodes can overlook the presence of tumour cells, occasionally due to sampling, but more often due to the failure to histologically distinguish tumour cells from other lymph node constituents (e.g., sinus histiocytes) (Cote *et al*, 1999). Furthermore, Gusterson and Ott (1990) calculated that a pathologist has only a 1% chance of identifying a metastatic focus of BrCa with a diameter of three cells in a lymph node. It has been clearly demonstrated that lymph node sections initially considered "negative" for tumour by routine histologic screen frequently show metastatic foci of tumour deposits after histologic re-examination, demonstrating that even when tumour cells are present in the section, they can be missed (Neville, 1991). There is now growing evidence that the detection of occult lymph node metastases not detected by standard pathologic examination identifies patients at increased risk for recurrence and reduced survival (Cote *et al*, 1999).

Almost all studies have demonstrated that lymph node metastases can be overlooked, but there is discrepancy regarding the prognostic importance of these occult tumour deposits. Many studies have concluded that the presence of occult lymph node metastases is not associated with recurrence in a statistically significant way (reviewed by Cote *et al*, 1999). This appeared to be largely the result of study design; most of the negative studies involved too few patients to address the issue with sufficient statistical power, and the use of less sensitive, standard methods of histopathological examination. Groshen and Cote (2000) have calculated that, given the relatively small differences in recurrence rates that might be expected between occult lymph node positive

versus negative patients (as for example would be expected comparing node negative patients with patients who have one or two positive nodes), there is no possibility that studies of the clinical impact of occult lymph node metastases involving so few patients will result in statistically significant data, even if the finding were prognostically important. In fact, Fisher *et al* (1978) were the first to clearly point out that large numbers of patients would be required to determine the true significance of occult lymph node metastases. In addition, reviews by Taylor and Cote (1997), Calaluce *et al* (1998), and Corey and Corey (1998), have illustrated that technical issues may have accounted for discrepancies in some studies. For example, the analysis of a few sections, 5-6μm thick, represented a relatively small random sample of a lymph node.

Furthermore, in general, the use of anti-CK Abs appeared to be a reliable and effective method for tumour cell detection, although normal lymph node (reticulum) cells can express CKs (e.g., CK19) (Moll *et al*, 1982). However, Abs against other epithelial antigens that are not present on normal lymph node cells have been used, including BerEP4, an Ab that recognises two glycoproteins of 34 and 49kDa present on the cell surface (Passlick *et al*, 1994a; Izbicki *et al*, 1997; Kubuschok *et al*, 1999), and an Ab against CEA (Hitchcock *et al*, 1996). Sensitive immunohistochemical detection of occult metastases in the lymph nodes of patients with node-negative cancer is being shown to be prognostically important in an increasing number of studies in many types of cancers, including BrCa (de Mascarel *et al*, 1992; McGuckin *et al*, 1996; Cote *et al*, 1999), colon cancer (Greenson *et al*, 1994; Liefers *et al*, 1998), gastric cancer (Maehara *et al*, 1996a), non-small-cell lung cancer (Passlick *et al*, 1994a; Cote *et al*, 1998; Kubuschok *et al*, 1999), oesophageal cancer (Izbicki *et al*, 1997), prostate Ca (Freeman *et al*, 1995; Edelstein *et al*,

1996), and melanoma (Cochran *et al*, 1988). These results emphasise the importance of verifying the lymph node status, which may improve tumour staging and may provide additional criteria for administering adjuvant therapy.

In addition to immunocytochemistry, molecular methods based on the PCR-mediated amplification of tumour cell DNA or of cDNA reverse transcribed from mRNA have been used to detect tumour cells in lymph nodes. However, the specificity of RNA-based markers, such as CEA mRNA, recently used for the analysis of lymph nodes in patients with colon cancer (Liefers *et al*, 1998), is not absolute because of the low-level illegitimate expression of the marker gene discovered in surrounding lymph node cells (Bostick *et al*, 1998b). Alternative, DNA-based markers, such as mutations in the p53 gene or Ki-ras gene, have been used in patients with colorectal cancer, lung cancer, or head and neck cancer to detect single tumour cells in a background of thousands of lymph node cells (Brennan *et al*, 1995; Hayashi *et al*, 1995; Ahrendt *et al*, 1997).

Investigators from the University of Southern California in Los Angeles, the Ludwig Institute for Cancer Research in London, the International Breast Cancer Study Group in Switzerland, and Harvard University in Boston, recently completed a multi-institutional study to ascertain whether immunohistochemical methods could improve the detection of occult metastases from primary BrCa patients whose axillary lymph nodes were considered to be disease free by conventional methods (Cote *et al*, 1999). The axillary lymph nodes from 736 patients with no evidence of tumour involvement by routine histology were examined for the presence of occult metastases using anti-CK Abs by immunohistochemical methods. Occult metastases could be detected in 20% of the patients. While patients with large tumours (>2cm) tended to have a higher

frequency of occult metastases (26%), those with smaller lesions (<2cm) also had a substantial rate of occult metastases (16%). Indeed, occult metastases were found in lymph nodes of 15% of patients with micro-invasive carcinoma. Of particular note is that occult metastases were detected in 40% of the cases in 64 patients who had invasive lobular or mixed invasive lobular-ductal carcinoma.

With respect to outcome, the presence of occult lymph node metastases was a significant predictor of disease-free and overall survival. Postmenopausal patients with occult lymph node metastases had 1.73 times the risk of recurrence and 2.09 times the risk of dying compared to occult node negative patients (Cote *et al*, 1999). In a multi-variant analysis, occult lymph node metastases remained an independent and highly significant predictor of outcome in postmenopausal patients, even after controlling for tumour grade, tumour size, oestrogen receptor status, vascular invasion, and treatment assignment ($p = 0.007$). Interestingly, the prognostic predictive power of occult lymph node metastases could not be demonstrated in premenopausal patients.

An important advance in the evaluation of regional lymph nodes has been the development of a more limited dissection, the sentinel lymph node dissection, that is based on the identification, with dyes or radioactivity, of the specific lymph node that drains the tumour and the removal of this lymph node for analysis. This approach was pioneered by Morton *et al* (1992) and Giuliano *et al* (1994, 1995) and has been extensively evaluated in patients with melanoma and BrCa (Morton *et al*, 1992; Giuliano *et al*, 1994, 1995; Veronesi *et al*, 1997; Krag *et al*, 1998). Because more limited material is available as a result of the sentinel lymph node dissection, detection of occult tumour cells may be an important adjunct to the use of limited lymph node dissection for

staging and for therapy (Van der Velde-Zimmermann *et al*, 1996; Min *et al*, 1998).

1.5 Biological Characteristics of Disseminated Carcinoma Cells

The detection of disseminated tumour cells has introduced a new opportunity to evaluate which of the diverse biological characteristics of the primary tumour might favour the early dissemination of its cells. Two groups (Fox *et al*, 1997; Maehara *et al*, 1998) have recently reported an association of tumour angiogenesis with BM micrometastases for breast and gastric cancers. In addition, McCulloch *et al* (1995) and, Choy and McCulloch (1996) found an association between tumour angiogenesis and tumour cell shedding into effluent venous blood during BrCa surgery. The metastatic potential to BM was not associated with the expression of p53 and retinoblastoma (RB) genes or the proliferative activity of the primary lesion of gastric cancer (Maehara *et al*, 1996b). In view of the malignant potential of CK^+ cells, a number of tumour-associated characteristics have been identified in CK^+ cells with ICC double-staining methods, including expression of urokinase plasminogen activator receptor, overexpression of the Her-2/*neu* oncogene, and deficient expression of major histocompatibility complex (MHC) class I molecules (Table 1.6).

Marker	Tumour Origin	Marker-positive or CK-positive Cells*. No. of Patients With Marker/No. of Total Patients (%)
Growth factor receptor		
Her-2/*neu*	Breast	48/71 (67.6)
	Colorectum	8/28 (28.6)
	Stomach	6/22 (27.3)
	Lung	5/6 (83.3)
Transferrin receptor	Breast	9/26 (34.6)
	Colorectum	7/17 (41.1)
MHC class I antigen[#]		
	Breast	9/26 (34.6)
	Colorectum	12/17 (70.6)
	Stomach	8/11 (72.7)
Adhesion molecule		
17-1A (EpCAM)[#]	Breast	23/31 (74.2)
ICAM-1[#]	Lung (NSCLC[#])	13/31 (41.9)
Plakoglobin	Lung (NSCLC[#])	4/12 (33.3)
	Colorectum	4/13 (30.8)
Proliferation-associated protein		
Ki-67	Breast	1/12 (8.3)
	Colorectum	0/13
	Stomach	0/8
	Lung	0/7
p120	Breast	1/11 (9.1)
	Colorectum	5/12 (41.7)
	Stomach	4/13 (30.8)
	Lung	3/10 (30)
Protease uPA[#] receptor	Stomach	20/44 (45)

Table 1.6: Phenotype of disseminated CK-positive tumour cells in BM.

*From (Schlimok *et al*, 1990; Pantel *et al*, 1991, 1993a, 1993b; Heiss *et al*, 1995; Allgayer *et al*, 1997; Braun and Pantel, 1998).
[#]uPA = urokinase plasminogen activator; MHC = major histocompatibility complex; ICAM-1 = intercellular adhesion molecule-1; NSCLC = non-small-cell lung cancer; and EpCAM = epithelial cell adhesion molecule.

1.5.1 Proliferation-associated Antigens

While the capacity of disseminated tumour cells to home in BM appears to be similar – as demonstrated by similar rates of detection throughout different tumour entities (Pantel *et al*, 1999) – their potential to outgrow in this new environment is under debate. Applying double labelling techniques, Pantel *et al* (1993a) investigated the proliferative fraction of CK^+ cells in BM as characterised by the proliferation markers Ki-67 and p120 antigen (Table 1.6); Ki-67 antigen is known to be present in all phases of the cell-cycle except G_0 and early G_1 (Gerdes *et al*, 1984), while p120 antigen can be detected during early G_1 with another peak in S phase (Freeman *et al*, 1988). Ki-67-positive/CK-positive cells in BM were only detected in one of 33 patients with carcinoma (breast, colorectum/stomach). Although CK^+ cells revealed p120 antigen expression in 10 (28%) of 36 cases, the fraction of double-positive CK^+ cells per specimen remained below 10%. Therefore, the authors concluded that the majority of disseminated tumour cells appeared to be non-cycling and at rest in the G_0 phase of the cell cycle, and proposed that the reduced proliferative activity observed in micrometastatic tumour cells at this early stage of dissemination was consistent with the well-known phenomenon of tumour cell dormancy.

1.5.2 Tumour Suppressor Genes

By delaying the transition of cells from a quiescent state into S phase, wild-type p53 protein allows DNA repair to take place, thereby exerting a control function sometimes also addressed as "guardian of the genome" (Lane and Benchimol, 1990). Loss of this function may therefore result in the destabilisation of the genome by an increased number of mutations that are

carried over to the next generation of cells. Since many mutations in the p53 gene lead to the expression of a stabilised mutant protein (Iggo *et al*, 1990), accumulation of this protein has been claimed as a marker of malignant disease in diagnostic cytopathology (Hall *et al*, 1991). Offner *et al* (1999) evaluated whether such accumulation could be detected in disseminated tumour cells by double marker analysis. Co-expression of p53 protein (TP53) was only found in 4 (3%) of 63 patients examined, and was found to be entirely absent in another series applying further epithelial markers for the detection of tumour cells, such as MoAbs to CK19 and to an epitope shared by various CK molecules (Offner *et al*, 1999). Thus, in the hands of this group, immunodetection of TP53 appeared of little value for the identification of individual micrometastatic carcinoma cells in BM. The study demonstrated that the rare detection of increased TP53 levels in these cells argues against the assumption that protein-stabilising mutations in the p53 gene provide a selective advantage for early tumour cell dissemination. This hypothesis has been confirmed by molecular analyses, providing direct evidence for the presence of wild type p53 gene sequence in micrometastatic cancer cell lines (Offner *et al*, 1999).

1.5.3 Histogenetic Marker Proteins and Intermediate Filaments

Aberrant CK expression in single BM cells was excluded by labelling of BM preparations with MoAbs CK2 to CK18 and either T29/33 to CD45 or V9 to vimentin, both of which are reliable markers for haematopoietic cells (Gatter *et al*, 1985). While all of the haematopoietic cells were either CD45-positive or vimentin-positive, CK^+ epithelial cells consistently lacked detectable expression of these mesenchymal marker proteins (Schlimok *et al*, 1987; Pantel *et al*, 1994).

In patients with prostate Ca, Riesenberg *et al* (1993) performed double marker assays employing MoAbs specific for PSA. Among the first 13 BM samples admitted to the study, co-expression of PSA on CK^+ cells was seen in five (38%) samples, thereby revealing the prostatic origin of tumour cells. This incidence is consistent with the rate of PSA expression in primary carcinomas and the LNCap cell line (Gallee *et al*, 1986). The malignant nature of CK^+ cells in BM of prostate Ca patients was supported by the recent work of Pallavicini *et al* (1995). Taken together, these findings suggested that CK^+ cells detectable in BM aspirates from patients with prostatic cancer are descendants of the primary tumour.

1.5.4 Proteins Relevant to the Immunological Anti-tumour Defence

The low frequency of epithelial tumour cells in BM and the localisation of epithelial cells in an organ that has such a good blood supply offer ideal conditions for the elimination of epithelial cells by immune effector cells. The clinical history of epithelial tumour cells shows, however, that micrometastatic tumour cells can be ignored for many years by the immune system (Riethmüller and Johnson, 1992). In a first attempt to address the problem of how CK^+ cells escape recognition by immune effector cells, Pantel *et al* (1991) applied their double marker assay to phenotype CK^+ cells for the expression of HLA class I molecules. In total, 25 (46%) of 54 patients yielded CK^+ cells that lacked a detectable expression of HLA class I molecules. In patients with BrCa, the percentage increased to 65% HLA-negative cells in BM as compared to only 27-29% in patients with gastrointestinal carcinomas (Pantel *et al*, 1991). This observation matched well with the high incidence of metastatic disease of the bone or BM occurring in BrCa patients. It was therefore suggested that

downregulation of HLA class I molecules is an effective mechanism to escape from the anti-tumoral immune defence mediated by cytotoxic T-lymphocytes. Moreover, the overall incidence of HLA negative tumour cells in the BM was higher than that reported for the respective primary tumours (Hämmerling *et al*, 1989), an observation which further supported the latter assumption that downregulation of HLA class I molecules may confer a selective advantage to CK^+ cells. The underexpression of HLA class I molecules could limit the prospects of tumour cell vaccines (Pardoll, 1993). Antibody-mediated tumour cell killing, on the other hand, is independent of tumour-cell HLA expression.

Several families of adhesion molecules participate in an effective immune response by mediating the tight binding of leukocytes to their target cells (Springer, 1990; Hogg and Landis, 1993). One of these molecules, the intercellular adhesion molecule-1 (ICAM-1) mediates leukocyte binding through its interaction with the integrins $\alpha_L\beta_2$ (LFA-1) and $\alpha_M\beta_2$ (Mac-1). ICAM-1 is normally not expressed by epithelial cells, though it can be induced by a variety of stimuli and is sometimes expressed by carcinoma cells *in vivo* (Johnson *et al*, 1989; Passlick *et al*, 1994b). Expression of ICAM-1 on CK^+ cells in the BM was found in 13 (42%) of 31 patients with completely resectable non-small cell lung carcinoma (Table 1.6) and appeared to be of prognostic significance (Pantel *et al*, 1992). Those patients with ICAM-1-positive micrometastatic cells had a reduced rate of tumour relapses and cancer-related deaths as compared to those patients with ICAM-1-negative tumour cells. Local and distant recurrence of lung cancer, or death directly related to the disease occurred predominantly in patients with ICAM-1-negative tumour cells, whereas only few patients with ICAM-1-positive tumour cells succumbed to distant metastatic disease. Even though the number of patients examined both immunocytochemically for ICAM-

1 expression (n = 31) and evaluated prospectively for the course of the disease (n = 19) is ~~considerably low,~~ SMALL Pantel *et al's* data (1992) suggest that ICAM-1 mediated binding of leukocytes may facilitate an effective immune response. This observation is consistent with studies on renal cell carcinoma in which the expression of ICAM-1 by the primary tumour was correlated with well differentiated tumours having a good prognosis (Tomita *et al*, 1990). A recent study unravelled the induction of ICAM-1 by protein kinase C after stimulation with neu differentiation factor (NDF), also called heregulin (Bacus *et al*, 1993). NDF is a 44kDa glycoprotein that stimulates tyrosine phosphorylation of the $p185^{Her-2}$ receptor and induces phenotypic differentiation of some breast cancer cell lines to growth-arrest and milk-producing cells. Thus, expression of $p185^{Her-2}$ may exert a dual role for the malignant potential of tumour cells, depending on the ligand present in the local environment.

An alternative approach used in unravelling structures involved in metastatic spread is to evaluate correlations between the phenotype of primary breast carcinomas and the presence of tumour cells. Expression of ICAM-1, known to facilitate metastatic spread of melanoma cells (Johnson *et al*, 1989), had no apparent influence on the metastatic behaviour of primary non-small cell lung cancer cells (Passlick *et al*, 1994b). On the other hand, Ménard *et al* (1994) revealed that the expression of the 67kDa laminin receptor on primary BrCa cells may support tumour cell dissemination into lymph nodes and BM (Martignone *et al*, 1993).

The downregulation of HLA class I molecules as well as the modulation of ICAM-1 expression appears to influence the recognition by immune effector cells and the survival of tumour cells. Downregulation of HLA class I antigens on residual tumour cells has implications for potential therapeutic approaches,

using genetically modified cancer vaccines (Pardoll, 1993). The effectiveness of such vaccines has been proven in animal models and the first clinical trials have begun to assess safety and efficacy in patients with advanced solid tumours (Pardoll, 1993). However, this active immunisation approach requires the residual tumour cells to be recognised as foreign by T-lymphocytes.

1.5.5 Proteases and Epithelial Cell Adhesion Molecules

To gain access to blood and lymphatic vessels, tumour cells need to emigrate from the primary tumour and invade the surrounding stroma, which requires co-ordinated proteolysis (Figure 1.1). Among several tumour-associated proteases the urokinase-plasminogen activator (uPA) system evoked special interest, since uPA-receptor expression in disseminated tumour cells in BM of patients with gastric cancer (Table 1.6) was significantly correlated with increasing tumour cell counts in BM and poor clinical prognosis (Heiss *et al*, 1995; Allgayer *et al*, 1997). This finding suggested that expression of uPA-receptor is not only involved in tumour invasion but also influences the survival and/or outgrowth of disseminated tumour cells in BM, which is in agreement with the current views of the role of proteinases in metastasis. In addition, two papers by Solomayer *et al* (1997, 1998) showed that the detection of the serine protease uPA and of cathepsin-D on disseminated cells indicated a poorer prognosis in comparison to patients with uPA/cathepsin-D-negative tumours.

Flexible adhesive interactions of metastatic cells with other cell types are important prerequisites for the initiation of tumour cell emigration from the primary location to secondary sites (Behrens *et al*, 1992); loss of homotypic adhesion is one of the first steps required for the successful dissemination of

tumour cells (Hart *et al*, 1989). In the case of epithelial organs, a network of intercellular adhesive junctions is responsible for the tight integration of an individual cell within the tissue (Schwarz *et al*, 1990). The adherens junction complex is organised around the transmembrane E-cadherin protein that organises a complex of cytoplasmic proteins, including α-catenin, β-catenin and plakoglobin, a β-catenin relative found in desmosomes (Schwarz *et al*, 1990). The cadherin-catenin complex mediates adhesion, cytoskeletal anchoring, and signalling. Catenins can also form a complex with the product of the tumour suppressor gene APC, which may mediate transmission of a growth regulatory signal (Pfeifer, 1993).

Employing a double marker assay, Pantel *et al* (personal communication) were able to study the expression of plakoglobin on tumour cells. Among the first 25 BM samples analysed in their study, they found that in 17 (68%), expression of plakoglobin appeared to be absent or at least below the ICC detection level in tumour cells (Table 1.6). In contrast, microaggregates of carcinoma cells present in BM expressed plakoglobin. Therefore, they suggest that downregulation of plakoglobin expression participates in mechanisms that determine the dissemination of an individual cancer cell, whereas the up-regulation of expression might be necessary for multicellular metastasis formation at the secondary site.

Another interesting homophilic cell-cell adhesion molecule is the epithelial cell adhesion molecule, EpCAM, also called 17-1A antigen, which is encoded by the GA-733-2 gene (Göttlinger *et al*, 1986). The protein is found in the vast majority of cancers and has attracted attention as a tumour marker (Göttlinger *et al*, 1986). Double marker immunoassays have demonstrated a, presumably, modulated differential expression of EpCAM on CK^+ cells.

Micrometastatic BrCa cells in BM were found to be EpCAM-positive in 23 (74%) of 31 cases (Table 1.6). Downregulation of EpCAM expression was thought to permit tumour cells to be released from contact-mediated controls within the primary tumour, while re-expression at the secondary site was thought to facilitate organ-specific homing of disseminated tumour cells.

The hypothesis that EpCAM-expressing CK^+ cells might therefore serve as targets for antibody-based therapy, has been recently sustained in Dukes' C colorectal cancer patients without clinical signs of overt distant metastases who were treated in a randomised clinical trial with the murine MoAb 17-1A (edrecolomab) specific for EpCAM (Riethmüller *et al*, 1998). Administration of this Ab, in this randomised study, was found to reduce the overall death rate by 32% and decrease the recurrence rate by 23% (Riethmüller *et al*, 1998) after a median observation time of seven years.

1.5.6 Phenotypic Profile of Micrometastatic Cancer Cell Lines

Because few tumour cells are initially present in each of the BM samples, both Pantel *et al* (1995) and Putz *et al* (1999) recently performed a detailed phenotypic analysis of nine unique cell lines established from metastatic tumour cells present in BM of patients with epithelial primary tumours. Although the *in vitro* expansion and subsequent immortalisation with SV40 T antigen cDNA might have altered the phenotype of tumour cells, the authors did not observe consistent differences between SV40 T antigen-positive and negative cells. Furthermore, the expression of CKs, Her-2 and ICAM-1 antigens and the downregulation of certain epithelial adhesion molecules (e.g., plakoglobin), which were observed in the cell lines, were also characteristic of *in situ* micrometastatic cancer cells (Pantel *et al*, 1993a, 1993b, 1999). This was

found to be consistent with a number of reports demonstrating retention of phenotypic properties following SV40 T antigen-induced immortalisation of different epithelial cells (Pantel *et al*, 1995). The authors regarded this approach as a means of defining therapeutic targets to evaluate new anti-cancer agents, as well as investigating metastasis-specific factors.

1.6 Monitoring of Therapeutic Efficacy and Implications for Adjuvant Anti-cancer Therapy

Adjuvant therapy for patients with early stage operable disease has been shown to be an important component in the management of many cancers. Conventional adjuvant chemotherapy has been modified and improved in various ways (van Triest *et al*, 1995; Connors, 1996). For colorectal cancer, it has been shown that adjuvant chemotherapy is effective in some patients and generally well tolerated (Haller, 1995); the benefit published to date is of an approximate 30% reduction in mortality (Haller, 1995). The success of adjuvant therapy is assumed to be due to its ability to eradicate occult metastases before they become clinically evident (Schabel, 1977).

The original concept of Paul Ehrlich of treating tumours with specific Abs ("magic bullets") is more than 100 years old. The hybridoma technique for making MoAbs in large quantities was described in 1975 (Kohler and Milstein) and has presented a vast array of potential therapeutic options (i.e., specific targets expressed by the cancer cells). Although MoAb therapy has been effective in various experimental systems, the clinical experience to date has been disappointing for patients with advanced stage solid tumours (Pantel and Riethmüller, 1996; Scott and Welt, 1997), as a result of the large cell burden and the lack of access that macromolecules have to cells in these tumours

(Jain, 1990). Complete remission has nevertheless been induced in some patients with metastatic colorectal carcinoma using combination therapy of MoAb 17-1A and granulocyte-macrophage colony-stimulating factor (Ragnhammar *et al*, 1993). Another approach is the use of antibody-toxin conjugates, or immunotoxins (Vitetta *et al*, 1993; McNeil, 1995), and promising effects with such agents, even in advanced solid tumours, have been reported (Pai *et al*, 1996).

The efficacy of adjuvant therapy can thus far be only assessed retrospectively in large scale clinical trials following an observation period of at least five years. Consequently, progress in this form of therapy is extremely slow and cumbersome and, in addition, therapy is difficult to tailor to the special needs of an individual patient.

The feasibility of follow-up BM aspirations during anti-cancer therapy, has been recently investigated in a prospective study on patients with cancer of the prostate (stage C) treated with androgen-deprivation (Pantel *et al*, 1997). The number of tumour cells determined before and after androgen-deprivation was compared to the standard serum tumour marker PSA, and the clinical features. After androgen-deprivation 20 of 21 previously CK^+ BM aspirates showed a reduced or non detectable tumour cell load. Since 7 patients with persistently high tumour cell counts had no detectable serum-PSA titres, the study further demonstrated that serum-PSA was an unsuitable marker to indicate the presence of disseminated tumour cells, and, therefore, permitted no conclusions on the therapeutic elimination of tumour cells under androgen deprivation (Pantel *et al*, 1997).

Cytotoxic Abs represent another promising therapeutic option for the specific treatment of residual cancer (Dillman, 1994). In a pilot study on 8 BrCa

patients with advanced stage disease, Braun *et al* (1998b) were able to show the feasibility of establishing a surrogate assay for therapeutic efficacy by the specific elimination of target-positive tumour cells. Bone marrow samples before and after the administration of a single dose of 500mg edrecolomab (17-1A Ab) revealed the reduction of both CK^+ and EpCAM-positive/CK^+ tumour cells in all cases examined. To exclude the possibility of any anti-tumour activity other than due to the applied Ab, the authors determined both the tumour cell number after 5-7 days treatment and excluded patients with any concurrent anti-tumour treatment. The observed reduction or eradication of CK^+ cells was a likely effect of the infused Ab.

In another pilot study by Schlimok *et al* (1995), 40 patients with breast or colorectal cancer were treated in a randomised study with 6 x 100mg Ab ABL 364 which is directed to the Lewis Y (Le^Y) blood group precursor carbohydrate antigen (Scholz *et al*, 1991) against a placebo infusion; CK^+ cells in BM were monitored on day 15 and 60 after initiation of treatment. Even in patients with low numbers of CK^+ cells (1-11 per 4 x 10^5 MNC), there was a trend for reduction of CK^+ cells seen after Ab therapy. Significant results, however, were obtained only from the 10 BrCa patients who displayed an initial cell count of more than 20 CK^+ cells per 4 x 10^5 MNC. Of the 7 patients treated with Ab, 5 showed a reduction or eradication of CK-positive/Le^Y-positive cells (96-100%), while in 2 patients with CK^+ but Le^Y-negative cells no response was found. Similarly, in the 3 patients receiving human serum albumin no significant tumour cell reduction was observed. Because of the marked Ab dependant cellular cytotoxicity and complement dependant cytotoxicity that the Ab ABL 364 initiated in *ex vivo* experiments with serum of treated patients (Scholz *et al*,

1991), Schlimok *et al* postulated that the observed disappearance of tumour cells from BM could be attributed to the action of the administered Ab.

Despite the preliminary character of these studies, they exemplified a new approach towards a more rational selection of Abs for adjuvant studies in minimal residual disease. The proposed use of CK^+ cells as surrogate markers for the prediction of therapeutic response, benefited from the recent improvements of the CK assay (Pantel *et al*, 1994) which allowed a more precise quantitation of the individual tumour cell load.

Unfortunately, no clinical study has investigated the natural course of tumour cell contamination in patients not receiving adjuvant systemic therapy. Only one single study was able to show that in 82 patients with BrCa the detection rate at repuncture after 18 months had fallen to 3% compared with 26% at the time of primary treatment (Mansi *et al*, 1989). However, this study included patients with or without adjuvant therapy. It is also unclear whether cytotoxic chemotherapy or hormone therapy lead to a reduction in the number of tumour cells. Studies in patients of neoadjuvant chemotherapy have not been able to demonstrate this effect (Diel *et al*, 1997).

Current cytotoxic chemotherapy regimens may fail to eliminate dormant, non-proliferating tumour cells, which may explain metastatic relapse even after high-dose chemotherapy (HDC). Two pilot studies on BrCa patients undergoing either ifosfamide-carboplatin-epirubicin (n = 18) or vinblastin-ifosfamide-carboplatin (n = 10) HDC with autologous stem cell transplantation described the presence of CK^+ cells in 15 (83%) and 3 (30%) BM specimens obtained after completion of treatment with the majority of patients being in complete remission (Hohaus *et al*, 1996; Hempel *et al*, 1997). Therefore, complementary strategies, such as antibody-based immunotherapy, were considered. Hempel

et al (1997) offered additional 17-1A Ab (edrecolomab) therapy to patients with disseminated CK^+ cells resistant to HDC, which led to elimination of these cells, avoiding early metastatic relapse in 2 of 3 patients. Interestingly, residual CK^+ cells in both patients showed co-expression of EpCAM, while the respective cells of the third patient were EpCAM-negative (Hempel *et al*, 1997).

Baselga *et al* (1996) and, more recently, Slamon *et al* (1998) reported clinical trials demonstrating the successful treatment of metastatic BrCa with a humanised MoAb directed against the $p185^{Her\text{-}2}$ growth factor receptor given in combination with chemotherapy. The importance of these trials is that they are among the first studies in BrCa patients which have shown a biological effect of unconjugated recombinant Ab against established solid tumours. However, the relatively low response rates clearly show that other aspects needed to be taken into account. Jain (1990) previously demonstrated that the relatively high intratumoral oncotic pressure represents a physiological barrier to deliver MoAbs or other macromolecules to solid tumours. Therefore, it is clear that a major consideration for the successful application of Ab therapy is the choice of the appropriate disease stage in which the tumour cells are accessible for intravenously administered immunoglobulins (Riethmüller and Johnson, 1992).

Occult tumour cells in the BM of patients with early stage cancer have been the target of another class of therapy, where it has recently been shown that the bisphosphonate clodronate can reduce the incidence of developing overt metastases in patients with early stage BrCa who have occult metastases detected in their BM (Diel *et al*, 1998). This therapy given to a population of patients at risk for developing metastases (i.e., those with occult systemic disease at the time of presentation) has been shown to be beneficial. One of the postulated effects of bisphosphonate therapy postulated is that the

bisphosphonates that have accumulated on the bone surface have an apoptotic effect on the individual tumour cells (Diel *et al*, 1998).

In addition, genomic instability of neoplastic cells leads to a considerable heterogeneity in the expression of potential immunotherapeutic target antigens (Dillman, 1994). In a recent study, Braun *et al* (1999) investigated the pattern of tumour-associated antigens, including EpCAM and $p185^{Her\text{-}2}$, on BM micrometastases in BrCa patients. Their analysis revealed that despite a relatively high incidence of antigen expression, the number of cells with antigen expression per total number of detectable tumour cells varied considerably, indicating a heterogeneous pattern of expression of the investigated antigens. To cope with this antigen heterogeneity, the authors concluded that a combination of Abs directed to independently expressed antigens might be more efficient than a single agent (Braun *et al*, 1999). Recent progress has enabled translation of antibody-based immunological research from the laboratory to the clinic, and the adjuvant trials reported to date have supported the potential for this selective targeting approach for cancer therapy (Scott and Welt, 1997).

1.7 Outline and Proposals of PhD Research Programme

Despite the progress made in clinical oncology in recent decades, the presence of metastatic cancer has limited the benefit in the clinic.

Although conventional tumour-staging parameters can provide reliable information about the proportion of patients who will experience a recurrence of the disease, these measures cannot predict which patients will have a recurrence of disease after primary therapy, particularly in the patient with early stage disease. New parameters need to be defined that better identify those

patients at the greatest (and at the least) risk of relapse, since this would provide information critical to the subsequent management of the patient and aid in the conduct of clinical trials by defining patient groups. The detection of the earliest manifestations of tumour dissemination is an extremely promising approach that should improve risk assessment and the identification of specific patients who would benefit from adjuvant treatment. During the last 10 years, new immunologic and molecular analytic procedures have been developed to diagnose minimal residual cancer. Improved methods for genomic analysis of single tumour cells and for the assessment of target molecule expression may increase the diagnostic precision of current detection techniques and optimise the therapy for individual patients.

The benefit of adjuvant therapy can be assessed only after an observation period of several years. The availability of a surrogate marker for monitoring the effectiveness of a treatment should speed the evaluation and development of new adjuvant therapies. Periodic examination of PB and BM during therapy could indicate whether the therapeutic approach being used was effective. Monitoring procedures of this type would be of considerable value. Because of their accessibility, PB and BM samples would be logical contenders for monitoring minimal residual cancer at the sub-clinical stage.

Therefore, I will address two hypotheses:

1. **The number of circulating/micrometastatic CK^+ tumour cells in PB/BM of patients with solid tumours can be used as a surrogate marker of response to adjuvant chemotherapy and subsequent risk of metastasis.**

2. Disseminated CK^+ tumour cells in patients with epithelial malignancies express predictive markers associated with metastatic potential.

With the aims at outset being:

- To establish, validate and employ a novel and sensitive ICC method for the detection of ultra-rare CK^+ tumour cells in PB/BM, to allow the rapid evaluation of the effect of adjuvant chemotherapy on minimal metastatic disease, and to determine if patients with persistent disease are candidates for alternative therapy.
- To compare the molecular phenotype of tumour cells detected in the PB/BM, with that of the primary tumour, to determine if disseminated tumour cells show any phenotypic differences, and whether the shed cells have markers historically associated with poor prognosis.
- To use data from these analyses to improve our ability to determine the risk of BM micrometastasis in patients with operable primary tumours of the breast.

Chapter 2 Materials and Methods

This chapter describes the methods and source of material used for the experiments (i.e.; seeding experiments, source of tumour cells and clinical samples) described in this thesis.

2.1 Tumour Cell Lines

Cells of the human BrCa cell lines MDA-MB-361, BT-474 and MCF-7 were used (all from American Type Culture Collection [ATCC], Rockville, MD). MDA-MB-361 cell line (adenocarcinoma, metastasis to brain) was maintained in culture in Leibovitz's L-15 medium with 15% foetal bovine serum (FBS), BT-474 (ductal carcinoma) in RPMI-1640 medium with 10% FBS and 10µg/ml bovine insulin (Boehringer Mannheim, Lewes, UK), MCF-7 (adenocarcinoma, pleural effusion) in Eagle's minimum essential medium (MEM) with non-essential amino acids and sodium pyruvate with 10% FBS. The Daudi B lymphoblastoid cell line was also used and propagated in RPMI-1640 medium with 20% FBS and subcultured at 48-72 hour intervals. All culture media were supplemented with 2mM L-glutamine, penicillin (100µg/ml) and streptomycin (100µg/ml).

Cells were maintained at 37°C with 5% CO_2 in a humidified atmosphere. Culture media and supplements were from Life Technologies Ltd. (Paisley, Scotland).

The adherent growing cells were sub-cultured as follows: MDA-MB-361: semi-suspension; transferred 1:5 weekly; BT-474: adherent patches of epithelial cells (compact, multi-layered colonies, rarely becoming confluent); transferred 1:2 weekly; MCF-7: epithelial-like monolayer; transferred 1:2 weekly. Epithelial cells were harvested by detaching the cells from the tissue culture flask by

exposure to a 1 X solution of trypsin-EDTA (0.5g/l trypsin; 0.2g/l EDTA; Life Technologies Ltd.) for 5-10 minutes at 37°C. Cells were then washed in the appropriate medium and viable cells counted by 0.4% Trypan blue (Boehringer Mannheim) exclusion test.

2.1.1 Cell Lines Derived From Disseminated Cancer Cells in Bone Marrow of Patients with Prostate Cancer

The micrometastatic cancer cell lines PC-R1 and PC-H1, (established from lymph node histopathologic-negative patients) (generously supplied by Dr. Klaus Pantel, Institute of Immunology, University of Munich, Munich, Germany), were initially plated in T25 culture flasks (Life Technologies Ltd.), and incubated in 5-10% CO_2. The culture medium contained RPMI-1640 supplemented with 10% FBS, 10μg/ml transferrin, 5μg/ml insulin, 2mM L-glutamine, 10ng/ml recombinant human epidermal growth factor (hEGF), synthetic androgen (R 1881) and 10ng/ml recombinant human basic fibroblast growth factor (hbFGF). All growth factors were from Boehringer Mannheim. The cells were cultured under reduced oxygen, the medium was changed twice a week, and at confluence, the adherent monolayer, comprised of epithelial cells, were removed by trypsinisation and passaged in new flasks. Again, culture media and supplements were from Life Technologies Ltd., unless otherwise stated.

2.2 Collection of Clinical Specimens

Studies were approved by the ethical practices sub-committee of the Royal Free Hospital and written informed consent was obtained from all patients. Venous blood samples from both normal controls and patients were obtained from peripheral veins or central venous catheters. Twenty to forty

millilitres of PB was collected in preservative-free, sodium heparin tubes (100U/ml) before processing. Bone marrow aspirates containing a minimum of 1 x 10^7 cells/ml were collected into sterile sodium heparin tubes for analysis. Bone marrow harvest collections and PBSC aphereses were performed at the Royal Free Hospital according to protocol. Bone marrow was collected when the leukocyte count was greater than 3 x 10^9/l. Marrow was collected from the posterior iliac crests under general anaesthesia. The processing technique has been previously described (Gilmore *et al*, 1982). Briefly, a buffy-coat concentrate was prepared using a Cobe 2991 cell washer (Cobe Industries, Lakewood, CO, USA), followed by light-density mononuclear cell (MNC [specific gravity, ≤1.077g/l]) separation from the buffy-coat fraction by layering over Ficoll-Hypaque (Nycomed Pharma, Oslo, Norway), again in the Cobe 2991. The MNCs were mixed with 40% autologous plasma, and 10% sterile dimethylsulfoxide (DMSO [Sigma, Poole, UK]); the concentrate was cryopreserved using a controlled-rate program technique. No BM purging was done. Marrow was stored in the liquid phase of nitrogen until thawing at the bedside prior to return to the patient. Thawing was done rapidly at 37°C in a sterile saline bath and infused over 10 minutes without further processing through a central venous catheter. A mean 0.96 x 10^8 (range, 0.25 to 1.58 x 10^8) nucleated cells per kilogram patient weight was infused.

To enhance the mobilisation of stem cells into the PB, patients were administered chemotherapy and growth factor (recombinant human granulocyte-colony stimulating factor [rhG-CSF]). As part of the Anglo Celtic Co-operative Oncology Group clinical trial of intensive chemotherapy for high risk BrCa, 14 patients (18-55 years of age) with stage II-IIIa BrCa involving 4 or more axillary lymph nodes were assigned randomly to receive a high-dose

regimen consisting of 4 courses of doxorubicin followed sequentially by high-dose cyclophosphamide (Cy) and by a single course of thiotepa/Cy. Five patients with stage IV metastatic BrCa were also treated with the same high-dose regimen used in the current study. Patients received a single dose of Cy ($4g/m^2$) followed 5 days later by G-CSF (300μg [daily subcutaneous injection]), and apheresis was performed when the absolute peripheral $CD34^+$ cell count exceeded 20 x 10^6/l (usually around 10-12 days after chemotherapy, on recovery from the nadir induced by Cy). Routinely, one or two aphereses were performed with the aim to collect ≥2.5 x 10^6 $CD34^+$ cells per kilogram patient weight. Each apheresis was performed via a central catheter using a Cobe Spectra (Cobe Industries) with an approximate processed volume of 15 litres over 4 hours. A MNC fraction of approximately 200ml was collected with each apheresis. The PBSC were mixed with 10% DMSO, controlled-rate cooled at -1°C/minute, and then stored in the liquid phase of nitrogen until use. In 6 patients, two consecutive apheresis collections were obtained. A mean 3.17 x 10^8 nucleated cells per kilogram patient weight was infused (range, 0.98 to 6.50 x 10^8 nucleated cells per kilogram patient weight). Representative aliquots containing at least 1 x 10^7 cells/ml from each apheresis/BM harvest session were similarly processed for ICC assay analysis.

2.2.1 Bone Marrow and Peripheral Blood Preparation

Unilateral BM samples were obtained under either general or local anaesthesia from the posterior iliac crest of each patient through a needle aspiration. The volume of the aspirates ranged from 3.5 to 12.0 ml (mean 8.5 ml). A maximum of 5 ml per tube was diluted with Hank's Balanced Salt Solution (HBSS) (without Ca^{2+}/Mg^{2+} pH 7.4) (Life Technologies Ltd.), and

centrifuged at 168 x *g* for 10 minutes at room temperature (RT). Ten millilitres of the upper layer (marrow fat) was removed and discarded, and the cell pellet resuspended. Diluted samples were layered over Ficoll-Hypaque and then subjected to density-gradient centrifugation at 900 x *g* for 30 minutes. The light-density MNC fraction was resuspended and washed twice in HBSS (500 x *g* for 10 minutes), tested for viability (0.4% Trypan blue exclusion), counted using a Neubauer chamber, and resuspended in HBSS at a dilution of nucleated cells 5 x 10^6 cells/ml. One million cells were then adhered onto each glass microscope slide (detailed in Chapter 3, section 3.2).

After discarding the supernatant, if the cell pellet was contaminated with red cells, it was resuspended in buffered NH_4Cl solution at 4 times the original volume, vortexed and incubated on ice for 10 minutes to allow lysis of the red blood cells. (The lysing agent was 0.8% NH_4Cl in water, 10μM Na-EDTA, buffered with $NaHCO_3$ with a final pH of 7.4-7.6, which was stored at 4°C). The cell suspension was centrifuged, the cells washed, tested for viability (0.4% Trypan blue dye exclusion), counted using a Neubauer chamber, and resuspended in HBSS at a dilution of nucleated cells 5 x 10^6 cells/ml. One million cells were then adhered onto each glass microscope slide (detailed in Chapter 3, section 3.2).

2.2.1.1 Preparation of Clinical Specimen Adhesion Slides

Prior to use, all microscope slides were cleaned with acid alcohol (i.e.; 1% HCl in 70% ethanol) and coated with the attachment factors, Cell-Tak® Cell and Tissue Adhesive (Universal Biologicals Ltd, London, UK), 0.1% solution of Poly-L-Lysine (Sigma) or Cel-Line HTC Super Cured® slides (Cel-Line Associates, Newfield, NJ, USA). (The reliability of sedimentation of cells to

allow a well-defined transfer [i.e. 10^6 cells per glass slide] is documented in Chapter 3, section 3.3.1). Isolated MNCs were attached to adhesion slides consisting of 2 spots each of 1.4 cm in diameter. Routinely, two slides (4 spots) were examined for each patient and for each time point. One hundred microlitres of the cell suspension was added to each spot (= 5 x 10^5 cells/spot). For optimal results, the cell suspension did not contain any free protein. After a 30 minute incubation period, the slides were gently rinsed with serum-free medium to remove cellular debris and non-attached cells. Subsequently, the slides were air-dried for 12-24 hours, (since slides not completely dry when subjected to the staining procedure, cells may wash off), and stored at RT for up to 3 days or -70°C until use.

2.3 Immunocytochemistry

The MoAb, A45-B/B3 (murine immunoglobulin G1; kindly provided by Dr. E. Felber, Micromet GmbH, Munich, Germany), directed toward a common epitope of CK polypeptides, including the heterodimers CK8-18 and CK8-19 (Stigbrand *et al*, 1998), was used at 1.0 to 2.0µg/ml to detect tumour cells on adhesive slide preparations. These antigens are highly specific for normal epithelial cells, as well as for cells derived from primary and metastatic carcinoma cells (Moll *et al*, 1982; Debus *et al*, 1984). The specificity of the antibody reaction was also controlled by an appropriate dilution of the unrelated mouse myeloma antibody MOPC21 (Sigma) as isotype control on patients' MNC specimens. The BrCa cell line MCF-7 served as the positive control for CK immunostaining. The specific reaction of the primary antibody was developed with the alkaline phosphatase anti-alkaline-phosphatase (APAAP) technique combined with the Fast Red/New Fuchsin method to indicate

antibody binding. The immunostaining procedure, which was a modification of the method described by Cordell *et al* (1984), was as follows: Slides freshly prepared or thawed and air-dried were permeabilised with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) pH 7.4 (Life Technologies Ltd.) containing 0.5% bovine serum albumin (BSA) (Sigma), 0.1% saponin (Merck, Lutterworth, UK) in a volume of 45ml for 5 minutes at RT and fixed upon addition of either 37% formaldehyde solution (Merck), stabilised with 10% methanol and calcium carbonate for 10 minutes at RT, or in acetone for 7 minutes at RT. After air-drying and blocking of unspecific binding sites with 10% blood group AB-serum (Sigma) in PBS for 25 minutes in a moist chamber, incubation was performed with the MoAb A45-B/B3 (diluted in 10% AB-serum/PBS). (Later clinical studies were performed with a conjugate consisting of F_{ab}-fragments of the pan-CK MoAb A45-B/B3 complexed with alkaline phosphatase molecules). There are no F_c-fragments that might lead to false positive results due to binding of F_c-receptors on MNCs from PB or BM). After removing the AB-serum/PBS, in a moist chamber, 150-220µl/spot of A45-B/B3 was added and the slides incubated for 45 minutes. The slides were washed 3 x 3 minutes in PBS and 150µl of the second layer, an unlabelled rabbit-anti-mouse-IgG bridging antibody (Dako Ltd., High Wycombe, UK) (160µg/ml [1:20 in 10% AB-serum/PBS]), was added and the slides incubated for 30 minutes in the humid chamber. The slides were washed in PBS as above. One hundred and fifty microlitres of mouse-anti-rabbit APAAP complexes (Dako Ltd.) (0.9µg/ml [1:100 in 10% AB-serum/PBS]) was then added and the slides were again incubated at room temperature for 30 minutes. After washing in PBS, the intensity of the final staining was greatly enhanced at this point by repeating the anti-mouse IgG and APAAP steps. When carrying out this cycle the incubation times for

these steps were reduced to 10 minutes. The slides were washed as before, the alkaline phosphatase substrate was added, and the slides incubated at RT for 20 minutes. The substrate solution for development of antibody-bound phosphatase contained 0.2mg/ml naphthol AS-MX phosphate (Sigma) dissolved in N.N-di-methyl-formamide (Sigma), 1% Fast Red TR salt 1mg/ml (Sigma) in 0.2M TRIS buffer (pH 8.2), or 5% New Fuchsin (AMS Biotechnology, Abingdon UK) in 2N HCl and levamisole (0.25mg/ml; Sigma) to block endogenous intracellular phosphatase activity (Cordell *et al*, 1984). Finally, the slides were washed in PBS and then rinsed in tap water, air-dried and mounted in an aqueous mounting medium (Kaisers glycerol gelatin [Merck]), and scored for positive cells using light microscopy. Stained cells – referred to as positive cells – appeared bright red with good contrast to unstained negative cells, particularly in the absence of counterstaining with haematoxylin, which facilitated the rapid screening of stained slides. For each patient, 2 x 10^6 cells were screened manually by bright-field microscopy; an identical number of cells served for immunoglobulin isotyping. Both morphologic criteria and the ICC staining of MNC were used.

2.4 *Fluorescence* In Situ *Hybridization (FISH)*

2.4.1 *Cells and Cell Preparation*

Cell lines and normal BM samples were treated either with Colcemid (0.05μg/ml; [Gibco BRL, UK]) for 20 minutes to obtain metaphase preparations or harvested at confluency to obtain G_1 phase enriched interphase nuclei. Cell suspensions were centrifuged for 5 minutes at 1,500 rpm. The supernatant was removed, and the cell pellet thoroughly resuspended in 10ml of 0.075M hypotonic KCl solution at RT for 20 minutes. Samples were centrifuged as

above and the supernatant removed. Ten millilitres of fixative (methanol:glacial acetic acid, 3:1) was added after resuspension of the pellet within the last minute of hypotonic exposure, with the first millilitre added slowly dropwise with thorough vortexing of the pellet. The cell suspension was incubated at RT for 30 minutes, centrifuged as above, and resuspended in a further 10ml of fixative. Cells were then kept at 4°C for at least 8 hours before slide preparation.

2.4.2 Slide Preparation

Microscope slides were washed sequentially in detergent, dilute HCl and methanol before use, air-dried, then stored at 4°C in ddH_2O before use. Fixed cells were centrifuged, the supernatant removed, and the cell pellet resuspended in fresh fixative at an optimum concentration (i.e. "milky appearance"). Using a glass pasteur pipette, one drop of cell suspension was applied to a slide. The slide was then slowly passed through the flame of a "spirit lamp", which causes the fixative to ignite and spread the metaphases. The slide was cooled and dried and the morphology, (well spread metaphases and no plasma membranes), and concentration of cells checked ~~under~~ USING a phase contrast microscope. If necessary, the cell concentration was adjusted and further slides prepared. Spare fixed cells were stored at -20°C. Each procedure was accompanied by a control preparation using normal DONOR MNCs.

2.5 Metaphase Fluorescence In Situ *Hybridization*

2.5.1 Probes

Breast cancer metaphase chromosome preparations were hybridized with solutions containing a biotinylated unique sequence cosmid probe for Her-2/*neu* (c-*erb*B-2) (located on chromosome 17q12-q21.1) (Oncor, Inc.,

Gaithersburg, MD, USA) or a whole chromosome 17 painting probe (Cambio, Cambridge, UK) (DNA from a chromosome 17 cosmid library), labelled, respectively, with biotin and Cyanine 3 (Cy3). Prostate cancer metaphases were hybridized with Cy3-conjugated whole chromosome 7 and 8 painting probes (Cambio).

2.5.2 Slide Pre-treatment

Slides carrying metaphase spreads were "aged" in 2 x SSC (1 x SSC is 0.15M NaCl/0.015M sodium citrate, pH 7.0) at 37°C for 60 minutes. Slides were treated with 100µl RNase A (Sigma) (100µg/ml in 2 x SSC for 1 hour at 37°C) under a 24 x 24mm coverslip in a humidified chamber. The coverslips were removed by immersion in 2 x SSC, the slides were dehydrated in a graded ethanol series (70% [v/v], 80%, 90%, 100%) for 2 minutes each at -20°C, and then air-dried.

2.5.3 Slide Denaturation

Denaturation was performed by incubating slides in 70% (v/v) formamide (35ml formamide plus 15ml of 2 x SSC [final concentration], pH 7.0), at 70°C for 2 minutes. It was critical that the 70% formamide had reached its temperature of 70°C before use and that the incubation time was not exceeded. Slides were immediately quenched in ice-cold 70% (v/v) ethanol and dehydrated in a 4°C-ethanol series as above. Slides were then treated with proteinase K (Boehringer Mannheim) (60ng/ml in 20mM Tris·HCl/2mM $CaCl_2$, pH 7.5, at 37°C for 7.5 minutes) and dehydrated as above through an ethanol gradient. (If Her-2/neu signals were weak, presumably due to low hybridization efficiency, hybridization was repeated using the same protocol with an increased

proteinase K concentration [up to 0.5µg/ml]. Proteinase K concentration was reduced if excessive nuclear damage was observed).

2.5.4 In Situ Hybridization

Whole chromosome-specific painting probes: were pre-warmed to 42°C for 5 minutes and then centrifuged for 5 seconds for thorough mixing. Fifteen microlitres of hybridization mixture, containing a total of 20-50ng of labelled probe, 0.5µg of unlabelled, sonicated (200-500 base pairs) human placental carrier DNA (Sigma) (alternatively, sonicated herring sperm DNA [Oncor, Inc.] was used) in 50% formamide/10% dextran sulphate (Oncor, Inc.)/2 x SSC, was denatured for 5 minutes at 70°C, and allowed to reanneal for 45-60 minutes at 37°C. Probes were pipetted onto denatured slides, overlaid with 22 x 22mm coverslips, sealed with rubber cement and incubated overnight at 37°C in a humidified chamber.

Biotinylated Unique sequence Her-2/*neu* cosmid probe: was pre-warmed to 37°C for 5 minutes and then centrifuged for 5 seconds. The DNA probe was premixed with blocking DNA in Hybrisol VII® (Oncor, Inc.) (50% formamide, 2 x SSC). This mixture was applied to the slides under a glass coverslip (3µl/cm^2), sealed with rubber cement and incubated overnight at 37°C in a humidified chamber.

2.5.5 Post Hybridization Wash

Whole chromosome-specific painting probes: Dried rubber cement was carefully removed and coverslips gently rinsed off in a coplin jar of 2 x SSC at 37°C. With a maximum of four slides per procedure, (rather than at once) and timing commenced when the last slide was immersed, slides were washed

twice in 50% formamide/2 x SSC, and twice in 0.1 x SSC for 5 minutes each at 45°C. Alternatively, a rapid wash procedure without formamide was also used following removal of coverslips. This consisted of washing slides twice for 5 minutes each in 0.1 x SSC at 60°C, twice in 2 x SSC at 42°C, and once in 4 x SSC/0.05% Tween-20 for 10 seconds.

Biotinylated Unique sequence Her-2/*neu* cosmid probe: Following the removal of coverslips and immersion of slides as above, slides were washed three times in 50% formamide/2 x SSC, and once in 0.1 x SSC for 10 minutes each at 45°C. Alternatively, the rapid wash procedure involved washing slides once for 5 minutes in 2 x SSC, and three times in 0.1 x SSC for 5 minutes each at 60°C. Slides were then immersed in BN buffer (0.1M sodium bicarbonate, 0.05% Nonidet P-40 [NP-40] {Vysis Ltd., Richmond, UK}, pH 8.0). The slides were not allowed to air-dry from this point.

2.5.6 Immunofluorescent Her-2/neu Cosmid Probe Detection

Slides labelled with the Her-2/*neu* probe were removed from the BN buffer and preblocked in 1% BSA/4 x SSC for 5 minutes under plastic coverslips. The coverslips were removed, the excess liquid was briefly drained, and fluorescein (FITC)-avidin DCS (5µg/ml in 1% BSA/4 x SSC) was applied (5µl/cm^2). The coverslips were replaced in their original positions and the slides were incubated for 20 minutes at 37°C in the dark. The slides were then washed in 4 x SSC, 4 x SSC/0.1% Triton X-100, 4 x SSC, and PN (a mixture of 0.1M NaH_2PO_4 and 0.1M Na_2HPO_4 (pH 8.0) and 0.1% NP-40) for 10 minutes each and preblocked with PNM (5% non-fat dry milk [Carnation] and 0.02% NaN_3 in PN buffer) for 5 minutes. The intensity of biotin-linked fluorescence was amplified by adding a layer of biotinylated goat anti-avidin antibody, 5µg/ml

in PN buffer followed by three PN washes (3 minutes each). After PNM block, a third immunocytochemical staining was carried out with another layer of FITC-avidin DCS. FITC-avidin DCS and goat anti-avidin were from Vector Laboratories (Burlingame, CA, USA). After a further three PN washes and draining the excess liquid from the slide, a fluorescence antifade solution, *p*-phenylenediamine (Vector Laboratories) (1.5µl/cm^2 of coverslip) was added. A thin layer produced optimal microscopic imaging. The DNA counterstain (4,6-diamidino-2-phenylindole [DAPI]) was included in the antifade solution at 80ng/ml.

2.5.7 Fluorescence Microscopy

Excitation of each fluorochrome was accomplished by using single-band-pass excitation filters of specific wavelengths in a computer-controlled filter wheel. This made it possible to collect sequential, accurately registered images of the three fluorochromes (DAPI, FITC, and Cy3). DAPI, a blue fluorescent DNA-specific stain excited by ultraviolet, was used as the counterstain to allow simultaneous observation of hybridized probe and total DNA. Images of the metaphases and nuclei were captured separately using a X100 Neofluar oil immersion objective and a cooled coupled device (CCD) camera (Photometrics, Tucson, AZ, USA) coupled to a Zeiss Axioskop fluorescence microscope equipped with a triple band-pass beam splitter and emission filters, and controlled by Smart Capture software (Vysis) on a Power Macintosh computer.

2.6 Fluorescence Immunophenotyping and Interphase Cytogenetics

2.6.1 Preparation of Slides

Before starting the combined *in situ* hybridization and fluorescence immunophenotyping procedure, the morphological quality of cells attached to Cell-Tak coated adhesion slides or cytospins were examined by phase contrast microscopy. It was only worthwhile to process those slides with good cell morphology (i.e. optimum density to fully evaluate the immunophenotype of individual cells). If the cells were too densely packed, it was difficult to evaluate the immunophenotype of individual cells. Moreover, it was then difficult to correlate the hybridization signals to individual nuclei.

2.6.2 Mastermixes and Hybridization Mixtures

2.6.2.1a Mastermix for Single Copy Probe

Five millilitres of deionised formamide was added to 2ml of 50% dextran sulphate and 0.5ml of 20 x SSC. The mastermix was vortexed thoroughly, adjusted to pH 7.5 with 2M HCl and made up to a final volume of 10ml with ddH_2O.

2.6.2.1b Mastermix for Single Copy Probe Plus Cot-1 DNA

Two hundred and fifty microlitres of 100% ethanol was added to 100µl of Cot-1 DNA (1µg/1µl [Gibco BRL]), in a 1.5ml Eppendorf tube, incubated for 30 minutes at –70°C and centrifuged at 14,000 rpm for 30 minutes. The supernatant was removed and 500µl of 70% ethanol was added, and the tube vortexed. Following a second centrifugation at 14,000 rpm for 15 minutes, the

supernatant was removed and the pellet dried in a speed vacuum or allowed to air-dry.

2.6.2.1c Hybridization Mixture for Indirectly Labelled Single Copy Probe (60µl)

The single copy Her-2/*neu* (c-*erb*B-2 [located on chromosome 17q12-q21.1]) DNA probe is a digoxigenin-labelled probe specific for genomic sequences including the Her-2/*neu* locus (Oncor). Originally, the probe was applied precisely according to manufacturer's instructions, without prior modifications. Initially, hybridization produced a high level of background noise. Dilution of the probe in "mastermix for single copy probe plus Cot-1 DNA" enhanced the hybridization quality and reduced background. Therefore the following modifications were adopted: three hundred microlitres of Cot-1 DNA was ethanol precipitated and 27µl of mastermix added to the air-dried precipitate. Following the addition of 3µl TE-buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) and 30µl Her-2/*neu* probe, the tube was vigorously vortexed.

2.6.2.2a Mastermix for Centromeric Probe

Five millilitres of deionised formamide was added to 2ml of 50% dextran sulphate, 0.5ml of 20 x SSC and 50µl of sonicated salmon sperm DNA (10mg/ml [Sigma]). The mastermix was vortexed thoroughly, adjusted to pH 7.5 with 2M HCl and made up to a final volume of 9ml with ddH_2O.

2.6.2.2b Hybridization Mixture for Centromeric Probe (10µl)

One microlitre of a directly conjugated Spectrum Green™ fluorophore, centromeric probe (CEP®), specific for chromosome 7 (Vysis Ltd.) was added to

9µl of mastermix and vortexed. Additionally, 1µl of a directly conjugated FITC centromeric (α-satellite) paint, specific for chromosome 17 (Cambio) was either premixed with 7µl of the supplied hybridization buffer (50% formamide, 2 x SSC, 10% dextran sulphate), and 2µl distilled water and vortexed, or added to 9µl of mastermix and vortexed.

2.6.3 Immunophenotyping

Slides were fixed in fresh acetone for 10 minutes at RT and then air-dried for 10 minutes at RT in order to allow the acetone to evaporate. Frozen slides were air-dried prior to fixation for at least one hour after being removed from the freezer. At this point it was possible to interrupt the staining procedure and continue the following day. One hundred microlitres of MoAb A45-B/B3 (1.5µg/ml) in PNM buffer, was applied to the area of the slide containing the cells, and incubated for 30 minutes at RT. Detection of MoAb was performed by sequential incubation with 100µl of Cy3-conjugated goat anti-mouse (1:200 dilution in PNM buffer), rabbit anti-goat (1:200 dilution in PNM buffer), and donkey anti-rabbit (1:100 dilution in PNM buffer) antibodies (Jackson/Dianova, Hamburg, Germany). Each antibody incubation was performed for 30 minutes at RT. All incubations were followed by 3 x 2 minute washes in PN buffer.

2.6.4 Monitoring of Immunophenotyping

At this point, the success of the immunostaining was monitored. The quality of immunostaining was found to be highly variable depending on the length of time the processed slides had been stored. It was important to proceed further with slides giving the best results. Slides could be adequately assessed under the fluorescence microscope by mounting in PN buffer.

Although the fluorescence intensity in PN buffer was relatively low, the cells were protected from hybridization artefacts produced by mounting in glycerol at this stage.

2.6.5 Fixation

Successfully immunophenotyped slides were dehydrated in a graded ethanol series (70, 85, 99 vol.%) and incubated with proteinase K (Sigma) (0.5µg/ml in 20mM Tris·HCl, 2mM $CaCl_2$, pH 7.5) for 7.5 minutes at 37°C. After 2 x 2 minute washes in 2 x SSC, the slides were fixed in freshly prepared, ice-cold Carnoy's fixative (methanol:acetic acid, 3:1) for 10 minutes, and then washed in ddH_2O for 2 minutes at RT. This was then followed by 1% paraformaldehyde (2 x SSC, pH 7.6) for 1 minute at RT, and a further wash in ddH_2O for 2 minutes at RT. Appropriate fixation was one of the most important steps of the procedure to maintain the immunostaining and the hybridization signals. Fixation in 1% paraformaldehyde for 1 minute was found to be optimal. With lower concentrations of paraformaldehyde and shorter fixation periods the immunostaining diminished during the harsh hybridization procedure. More intensive fixation diminished the hybridization signals. The slides were dehydrated in a series of 70%, 85%, and 100% ethanol at RT, for 2 minutes each, and the slides then air-dried for 10 minutes at RT.

2.6.6 Hybridization

The selected hybridization mixture (detailed in 2.6.2) was applied to the cell containing area of the slide, covered with a coverslip and sealed with rubber cement. The quantity of hybridization mixture and size of coverslip were dependent on the size of the area to be hybridized. For hybridization of

cytospin slides, 1.5µl was sufficient under 8-10mm round coverslips. After sealing with rubber cement, DNA and probe together were denatured at 75°C for 5 minutes on a "hot-plate", and then immediately transferred into a humidified chamber at 37°C. Centromeric probes, were hybridized for 2-72 hours and the single copy DNA probe, for 24-72 hours. Variable hybridization times neither diminished the immunostaining or the quality of *in situ* hybridization.

2.6.7 Post Hybridization Washes

Post-hybridization washes were carried out in three changes of 50% formamide in 2 x SSC, pH 7, at 45°C for 2 minutes each, followed by brief 2 minute equilibration in phosphate buffer at RT. Alternatively, a rapid wash procedure without formamide was also used following removal of coverslips. This consisted of prewarming three Coplin jars containing 0.1 x SSC to 60°C in a waterbath. Following removal of rubber cement from the slides, coverslips were soaked off by gently agitating the slides in the first Coplin jar containing 0.1 x SSC at 60°C. The slides were then washed by gentle agitation, three times in 0.1 x SSC at 60°C for 5 minutes each, followed by a brief wash in phosphate buffer at RT.

2.6.8.1 Detection of Digoxigenin Labelled Probe With FITC

For detection of digoxigenin labelled DNA, the slides were incubated for 30 minutes at RT with FITC-conjugated monoclonal mouse anti-digoxigenin antibody (1:200 in PNM buffer [Boehringer Mannheim]), FITC-conjugated donkey anti-mouse antibody (1:200 in PNM buffer [Jackson]), monoclonal mouse anti-FITC antibody (1:200 in PNM buffer [Dako]), followed by a second

incubation with FITC-conjugated donkey anti-mouse antibody (1:200 in PNM buffer). After each incubation-step the slides were washed three times for 2 minutes each in PN buffer. In order to enhance the signal intensity the last two incubation steps were repeated.

2.6.8.2 Detection of FITC Labelled Probes

Hybridization signals were detected with monoclonal mouse anti-FITC antibody, followed by FITC-conjugated donkey anti-mouse antibody. Both antibodies were diluted 1:200 in phosphate buffer with 5% milk powder. All incubations were followed by three 2-minute washes in phosphate buffer. If necessary the fluorescence intensity was amplified by a further repetition of both detection steps. Finally, slides were covered with antifade medium containing glycerol and *p*-phenylenediamine (1.5µl/cm^2 of coverslip) containing the DNA counterstain DAPI (4,6-diamidino-2-phenylindole) at 80ng/ml. A thin layer produced optimal microscopic imaging at high magnification.

Unspecific antibody binding was prevented by replacing the MoAb with IgG1 class-matched murine MoAbs (Immunotech).

2.6.9 Evaluation

Immunophenotype and hybridization signals were evaluated simultaneously on a Zeiss Axioskop fluorescence microscope equipped with a X100 objective and filter sets for Cy3, FITC and DAPI, and the stringent criteria proposed by Hopman *et al* (1988) were applied. The portion of zero-spot cells (inversely correlated with hybridization efficiency) was below 1% in all control and clinical specimens. Morphologic features, such as the shape of cells or the presence of nucleoli were examined simultaneously by phase contrast

microscopy. In addition, CK^- cells were examined morphologically for the presence of numerical chromosome aberrations within lymphocytic bystander cells. It was difficult to evaluate the hybridization signals if the cells were located very close to each other. In such cases it was feasible to switch to bright-field microscopy in addition to fluorescence, and to assign the hybridization signals to individual nuclei with the benefit of phase contrast.

Diagnostic thresholds were defined for each probe. Each hybridization was accompanied by a control hybridization using normal DONOR lymphocytes. This was achieved by analysing control cases with a minimum of 1000 well-defined interphase nuclei with intact morphology on the basis of DAPI counterstaining. Broken, torn, squashed, smeared, or overlapping nuclei were ignored. For each slide preparation process these controls were performed independently as there are significant differences in the false positive and negative rates according to the probes and protocols used. For an unequivocal definition of true aneuploidy in clinical specimens, mean percentages plus/minus three standard deviations of control cells with non-disomic signal numbers were set as cut-off levels (Bentz *et al*, 1993; Drach *et al*, 1995; Fiegl *et al*, 1995). Positive controls of CK^+ tumour specimens with known karyotype were evaluated to verify the specificity of each assay. For the evaluation of clinical specimens the same procedures were applied as for the controls. Only good quality slides and clearly analysable cells were counted. In cases with low percentages of aberrant cells, at least 200 nuclei were counted (Kibbelaar *et al*, 1993).

2.7 Negative Depletion of Epithelial Tumour Cells Using StemSep™ Labelling of Human Cells

(This procedure was carried out following instructions by Drs. Terry E. Thomas and Carrie E. Peters, StemCell Technologies, Vancouver, BC, Canada).

2.7.1 Sample Preparation

2.7.1.1 Recommended Media

Buffered salt solutions without Ca^{2+} or Mg^{2+}, such as PBS or Hank's, modified with 4% FBS, were used unless otherwise stated. The recovery of circulating epithelial tumour cells was further improved and optimised, with the addition of EDTA to a final concentration of 2mM to all media for cell washing and labelling.

2.7.1.2 Types of Cell Suspensions

2.7.1.2.1 Fresh Whole Blood and Bone Marrow

These cell suspensions required a Ficoll-Hypaque step to remove most of the mature erythrocytes (to less than 20 erythrocytes per nucleated cell or haematocrit of 5%). Cells were washed with PBS or Hank's. When using a 0.1" (0.1 inch), or 0.3" (0.3 inch) column, nucleated cells were resuspended at 50 x 10^6 per ml (a range of 20-80 x 10^6 was suitable) in PBS or Hank's. Cell suspensions were ready for antibody labelling.

2.7.1.2.2 Mobilised Peripheral Blood – leukopheresis Preparations

Cells were washed twice with PBS or Hank's. As above, when using a 0.1", or 0.3" column, nucleated cells were resuspended at 50 x 10^6 per ml (a

range of 20-80 x 10^6 was suitable). Cell suspensions required no further manipulations, and were ready for antibody labelling.

Ficolled cells were treated as leukopheresis preparations but column capacities (see below) were doubled.

Column Size	Optimum # of Cells	Extended Range of Cell #
0.1"	10^6	10^5–20 x 10^6
0.3"	50 x 10^6	20–80 x 10^6

2.7.1.3 Previously Cryopreserved or Clumped Samples

Previously cryopreserved aliquots of cells were quickly thawed in a 37°C water bath for approximately one minute. Cells (0.5-5.0ml) were transferred to a 50ml falcon tube, and PBS (without Ca^{2+} or Mg^{2+}) was added dropwise while gently mixing. The tube was filled to 50ml, gently inverted to mix and centrifuged at 1,200 rpm for six minutes. The supernatant was discarded, the tube gently agitated to resuspend the pellet, and the cells were further resuspended at the desired concentration in the appropriate medium.

Due to clumping of the cells, 0.25-0.5ml of 1mg/ml Deoxyribonuclease I (DNase [from bovine pancreas]) (2,000 Kunitz units/ml in 0.15M NaCl [Sigma]), was added dropwise per millilitre of cells, while gently mixing. Following the first wash, the cell pellet was resuspended in 1ml of 1mg/ml DNase. DNase was finally added to the cell suspension at 0.1mg/ml (80 Kunitz units/ml). In the case of gravity feed separations of excessively clumped cell suspensions, cells were filtered through a Falcon 70μm nylon cell strainer (Marathon Laboratory Supplies, London, UK).

2.7.2 Immunomagnetic Labelling

One hundred microlitres of bispecific tetrameric antibody complexes (CD2, CD14, CD16, CD19, CD36, CD38, CD41, CD45, CD66b and glycophorin-A), was added for each millilitre of cells and thoroughly mixed. The cell suspension was incubated on ice for 30 minutes or for 15 minutes at RT. Sixty microlitres of a colloidal suspension of magnetic dextran iron particles in USP saline, was then added for each millilitre of cells, thoroughly mixed and incubated on ice for 30 minutes or for 15 minutes at RT. During this incubation period, columns were prepared as described below. Cells were then ready for magnetic cell separation.

2.7.3 Magnetic Cell Separation

2.7.3.1 Column Assembly – Gravity Feed

An appropriate magnet was placed in its stand and a suitably sized sterile StemSep™ column was placed in the magnet. (For 0.1" columns that were being used with a MiniMACS [Miltenyi Biotec Ltd., Bisley, UK]: the whole column was set in the gap of the magnet with the column rim resting on the top of the magnet). A 3-way stopcock (Uno Plast AS, Denmark) was then connected to the column or column extender (0.1" column only). This was followed by attaching the hub of a 23-gauge blunt end needle, to the luer lock fitting on the stopcock directly below the column, whilst keeping the cover on the end of the needle until Column Preparation (washing the column). All connections were then checked.

2.7.3.2 Column Preparation – Gravity Feed

From this point, the column was never allowed to run dry at any time during the priming, washing, or loading steps. The plug was removed from the top of the column (0.3" column only), and the 3-way stopcock was set to allow flow from the side connection into the column. A sterile syringe was filled with PBS (without FBS or other protein), air bubbles removed, and attached to the side connection of the 3-way stopcock.

For 0.3" column: the plunger of the syringe was slowly depressed to deliver PBS up into the column until the level was above the stainless steel matrix, while avoiding the introduction of air bubbles into the mesh matrix. Any air bubbles in the matrix were dislodged by sharply tapping the side of the column. Additionally, if an air bubble was lodged in the 3-way stopcock, PBS was added to the top of the column and the bubble pulled out into the side syringe.

For 0.1" column: the plunger of the syringe was depressed with firm even pressure to deliver the entire 1ml of PBS up through the column. Once again, any air bubbles trapped in the column matrix were removed by gently moving the plunger of the syringe in and out. This was repeated 5 to 10 times making sure that the level of PBS did not fall below the top of the column matrix (more PBS was added to the top if necessary). Pulling back 500µl of PBS and any air bubbles into the side syringe ended the priming process. If the column stopped running at any point, a small volume of PBS was introduced from the side syringe into the column. This usually removed any air bubbles that were trapped at the top of the column and thereby disrupting the flow. An air lock in the needle was removed be redirecting the flow of buffer from the side syringe out through the needle.

In order to wash the column, the cover of the blunt end needle was removed, and the recommended medium was added to the top of the column (PBS or Hank's plus FBS). (For enrichment of circulating epithelial tumour cells, EDTA was added to a final concentration of 2mM). The 3-way stopcock was turned so that the flow was from the column down through the needle (side exit closed). Media was added continuously until three column volumes were collected (0.3" column – 8ml collected; 0.1" column – 1.0ml collected). The stopcock was turned to stop the flow of media from the column when the fluid level was just above the column matrix. The column was now ready for the separation procedure.

2.7.3.3 Separation Procedure – Gravity Feed

Sample was loaded into the top of the column, and the stopcock was turned to start the flow of media down through the needle into the collection tube. The sample was then allowed to run into the column matrix. The recommended medium, (PBS or Hank's plus FBS, and EDTA [added to a final concentration of 2mM]), was added to the top of the column until three column volumes were collected (0.3" column – 8ml collected; 0.1" column – 1.5ml collected) plus the volume of the start sample. The stopcock was turned to stop the flow of media. This collected flowthrough fraction was the enriched epithelial tumour cell fraction.

2.7.4 Preparation of Cytospin Slides

The enriched epithelial tumour cell fraction was centrifuged for 10 minutes at 250 x *g* (RT). The cell pellet was then resuspended in a small

volume of PBS, and the viability of the cells was determined with 0.4% Trypan Blue.

Cells were cytocentrifuged onto poly-l-lysine coated glass slides (1,030 rpm for 3 minutes at RT) at a density of 2000 cells per mm^2, using a Shandon Cytospin 2 cytocentrifuge (Shandon Scientific Ltd., Runcorn, UK) (~40,000-50,000 cells per cytospin). Approximately 2 x 10^6 cells were adhered onto two Cell-Tak Cell and Tissue Adhesive coated slides from the pre tumour enrichment fraction. Following cytocentrifugation and adhesion, slides were air-dried for 1 to 24 hours. Slides were either stained immediately or stored at -20°C (up to four weeks) or at -70°C (up to one year).

2.8 Cancer Cell Enrichment by Prototype Avidin-Biotin Based Tumour-Enrichment Column (TEC)

2.8.1 Reagent Preparation

(This procedure was carried out following instructions by Dr. Amy A. Ross, MRD_X^{TM} Diagnostics, CellPro, Inc., WA, USA).

The avidin column, 5% BSA/PBS, PreGel (uncoated beads) and sample/wash chamber were all brought to RT. The antibody reagent (25µg at 1.0mg/ml) was refrigerated until ready for use. Fifty millilitres of 1% BSA in PBS was prepared for each enrichment procedure. Ten millilitres of 5% BSA (provided by CellPro, Inc., [reagent selected to provide optimal performance]), was mixed with 40ml of Ca^{2+}-Mg^{2+} free Dulbecco's phosphate buffered saline (D-PBS) (Life Technologies Ltd.)

2.8.2 Sample Preparation

MNCs from BM samples were isolated following density gradient separation on Ficoll-Hypaque and washed twice in 10-20ml of Ca^{2+}-Mg^{2+} free D-PBS containing 1% BSA, at 300 x *g* for 10 minutes. Cryopreserved vials of cells were thawed in a 37°C water bath and resuspended in Ca^{2+}-Mg^{2+} free D-PBS containing 1% BSA and 100U/ml of DNase (Sigma). Trypan blue viability cell counts on samples were then recorded. Cells were counted using a haemocytometer, and resuspended at 100-200 x 10^6 cells/ml in 1% BSA in D-PBS with a minimum volume of 1ml. A maximum of 500 x 10^6 cells and a minimum of 50 x 10^6 cells were required for processing on the column. Approximately 2 x 10^6 cells per sample were reserved for tumour-cell detection prior to using the TEC.

2.8.3 Antibody Incubation

The TEC PAN-05 anti-epithelial cell biotinylated MoAb reagent was added to the cell suspension at a final concentration of 1:1000 and gently mixed. (These MoAbs have been shown to react with cell membrane antigens in breast, prostate, ovarian, colon, small cell and non-small cell lung tumours [Dr. Amy A. Ross, personal communication]). The cell suspension was then incubated for 30 minutes at RT. Cells were resuspended after 15 minutes to promote uniform antibody distribution. (Sections 2.8.4 and 2.8.5 were proceeded with, while the cells were incubating with antibody). Following incubation, the cells were diluted to a final volume of 14ml in D-PBS with 1% BSA, washed (300 x *g* for 7-10 minutes) to remove unbound antibody, and resuspended in D-PBS with 5% BSA at 1ml per 1 x 10^8 cells for addition to the TEC device (Cell Enrichment [section 2.8.6]).

2.8.4 Assembling Avidin Column and Sample/Wash Chamber

The sample/wash chamber was removed from its sterile pouch, and the 3-way valve "snapped" into the black guide on the operating platform. The luer cap was separated from the top of the avidin column, and the column was then attached to the 3-way valve on the sample/wash chamber.

2.8.5 Priming the Avidin Column

The 3-way valve was turned to the left (9 o'clock position) and the avidin column slowly squeezed and held to force liquid into the sample chamber and through the disc filter in the bottom of the chamber. While the liquid level was above the filter, 5ml of PBS was added to the sample chamber, and the avidin column released. If air bubbles remained in the column, they were forced through the filter by squeezing the avidin column again. The outlet clamp was closed and the outlet tubing cut and placed into a 15ml centrifuge tube. The 3-way valve was turned down (6 o'clock position), allowing PBS to flow down from the sample chamber (right side) into the wash chamber (left side). This removed air from the connection between the sample chamber and the wash chamber. The 3-way valve was then turned to the left (9 o'clock position), while bringing the PBS volume in the sample chamber to 5ml and in the wash chamber to 10ml. Using a pipette, the entire volume of the PreGel (300μm avidin-conjugated beads) was mixed and transferred to the sample chamber, resulting in an approximate PreGel bed volume of 0.7ml. The outlet clamp was opened and PBS allowed to pass through the avidin column. As the PBS flowed through the avidin column, it was squeezed 5-10 times along its length, until the level reached the top of the PreGel and the outlet clamp was closed. The level of PBS was never allowed to enter the PreGel, as this introduced air

bubbles into the avidin column. However, if air was introduced into the system, section 2.8.5 (priming the avidin column) was repeated. Five millilitres of 5% BSA solution was then added to the sample chamber and the outlet clamp opened. As the 5% BSA solution flowed through the avidin column, it was squeezed 5-10 times along its length, until the level reached the top of the PreGel and the outlet clamp was closed. The avidin column remained in this state until the antibody labelling was completed.

2.8.6 Cell Enrichment

Antibody-labelled cells were layered on top of the PreGel in the sample chamber. Excessive resuspension of the PreGel was avoided and the cell concentration did not exceed 1 x 10^8 cells/ml. If the sample volume exceeded 4ml, the remaining sample was added after a sufficient volume flowed into the avidin column. The outlet clamp was opened and when the sample volume reached the top of the PreGel, 1-2ml of 5% BSA was immediately added to rinse the remaining cells into the PreGel. (The avidin column was not squeezed or manipulated during cell loading and column washing, as this would have reduced recovery of target cells). When the 5% BSA reached the top of the PreGel, the 3-way valve was turned up (12 o'clock position) to allow PBS to flow from the wash chamber into the avidin column. After 3ml of PBS had drained through the avidin column, the outlet clamp was closed for 60 seconds. The clamp was then opened to resume fluid flow, and the avidin column was washed with an additional 2ml of PBS. The clamp was then closed again, whilst 5ml of PBS was redirected to the wash chamber. The centrifuge tube containing the flow through (unadsorbed) cells was removed and replaced with a new tube containing 1ml of 5% BSA. The outlet clamp was re-opened and

the avidin column squeezed 5-10 times along its length. When the PBS was at the 2ml mark, the column was squeezed a further 3-5 times along its length. Avidin gel was resuspended into the wash chamber during squeezing, however, this did not affect separation performance. More than 4ml was collected to ensure all target cells were recovered. This fraction constituted the adsorbed, or enriched cells.

The biotin adhered to the avidin on the beads and the target cells (epithelial tumour cells) were retained in the column. Non-target cells (normal haematopoietic cells) washed through the column. Gentle agitation of the column bed released the target cells, while the biotinylated antibody remained behind, firmly attached to the avidin beads. The released cell population was highly enriched for the target cells. Because the biotinylated antibody remained firmly attached to the avidin beads, there was very little residual antibody on the cell surface (Dr. Amy A. Ross, personal communication). Cell counts and viability for both fractions were determined using a haemocytometer and Trypan blue exclusion.

ICC staining was performed on all cell fractions (pre-TEC fraction, and both the unadsorbed/flow-through fraction and the adsorbed fraction). Cytospin slides were only prepared from the adsorbed fraction using the Shandon Cytospin 2 cytocentrifuge (Shandon Scientific Ltd.) The cells were centrifuged at 1,200 rpm for 4 minutes onto poly-l-lysine coated slides. Approximately 2 x 10^6 cells were adhered onto two Cell-Tak Cell and Tissue Adhesive coated slides each from the pre-TEC fraction and the unadsorbed fraction. All of the cells eluted from the adsorbed fraction were centrifuged onto 1-2 slides, depending on the number of cells recovered. The slides were then stored desiccated at -70°C, prior to ICC staining.

Chapter 3 Immunocytochemical Detection of Breast Cancer Cells – A Comparison of Three Attachment Factors

3.1 Introduction

Patients with BrCa are at risk of relapse after surgical/radiotherapy treatment of the primary disease despite the lack of detection of metastases. This risk can be estimated using a combination of tumour factors including the number of lymph nodes involved, size of the primary tumour and histological grade. Best current practice is to administer adjuvant chemotherapy for 6 months and/or adjuvant endocrine therapy for 5 years following primary treatment for localised BrCa. Patients are subsequently followed thereafter with overt metastatic disease developing in many. No patients survive metastatic disease and the median survival is approximately 9 months. There is a stark contrast between outcome of treatment in the adjuvant and metastatic situation. Following adjuvant therapy, 17-23% more patients can be expected to survive free of disease, compared with those who receive no adjuvant therapy (Early Breast Cancer Trialists' Collaborative Group [EBCTCG], 1998). Chemotherapy and/or endocrine therapy, whilst capable of extending the life of patients with metastatic disease, is not able to cure patients. The development of metastasis is thought to be due to undetected microscopic disease in PB/BM. Previous studies have demonstrated tumour cells with clonogenic potential in the BM of patients with BrCa (Ross *et al*, 1993) and it has been recognised that these patients relapse sooner than those with a negative BM (Schoenfeld *et al*, 1997; Mansi *et al*, 1999).

One of the most controversial issues in medical oncology has been the use of high-dose chemotherapy (HDC) and autologous progenitor-cell transplantation in the treatment of BrCa (Weiss, 1999). Historically controlled phase II trials in patients with metastatic disease (Peters *et al*, 1988) or high-risk primary disease (Peters *et al*, 1993; Gianni *et al*, 1997) have suggested significant disease-free and overall survival benefits from this approach, however several randomised studies have yet to confirm an advantage. In December, 1990, randomised trials assessing the efficacy of HDC in metastatic and high-risk primary BrCa were begun at the University of Witwatersrand, Johannesburg, South Africa, by Werner Bezwoda. The protocols involved two cycles of HDC (tandem transplants) with progenitor-cell support and no intervening standard-dose chemotherapy (Bezwoda *et al*, 1995; Bezwoda, 1998; 1999). Patients with metastatic and high-risk, primary BrCa were reported to have survival in the HDC groups superior to that in the standard-dose groups. This trial was the only randomised study presented at the plenary session of the 1999 meeting of the American Society of Clinical Oncology (ASCO) meeting that showed an overall survival advantage for HDC in women with BrCa. To corroborate the Bezwoda study results before starting a large international confirmatory study, a team of US oncologists did an on site review of records for patients in the high-risk study. The US team found disparity between the reviewed records (Weiss *et al*, 2000) and the data presented at two international meetings. In addition, the reviewers saw no evidence of signed informed consent, and the institutional review committee had no record of approval for the investigational therapy. These studies are suspected to be fraudulent. Although HDC/stem cell rescue has not to date fulfilled its initial promise, we were able to study the presence/absence of contaminating

micrometastases as part of a UK prospective, randomised evaluation of high-intensity chemotherapy with PBSC support in patients with high-risk BrCa begun prior to reporting of the Bezwoda study (treatment regimens detailed in Chapter 2, section 2.2), and to correlate this with clinical relapse.

One limitation of the value of autologous transplantation is the inadvertent reinfusion of tumour cells contaminating the preparations of stem cells used for haematological rescue (either from BM harvest or PBSC collections). This has been shown in bcl-2-positive lymphomas undergoing autologous BM transplantation (Gribben *et al*, 1991), where higher relapse rates were found in patients transplanted with bcl-2-positive stem cell preparations (26/57 [46%] patients) when compared to those receiving bcl-2-negative products (4/57 patients [7%]) ($p<0.00001$). In patients with acute myeloid leukaemia (Brenner *et al*, 1993), chronic myelogenous leukaemia (Deisseroth *et al*, 1994) and neuroblastoma (Rill *et al*, 1994), the use of gene marking techniques showed that the reinfused BM tumour cells were contributing to disease relapse.

The detection of occult breast tumour cells in BM has relied mainly upon immunological techniques, with sensitivity levels which range from one tumour cell in 10^4-10^5 normal BM cells for immunocytochemistry (Ross *et al*, 1993; Brugger *et al*, 1994) up to one in 5×10^5 for flow cytometry (Simpson *et al*, 1995). In a comparative study (Vredenburgh *et al*, 1996) of three different immunodetection techniques using a panel of four murine monoclonal anti-breast cancer cell antibodies (260F9, 317G5, 520C9 and BRE-3), a two-colour immunofluorescence assay was shown to be the most sensitive and able to detect one tumour cell among 10^6 normal BM cells. Higher sensitivity may be obtained by four-colour immunofluorescence (Gross *et al*, 1995).

The detection by RT-PCR of CK19 (K19) messenger RNA (mRNA), a protein expressed by normal epithelial cells and epithelial-derived tumours, has been proposed as a sensitive and specific marker for breast tumour cell contamination (Datta *et al*, 1994). However, other studies advocate caution in the use of K19 RT-PCR assay, due to an observed low specificity in the evaluation of haematogenous dissemination in patients with lung tumours (Krismann *et al*, 1995). Krismann *et al* (1995) provided evidence for an illegitimate expression of CK19 mRNA in BM and blood obtained from a group of non-carcinoma control patients. This limitation of the RT-PCR approach is that ectopic expression of small amounts of epithelial mRNA in mesenchymal BM cells might also be detected if the amplification of the transcribed cDNA is extensive. Moreover, this approach might also be hampered by the possibility that unknown processed pseudogenes in myeloid cells of CK genes might exist or that CK debris-containing phagocytic cells register as RT-PCR positive.

The potential value of a sensitive and reproducible method for the detection and enumeration of micrometastases in blood or BM include:

- The ability to follow response to therapy;
- Allow an earlier change from ineffective treatment;
- Detect relapse earlier;
- Monitor mobilisation of tumour cells during growth factor administration.

The aim of this study was to establish a sensitive, specific and reproducible immunocytochemical (ICC) assay capable of detecting very low levels of tumour cells (at least comparable to conventional ICC assay detection sensitivity [$1:10^5 - 1:10^6$]) by attachment to defined areas on microscope slides coated with adhesive factors. The principle employed to achieve this was to firstly optimise the distribution of the cells into a single cell layer and also to

ensure minimal cell loss during processing so that the number of positive cells could be related directly to the number of cells fixed onto the slides. The specificity-proven pan-CK antibody A45-B/B3 and the alkaline phosphatase-anti-alkaline phosphatase (APAAP) staining technique in the absence of counterstaining to optimise the signal:noise ratio were used.

3.2 Materials and Methods

3.2.1 Seeding Studies

Breast cancer cells (MCF-7 cell line) were isolated by trypsinisation, washed twice in PBS, (as were Daudi suspension culture cells), and re-suspended at 5 x 10^6 cells/ml in PBS. Breast cancer cell samples processed as described above were mixed with different concentrations of Daudi cells and divided into several tubes at dilutions of 1 MCF-7 cell:10, 1:100, 1:1,000, 1: 10,000, 1:100,000 and 1:1,000,000. One hundred microlitres of each cell suspension (5 x 10^5 cells) were attached by sedimentation onto defined areas (spots) (1.4cm in diameter) on microscope slides coated with one of three adhesive factors. Triplicate samples of 2 x 10^6 cells were immunostained with MoAb A45-B/B3.

3.2.2 Attachment Factors

Before use, all microscope slides were cleaned with acid alcohol (i.e.; 1% HCl in 70% ethanol) and coated with one of the following attachment factors:

1) Cell-Tak® Cell and Tissue Adhesive (Universal Biologicals Ltd, London, UK)
2) 0.1% solution of Poly-L-Lysine (Sigma, Poole, Dorset)

3) Cel-Line HTC Super Cured® slides (Cel-Line Associates, Newfield, NJ, USA).

3.2.2.1 Cell-Tak®

Cell-Tak® is a formulation of polyphenolic proteins from the anchoring glue secreted by the marine mussel, *Mytilus edulis* (Waite and Tanzer, 1981). Glass microscope slides were cleaned and after drying, defined areas were covered with a variable volume, (dependent on the stock concentration), of freshly prepared solution of Cell-Tak®, (determined after a series of preliminary experiments to confirm optimal density to be 5.0µg/cm^2 of surface area), in 0.1M $NaHCO_3$, pH 8.0 or 0.1M HEPES (N-2-hydroxyethylpiperazine-N2 ethanesulphonic acid) buffer, pH 8.0. The adhesive is provided in a dilute acetic acid solution and is poorly soluble around neutral pH, causing it to be adsorbed to the slide. After 20 minutes at RT the slides were rinsed in sterile water. Cells in suspension were tested for viability (0.4% Trypan blue exclusion), counted using a Neubauer chamber, resuspended in HBSS and 5 x 10^5 cells were applied to each spot. Following a 30 minute incubation period, the slides were gently rinsed with serum-free medium to remove dead cells and cellular debris. Subsequently, cells were air-dried for 12-24 hours and stored at -70°C until used.

3.2.2.2 Poly-L-Lysine

The principle behind this method is that the polycationic polylysine molecules attach strongly to glass surfaces, leaving cationic sites which combine with the anionic sites on cell surfaces. A working concentration of Poly-L-Lysine solution was prepared by diluting Poly-L-Lysine solution at the manufacturer's concentration of 1:10 with deionised water prior to coating defined areas on cleaned glass microscope slides. Surfaces were prepared by

covering them briefly (5 minutes) with the 0.1% solution of Poly-L-Lysine in water. The slides were then washed sequentially with running water and serum-free medium. They were dried in a 60°C oven for 1 hour or at RT overnight and either stored at 4°C or used immediately. As above, after a 30 minute incubation period with the cell suspension, they were gently rinsed with serum-free medium, air-dried for 12-24 hours and if not stained immediately, were stored at -70°C until use.

3.2.2.3 Cel-Line

To perform the ICC assay within this system, cells (5 x 10^6 cells/ml) were seeded into the wells (5 x 10^5 cells per well) of specially designed, Cel-Line HTC® (heavy polytetrafluoroethylene [Teflon®]-coated) Super Cured® slides (14-mm wells), by sedimentation. The slides were air-dried and, if not stained immediately, were stored at -70°C until use.

3.2.3 Immunocytochemistry

Using the APAAP staining technique, a strong colour reaction without a background reaction was observed (Figure 3.1). Cells containing CK components were stained bright red. This meant that counter-staining could be omitted, allowing for a faster screening for stained cells occurring at low frequencies.

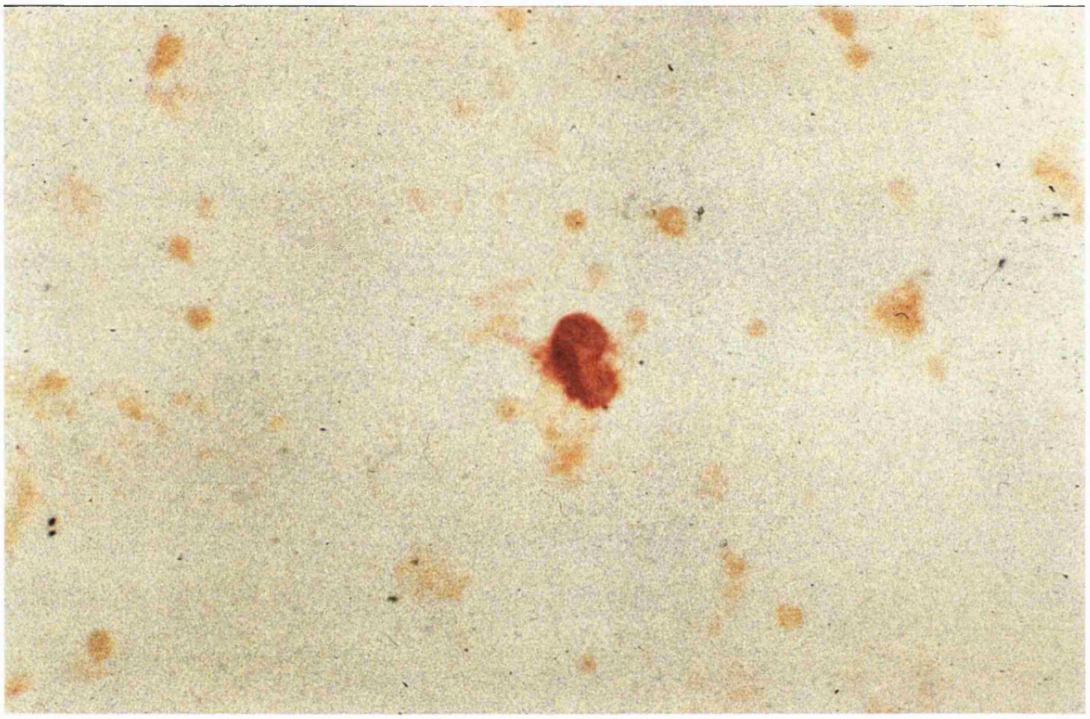


Figure 3.1: Immunocytochemical detection of tumour cells.

Cytokeratin immunostaining of tumour cells shows intense immunoalkaline phosphatase reaction, whereas Daudi cells show no immunostaining reaction. Original magnification, X100.

Each immunostaining assay contained a negative as well as a positive control. Two million cells incubated with non-immune mouse serum served as a negative control, one spot with the human BrCa cell line MCF-7 served as a positive control. Blood and BM samples from patients with haematological malignancies, incubated with the breast-reactive MoAb in parallel served as additional negative controls. Nonreactive normal haematopoietic cells on the patient preparations served as additional internal negative controls for APAAP staining. After seeding into the Daudi cell line, at each dilution, a total of 12 spots with at least 6.0×10^6 cells in total were analysed for the presence of BrCa cells. A total of 4 spots with at least 2.0×10^6 total cells were analysed for the presence of tumour cells in clinical specimens using a phase contrast microscope with bright field illumination at low magnification. The total number of either epithelial or Daudi cells present on control slides, on each spot within each adhesive system were calculated by counting the cell number within 20 quadrants out of 100 total quadrants five times from different fields using a graticule within the ocular.

3.2.4 Statistical Methods

χ^2 tests were used to compare the observed number of BrCa cells with the number of cells seeded in all three adhesive systems. Results were expressed as percentage ± coefficient of variance (c.v.). χ^2 tests were also used to compare the total number of either BrCa or Daudi cells present on control slides, on each spot within each adhesive system, with the expected total number. Difference in rates of tumour cell contamination of PBSC and BM was tested using Fisher's exact test of concordance. The quantity of tumour cell involvement in PBSC and BM was compared using Mann-Whitney test.

The calculated number of tumour cells reinfused in PBSC and BM was also compared using Mann-Whitney. A p value of less than 0.05 was considered to indicate a statistically significant difference. For statistical analysis, GraphPad Prism® software (San Diego, CA, USA) (version 2.01) was used.

Complete response was defined as the lack of detectable disease by radiography and physical examination. The probability of survival free of distant metastases was calculated from day 0 (reinfusion of autologous haematopoietic stem cell source) until the day of death or last follow-up. Patients who did not die were censored at the last day of follow-up. The probability of disease-free survival (DFS) was calculated using the method of Kaplan and Meier (1958) and compared using a log-rank test.

3.3 Results

3.3.1 Quantitation of Breast Cancer Cells in Daudi Cells Using Immunocytochemistry

Cultured BrCa cells were added to Daudi cell suspensions to result in final concentrations of between 1-1000 per 1,000,000 Daudi cells. These mixtures were then stained and analysed as described, and the number of tumour cells (observed) was plotted against the number seeded (expected) for each adhesive system investigated. Highly significant correlations of expected versus observed values were found. Linear regression analyses were performed for all three adhesive systems where the ranges examined were 0.0001% to 0.1%, indicating strong correlation between expected and measured numbers of tumour cells ($p<0.0001$) present in all test systems. Intra-assay reproducibility for each system, (Figure 3.2A-C), (median c.v. = Cell-Tak® [8.81%], Cel-Line [7.47%], and Poly-L-Lysine [9.72%]) is illustrated. All

test samples were re-tested on 4 occasions to assess inter-assay reproducibility (Figure 3.2D) which was also well within accepted limits for a clinical assay (median c.v. = Cell-Tak® [2.42%], Cel-Line [6.84%], and Poly-L-Lysine [6.83%]). There were no significant differences between the observed and expected numbers of BrCa cells in this system down to $1:10^5$ dilution, irrespective of the adhesive used (Table 3.1). Below $1:10^5$ the Cell-Tak® and Cel-Line slides continued to show significant correlation between expected and observed and no significant difference between the number of BrCa cells detected and the number seeded. In contrast the Poly-L-Lysine slides showed significant difference between observed and expected (χ^2 42.2 $p<0.10$) at the $1:10^6$ dilution. Despite this, the overall correlation for Poly-L-Lysine slides remained highly significant ($p<0.0001$).

3.3.2 Graticule Data

After calculating the total number of either epithelial or Daudi cells present on each spot within each adhesive system by counting the cell number within quadrants, no significant difference ($p>0.1$) was observed between the number of cells applied and the number retained after processing (Table 3.2). Consequently, it was established that significant cell numbers were not lost after completion of the ICC staining method. This was also found to be the case, after calculating the total number of MNCs from clinical material studied, present on each spot within the Cell-Tak® system.

Immunocytochemical and additionally morphologic analyses also showed that cells from the epithelial tumour cell lines were larger than Daudi cells, which are comparable to PB MNCs. This size differential was also noted between circulating breast tumour cells and MNCs in the clinical samples studied.

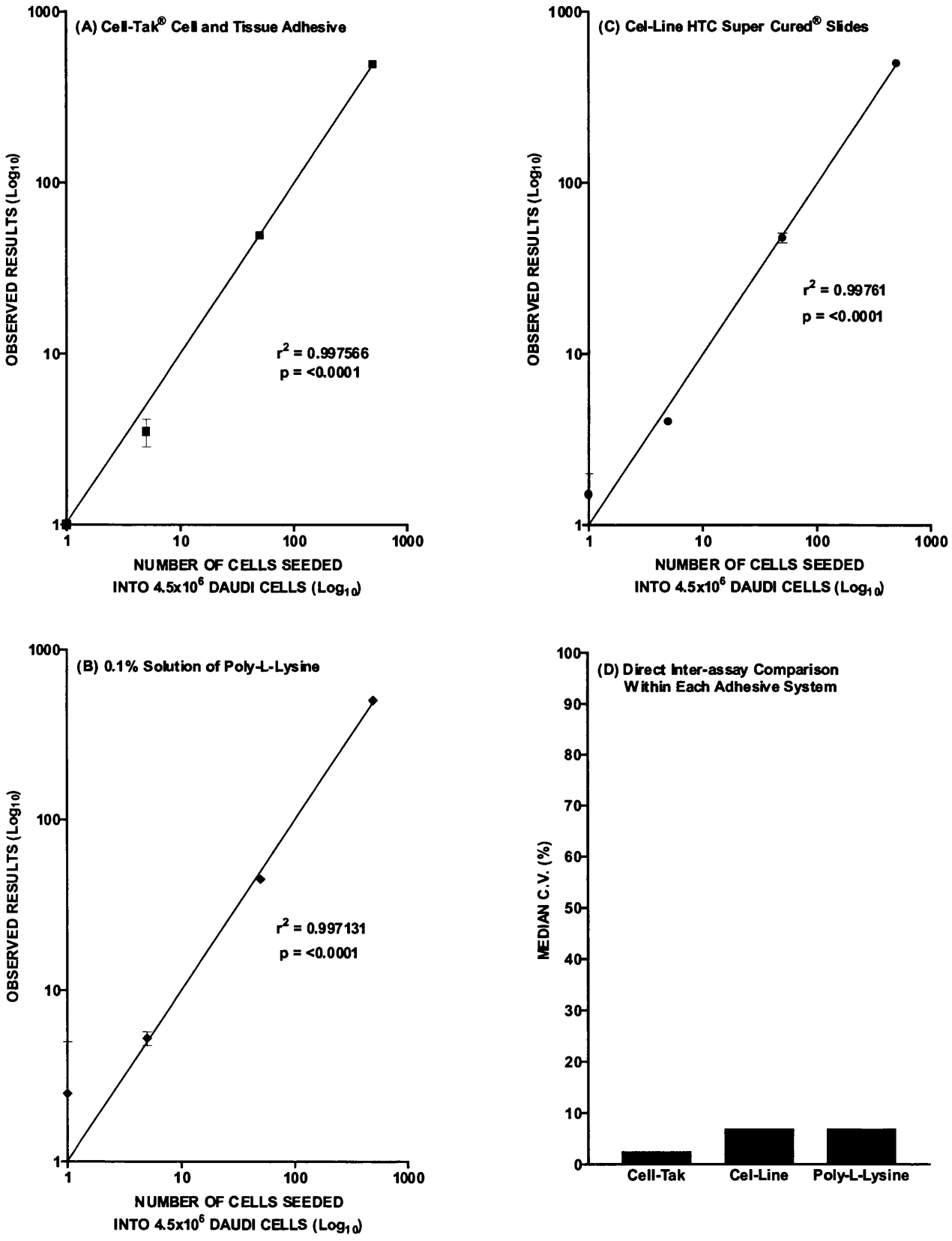


Figure 3.2: Comparison of adhesion factors in the detection of seeded BrCa cell lines into Daudi cells.

The observed number of BrCa cells is plotted against the number of cells seeded (Log_{10} mean of replicates ±SEM) to illustrate intra-assay variation for each adhesive system).

Slide Coating	% Tumour Cell Dilution	% Pos. Detected Tumour Cells $\bar{x}$ ± %c.v.
Cell-Tak®	0.1	0.1001 ± 0.0039
	0.01	0.0098 ± 0.0009
	0.001	0.0009 ± 0.0003
	0.0001	0.00008
Poly-L-Lysine	0.1	0.1005 ± 0.0047
	0.01	0.0097 ± 0.0010
	0.001	0.0010 ± 0.0002
	0.0001	0.00017
Cel-Line Slides	0.1	0.1000 ± 0.0035
	0.01	0.0095 ± 0.0011
	0.001	0.0010 ± 0.0002
	0.0001	0.00010

Table 3.1: Detection of BrCa cell lines in Daudi cells using ICC in all three adhesive systems[a].

[a]Triplicate samples of identical sizes (2 x 10^6 cells), at each dilution, were stained with MoAb A45-B/B3 using the APAAP technique.

Slide Coating	Cell Type	Total No. Cells Applied (x 10^5)	Total No. Cells Observed (x10^5)	(χ^2)	p
Cell-Tak®	Tumour	5.00	4.65	2.169	>0.50
Poly-L-Lysine	Tumour	5.00	4.53	2.093	>0.50
Cel-Line Slides	Tumour	5.00	4.68	2.155	>0.50
Cell-Tak®	Daudi	5.00	5.51	3.292	>0.50
Poly-L-Lysine	Daudi	5.00	5.74	3.931	>0.1
Cel-Line Slides	Daudi	5.00	5.22	1.703	>0.50

Table 3.2: Graticule data illustrating that significant celi numbers were not lost after the ICC staining method from control slides in both BrCa and Daudi ceii lines, in ail three adhesive systems.

3.3.3 Characteristics of Patients With Bone Marrow Harvest or Peripheral Blood Stem Cell Involvement

The presence of CK^+ cells was evaluated in PBSC harvests collected after Cy/rhG-CSF (doses and schedules defined in Chapter 2, section 2.2); in 13/19 (68.4%) patients, one apheresis was considered satisfactory for haematological rescue, whereas in the remaining 6 a second apheresis was necessary to obtain the target number of stem cells ($\geq 2.5 \times 10^6$ $CD34^+$ cells per kilogram patient weight). Eleven of nineteen patients (57.9%) had a pheresis product which was CK^+; in only one of the eleven patients requiring two aphereses were consecutive pheresis products shown to contain CK^+ tumour cells (patient with lung metastasis). The percentage of aphereses positive for CK^+ cells was 53% and 33%, respectively, for PBSC suspensions collected on the first and second day, following growth factor administration ($p = 0.6447$, Fisher's exact test).

PBSC collections were less likely than BM to contain tumour cells in patients with BrCa, (however, no patient in this part of the study had paired BM/PBSCs collected). In patients with localised BrCa PBSC tumour involvement was found in 64% (9/14). In contrast, BM collections from patients with similarly localised disease consistently contained immunocytochemically detectable tumour cells (2/2 patients) although the number of samples was low. Patients with advanced stage disease (metastatic sites included axilla, liver, lung and lymph nodes) at the time of collection also were less likely to have PBSC involvement (40%, 2/5) than BM (100%, 8/8). The differences in PBSC and BM tumour cell involvement was significant in the cohort of patients with metastatic disease ($p=0.0350$, Fisher's exact test). Among the patients with localised BrCa there was no significant difference in PBSC and BM tumour cell

involvement, possibly because of the small number of patients in the BM harvest arm.

3.3.4 Comparison of Tumour Involvement in Bone Marrow and Peripheral Blood Stem Cell Collections

Twelve of 25 PBSC collections (48%) obtained from 11/19 patients (57.9%) had tumour cells detected by immunocytochemistry. On the other hand, tumour cells were detected in 10/10 (100%) BM collections. The difference in PBSC and BM involvement was significant ($p = 0.0001$, Fisher's exact test). (To note, there was no overlap between these two mutually exclusive groups of patients).

The numbers of specimens analysed per patient was 1 for BM and 1 to 2 for PBSC. In patients with two consecutive collections, tumour cells were not detected in all serial samples. The concentration of tumour cells detected in the immunocytochemically positive PBSC collections ranged from 1 to 6 per 2×10^6 mononuclear cells (median concentration of 1.5 per 2×10^6 haematopoietic cells, $n = 11$ patients). In contrast, the concentration of tumour cells in the immunocytochemically positive BM specimens ranged from 1 to 62 with a median of 14 per 2×10^6 cells ($n = 10$ patients). The difference in tumour cell concentration was statistically significant ($p = 0.0012$, Mann-Whitney test). A comparison between all PBSC and BM autograft results obtained with immunocytochemistry is illustrated in Figure 3.3.

Because patients undergoing PBSC transplantation received a greater number of infused haematopoietic cells than patients receiving autologous marrow (0.98 to 6.50×10^8 nucleated cells per kilogram patient weight for PBSC vs. 0.25 to 1.58×10^8 nucleated cells per kilogram patient weight for BM; in these patient groups), this resulted in the reinfusion of a comparable number of

tumour cells (median tumour cell load of 1.42 x 10^4 per kilogram patient weight for PBSC vs. 3.48 x 10^4 per kilogram patient weight for BM [Figure 3.4]). The difference in the total number of tumour cells reinfused was not significantly different ($p = 0.0503$, Mann-Whitney test).

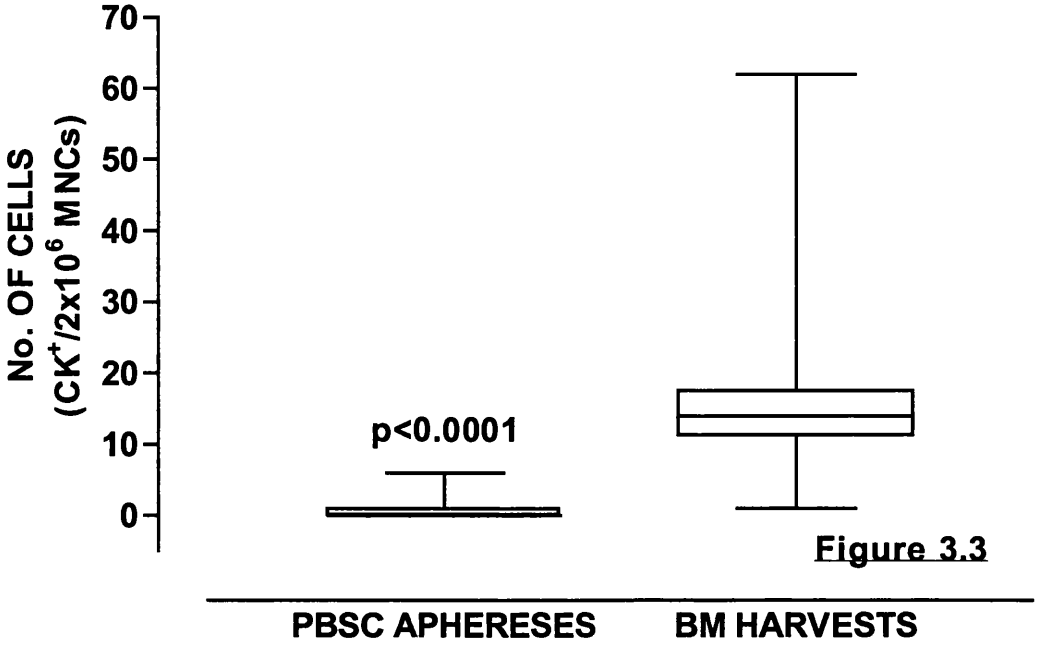


Figure 3.3

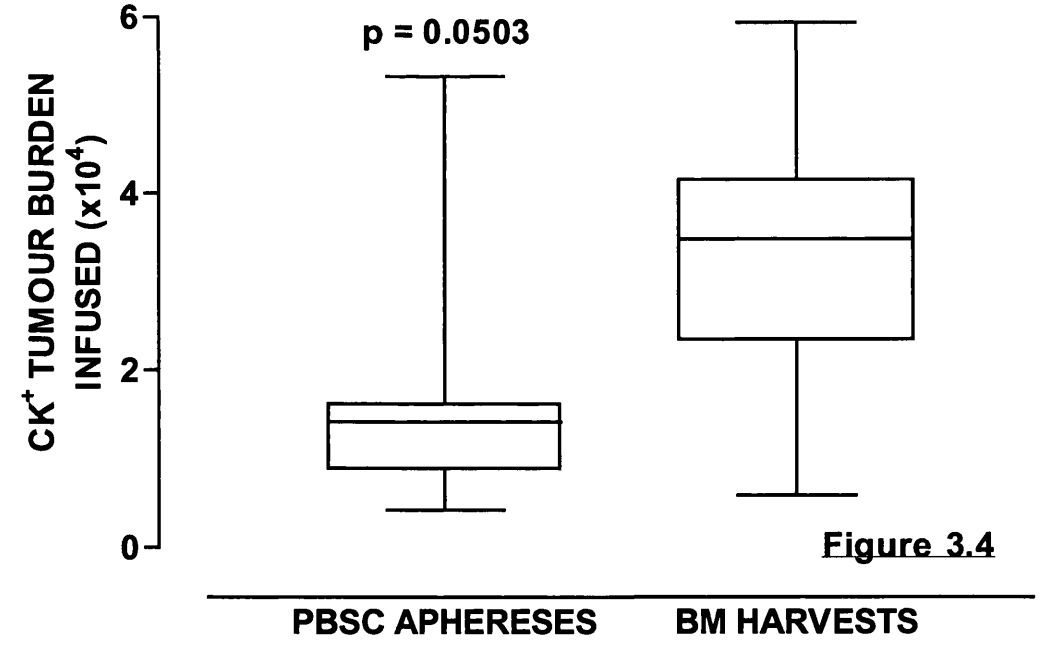


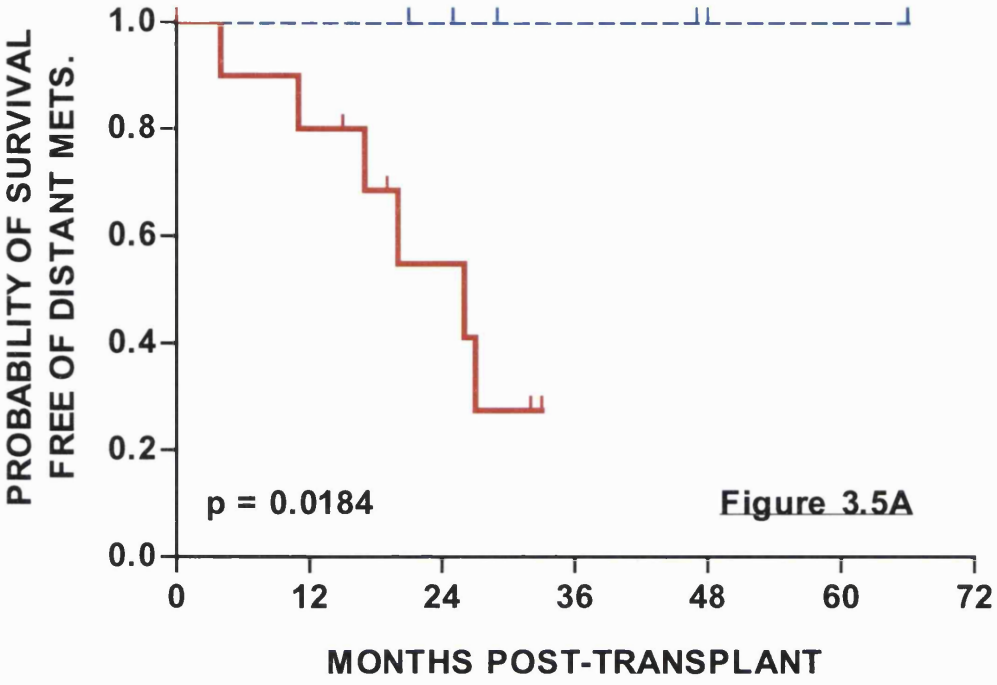
Figure 3.4

Figure 3.3: ICC detection of tumour cells in unpaired autograft collections from BrCa patients.

Figure 3.4: Absolute number of CK^+ tumour cells reinfused in two unpaired groups of BrCa patients.

3.3.4 Clinical Outcome Data

There were no treatment-related deaths in the patients included in this study. After a median follow-up of 25.5 months (range 4-66), 10 patients remain alive and disease-free in complete remission, but 6 patients experienced a tumour relapse; of these, all 6 died of their disease (Figure 3.5A). Of the 6 patients who relapsed, all demonstrated CK^+ cells in the stem cell transplants. Altogether, 60% of the patients with tumour cell positive transplants (6/10) had a relapse (median survival = 26 months), whereas recurrence of disease was not seen in any of the 6 patients with CK-negative transplants (median survival to-date – undefined). A trend towards a superior disease-free survival is present in the patient group with CK-negative transplants. Kaplan-Meier analysis yields a better disease-free survival for the patients with CK-negative transplants ($p = 0.018$). This analysis was based on a patient cohort with stage II-IIIa localised BrCa. If the analysis incorporated an additional 3 patients with stage IV metastatic disease, transplanted with CK-negative stem cell components, with all 3 patients experiencing a tumour relapse and death within 8 months post-transplant, Kaplan-Meier analysis (Figure 3.5B) is no longer statistically significant ($p = 0.3619$).



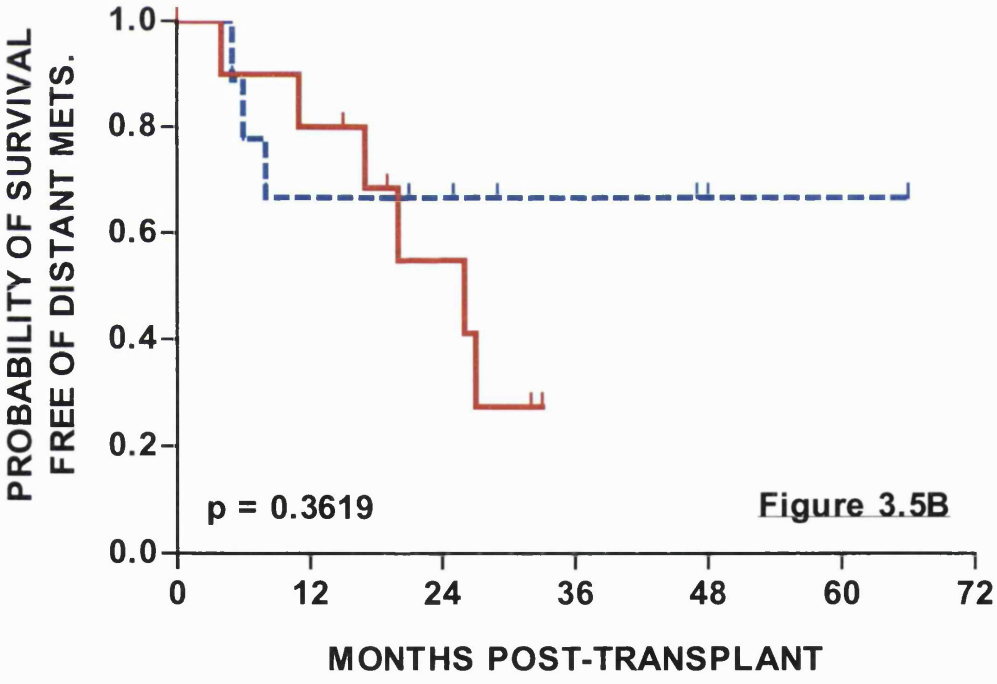


Figure 3.5A & 3.5B: Kaplan-Meier analysis of the probability of survival, free of distant metastases.

DFS for patients with stage II-IIIa localised BrCa (A), in all patients, including stage IV advanced metastatic disease (B). The broken blue lines represent patients without ICC evidence of tumour cell contamination in their autograft. The solid red lines represent those patients with ICC evidence of contamination.

3.4 Discussion

A sensitive and reproducible method for detection and enumeration of epithelial tumour cells requires rigorous evaluation in model systems before application in the clinical setting to ensure both reliable and accurate results.

The routine ICC analysis of BM from patients with BrCa can present some problems. For example, in previous studies, ICC techniques have been used that require the examination of BM spread over several slides (Schlimok *et al*, 1987; Cote *et al*, 1988). The technique used here has some possible advantages over previous ICC techniques. The addition of predetermined numbers of cells to defined areas of coated adhesive microscope slides allowed cells to sediment and attach and may lead to better distribution of cells than previously experienced with cytocentrifuge preparations. These slides are easier to examine and as a consequence, a larger number of cells can be applied per slide. The adhesive nature of the test surfaces prevented significant cell loss during extensive liquid incubation and washing procedures. The data illustrate that in the multiple seeding experiments performed, one BrCa cell in 1,000,000 Daudi cells could reliably and reproducibly be detected in the Cell-Tak® and Cel-Line systems and one in 100,000 with the Poly-L-Lysine system. Each adhesive system offered enhanced cell attachment and spreading capabilities within the wells. Furthermore, up to 2 x 10^6 cells could readily be screened from clinical material obtained, without the need to examine multiple cytospin preparations, and the data further demonstrate the feasibility of ultra-high sensitivity detection of tumour cells in haematopoietic samples.

The technique also allows subsequent morphological characterisation of the malignant nature of CK^+ cells, achieved by counterstaining stored frozen slides prepared at the time of sample processing, or by the application of FISH

technology (Chapter 6). Fluorescence *in situ* hybridization could facilitate the further biological characterisation of contaminating/disseminated tumour cells, with respect to metastatic potential, and may provide a powerful approach that can be used to detect over amplification of various genetic prognostic markers, and new insights into the course of an individual tumour. The sensitivity of the method is high, and clonal variation among the carcinoma cells can be revealed. Because FISH depends on molecular marker probes that give clear cut qualitative results, only a small number of cells are needed for analysis.

By applying ICC techniques, breast tumour cells have been detected in BM biopsies of 20-45% of patients with local disease at the time of surgery, and of up to 70% in those with metastases (Schlimok *et al*, 1987; Mansi *et al*, 1991). A higher likelihood of early relapse and reduced overall survival are associated with the presence of BM micrometastasis (Cote *et al*, 1991; Diel *et al*, 1992; Braun *et al*, 2000b). In multivariate analysis of prognostic factors, the results of BM biopsy behaved as an independent factor and positivity was significantly associated with early disease relapse (Harbeck *et al*, 1994) and reduced overall survival (Braun *et al*, 2000a). In other studies the best predictive value was from the combination of BM micrometastasis, nodal status and progesterone receptor status (Diel *et al*, 1992). Breast tumour cells in BM and peripheral blood are also capable of *in-vitro* clonogenic growth (Ross *et al*, 1995a), and in previous studies, tumour contamination of either BM grafts or PBSC collections has been associated with shorter disease-free and overall survival in stage II-III patients subjected to autologous transplantation (Harbeck *et al*, 1994; Hurd and Peters, 1995; Pedrazzoli *et al*, 1997; Vredenburgh *et al*, 1997). In follow-up studies of a subset of advanced-stage BrCa patients reported initially by Ross *et al* (1993), preliminary analysis at 30 months indicates that no correlation was

found with tumour contamination of the PBSC collection and time to disease progression, sites of relapse, or overall survival (Ybanez *et al*, 1995). Another retrospective study of a small cohort (n = 26) of advanced-stage BrCa patients with tumour-contaminated BM found a trend toward decreased overall survival (p = 0.11) at 84 months follow-up (Brockstein *et al*, 1996). Insufficient power as a result of the low sample size precluded any subset analyses in this cohort. Similar trends toward decreased progression-free and overall survival in advanced-stage BrCa patients who received tumour-contaminated PBSC collections have been reported by Gluck *et al* (1996).

In contrast, the retrospective study of MRD in BM by Fields *et al* (1996) concluded that the probability of relapse at 36 months posttransplant was 32% for stage II-III and 94% for stage IV CK19 RT-PCR-positive patients. Conversely, the probability of relapse was 10% for stage II-III and 14% for stage IV CK19 RT-PCR-negative patients. The difference was significant (p = 0.0002) for stage IV patients. In a similar retrospective study, investigators at Duke University found that stage II-III BrCa patients with ICC-positive BM harvests had significantly shorter disease-free and overall survival (Vredenburgh *et al*, 1997). Unfortunately, too few patients had tumour-contaminated PBSC for comparable statistical analysis. Moreover, Moss *et al* (1997) reported that tumour contamination of autologous BM grafts in stage IV BrCa patients was significantly associated with reduced disease-free survival (p = 0.0001). Patients who had both BM and PBSC contaminated with tumour had a worse posttransplant clinical course than did all other patients (p = 0.015). However, this study was a retrospective analysis of patients from a number of transplant centres and only included univariate statistical analysis. However, with reference to the studies cited (including this one), without multivariate analyses,

it is difficult to determine if tumour contamination of the graft is truly predictive of posttransplant outcome, or if it is a very accurate surrogate marker for treatment-resistant disease.

Some of the aforementioned studies indicate that the reinfusion of occult tumour cells in autografts, as detected by ICC, do not ~~contribute~~ CORRELATE significantly to relapse following HDC in patients with BrCa. There are several possible explanations for these observations. First, the number of tumour cells may be insufficient to result in relapse and not all occult tumour cells may be clonogenic *in vivo*. It is possible that freezing and thawing could affect the number and clonogenicity of tumour cells. The immune system of the patient might eliminate the relatively small number of tumour cells infused and/or ultrasensitive ICC or other testing methods will need to be employed to reduce the number of possible false negative results. Any one or a combination of these mechanisms could explain the apparent lack of effect of infused tumour cells on relapse.

Recently, an RT-PCR assay for the detection of CK mRNA has been proposed as a marker of cancer cell contamination in BrCa patients. Cytokeratins are cytoskeletal intermediate filaments (Moll *et al*, 1982) that are selectively expressed in cells of epithelial origin; however, CK8 and CK18 mRNA were found to be expressed also in samples of normal PB (Traweek *et al*, 1993), although data for K19 were more conflicting. In fact Traweek *et al* (1993) and Datta *et al* (1994) failed to detect K19 mRNA in the PB and BM samples from normal subjects or patients with haematological disease, whereas false positive results in 38-50% of normal PB samples have been reported by others (Burchill *et al*, 1995; Krismann *et al*, 1995; Hildebrandt *et al*, 1997).

Although there are several studies in which histology and/or ICC have been used for the evaluation of BM biopsies in BrCa patients, no comprehensive comparative analysis of these morphological techniques with RT-PCR for K19 has been undertaken. In accord with other studies (Fields *et al*, 1996), conventional histology proved inadequate to evaluate BrCa cell contamination, since >50% of BM samples with a positive result by both RT-PCR and ICC were considered to be normal by the pathologist. Conversely ICC and RT-PCR showed a high degree of concordance; additionally, it is conceivable that ICC might help in identifying cancer cells in occasional samples with negative RT-PCR results as a consequence of the uneven pattern of BM infiltration and sampling. Therefore, ICC and RT-PCR for K19 could possibly be combined. By using these sensitive techniques, the rate of detection of BM contamination may be improved in women with high-risk, localised, non-metastatic BrCa at the time of primary diagnosis.

Although PBSC collections are believed to have a lower incidence of tumour involvement than BM in BrCa patients, there are few studies to support this contention (Sharp *et al*, 1992a; Ross *et al*, 1991). To address this issue, a highly sensitive ICC assay was developed and validated by quantifying the number of occult tumour cells in unpaired BM and PBSC specimens collected from 19 patients with either advanced-stage metastatic or high-risk, localised, non-metastatic BrCa.

The findings indicate that occult BrCa cells are present less frequently in PBSC than in BM ($p = 0.0001$, Fisher's exact test). Furthermore, the concentration of tumour cells in immunocytochemically positive PBSC collections was significantly lower than in immunocytochemically positive BM ($p = 0.0012$, Mann-Whitney test). In patients with metastatic disease, the

incidence of circulating tumour cells detected in PBSC collections was independent of sites of metastatic involvement. Because patients undergoing PBSC infusion received a greater number of infused haematopoietic cells than patients receiving autologous marrow, and despite this lower contamination, this resulted in the infusion of a comparable number of tumour cells ($p = 0.0503$, Mann-Whitney test). Therefore, it may be postulated that not only tumour burden but phenotypic differences between BM-derived and PBSC-derived tumour cells may govern the risk of metastatic relapse following dose intensification. In addition, the data indicate that tumour cells may not be present in all PBSC pheresis collections from a single patient. Taken together, these data support the former view that PBSC collections are a less-contaminated source of haematopoietic stem cells compared to BM (Moss and Ross, 1992).

In this retrospective analysis of a small number of localised BrCa patients, a trend was found towards a statistically significant difference in DFS in patients who were inadvertently infused with contaminating tumour cells. There are at least two possible explanations. The first, and perhaps the most obvious, is that reinfused cancer cells contribute to disease relapse; the other, and perhaps the most pertinent, is that stem cell contamination reflects only a greater overall tumour burden present at diagnosis, with reinfused cancer cells having no major role on disease relapse. Additionally, with reference to the second explanation and to the patients with advanced stage IV metastatic disease, transplanted with CK-negative components, the shorter DFS observed may indicate relapse from residual endogenous tumour (greater tumour burden and/or more resistant disease). These observations suggest that when treating a subgroup of patients with a relatively high and chemoresistant residual tumour

burden, the inadequate tumour cytoreduction makes it difficult to detect the potential influence of the infusion of tumour. In contrast, when analysing patients with high risk of relapse stage II and III BrCa, potentially with a more limited and chemosensitive residual tumour burden, the presence of tumour cells in the infused haematopoietic support may more accurately predict relapse. Furthermore, with regard to relapse, preliminary evidence in patients with non-Hodgkin's lymphoma (Sharp *et al*, 1992b; Vose *et al*, 1992) and leukaemia (Miller *et al*, 1991) suggests that the removal of tumour cells either by BM purging techniques or the use of tumour-uninvolved PBSC may improve survival. The presence of contaminating tumour cells in pheresis products has been reported in recent studies to occur at a frequency of 4-20% (Brugger *et al*, 1994; Fields *et al*, 1996; Passos-Coelho *et al*, 1996; Vogel *et al*, 1996; Schulze *et al*, 1997); the higher figure reported here (58% of patients) may be due to the improved sensitivity of this novel ICC assay. However, in another study (Mapara *et al*, 1997) it was shown that as many as 17/21 (82%) pheresis products analysed with a pancytokeratin monoclonal antibody were found to contain tumour cells. Therefore, the use of techniques such as purging with chemotherapy (Kennedy *et al*, 1991; Shpall *et al*, 1991a; Passos-Coelho *et al*, 1994), MoAbs (Shpall *et al*, 1991b), or enriching for $CD34^+$ cells (Berenson *et al*, 1991) were explored, in the hope that this might reduce the tumour load. The immunoselection of $CD34^+$ cells employed by Vannucchi *et al* (1998) in four cases with micrometastasis in bilateral BM biopsies at diagnosis (as demonstrated by histology, ICC and RT-PCR) and in PBSC collections (by RT-PCR) was able to yield a stem cell suspension devoid of cancer cells. Recent experience with ICC analysis of purified $CD34^+$ cells (Hildebrandt *et al*, 1997), even after *ex-vivo* expansion using a cocktail of haemopoietic growth factors

(Vogel *et al*, 1996), is in line with the observations of Vannucchi *et al* (1998). However, Mapara *et al* (1997) still found a significant tumour cell contamination after $CD34^+$ cell selection using both ICC and RT-PCR for K19 (single-round RT-PCR reaction) and the epidermal growth factor receptor (EGF-R); although the reasons for these discrepancies are unknown, the use of a different method for $CD34^+$ cell separation (an immunomagnetic-based procedure in that study versus an avidin device in the Vannucchi *et al*, [1998] study) might have been, at least in part, responsible.

If ICC techniques are to be used for the detection of BrCa cells, methods must be developed to deal with the heterogeneity of antigen expression within and between different tumours. Substantial phenotypic heterogeneity has been observed in different breast cancers. However, the use of the broad-spectrum MoAb A45-B/B3 explored in this study supports its use for detecting cancer cells in haematopoietic samples from BrCa patients, and these assays were further utilised to study clinical material from patients with other epithelial malignancies.

In conclusion, this study shows a good correlation between the number of tumour cells added and the number of cells detected, by ICC, and this correlation has been shown to be linear over a range of several logs in all three adhesive systems. This technique will allow quantitation of tumour cells in BM samples, and it may prove useful in monitoring the response of metastatic disease to cytotoxic or hormonal therapy and this application is explored below. The sensitivity and specificity of this ICC method supports its introduction into tumour staging classifications. Results obtained by this method may then serve as a new standard for alternative approaches to substantiate the possible

increased sensitivity over existing immunocytology methods using MoAbs to detect CK^+ cells.

Chapter 4 Detection of Circulating/Micrometastatic Tumour Cells – Response to Adjuvant Chemotherapy

4.1 Introduction

Many previously unmanageable cancers can now be cured by modern treatment methods, provided that a diagnosis is made early. Most current methods of cancer diagnosis and detection of metastasis rely on biopsy analysis and imaging principles, including radiography, computed tomography (CT), magnetic resonance imaging (MRI), bone scintigraphy, and sonography. These techniques require a degree of subjective interpretation and may be subject to error. Furthermore, the lower limit of tumour size detection of these techniques is ~1cm, which represents 10^9 cells or a mass of 1g. A more sensitive detection method would help early diagnosis and could improve the rate of survival.

Metastatic spread, via the blood stream is probably the single most important factor affecting the prognosis of patients with cancer. Patients with primary tumours such as breast, colon, or lung carcinomas who have undergone radical, potentially curative surgery have a recurrence rate in the range of 20-60%. Currently, lymph node involvement is the most important prognostic factor for tumour recurrence in these patients. However, 30-50% of patients with cancer who show no evidence of disease in the locoregional lymph nodes will still have a recurrence at a distant site. Therefore, novel diagnostic methods that separate patients into low-risk and high-risk prognostic groups for recurrence, and need for adjuvant therapy, are required.

The metastatic process is a complex cascade of events: tumour cells in the primary site erode the endothelial basement membrane; penetrate blood vessels; and spread to distant sites or via lymphatics. Thus, detection of cancer cells in the blood could be important to identify patients at high risk of relapse. Immunocytochemical detection of micrometastases in the BM of patients with breast, colon, and gastric cancer has been shown to correlate with early disease relapse (Schlimok *et al*, 1991; Diel *et al*, 1992; Lindemann *et al*, 1992). Several markers have been used to detect circulating cancer cells. In particular, CKs and CEA have been proposed as useful markers to detect circulating tumour cells of the ~~gastrointestine,~~ GASTROINTESTINAL TRACT breast, and/or lung (Datta *et al*, 1994; Gerhard *et al*, 1994; Soeth *et al*, 1996; Castaldo *et al*, 1997). Few reports have suggested that a correlation between expression of these markers in the PB and/or in the BM and patient outcome might exist (Fields *et al*, 1996; Castaldo *et al*, 1997; Soeth *et al*, 1997; Mori *et al*, 1998). The identification of circulating tumour cells in the PB of patients with BrCa has the potential to provide an important prognostic indicator for survival, since early dissemination of tumour cells is one of the main causes for disease progression (Frost and Levin, 1992).

Death from carcinoma of the breast is principally caused by distant metastases. More than 95% of patients with breast cancer will have no evidence of metastatic disease on clinical, radiologic, and biochemical examination at presentation. The presence of BM metastases has been correlated with early recurrence and shorter overall survival. However, a proportion of patients relapse at these sites in the absence of histologic or immunohistochemical evidence of BM micrometastases after resection of the primary tumour (Mansi *et al*, 1991). In advanced BrCa, the rationale for

adjuvant therapy is based on the assumption that clinically undetectable haematogenous dissemination of viable tumour cells has already occurred, as indicated by a number of risk factors, including tumour size greater than 2cm, cutaneous lymphangioitis carcinomatosa, and regional lymph node metastases. Unfortunately, little is known about the natural history of micrometastases, and it has been suggested by some studies that the monitoring of such "minimal residual disease" (MRD) could be used to improve disease staging, as a marker for evaluating new therapeutic strategies, and to assess treatment response in individual patients. Most current methods are not sensitive enough to detect circulating cells in significant numbers of patients with early-stage carcinomas (Redding *et al*, 1983; Leather *et al*, 1993; Datta *et al*, 1994). However, this may be due not only to the inadequate sensitivity of the assays used but also to the possibility that such cells may only appear periodically in the circulation during early tumour development. There may be intermittent shedding of tumour cells into the circulation corresponding with microinvasive events within the tumour. Previous studies have shown detection rates of MRD in the PB of patients with solid tumours in the order of 0% to 27% using PCR methodology (Datta *et al*, 1994; Schoenfeld *et al*, 1997) and 0% to 5% using ICC (Aihara *et al*, 1997; Schoenfeld *et al*, 1997) in patients with early-stage disease. Patients with advanced disease have a much greater tumour burden and are more likely to have tumour cells present at blood sampling and as such represent a group of patients who can more readily be studied to determine the effect of therapy on disease.

A reliable indicator of the efficacy of adjuvant therapy requires trials with large numbers of patients observed over several years (Sloane, 1995), especially in BrCa, because residual tumour cells may manifest and impact on

survival many years later (Overgaard *et al*, 1997). Because adjuvant treatment is usually delivered to patients with clinically occult micrometastatic disease, after the successful resection of the primary tumour, the efficacy of therapy can be only assessed retrospectively from the rate of disease-free survival. Consequently, progress in this form of therapy is extremely slow and cumbersome, and therapy is difficult to tailor to the special needs of an individual patient. The importance of a surrogate marker assay that would permit the immediate assessment of therapy-induced cytotoxic effects on residual cancer cells is therefore apparent.

The ICC detection of haematogenously disseminated tumour cells may be such a surrogate marker assay since numerous studies have demonstrated the prognostic impact of such early tumour cell dissemination in BrCa patients (e.g., reviewed by Braun and Pantel, 1998). In this study, I have therefore applied the MoAb A45-B/B3 directed against the heterodimers CK8/18 and CK8/19 as well as a common epitope of several CK polypeptides (Braun *et al*, 1998a; Stigbrand *et al*, 1998), to demonstrate the validity of the Cell-Tak® Cell and Tissue Adhesive immunoassay with the MoAb directed against CK as specific marker antigen of extrinsic epithelial cells in the background of mesenchymal cells (Delsol *et al*, 1984; Schlimok *et al*, 1987; Pantel *et al*, 1994; Braun *et al*, 1998).

The primary objective of the study was to (*a*) validate the CK8/18 and CK8/19 ICC approach by quantitating tumour cells in the venous blood of women with BrCa (both metastatic and non-metastatic disease); (*b*) to correlate tumour cells detected in the venous blood with tumour cells detected in the BM of women with cytologically or histologically confirmed primary BrCa, to elucidate whether primary carcinoma patients are already afflicted with

disseminated disease; (*c*) to evaluate the impact of therapy (surgery and/or chemotherapy) on the presence of BrCa cells detected in the venous blood (and/or BM); (*d*) to determine whether the Cell-Tak® Cell and Tissue Adhesive immunoassay is a sufficiently powerful detection method ~~in~~ APPLIED TO venous blood; (*e*) to assess the use of venous blood as an alternative to BM; (*f*) to determine whether CK ICC detection in BM and venous blood have a prognostic value with respect to the survival of the patients.

4.2 Materials and Methods

4.2.1 Patients

From June 1995 to October 1999, samples of peripheral venous blood and/or BM aspirates taken from the posterior iliac crest, were obtained from 94 BrCa patients admitted to the Department of Clinical Oncology, at the Royal Free Hospital in London, after providing their written informed consent. The procedures were approved by the institutional review board. The stage and grade of the tumour were classified according to the tumour-node-metastasis classification of the Union Internationale contre le Cancer [UICC (Sobin and Wittekind, 1997)] by investigators unaware of the ICC findings in BM and/or PB. In addition, ICC analysis of the BM and/or PB specimens was performed without knowledge of the histopathological results.

In the preliminary phase of the analysis, samples of PB were first obtained from 25 unselected patients with primary BrCa. All patients had cytologically confirmed primary BrCa and no evidence of distant metastatic disease on chest radiology and bone and liver scanning. In comparison, PB samples from 25 patients being treated for advanced BrCa were also obtained. Inclusion criteria were histologically or cytologically proven progressive

metastatic disease, and clinically or radiologically assessable disease. No patient had received any previous endocrine or other treatment for at least 1 week before the blood samples were taken. All patients were ~~clinically examined~~ EXAMINED CLINICALLY and had chest radiology, liver computed tomography, and bone scan with radiologic confirmation of any areas of increased tracer uptake. The distribution of metastases for patients in this group included: liver (5), ovary (1), nodal (2), skin (2), multiple sites (4 [lung/bone, liver/bone, lung/nodes, liver/nodes]), ascites (1), bone (8), breast (1) and pancreas (1).

Since the main aim of the subsequent phase of the investigation was to determine the relationship between efficacy of adjuvant chemotherapy and circulating tumour cells, 26 patients commencing treatment with doxorubicin/cyclophosphamide, methotrexate, and 5-fluorouracil (Dox./CMF) or with classical CMF, were studied. Samples were obtained before the start of chemotherapy and then at 3- to 4-week intervals, within 48 hours of the beginning of the next cycle of chemotherapy. In addition, 18 patients with high-risk primary tumours who were undergoing chemotherapy (adriamycin, cyclophosphamide [AC]) prior to definitive surgery, had BM and PB samples taken at the time of diagnostic surgery. These patients then had serial PB samples taken as described above for adjuvant patients, throughout their chemotherapy, and at the time of definitive surgery had a further BM and PB sample taken. This, therefore, allowed a comparison of the quantification of tumour cells in BM and PB, and an assessment of the effect of chemotherapy on primary tumour and micrometastases (if present). The primary surgical treatment consistent of either breast conservation or modified radical mastectomy, leading to R_0 resection. Routine axillary dissection included levels

I to III for the high-risk study population. The main characteristics of the 44 assessable patients with BrCa are listed in Table 4.1.

4.2.2 Treatment Dosage and Schedules

In the 26 patient adjuvant group with BrCa, the primary surgical treatment consisted of radical mastectomy in 13, segmental mastectomy in 12 and wide local excision of the primary tumour in one patient. The tumour was completely resected in all patients, and the procedures included dissection of axillary lymph nodes. A total of 9 premenopausal adjuvant patients with 0-3 involved axillary lymph nodes received six cycles of CMF chemotherapy consisting of cyclophosphamide (600 mg per square metre of body-surface area), methotrexate (40 mg/m^2), and 5-fluorouracil (600 mg/m^2) every 21 days. In 11 patients who had at least four involved regional lymph nodes, four cycles of induction single-agent doxorubicin chemotherapy (75 mg/m^2) were administered at 3 week intervals, followed by eight cycles of cyclophosphamide, methotrexate, and 5-fluorouracil at the dose and schedule intensity of the above stated regimen. Following induction chemotherapy of four courses of single-agent doxorubicin (75 mg/m^2) and collection of peripheral blood stem cells (PBSC [detailed in Chapter 2, section 2.2]), 6 patients were administered high-dose sequential therapy (HDST) over 4 days, consisting of thiotepa at 200 mg/m^2/day and cyclophosphamide at 1.5 g/m^2/day (TC). Peripheral blood progenitor cells were administered 72 hours following the TC cycle. Irradiation of the chest wall followed mastectomy in 13 patients. The median absorbed dose in the target area was either 50.0 Gy, given in 25 fractions, or 50.4 Gy, given in 28 fractions. Following completion of adjuvant chemotherapy, 10 patients with oestrogen receptor-positive tumours (tested immunocytologically)

received 20 to 30 mg of tamoxifen daily, and it was recommended that therapy last two to five years. In addition, three patients with oestrogen receptor-negative tumours, and 4 patients with unknown oestrogen receptor status also received tamoxifen.

Primary medical therapy patients with high-risk tumours (n = 18) received chemotherapy, which consisted of repeated cycles of adriamycin (60 mg/m^2) plus cyclophosphamide (600 mg/m^2) (AC), followed by definitive surgery. Treatment was repeated at 3-week intervals for a total of six courses. Definitive surgical treatment consisted of mastectomy in 16 patients and segmental mastectomy in 2, including routine axillary lymph node clearance, in this high-risk study population. Five patients with detectable nodal involvement (single embedded lymph nodes screened at up to three levels) following surgery were treated with second-line chemotherapy, with four patients receiving additional CMF and one patient continuing onto HDST and PBSC. Four patients also received additional treatment consisting of radiation therapy post-surgery (mastectomy in 3 patients and segmental mastectomy in one patient). After surgery, 7 patients with oestrogen receptor-positive tumours (tested immunocytologically) received 20 to 30 mg of tamoxifen daily, and it was recommended that therapy last two to five years. In addition, two patients with oestrogen receptor-negative tumours, and one patient with unknown oestrogen receptor status also received tamoxifen.

At the time of primary surgery, the base-line diagnostic evaluation for distant metastases included plain chest radiography, mammography of the contralateral breast, ultrasonography of the liver, and bone scanning of the entire body. These examinations showed no evidence of distant metastases in any of the patients.

4.2.3 Treatment Response

Response was defined in accordance with standard UICC criteria (Miller *et al*, 1981) as follows: complete response (CR) was defined as the complete disappearance of all clinical disease by physical or radiologic examination for a period of at least 4 weeks. Partial response (PR) was defined as a greater than 50% reduction of tumour size as determined by two measurements not less than 4 weeks apart. Stable disease was defined as less than 25% progression or less than 50% tumour reduction, and progressive disease was defined as a greater than 25% increase in tumour measurements at any time.

4.2.4 Preparation of Blood and Bone Marrow Samples

The procedure for PB and BM preparation has been described previously in Chapter 2, section 2.2.1. In short, the mononucleocytes were separated from the PB and BM over Ficoll-Hypaque at 900 x *g* for 30 minutes. The interface cells were then removed and washed in 50 ml of HBSS. The cell pellet was resuspended in HBSS, and 1 x 10^6 cells were then adhered onto each glass microscope slide (detailed in Chapter 3, section 3.2).

4.2.5 Immunocytochemical Analysis

For each patient, 2 x 10^6 cells were screened by bright-field microscopy; an identical number of cells served as a control for staining with an irrelevant immunoglobulin. Both morphologic criteria and ICC staining were used to identify cells. There were no indeterminate results encountered, because there was no background staining.

Monoclonal antibody A45-B/B3 was used, which is directed against a common epitope on CK polypeptides, including the CK heterodimers 8-18 and

8-19 (Stigbrand *et al*, 1998), was used at 1.0 to 2.0μg/ml to detect tumour cells on adhesive slide preparations of PB and BM. The specificity of the MoAb reaction in the specimens was confirmed by the addition of an unrelated mouse myeloma immunoglobulin at an appropriate dilution. The BrCa cell line MCF-7 served as a positive control for CK immunostaining. The reaction of the primary MoAb was developed with the alkaline phosphatase anti-alkaline phosphatase technique combined with the fast red/new fuchsin stain (Cordell *et al*, 1984) to indicate MoAb binding.

4.2.6 Statistical Analysis

Differences in positivity rates were analysed by Fisher's exact test for contingency tables. The Mann-Whitney U test was used to assess the differences in the medians, differences between medians of independent samples with continuous variables were calculated from the *t* test. A p value of less than 0.05 was considered to indicate a statistically significant difference. All tests were two-tailed. For statistical analysis, GraphPad Prism® software (San Diego, CA, USA) (version 2.01) was used.

Characteristic	Primary Medical Therapy (n = 18)	Adjuvant (n = 26)
Mean age – yr. (range)	48 (39 – 64)	46 (30 – 67)
Menopausal status – no. (%)		
Premenopausal	11 (61)	15 (58)
Perimenopausal	0	3 (11)
Postmenopausal	7 (39)	8 (31)
Tumour stage – no. (%)‡		
≤2cm (pT1a, b, or c)	0	5 (19)
>2cm (pT2)	2 (11)	13 (50)
>2cm (pT3)	15 (83)	6 (23)
Infiltration of skin or chest wall (pT4a, b, c, or d)	1 (6)	2 (8)
N0	8 (44)	4 (15)
N1	10 (56)	22 (85)
Tumour grade – no. (%)‡		
1	1 (6)	0
2	7 (39)	13 (50)
3	6 (33)	12 (46)
NK	4 (22)	1 (4)
ER status – no. (%)		
Positive	11 (61)	14 (54)
Negative	6 (33)	8 (31)
NK	1 (6)	4 (15)
PR status – no. (%)		
Positive	8 (44)	14 (54)
Negative	7 (39)	6 (23)
NK	3 (17)	6 (23)
Histologic type – no. (%)		
Ductal breast cancer	14 (78)	23 (88)
Other¶	4 (22)	3 (12)
Chemotherapy – no. (%)		
AC	18 (100)	0
Dox./CMF	0	11 (42)
Classical CMF	0	9 (35)
HDST + PBSCT	0	6 (23)
Response to chemo. – no. (%)		
CR	2 (11)	Not Applicable
PR	15 (83)	
NC	1 (6)	
Intratumoral VI – no. (%)		
Present	6 (33)	9 (35)
Absent	11 (61)	16 (61)
NK	1 (6)	1 (4)
Lymphatic invasion – no. (%)		
Present	4 (22)	10 (38)
Absent	13 (72)	15 (58)
NK	1 (6)	1 (4)

Table 4.1: Details of patient characteristics.

‡The tumour-node-metastasis classification of the Union Internationale contre le Cancer was used (Sobin and Wittekind, 1997). The abbreviation pT denotes pathologically confirmed tumour.

¶Other histologic types were mixed carcinoma (3), lobular carcinoma (2), inflammatory carcinoma (1), and one unknown type.

Abbreviations: ER, oestrogen receptor; PR, progesterone receptor; AC, adriamycin, cyclophosphamide; Dox./CMF, doxorubicin/cyclophosphamide, methotrexate, 5-fluorouracil; HDST + PBSCT, high-dose sequential therapy + peripheral blood stem cell transplantation; CR, complete response; PR, partial response; NC, no change; VI, vascular invasion; NK, not known.

4.3 Results

Details of patients assessed by means of serial PB sampling are listed in Table 4.1. Peripheral blood samples from 150 individuals (50 normal controls from volunteer donors, 6 additional controls from patients with advanced ovarian cancer, 69 patients with primary BrCa, and 25 patients with metastatic disease) were studied.

4.3.1 Detection of Occult Mammary Carcinoma Cells in Peripheral Blood

Peripheral blood samples were obtained from 25 patients with primary, non-metastatic BrCa and from 25 patients with metastatic disease. All of the patients with primary BrCa had clinically localised disease and no evidence of distant metastases on routine bone and liver scans. All patients with metastases had clear evidence of progressive disease. Control PB samples were obtained from laboratory staff, volunteers attending the hospital for blood tests for a variety of other reasons, and patients who had breast surgery for benign conditions. The age range was 18 to 78 years (mean, 49 years). None of these patients had evidence of carcinoma at any site.

The results of circulating cancer cell detection in the BrCa patients are summarised in Figure 4.1. The incidence of tumour cell contaminated clinical PB samples was significantly higher in patients with metastatic disease than patients without overt metastatic disease ($p<0.0001$). Circulating tumour cells were detected in 23 of 25 (92%) blood specimens drawn from patients with metastatic disease. The frequency of tumour cells in these positive samples, ranged from 1 to 38 per 2 x 10^6 MNCs screened (median 6.5 per 2 x 10^6 MNCs). Of the 25 PB samples of primary BrCa patients without overt metastatic disease, 11 (44%) were positive for tumour cells by ICC. The

frequency of tumour cells in positive samples, ranged from 1 to 6 per 2 x 10^6 MNCs screened (median 1.0 per 2 x 10^6 MNCs) ($p<0.0003$ in comparison to positive metastatic PB samples).

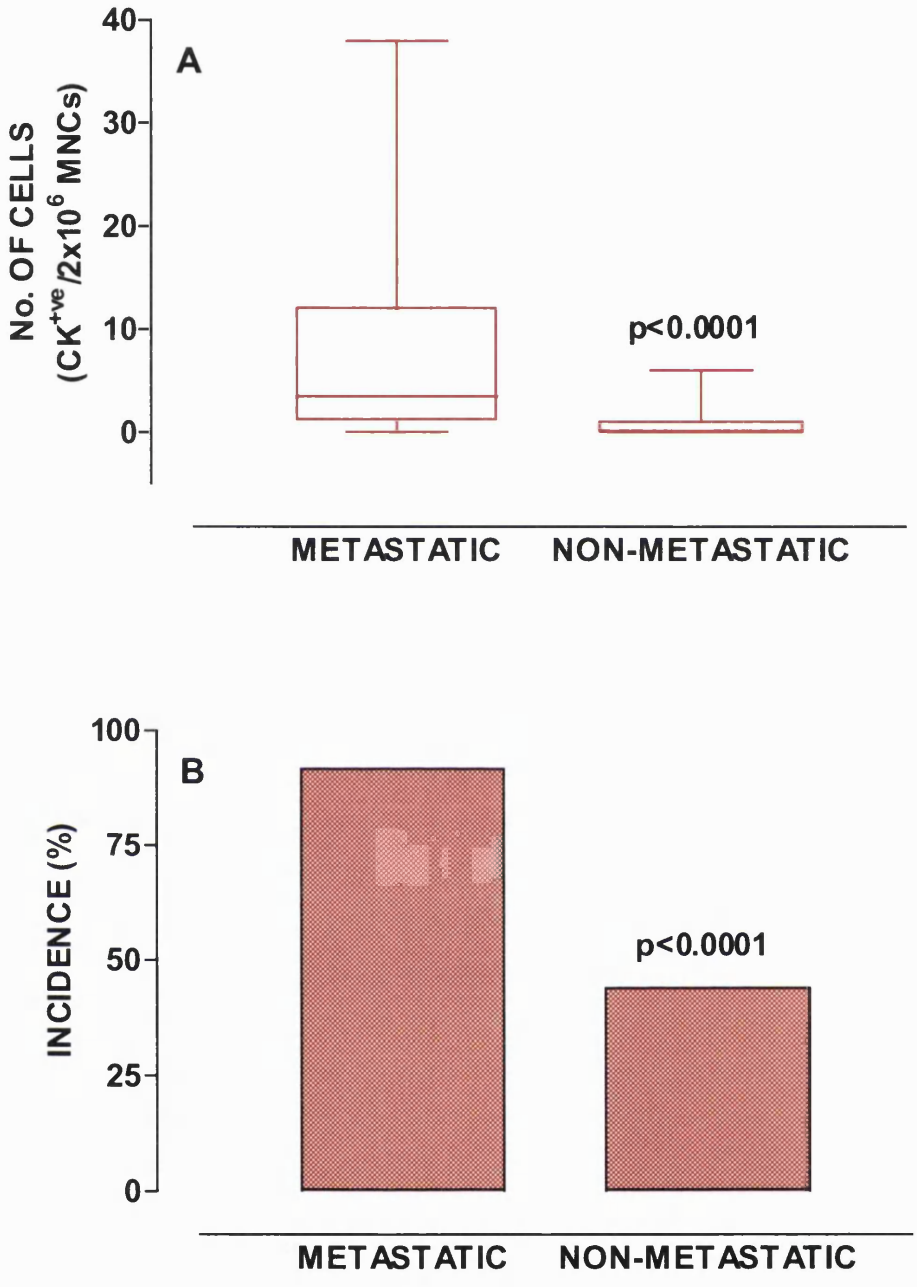


Figure 4.1: A summary of all detection results for patients with various stages of BrCa.

A, ICC detection of CK^+ tumour cells in unpaired PB samples from patients with BrCa (p value calculated by the Mann Whitney test).

B, incidence of all CK^+, unpaired PB samples from patients with BrCa (p value calculated by Fisher's exact test).

4.3.2 Detection of Cytokeratin-Positive Cells in Control Peripheral Blood Samples

In contrast, 50 PB samples from volunteer donors, as well as 6 PB samples from patients with advanced ovarian cancer, (since this tumour metastasises primarily by direct extension into body cavities, it was assumed that tumour cell contamination of PB would be non-existent), were also analysed in a blinded fashion. All samples in this group consistently tested negative for specifically stained CK^+ tumour cells by ICC.

4.3.3.1 Effect of Conventional-Dose Chemotherapy on Tumour Cells in Peripheral Blood of High-Risk Breast Cancer Patients

A total of 112 serial PB samples were obtained from 33 (17 primary medical therapy and 16 adjuvant [10 receiving Doxorubicin/CMF and 6 classical CMF chemotherapy]) patients over a period of up to 36 months. Patient clinical characteristics are listed in Table 4.1. All patients had local disease with no evidence of distant metastases on scanning or radiology. Before chemotherapy (steady state), samples from all but 9 patients (24/33 [73%]) tested positive for the presence of CK^+ tumour cells (illustrated graphically in Figure 4.2B). Of the 9 patients who tested negative, 7 were undergoing adjuvant and 2 primary medical therapies. The median number of PB-derived tumour cells detected in these positive samples pre-chemotherapy in this high-risk primary non-metastatic group of BrCa patients was 2.0 per 2 x 10^6 MNCs (range 1 – 6 per 2 x 10^6 MNCs screened) (Figure 4.2A). Thirty-two of 79 (41%) (Figure 4.2B) serial PB samples were positive for CK^+ tumour cells as measured by ICC in the course of assessable chemotherapeutic treatment cycles. A reduction in the absolute frequency of circulating CK^+ cells was observed in all 33 patients

during successive courses of chemotherapy (anthracyclines and/or taxanes). The median number of PB-derived tumour cells detected in these positive samples was 1.0 per 2 x 10^6 MNCs (range 1 – 3 per 2 x 10^6 MNCs screened) ($p<0.0005$ in comparison to pre-chemotherapy data) (Figure 4.2A).

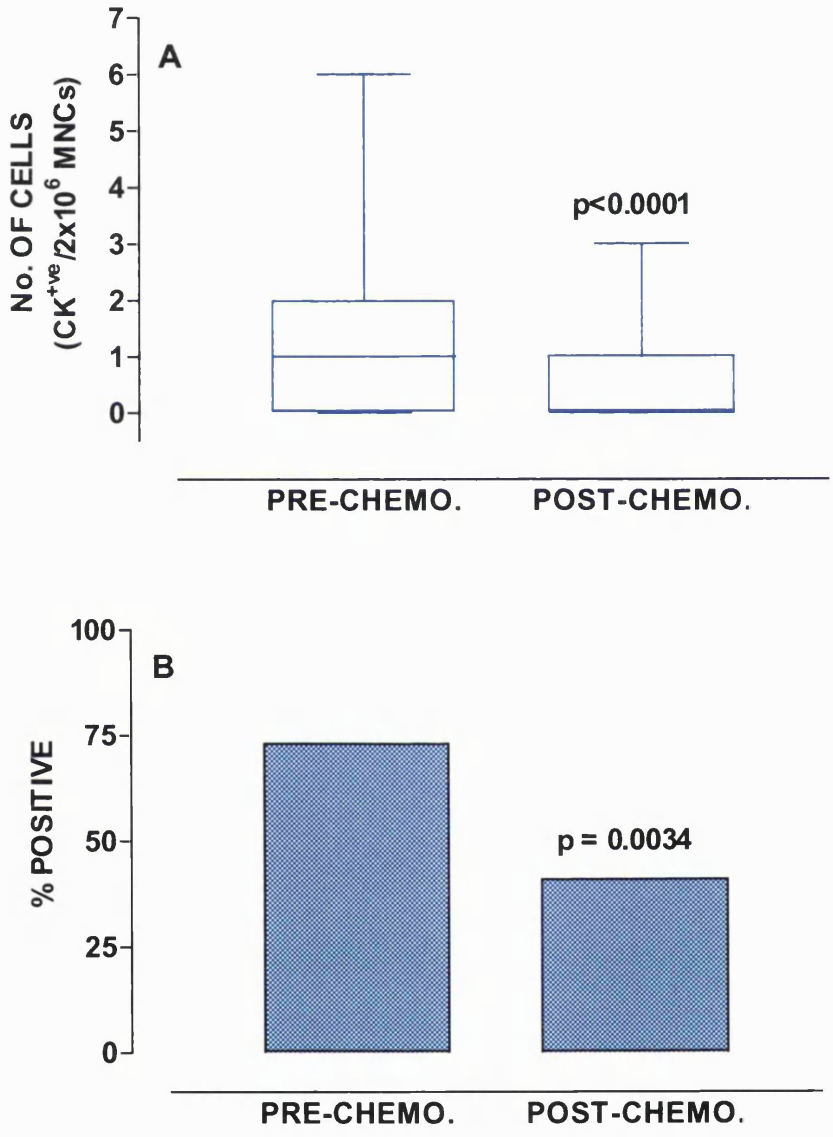


Figure 4.2: Assessment of anticancer therapy efficacy by determination of the relative number of circulating breast carcinoma cells.

A, Monitoring of the elimination of PB-derived CK^+ tumour cells during chemotherapy in high-risk, non-metastatic BrCa patients (p value calculated by the Mann Whitney test). B, incidence of CK^+ PB samples during AC/Doxorubicin, CMF chemotherapy (p value calculated by Fisher's exact test).

4.3.3.2 Response of Circulating Tumour Cells to Therapy – by Trend

Group A: Five patients who had tested negative before chemotherapy retained a negative status for CK^+ tumour cells during repeated PB sampling for the duration of successive, assessable courses of chemotherapy. None of these 5 patients changed to a positive test result following subsequent courses of therapy. Despite the absence of CK^+ cells from their PB, two patients (one adjuvant and one primary medical therapy) in this group, showed signs of disease progression and relapse of the tumour. In both cases however, CK^+ cells had been detected in BM aspirates at diagnosis and, in the case of the primary medical therapy patient, also after definitive surgery (detailed in section 4.3.4). The adjuvant therapy patient underwent autologous BMT, receiving harvested peripheral-blood progenitor cells that were CK^+ by ICC following preablative chemotherapy. The patient died of relapse of the tumour 20 months post-transplant, and had evidence of distant metastases (bone [13 months post-transplant], liver, pleural effusion, and BM by routine histology [18 months post-transplant]).

Group B: In 7 patients who tested positive for circulating CK^+ cells at baseline, further CK^+ cells were undetectable in serial PB samples taken during repeated, assessable courses of chemotherapy. Two relapses were observed in this particular group of patients with limited disease, who were treated with primary medical AC chemotherapy. One patient had persistent marrow infiltration at the time of re-staging at definitive surgery, compared with that of the first (baseline) aspirate taken at the time of diagnostic surgery (also detailed in section 4.3.4).

Group C: In 3 primary medical therapy patients who tested positive for circulating CK^+ cells at baseline, further CK^+ cells were detected in PB only after

the second course of therapy in one patient, and after the third course of AC chemotherapy in the remaining 2 patients (1 and 2 CK^+ tumour cells per 2 x 10^6 MNCs screened, documented before the 8^{th} course of chemotherapy). One adjuvant patient with persistent circulating CK^+ tumour cells during Doxorubicin/CMF chemotherapy, was shown to have a progressive increase in disease load by ICC, preceding conventional clinical confirmation of recurrence of disease. Following relapse of the tumour, distant metastasis to the liver occurred 27 months from the time of diagnostic biopsy, followed by the subsequent death of the patient.

Group D: In 7 patients who tested positive for circulating CK^+ tumour cells pre-chemotherapy, further persistent CK^+ cells were detected in PB, even after the 6^{th} course of adjuvant chemotherapy in one patient who ultimately relapsed. All 7 patients had at least two positive tests (median, three positive tests; range, two to four positive tests), before achieving a "negativity" in the PB. In addition, two further relapses were observed in this group of patients with localised disease who were treated with primary medical therapy, with one patient having detectable CK^+ micrometastatic tumour cells in the BM at the time of initial diagnostic biopsy of the primary tumour.

Group E: In this group of 4 BrCa patients, all showed evidence of persistent CK^+ tumour cells during PB sampling, including one primary medical therapy patient testing positive in the PB after 7 courses of chemotherapy. Continuous "positivity" was documented by ICC, however quantitative analysis of CK^+ cell numbers showed an overall decrease in disease load as compared to steady-state, pre-chemotherapy levels. Two of the 4 patients (one adjuvant and one primary medical therapy) showed clinical signs of disease progression, rapidly relapsed off therapy and ultimately died from distant metastases. The adjuvant

therapy patient relapsed 8 months from the time of initial diagnosis, having developed carcinomatous metastases in the distant site of the meninges.

The results of serial PB sampling for the detection of CK^+ circulating tumour cells as measured by ICC, in a total of 33 chemotherapy-naïve BrCa patients, (including the categorisation of patients based on their response to therapy through clearance of circulating carcinoma cell load), who underwent conventional chemotherapy without haematopoietic colony-stimulating factor support, is illustrated graphically below:

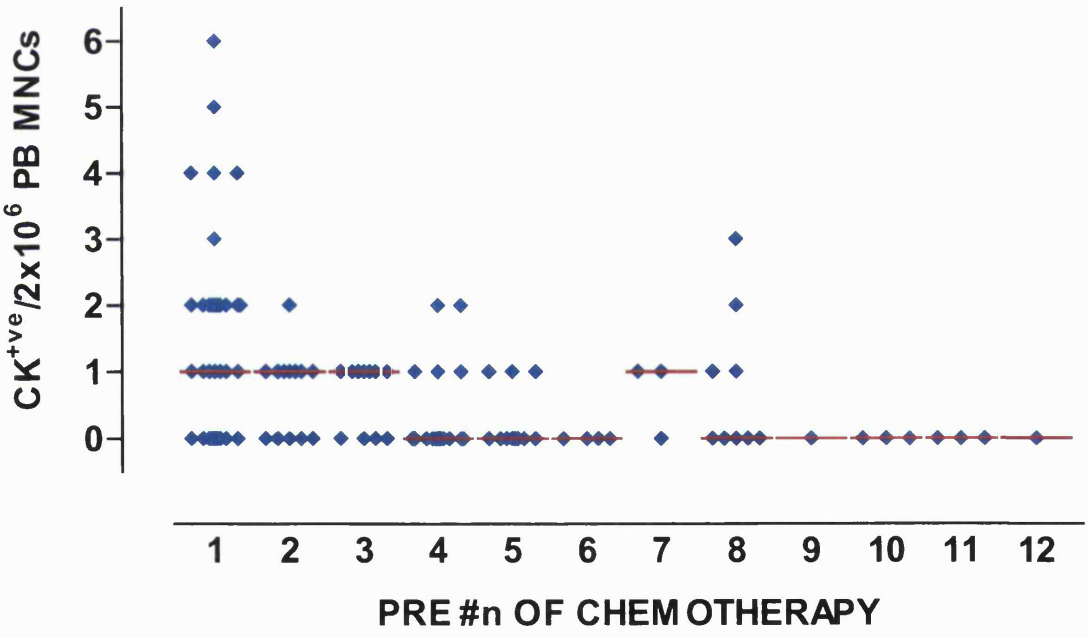


Figure 4.3: Overall impact of 12 successive courses of chemotherapy on the presence of circulating tumour cells in 33 patients with primary, non-metastatic disease – all data points + medians (red).

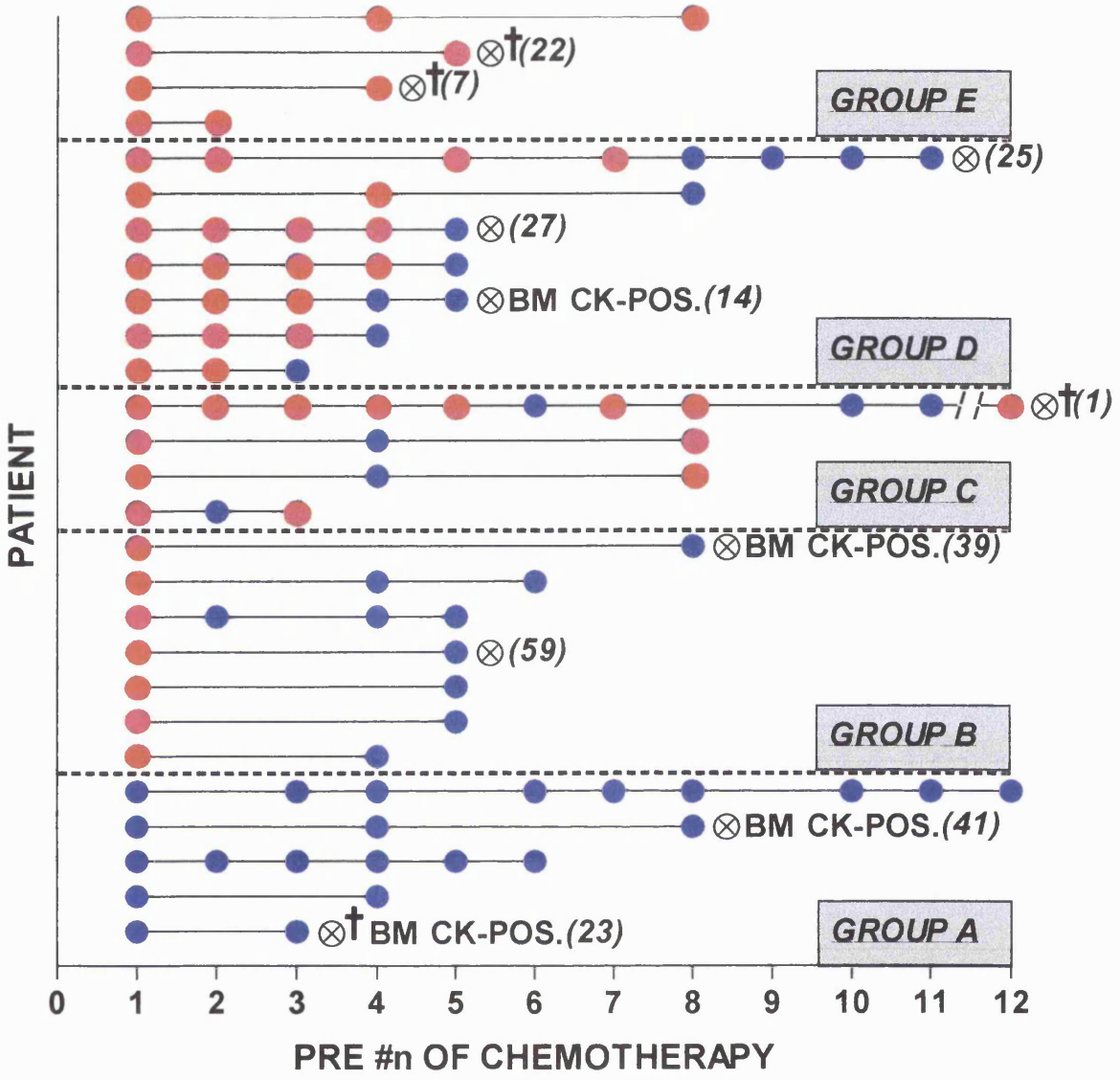


Figure 4.4: Monitoring therapeutic efficacy and elimination of circulating CK⁺ tumour cells by PB sampling during chemotherapy in individual high-risk patients – by trend.

Figure Legend: CytoKeratin-positive PB sample –●

CytoKeratin-negative PB sample –●

Clinical relapse –⊗

Patient death –†

Note: Numbers in parentheses denote the time interval (months) between the last PB sample analysed and the subsequent clinical relapse.

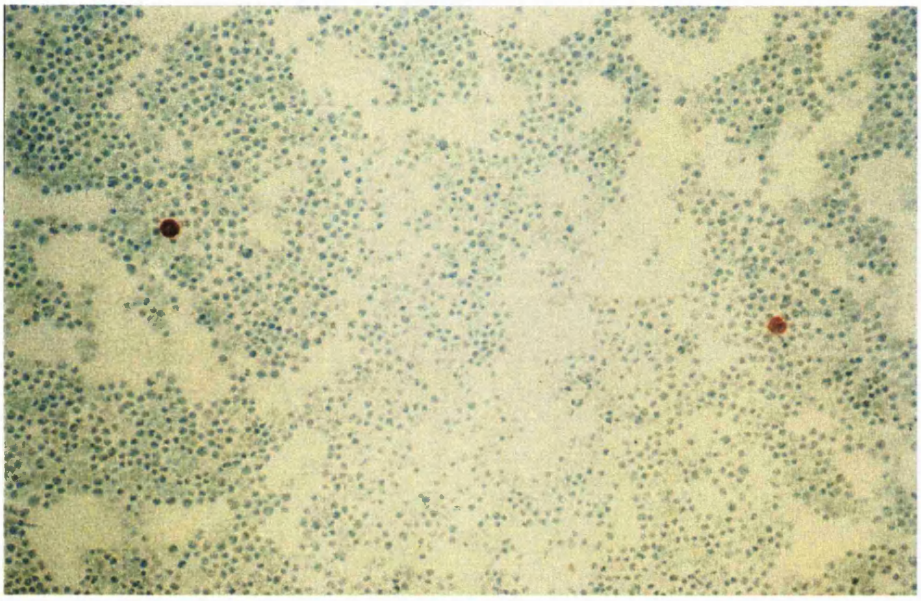
4.3.4.1 Immunocytochemical Assessment of Bone Marrow Aspirates for Monitoring Response to Chemotherapy in High-Risk Breast Cancer Patients

Bone marrow samples in this part of the study were taken from 14 newly diagnosed high-risk BrCa patients (8 primary medical therapy [all with ductal carcinoma *in situ*, with the exception of one patient with mixed histologic type], and 6 adjuvant therapy patients [all ductal carcinoma *in situ*]). The applied immunoassay identified disseminated CK^+ tumour cells in 8 patients (57.1%) at the time of first diagnosis, and in 5 of 6 patients (83.3%) at the time of surgery, post-chemotherapy. All of the BM aspirates tested immunocytochemically, were negative for mammary carcinoma cells by routine histology. In most specimens (10 of 13 [77%]) the occult cells were present as dispersed cells (Figure 4.5A); clusters of cells (Figure 4.5B) were found in only 23% of specimens (3 of 13). Further evidence for the reliable specificity of the applied MoAb was available from BM aspirates from 75 normal volunteer donor controls, which demonstrated the absence of specifically stained CK^+ cells in all samples tested, as a result of the lack of cross-reactivity with autochthonous BM cells.

Seven patients (50%) of the whole study population had metastasis to regional axillary lymph nodes (pN_{1-2}) but no evidence of manifest metastases (stage M_0). In patients analysed before chemotherapy, isolated CK^+ tumour cells were found in 5 (71.4%) of 7 node-positive cases and 3 (42.9%) of 7 node-negative cases. After chemotherapy, CK^+ BM findings were assessed in 6 (42.9%) of 14 patients; 2 (28.6%) of node-positive cases and 4 (57.1%) of node-negative cases. In node-positive BrCa, two (40%) of 5 patients (primary

medical therapy patients) yielded CK^+ BM aspirates before and after chemotherapy.

A:



B:

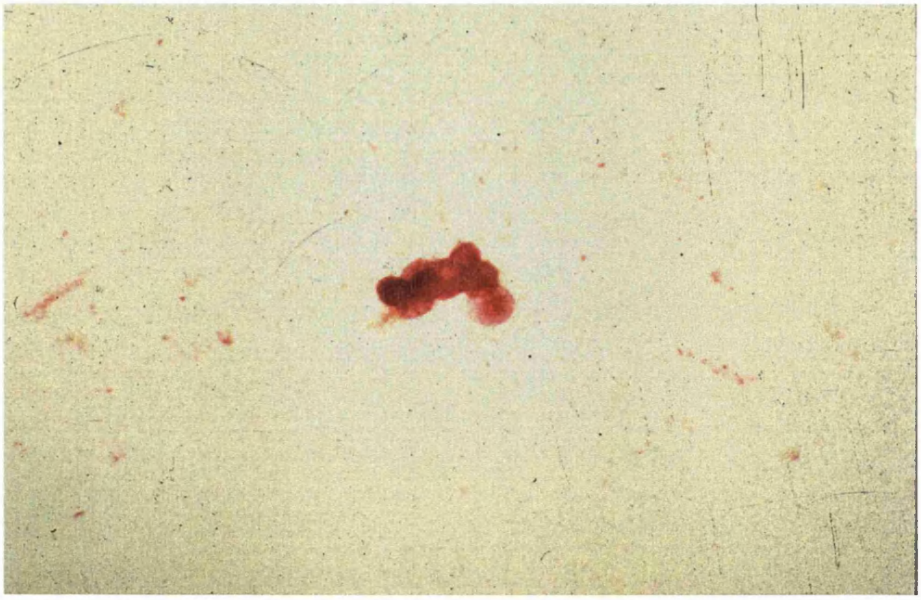


Figure 4.5: Immunostaining of occult metastatic cells in BM with MoAb A45-B/B3 (X 100).

Panel A shows two single dispersed metastatic cells. Panel B shows a cluster of micrometastatic cells, highlighted by strong labelling for CK along the entire cell membrane, with a cell morphology consistent with that of tumour cells; normal surrounding BM MNCs cells are unstained.

At the time of re-staging, BM infiltration in these patients, compared with that of the first aspirate, showed a decrease in the number of contaminating tumour cells. In node-negative patients, 1 of 3 initially CK^+ had a positive aspirate after chemotherapy (the number of contaminating cells were reduced between the first and the second aspirate); whereas 2 of 4 previously cytokeratin-negative (CK^-) patients became CK^+. Moreover, matched BM aspirates from 6 primary medical therapy patients at onset and at re-staging, showed no significant difference between the mean number of tumour cells before (6 CK^+ tumour cells per 2 x 10^6 MNCs screened) and after chemotherapy (3 CK^+ cells per 2 x 10^6 MNCs screened) ($p = 0.2060$; paired *t* test, $t = 1.453$) (illustrated graphically in Figure 4.6A). Overall, BM aspirates were positive in 3 of 6 (50%) patients before therapy and in 5 of 6 (83.3%) after therapy ($p = 0.5455$, 95% confidence interval (CI) 0.1734-1.441) (Figure 4.6B).

Data were also obtained from sets of samples ($n = 13$ [before chemotherapy] and $n = 6$ [after chemotherapy]), in which BM and venous blood were tested in parallel. Specimens received from patients at diagnosis revealed 7 of 13 equivalent results (BM+/PB+ or BM-/PB-) in both compartments, with 5 positive pairs and 2 negative pairs. Two of 13 divergent sets (BM+/PB- or BM-/PB+) exhibited a positive signal in the BM but tested negative in venous blood, and 4 of 13 pairs with divergent results were positive in PB and negative in BM. Specimens tested from the 6 primary medical therapy patients at definitive surgery revealed 2 of 6 equivalent results in both compartments (both positive pairs). Three of 6 divergent sets exhibited a positive signal in the BM but tested negative in PB, and 1 of 6 pairs with a divergent result was positive in PB and negative in BM. Furthermore, matched PB samples, from the 6 primary medical therapy patients treated with 8 cycles of AC chemotherapy, at diagnostic and

definitive surgery, showed no significant difference between the mean number of tumour cells before (2 CK^+ tumour cells per 2 x 10^6 MNCs screened) and after chemotherapy (1 CK^+ cells per 2 x 10^6 MNCs screened) ($p = 0.2387$; paired *t* test, $t = 1.337$) (Figure 4.6C). Overall, PB samples in this particular group were positive in 5 of 6 (83.3%) patients before therapy and in 3 of 6 (50%) after therapy ($p = 0.5455$, 95% confidence interval (CI) 0.4213-14.83) (Figure 4.6D). In general, the absolute tumour cell load between the two compartments differed significantly ($p = 0.0122$; unpaired *t* test with Welch's correction, $t = 2.769$) (Figure 4.7). Differences of CK-reactive tumour cells between the first and second BM aspirate, and corresponding venous blood sampling in all 14 patients in shown in Figure 4.8.

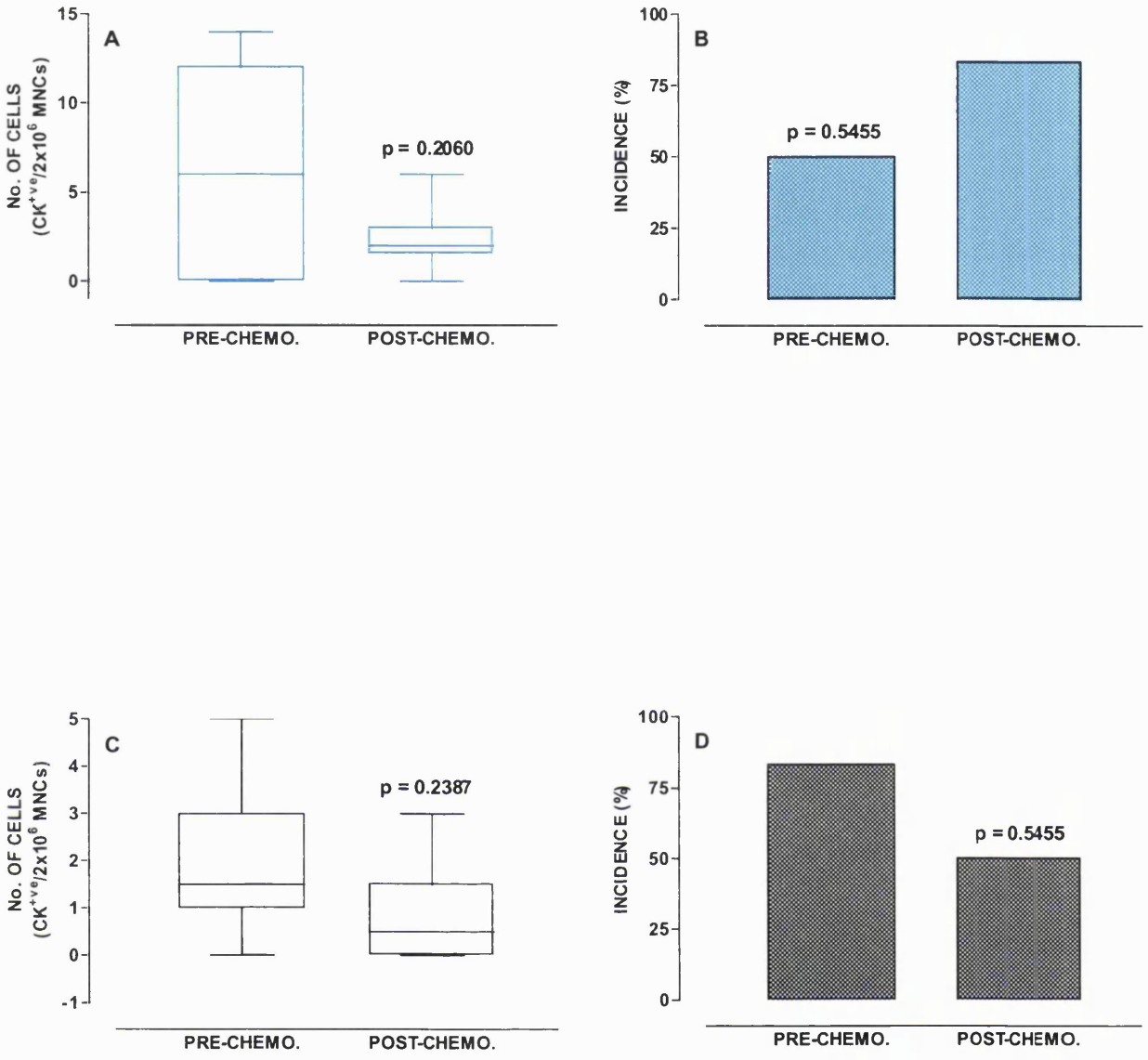


Figure 4.6: Monitoring of the elimination of CK^+ tumour cells during chemotherapy in high-risk BrCa patients.

Direct ICC monitoring of therapeutic efficacy and incidence of CK^+ samples, by paired BM (A and B) and PB (C and D) analysis at initial diagnosis and at the time of definitive surgery following 8 cycles of Adriamycin, Cyclophosphamide (AC) chemotherapy, in 6 high-risk primary medical therapy BrCa patients.

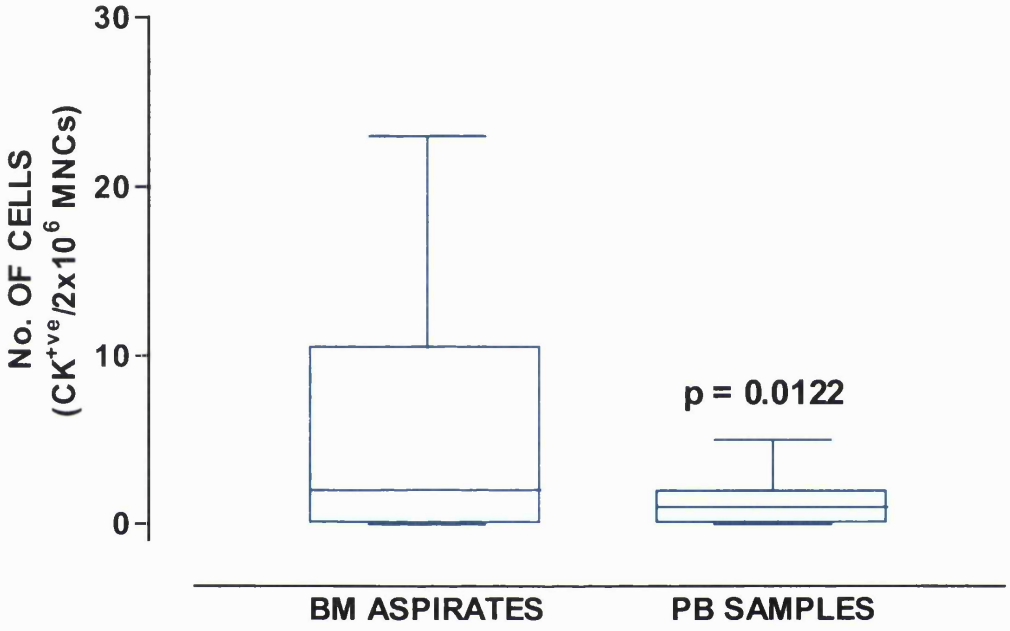


Figure 4.7: Comparison of the two separate compartments – BM aspirates (diagnostic and definitive surgery) and PB samples (pre-, during, and post-chemotherapy [AC/Doxorubicin, CMF]), in the 14 high-risk primary BrCa patients.

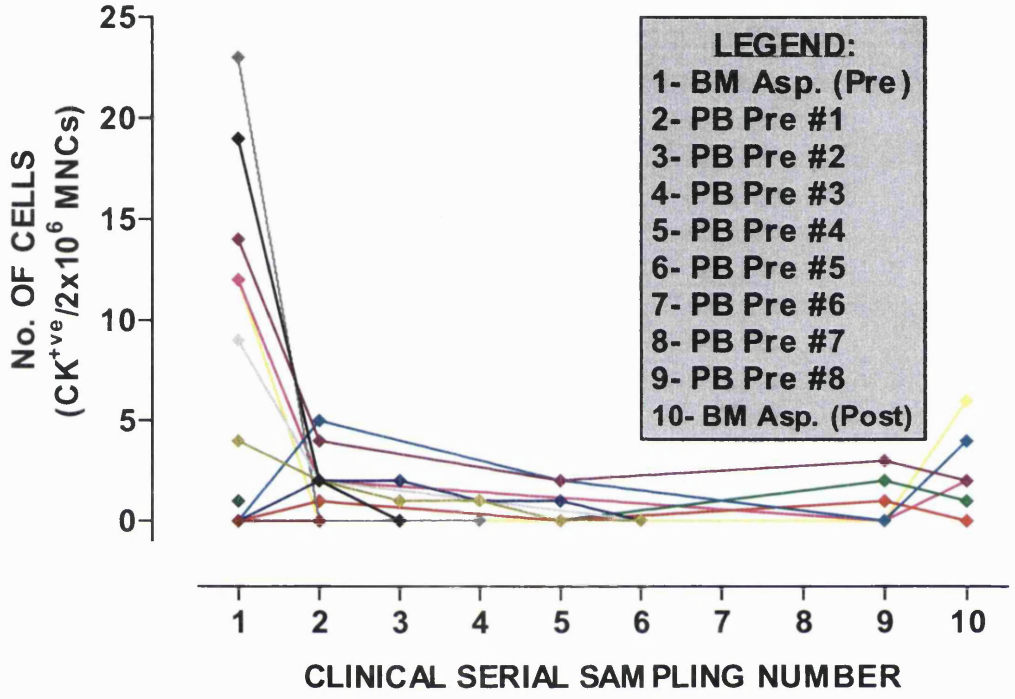


Figure 4.8: Results from the ICC assessment of anticancer therapy efficacy by determination of the relative number of micrometastatic and circulating CK^+ cancer cells.

Every symbol represents a separate data point (the number of CK^+ cells per 2 x 10^6 MNCs screened), from both BM aspirates and PB sampling, pre-, during, and post-chemotherapy, in the 14 non-metastatic, high-risk primary BrCa patients.

4.3.4.2 Clinical Outcome

A higher incidence of relapse was observed in patients with persistent CK^+ micrometastatic tumour cells in BM after chemotherapy (3 of 3 primary medical therapy patients) compared with patients who either remained CK^- during therapy (one patient) or turned CK^+ after therapy (2 patients). In addition, 2 patients (one adjuvant and one primary medical therapy) in this group, showed signs of disease progression and relapse of the tumour, following the detection of micrometastases in BM aspirates performed at the time of first diagnosis. The adjuvant therapy patient underwent autologous BMT, receiving harvested peripheral-blood progenitor cells that were CK^+ by ICC following preablative chemotherapy. The patient died of relapse of the tumour 20 months post-transplant, and had evidence of distant metastases (bone [13 months post-transplant], liver, pleural effusion, and BM by routine histology [18 months post-transplant]).

4.4 Discussion

In this part of the study of the haematogenous dissemination of breast-cancer cells, a monoclonal antibody (A45-B/B3) was used ,that binds to an antigen on cytokeratins 8, 18, and 19. These CKs are expressed by normal and transformed epithelial cells (Stigbrand *et al*, 1998) but not autochthonous BM cells (Pantel *et al*, 1994; Braun *et al*, 1998a). As compared with MoAbs against single members of the CK family, A45-B/B3 is more sensitive in the detection of epithelial tumour cells (Pantel *et al*, 1994; Braun *et al*, 1998a), perhaps in part because its broad specificity overcomes the down-regulation of individual CK polypeptides in some transformed cells (Schaller *et al*, 1996). Furthermore, this high specificity is in contrast to the considerable rate of false-positive results obtained with MoAbs directed against epithelial membrane antigen, milk fat globule, human epithelial antigen-125, and other cellular mucins, including tumour-associated glycoprotein-12 (Delsol *et al*, 1984; Taylor-Papadimitriou *et al*, 1985; Pantel *et al*, 1994; Braun *et al*, 1998a; Brugger *et al*, 1999).

Recently, the RT-PCR method has been proposed as a rapid screening procedure that showed a sensitivity 10- to 100-fold higher than routine immunologic methods. In particular, CK RT-PCR has been reported as an assay system for detection of circulating tumour cells in patients with BrCa (Datta *et al*, 1994). Some authors reported a specificity of up to 97% and a sensitivity of 10^{-5} to 10^{-7} in the detection of tumour cells in PB or BM. However, using primers specific for CK, one can obtain false-positive findings in blood and marrow from non-carcinoma patients. In particular, it has been reported that RT-PCR methods detected an illegitimate expression of CK19 mRNA in PB cells in a significant number of healthy controls (Krismann *et al*, 1995). Similarly, Zippelius *et al* (1997) have shown a constitutive low-level expression

of CK18 mRNA in normal BM cells using the RNA template-specific PCR assay. A further possible explanation for these false-positives includes amplification of the processed CK19 pseudogene from contaminating genomic DNA, because the CK19 pseudogene is virtually identical to the CK19 cDNA sequence (Bader *et al*, 1988). Thus, due to the variable levels of expression of CK mRNA in epithelial tissues or to ectopic expression of small amounts of epithelial mRNA in mesenchymal cells, the disease specificity of CK RT-PCR assays remain largely unsatisfactory. The complete absence of false-positive results and the high incidence of positive findings in patients at risk of metastatic disease associated with the immunoassay described herein, suggests that it is a reliable tool to detect clinically occult haematogenous tumour-cell dissemination to BM through venous PB with the added benefit of quantitation.

The controversy over the prognostic relevance of the presence or absence of CK^+ cells in the marrow (Salvadori *et al*, 1990; Cote *et al*, 1991; Courtemanche *et al*, 1991; Harbeck *et al*, 1994; Diel *et al*, 1996; Molino *et al*, 1997; Landys *et al*, 1998; Mansi *et al*, 1999) may be explained by the use of different MoAbs, staining techniques, and criteria for defining positively stained cells. The absence of detectable CK^+ cells in 131 of 131 specimens from control subjects with non-malignant disease in this study demonstrates the specificity of A45-B/B3. The specificity of CK as a marker of epithelial cancer cells seems clear (Pantel *et al*, 1999), but there remains the problem of the sampling error inherent in examinations of small volumes of aspirated BM. Moreover, *in vitro* experiments showed that the Cell-Tak® Cell and Tissue Adhesive ICC immunoassay reproducibly detected a single tumour cell among 1,000,000 CK^- cells (detailed in Chapter 3). In this part of the study, 2,000,000 MNCs were examined from each patient at each sampling time point.

The shortcomings of current tumour-staging practices are revealed by the fact that distant metastases eventually occur in up to 30% of patients with node-negative cancer (DeVita, 1989) and that approximately 40% of patients with node-positive cancer survive for 10 years or more (Ragaz *et al*, 1997; Overgaard *et al*, 1997). Ménard *et al* (1994) reported that the presence of lymph node metastases was not a reliable prognostic indicator in biologically defined subgroups of patients, suggesting that lymph node metastases are not necessarily associated with haematogenous spread of cancer. In contrast to the presence of residual tumour cells, which can be monitored at any time after administration of adjuvant treatment and may provide information on the kinetics of metastatic cells for the individual patient, the predictive value of the extent of lymph node metastasis is limited to the time of surgery. Most notably, Braun *et al* (2000b) have recently shown that early haematogenous dissemination to BM in BrCa patients seems to be a prognostic factor independent of lymphatic spread, and is associated with an increased frequency of bone and multiple metastases. In particular, skeletal relapse was strongly related to the presence of micrometastases, suggesting that precursor cells of overt metastases may indeed be present among the dispersed CK^+ cells detected in the BM at the time of diagnosis.

In the preliminary phase of the study, the ICC based assay was able to detect circulating CK^+ cells in the venous blood of 44% of patients (11 of 25) with localised disease and 92% of patients (23 of 25) with more advanced (M+) disease ($p<0.0001$). The increased median frequency of tumour cells in positive clinical samples from women with metastatic BrCa (6.5 versus 1.0 per 2 x 10^6 MNCs screened [$p<0.0003$]) could reflect the higher tumour burden and the advanced stage of disease. In contrast, all controls tested were CK^-, which

further indicates the specificity of the technique reported. Lack of CK ICC positivity in the blood of the two metastatic patients may have been due to consistently low numbers of circulating carcinoma cells below the threshold of detection by the CK ICC assay, or to sequestration of tumour cells at other distant sites. In addition, it may be possible to use the assay in the area of metastatic BrCa, because it is often difficult to assess response to chemotherapy in these patients. Biochemical markers are frequently misleading (Seckl *et al*, 1992), and radiologic techniques are insensitive.

In the follow up phase of the study, venous blood samples were examined from primary medical therapy and adjuvant therapy BrCa patients before and after conventional-dose chemotherapy. Applying the validated immunoassay to these high-risk patients, it was found that cytotoxic therapy with taxanes and anthracyclines significantly reduced, but did not completely eliminate, the number of circulating CK^+ tumour cells. Overall, the number of tumour cells identified per patient using the described immunoassay was low, usually between 1×10^{-6} and 6×10^{-6} CK^+ cells. Interpretation of the appearance or disappearance of a few cells as either success or failure of the applied therapy seems to be possible but should be performed repeatedly to reduce sampling error because false-negative results cannot be completely excluded. Using similar assay conditions, a recent monitoring study on stage C prostate cancer patients demonstrated that a therapeutic depletion of CK^+ cells under androgen deprivation is measurable (Pantel *et al*, 1997). In this study, the recent recommendations of the Tumour Cell Detection Committee of the International Society of Haematotherapy and Graft Engineering were adhered to, to minimise the methodological influence of a sampling error (Borgen *et al*, 1999). Changes in circulating tumour cell levels in particular patients with

locally advanced disease who had detectable disease using ICC reflected the clinical progression/regression of the disease, which is in agreement with other groups that have studied comparable numbers of patients (Peck *et al*, 1998; Racila *et al*, 1998). Although the absolute values did not always reflect the clinical course at a single point during therapy, the trend over a period of months was more important. Therefore, although these results are somewhat preliminary, it may be possible to apply such an immunoassay to monitor disease response from PB samples in patients who are considered to be disease-free. Such a monitoring system could be used as a surrogate marker in identifying patients who are at increased risk of relapse over a prolonged follow-up period, since the above results indicate that circulating tumour cell levels may reflect changes in disease load. Patients who are found to have increasing CK^+ tumour cells could be candidates for further systemic adjuvant therapy. The results reported here also, controversially, suggest that conventional-dose chemotherapy may have a tumour cell-mobilising effect into the PB in patients with primary BrCa (section 4.3.3.2 [group C patients]). Although the mobilising effect of high-dose chemotherapy, ranging between 5% and 50% in different conditions (Brugger *et al*, 1994), has been reported, clinical issues concerning the role of recruitment of tumour cells into the PB exerted by conventional chemotherapy have not been clarified. In this prospective part of the study, the presence of CK^+ circulating cells was evaluated in a total of 33 chemotherapy-naïve BrCa patients who underwent conventional chemotherapy without haematopoietic colony-stimulating factor support. Cytokeratin ICC was carried out on samples of PB at baseline and prior to assessable, successive chemotherapeutic treatment cycles at different times. In all but 9 pre-chemotherapy PB samples (73%), the use of CK ICC could detect circulating

tumour cells. When the samples of patients were analysed after the first course of chemotherapy, CK ICC was positive in 32 (41%) of 79 serial PB samples. None of the 5 patients who tested negative pre-chemotherapy changed to a positive test result after assessable chemotherapeutic treatment cycles, although persistent circulating tumour cells were observed in patients who tested positive after the first course, which suggests a limited "purging" *in vivo* effect of the first courses of chemotherapy.

Whether patients with BM micrometastases respond differently to adjuvant chemotherapy compared with patients without micrometastases remains to be studied. Pantel *et al* (1993a) have demonstrated that the proliferation rate of micrometastases appears to be low which might influence their sensitivity to chemotherapy. The lack of effect of chemotherapy on the elimination of residual CK^+ tumour cells in BM was quantifiable in this study. The short observation period of the study precluded the analysis of late recurrences but analysis at the time of follow-up showed that the detection of CK^+ cells after adjuvant chemotherapy is already associated with reduced overall survival. Additional long-term follow-up BM aspirations might reveal a therapeutic benefit of tamoxifen in oestrogen receptor-positive patients; this, however, was not within the scope of the present study. Thus, CK-positivity of BM after chemotherapy appears to be predictive of response to systemic chemotherapy.

The increased number of tumour cells in some patients after chemotherapy may indicate that chemotherapy has mobilised tumour cells to or from the BM, as suggested in previous studies (Brugger *et al*, 1994; Shpall and Jones, 1994). The present micrometastasis data point out that at least a redistribution of the residual tumour load by chemotherapy-dependent

mobilisation and therapy-induced elimination is to be considered as an explanation for the varying numbers of CK^+ tumour cells detected in BM after chemotherapy. However, the consequence of CK^+ tumour cells in BM after chemotherapy supports the notion that additional biologic factors influence persistent BM CK positivity in the face of adjuvant chemotherapy. Because micrometastatic cells rarely express proliferation-associated markers, such as Ki-67 and p120 (Pantel *et al*, 1993a), they might be relatively resistant to some chemotherapeutic agents. Overexpression of the Her-2/*neu* proto-oncogene might be regarded as another factor mediating resistance to cytotoxic drugs (Hancock *et al*, 1991) and, as detailed in Chapter 6, overexpression of the Her-2/*neu* proto-oncogene may characterise an "aggressive" subset of metastatic BrCa stem cells; such overexpression being associated with poor survival.

Therefore, the above data do not confirm the efficacy of chemotherapy as a method for *in vivo* "purging" of BM, even in responsive patients with localised disease. There is a growing consensus about the use of standard chemotherapy for both tumour debulking and BM purging before HDC (Brugger *et al*, 1994; Elias, 1997). Therefore, these data support the use of further manipulation of BM or peripheral blood progenitor cell products (i.e. "*ex vivo*" purging, selection of $CD34^+$ cells), in the appropriate clinical setting, in order to ensure a further reduction of contaminating tumour cells.

Comparative analyses of paired BM aspirates and venous blood samples from the same patient are uncommon. Investigations of neuroblastoma patients (n = 14) and more recently a study for CK19 mRNA in BrCa patients (n = 13) were reported (Miyajima *et al*, 1995; Krüger *et al*, 1996). Nineteen sets of BM and PB from BrCa patients were investigated in the present study in parallel (n = 13 [before chemotherapy] and n = 6 [after chemotherapy]). Results obtained

from the analyses of the sets of samples strongly suggested that in BM and PB, the same type of cells were detected. The detection rate in BM was higher than in the corresponding PB sample. It can be concluded that the tumour cells survive in blood less well and/or that the BM may function as a filter. A recent study on a larger group of prostate Ca patients investigated for disseminated disease by a PSA RT-PCR in both tissue compartments reached a similar conclusion (Melchior *et al*, 1997).

The detection of the earliest manifestations of tumour dissemination is a promising approach that should improve risk assessment and the identification of patients who might benefit from adjuvant therapy. New parameters need to be defined that better identify those patients at the greatest risk of relapse, since this might provide information critical to the subsequent management of the patient. In addition, the genotype/phenotype of these cells may have a role in determining whether overt metastases will develop. A highly sensitive assay combining tumour cell enrichment with immunolabelling and fluorescence *in situ* hybridization (FISH) was developed, to detect, enumerate, and cytogenetically characterise tumour cells in the PB and BM. The above approaches shall also be utilised to quantify the frequency of epithelial tumour cells in BM aspirates from prostate Ca patients, collected at the time of radical prostatectomy, and to apply genotypic analyses to determine whether detectable cells contain numeric chromosomal aberrations. Improved methods for cytogenetic analysis of single tumour cells may increase both the diagnostic precision of current detection techniques and optimise the therapy for individual patients, (possibly including the use of recombinant humanised monoclonal antibody directed against Her-2/*neu*, trastuzumab [Herceptin™]).

Chapter 5 Fluorescence In Situ Hybridization (FISH) – Model Systems

5.1 Introduction

5.1.1 Breast Cancer

In 1977 Bernard Fisher described BrCa as a systemic disease. Since that time the criteria, in addition to lymph node and hormone receptor status, which may be used as prognostic indicators, have been the subject of active investigation. Gene amplification is a feature of some cancer cells that allows increased production of specific proteins needed for acquisition and maintenance of the malignant phenotype. Amplification of certain oncogenes has been shown to have an important role in the progression of many tumours (Alitalo and Schwab, 1986; Schwab and Amler, 1990; Bishop, 1991). For example, the N-*myc* oncogene is amplified in neuroblastomas, *myc* and L-*myc* are amplified in small cell lung cancer, and Her-2/*neu* is amplified in a proportion of breast and ovarian cancers. Detection of such amplifications may, in some instances, assist in both the diagnosis and in prognostic assessment. Gene amplification may also contribute to the generation of resistance to cytotoxic drugs. When the Her-2/*neu* gene was discovered (Bargmann *et al*, 1986), it was rapidly adopted as a promising marker for BrCa prognosis, and prediction of response to therapy (Ravdin and Chamness, 1995). The gene Her-2/*neu* (also known as c-*erb*B-2) encodes a 185-kD transmembrane glycoprotein receptor tyrosine kinase that contains sequence homology to members of the type-1 receptor tyrosine kinase family, which includes Her-1, Her-3, and Her-4 (Tzahar and Yarden, 1998). Growth factors (such as

epidermal growth factor [EGF] or Neu differentiation factor) interact with the extracellular domains of different type-1 tyrosine kinase family members, which facilitates the formation of homodimers and heterodimers of various receptor family members. The heterodimerisation of Her-2/*neu* with other receptor family members leads to intracellular tyrosine autophosphorylation at multiple tyrosine residues on the carboxyl terminal region and provides docking (binding) sites for Src homology 2 adapter proteins (Kavanaugh and Williams, 1994; Cohen *et al*, 1995; Ricci *et al*, 1995). The docking proteins then relay the activation signal to proteins, such as ras, raf, mitogen-activated protein kinase (MAPK), c-Jun kinase, or ribosomal protein S6 kinase, which eventually leads to alterations in cell growth and transformation (Pierce *et al*, 1991; Ming *et al*, 1994; Karunagaran *et al*, 1996; Tzahar and Yarden, 1998). In cellular models, Her-2/*neu* appeared capable of inducing malignant transformation (Bargmann *et al*, 1986; Yamamoto *et al*, 1986). Yu *et al*, (1992, 1994) and Yu and Hung (1991) have also shown that Her-2/*neu* transfected cells can acquire a metastatic potential in animal models. The Her-2/*neu* gene is amplified and overexpressed in 20-30% of invasive, and in 40-60% of intraductal breast carcinomas (van de Vijver *et al*, 1988; Slamon *et al*, 1989).

Genetic changes lead to the amplification of Her-2/*neu* which occurs in the early stages in the development of BrCa. Her-2/*neu* amplification is generally considered to be a significant adverse prognostic factor in patients with BrCa, independent of conventional prognostic factors on multivariate analysis, however, its applicability continues to be controversial, in part because of analytical discrepancies associated with the methods traditionally used to evaluate its amplification and/or overexpression. These techniques include Southern blotting, slot blot analysis, and FISH (illustrated in Figure 5.1) for

detection of amplification, while ELISA, Western blotting, immunohistochemistry, and immunofluorescence are used to evaluate overexpression (van Dienst *et al*, 1991; Tiwari *et al*, 1992; Dawkins *et al*, 1993; Cuny *et al*, 1994). Because FISH allows the observer to accurately quantify the level of amplification in small subpopulations of cells, it has an increased sensitivity compared with blotting techniques. In addition, FISH accurately identifies the location of such amplification in tumour specimens (Pauletti *et al*, 1994). Immunofluorescence provides more accurate quantification because immunohistochemically stained slides provide data that are difficult to quantify and because ELISA and Western blotting data do not provide information concerning heterogeneity (Pauletti *et al*, 1994; Stal *et al*, 1994).

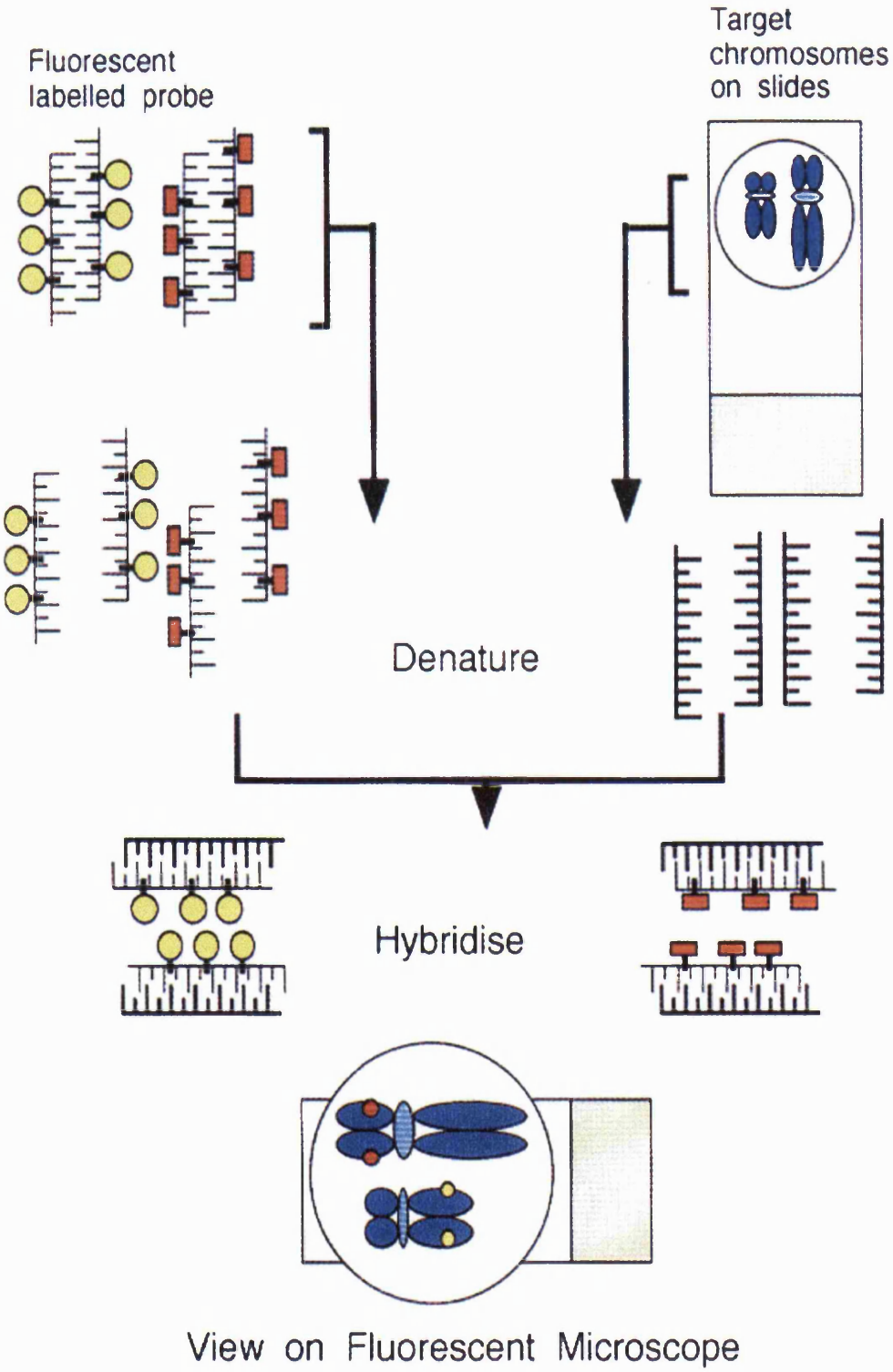


Figure 5.1: Schematic illustration of the principles of FISH.

Dual colour FISH for detection of two single locus sequences on metaphase chromosomes is shown. (Refer to Chapter 2, sections 2.4-2.6 for technical details).

Extensive clinical studies have shown that Her-2/*neu* amplification and overexpression is inversely correlated with both disease-free and overall survival in node-positive BrCa patients (Slamon *et al*, 1989; Gusterson *et al*, 1992). Patients with Her-2/*neu* overexpressing tumours do not benefit as much from adjuvant therapy with CMF (Gusterson *et al*, 1992) and require more aggressive anthracycline regimens (Muss *et al*, 1994) than other patients. Within prognostically favourable node-negative subpopulations of BrCa patients, a small cohort of patients with early haematogenous spread could be identified by measuring Her-2/*neu* amplification and overexpression (Allred *et al*, 1990; Paik *et al*, 1990; Brandt *et al*, 1995).

Marked heterogeneity has been described in primary BrCa in both the copy number of Her-2/*neu*/cell and in the level of $p185^{Her-2}$ protein (Borg *et al*, 1991; Allred *et al*, 1993; Barnes, 1993). Although cell-to-cell differences may be due in part to analytical variation, genetic and epigenetic dispersion may also play significant roles. This heterogeneity provides a potential source for the selection of subclones with increased malignant and metastatic potential, especially in the context of therapeutic targeting based on Her-2/*neu* expression.

Various methods for immunophenotypic characterisation of epithelial cells in BM have been described (Pantel *et al*, 1991; Riesenberg *et al*, 1993). Here, a double labelling method is described in which the cytokeratin labelling using a MoAb is performed first and the sample screened for the presence of epithelial cells. After the immunophenotypic characterisation, FISH was applied to the same specimens, allowing the detection of amplifications and other genetic alterations (genotyping) in individual morphologically and immunocytochemically defined tumour cells. The sensitivity of the method is

high, and clonal variation among the cancer cells can be revealed. The results of studies to demonstrate Her-2/*neu* copy number and the chromosome 17 centromere number in BrCa cell lines, by multicolour FISH are reported. It is also shown that FISH allows assessment of the level of Her-2/*neu* amplification as well as the spatial distribution of oncogene copies in established cultured breast cancer cell lines. The quantification and evaluation of spatial distribution and the heterogeneity of Her-2/*neu* amplification on a "cell by cell" basis, represents an advantage of FISH over ICC. This simultaneous study of individual cells for oncogene amplification and centromere copy number may provide more meaningful results than do studies of whole-tissue cell populations. In addition, studies of individual cells in a tumour sample may give new information leading to a better understanding of the malignant process and may also be useful in early diagnosis and/or establishing prognosis in BrCa. This approach was then further validated in a second epithelial malignancy (prostate Ca) that also metastasises to mesenchymal organs such as the BM. The two micrometastatic cell lines derived from disseminated cancer cells in BM of patients with prostate Ca were used as model systems.

5.1.2 Prostate Cancer

Prostate Ca is the most commonly diagnosed noncutaneous malignancy in men, and is the second leading cause of cancer-related death in men in Western industrialised countries. The incidence is rising, with over 200,000 new cases diagnosed each year, resulting in 40,000 deaths annually (Landis *et al*, 1998). From estimates based on *post-mortem* studies, it is known that approximately 25% to 50% of men over the age of 50 years, who have no clinical evidence of prostate Ca, harbour microscopic foci of well-differentiated

cancer within the prostate. When these numbers are extrapolated to the American male population, approximately 11,000,000 American men over 50 years of age have latent prostate Ca (Gittes, 1991). These data suggest that a large portion of prostate Ca is a clinically indolent disease and that only a minority of men with a histological diagnosis of prostate Ca will develop clinically significant, life-threatening tumours (Scardino *et al*, 1992). Population-based PSA screening has resulted in a marked increase in the early detection of prostate Ca (Potosky *et al*, 1995). However, at present it remains unclear what percentage of these early-detected cases represents sub-clinical disease, which might not require aggressive treatment. Therefore, the ability to discriminate indolent prostate Ca from tumours having the potential to progress to lethal metastatic disease has become important. Current methods for assessing the prognosis of prostate Ca, such as clinical and pathological staging and histopathological grading, fail to provide consistent predictive information regarding clinical outcome and therapeutic strategy in individual cases (Gittes, 1991; Gleason, 1992). Hence, there is a need for the identification of biological markers for tumour aggressiveness. An understanding of the molecular genetic changes that accompany both the initiation and the progression of prostatic cancer may provide these.

As with most types of human cancer, the stepwise accumulation of genetic alterations in genes important for regulation of normal growth (tumour suppressor genes), cell death (involving apoptotic pathways), and/or activation of oncogenes, contribute to the process of carcinogenesis. Molecular (cyto)genetic studies have identified multiple, non-random genetic alterations in prostate Ca (reviewed by Alers *et al*, 2000). However, the precise genetic basis of the development and progression of this disease remains largely unknown.

Recent cytogenetic and molecular studies have suggested that deletion or rearrangement of sequences that map to the short arm of chromosome 8 (8p) may be permissive for tumorigenesis in several organ systems. Cytogenetic studies have described deletions or translocations involving 8p12 in adenocarcinomas of the colon, kidney, and breast and involving 8p21-23 in adenocarcinomas of the pancreas, stomach, colon, breast, and uterus (reviewed by Mitelman, 1990).

Sequence deletions within the 8p chromosomal region have also been reported in prostate tumours. Cytogenetic studies reported by Lundgren *et al* (1992) cited alterations of 8p in 3 of 15 cases examined, involving regions 8p22, 8p21, or 8p11. Bergerheim *et al* (1991) observed loss of sequences on 8p in 65% of prostate tumours examined. Subsequent work has reported high frequencies of deletion involving the 8p22 chromosomal region in human prostate tumours (Bova *et al*, 1993). However, such studies suffer from an inability to examine homogeneous tumour cell populations (or pure populations of other cell types, e.g., hyperplastic epithelium) secondary to the histological heterogeneity of prostate tissue itself. Therefore, to date, no consistent chromosomal abnormality has been shown to be associated with prostate Ca and its metastatic progression to other sites.

Currently, the most useful clinical parameters for predicting the metastatic potential of prostate Ca are the grade, volume, and DNA ploidy of the tumour (Visakorpi *et al*, 1991). Although DNA ploidy is currently accepted as the most accurate measure of metastatic potential, it does not categorically predict metastasis (Whitmore, 1990); metastatic potential is confirmed only by identifying metastases with staging lymphadenectomy. Therefore, new prognostic cytogenetic markers would be clinically useful and desirable.

Fluorescence *in situ* hybridization was chosen for the present study to determine if trisomy 7 is associated with tumour progression in human prostate Ca for several reasons. First, previous work by Freeman *et al* (1991) and Isaacs and Hukku (1988) using rodent prostate Ca models demonstrated that three copies of the rodent chromosome 4 was a common feature during tumour progression. Because rat chromosome 4 has DNA sequences homologous to human chromosome 7, trisomy 7 may be important for progression of prostate Ca in humans. Second, the gain of all or part of chromosome 7 has been implicated in several prostatic tumours. Third, trisomy 7 has been shown to be associated with the progression of bladder cancer (Waldman *et al*, 1991) and the more aggressive clinical course of renal cell carcinoma (Weaver *et al*, 1988).

Therefore, in the present study, FISH was applied to examine in more detail the specific involvement of chromosome 7 and 8 alterations during prostatic tumour progression to the BM, using cell lines PC-R1 and PC-M1 as models, and to assign possible candidate genes underlying prostate Ca.

5.2 Materials and Methods

5.2.1 Cells and Cell Preparation

Breast cancer cell lines BT-474, MDA-MB-361, and MCF-7, and prostate Ca cell lines PC-R1 and PC-H1 were isolated by trypsinisation, washed twice in PBS and re-suspended at 5 x 10^6 cells/ml in PBS. Normal haematopoietic MNC samples processed as described in Chapter 2, section 2.2.1 were divided into several tubes and mixed with the different epithelial cell lines at a dilution of 1:250 to approximate conditions anticipated for detection of solid tumour micrometastases in marrow and blood specimens. One hundred microlitres of

each cell suspension (5 x 10^5 cells) were attached by sedimentation on 1.4cm diameter spots on cleaned microscope slides coated with Cell-Tak® Cell and Tissue Adhesive. Cell processing and immunofluorescent labelling were further optimised to detect low frequency tumour cells.

5.2.2 Control Studies: Specificity of Immunophenotyping

Certain haematopoietic cell types, carry F_c-receptors on their membranes, which bind to the so-called F_c-fragment of antibodies. Therefore, the F_c-fragments of monoclonal primary and polyclonal secondary antibodies (e.g. the Cy3-conjugated antibodies employed) may bind to the F_c-receptors of human haematopoietic cells.

Two different approaches were employed to negate this effect. Slides were incubated with the alkaline phosphatase-conjugated F_{ab} fragment of the anticytokeratin A45-B/B3 MoAb (centromeric DNA probe only). (The MoAb A45-B/B3 [immunoglobulin G1 form], was used in combination with either the indirectly labelled single copy DNA probe or centromeric DNA probe). However, the disadvantage of using $F_{(ab)2}$ fragments of second layer antibodies, is the reduced fluorescence intensity obtained. Therefore, whole molecule second layer antibodies were employed, which were selected by the manufacturers for minimal crossreactivity with human tissue. Nevertheless, specific antibody binding was verified by the following tests:

1. One A45-B/B3-labelled slide was not processed further and served as control for autofluorescence.
2. One A45-B/B3-labelled slide was stained immediately after finishing the CK labelling with FITC labelled goat anti-mouse immunoglobulin (F_c specific)

(Sigma, dilution 1:100 in 10% AB-serum). This slide served as a negative control.

3. As isotype control, one A45-B/B3-labelled slide was stained with FLOPC-21, a mouse IgG3 immunoglobulin (Sigma, dilution 1:200 in 10% AB serum) and goat anti-mouse immunoglobulin (F_c specific).
4. One A45-B/B3-labelled slide was placed in PBS for 3 hours at RT but not processed further to assess the autofluorescence of the cells after incubation in PBS.
5. and 6. Staining was analogous to slides 2 and 3 except that after CK labelling, specimens were placed in PBS for 3 hours at RT. To simulate the microscopic scoring process of real samples, slides were removed from PBS once during this 4 hour period and kept at RT for 5 minutes.

5.2.3 Analysis of Interphase Cells by Fluorescence Microscopy and Definition of Aneuploidy

Fluorescent DNA probe signals of at least 1000 nuclei from control samples were scored to increase accuracy and to evaluate hybridization efficiency and false positive rates. Whenever possible, 200-800 nuclei from epithelial cancer cell lines were scored, again to improve accuracy, with high-number cell counting in samples with low frequency of aneusomic cells (Kibbelaar *et al*, 1993).

For an unequivocal definition of true aneuploidy in clinical samples, mean percentages + 3 S.D. of normal control cells from healthy donors with non-disomic signal numbers were set as cut-off levels (Bentz *et al*, 1993; Drach *et al*, 1995; Fiegl *et al*, 1995). Percentages of monosomic, trisomic and up to hexasomic cells in clinical samples were thus corrected for background

aneuploidy, as derived from control samples (Table 5.1). Cells with more than three signals were considered as unambiguously aneuploid. To quantitate truly aneuploid cells in clinical samples, aneuploidy rate was calculated as the sum of percentages of monosomy and ≥ trisomy above the cut-off levels. (For further details refer to Chapter 2, section 2.6.9).

5.3 Results

5.3.1 Evaluation of Immunophenotype

Some cells showed autofluorescence, which was discriminated from non-specific Ab binding; (the latter was avoided by staining one slide of each case with the appropriate class-matched mouse Ab). Discrimination of true fluorescence from autofluorescence and non-specific binding is essential for accurate interpretation of results. A typical feature of autofluorescence was that it was observed with each filter set: with the Cy3 filter it appeared red, with the FITC filter yellow, and with the AMCA filter white (not the expected blue). With a simple check it was possible to distinguish a Cy3-signal from the immunostaining and autofluorescence: true Cy3 fluorescence was not visible if the AMCA filter set was used. Moreover, the appearance of true Cy3 fluorescence was different from autofluorescence. Cyanine 3 fluorescence was brilliant, while autofluorescence appeared dull red.

After labelling with the A45-B/B3 anticytokeratin F_{ab} fragment and Fast Red, tumour cells could be easily detected by bright-field microscopy. They expressed a strong dark red cytoplasmic labelling.

The goat anti-mouse immunoglobulin specific for the F_c fragment of mouse immunoglobulins did not react with the F_{ab} fragment of the A45-B/B3 anti-CK Ab. Addition of the anti-mouse immunoglobulin to A45-B/B3-stained

slides showed that background fluorescence was identical to A45-B/B3-labelled slides without any anti-mouse immunoglobulin irrespective of whether the anti-mouse immunoglobulin was added immediately after anti-CK labelling or after keeping the slides in PBS for 3 hours. The A45-B/B3 signal was not altered during this 3 hour storage period and background fluorescence and autofluorescence of the tumour cells did not change. In all of these control specimens, no double positive cells could be detected. Addition of an isotype control Ab did not change these results.

5.3.2 Evaluation of Hybridization Signals in Interphase Cells From Control Specimens

Occasionally, it was difficult to evaluate the hybridization signals if the cells were located very close to one another. In such cases, the switch over to bright-field on the microscope in addition to the fluorescence, was used to assign the hybridization signals to individual nuclei with the advantage of phase contrast.

This part of the study was performed to determine individual centromeric (α-satellite) copy numbers of chromosomes 17, 7, and individual Her-2/*neu* copy numbers, in MNCs from a cohort of 26 normal controls (Table 5.1).

DNA Marker	Signal Number per Nucleus				
	1	2	3	4	5
Chromosome 17	5.30 ± 1.39	93.85 ± 0.92	0.52 ± 0.38	0	0
Her-2/*neu*	7.59 ± 2.28	91.73 ± 1.88	0.34 ± 0.29	0	0
Chromosome 7	5.11 ± 1.08	94.41 ± 1.11	0.23 ± 0.11	0	0

Table 5.1: FISH results of PB and BM MNCs from normal healthy volunteer donors (n = 26).

Data are given as mean percentage ± S.D. of centromeric/unique sequence signal numbers.

At least 1000 nuclei were analysed per sample and DNA marker.

Mean percentages + 3 S.D. were calculated for definition of the cut-off levels.

Percentages of monosomic and trisomic cells were in accordance with previous observations in normal leukocytes (Fiegl *et al*, 1995; Herrington *et al*, 1995). Cells with more than three signals were not detectable in any control and therefore considered as unambiguously aneuploid in cancerous specimens. The slightly higher percentage of monosomy observed with the Her-2/*neu* unique sequence probe, may be due to smaller, less intense fluorescent signals compared to the centromeric probes and to overall hybridization efficiency.

5.3.3 Her-2/neu Amplification in Breast Cancer Cell Lines

Three BrCa cell lines, MCF-7, MDA-MB-364, and BT-474, were studied for distribution of Her-2/*neu* gene copy number and chromosome 17 centromere copy number (Figure 5.2). After *in situ* hybridization, the A45-B/B3 Fast Red or Cy3 signal was retained very well, thus facilitating relocation of the tumour cells and allowing fluorescent images to be superimposed (Figure 5.3).

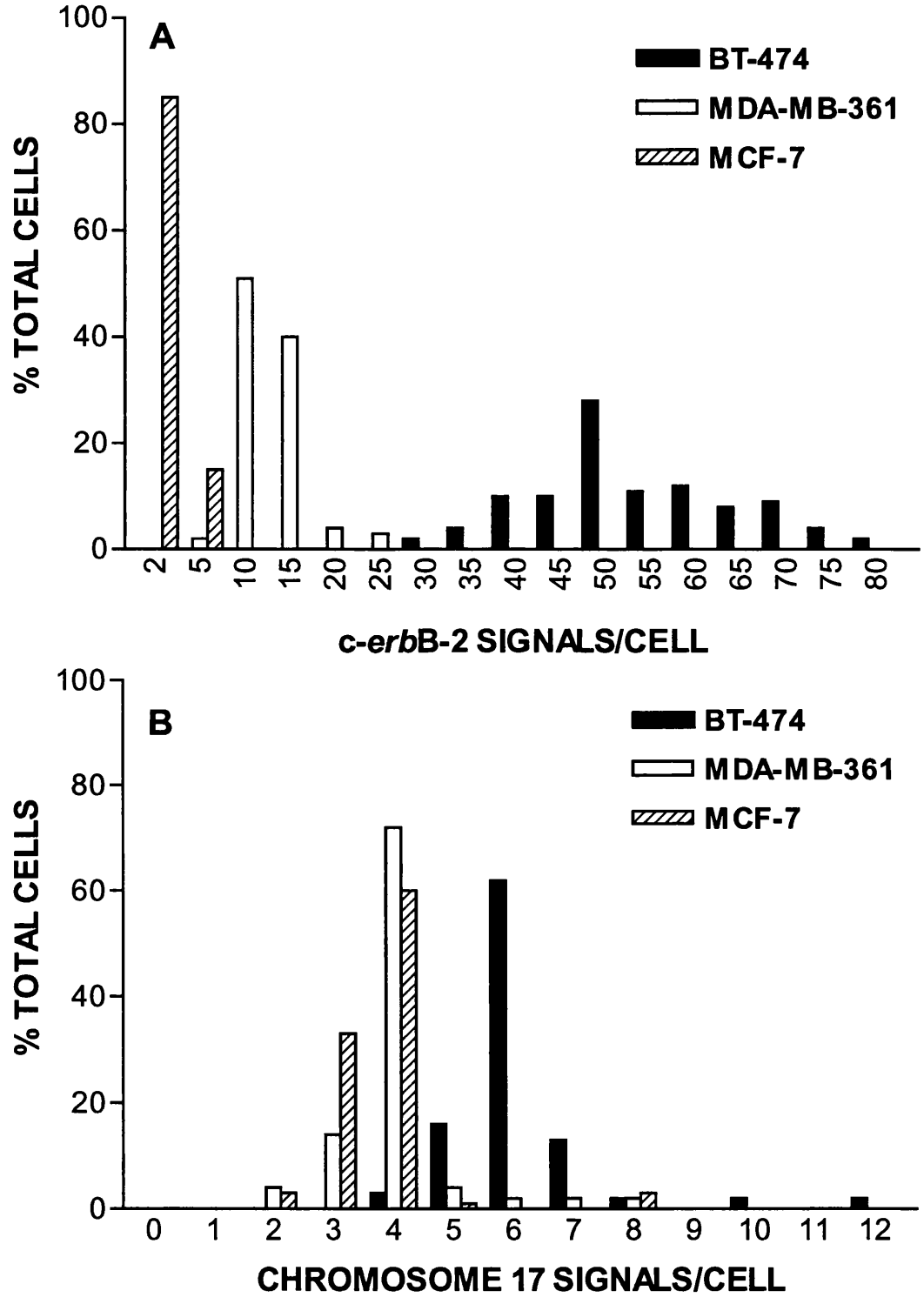


Figure 5.2: Number of Her-2/*neu* and chromosome 17 centromere copies in three breast cancer cell lines.

A, frequency distribution of Her-2/*neu* signals/cell; B, chromosome 17 signals/cell. Note the wide heterogeneity present in all but the MCF-7 distributions.

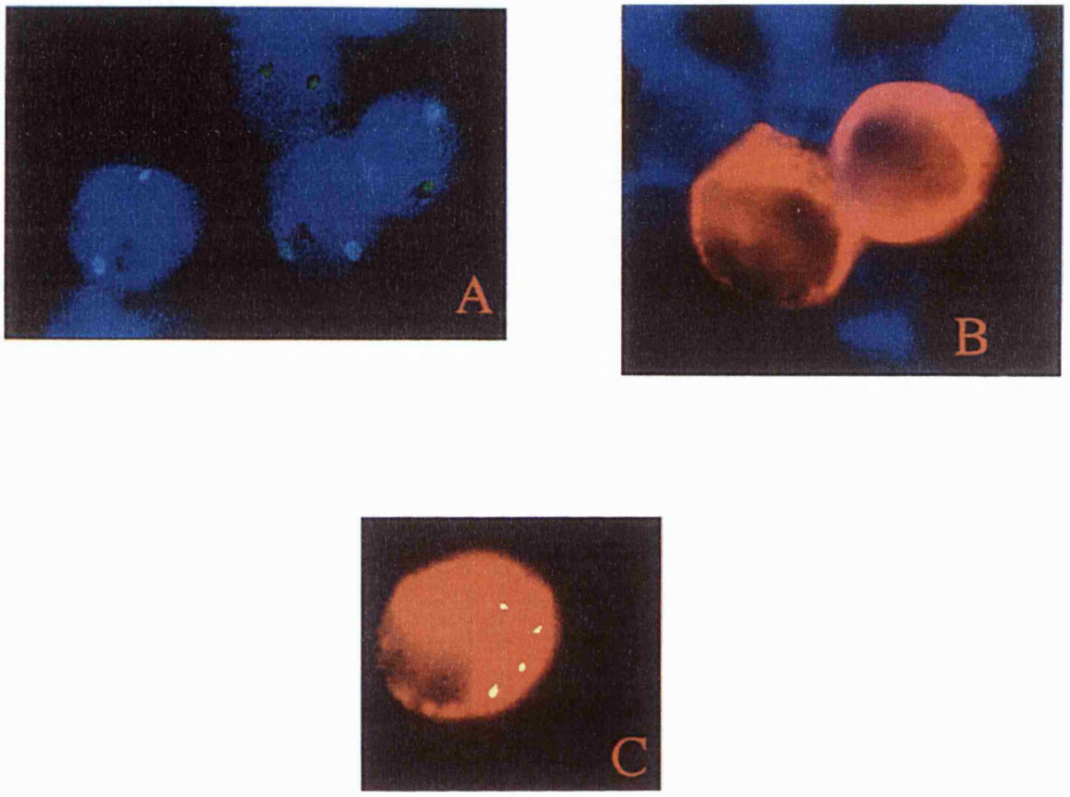


Figure 5.3: Simultaneous fluorescent Ab labelling and interphase FISH on BrCa cell lines (X 100).

A, single CK-negative BM MNCs from a normal donor, showing two hybridization signals for chromosome 17; B, cluster of two fluorescently immunolabelled CK^+ MCF-7 tumour cells (prior to the application of FISH); C, single polysomic CK^+ MDA-MB-361 tumour cell, showing 4 hybridization signals for chromosome 17.

Amplification of the Her-2/*neu* gene was expressed as copy number/cell, rather than as copy number relative to chromosome 17 copy number because I expected to find increased chromosome 17 copy numbers. Amplification of the Her-2/*neu* gene was observed in MDA-MB-361, and BT-474 cell lines, using the definition of aneuploidy as total Her-2/*neu* copies/cell. There was marked heterogeneity for Her-2/*neu* copy number, and chromosome 17 copy number, in the two cell lines with Her-2/*neu* amplification. In MCF-7, the Her-2/*neu* gene appeared to be deleted (Her-2/*neu* gene copy number was less than the chromosome 17 copy number/cell) and there was less heterogeneity in Her-2/*neu* gene copy number/cell. The mean values and the S.D.s of the copy number distributions are summarised in Table 5.2.

Breast Cancer Cell Lines	Her-2/*neu*[a]	Chromosome 17[b]
MCF-7	2.2 ± 0.5[c]	3.8 ± 1.0
MDA-MB-361	11.0 ± 3.9	4.1 ± 1.6
BT-474	52.0 ± 11.3	6.0 ± 1.1

Table 5.2: Her-2/*neu* amplification and expression in breast cancer cell lines.

[a]Her-2/*neu* copy number/cell.

[b]Chromosome 17 copy number/cell.

[c]Data expressed as mean, ± S.D.

In 95% of the tumour cells, hybridization was successful for the α-satellite probe and in >90% hybridization was of sufficient quality to evaluate the signal for Her-2/*neu*. The immunophenotyping with A45-B/B3 did not alter the

hybridization results compared to unlabelled tumour cells (Table 5.3 [MCF-7 data presented to illustrate model]).

	Number of Fluorescent Signals			
	1	2	3	>3
Chromosome 17				
w/o A45-B/B3	4.0 ± 4.2	6.0 ± 6.0	41.0 ± 9.1	50.3 ± 13.6
With A45-B/B3	4.5 ± 0.7	6.0 ± 5.6	38.0 ± 9.6	49.7 ± 10.5
Her-2/*neu*				
w/o A45-B/B3	29.3 ± 9.3	39.7 ± 3.8	29.3 ± 11.6	1.7 ± 1.5
With A45-B/B3	29.7 ± 5.5	48.0 ± 10.1	20.3 ± 13.8	2.0 ± 1.7

Table 5.3: FISH results for chromosome 17 and Her-2/*neu* In unlabelled MCF-7 cells or MCF-7 cells labelled with A45-B/B3.
A total of 500 cells were scored for unlabelled and labelled cells each.
Data expressed as mean, ± S.D.

Gains of chromosome 17 were seen in more than 50% of A45-B/B3-labelled MCF-7 tumour cells. This corresponded to the rate of gain of chromosome 17 in the MCF-7 line maintained in the laboratory, in which, more than 50% of tumour cells showed polysomy of chromosome 17. Amplification of Her-2/*neu*, was seen in a minority of MCF-7 cells by FISH in A45-B/B3-labelled tumour cells. This was reflected in control cells of MCF-7 without anti-CK staining, where Her-2/*neu* amplification was also seen in only a minority.

Analysis of metaphase preparations from the two BrCa cell lines with Her-2/*neu* amplification indicated that amplified genes were always integrated in chromosomes (Figures 5.4 and 5.5), compared to metaphase chromosomes prepared from normal BM with 2 normal signals (Figure 5.6).

Identification of chromosomes with Her-2/*neu* copies was based on DAPI-stained metaphase preparations and dual-colour FISH with Her-2/*neu* probe (green) (images labelled B) and chromosome 17 whole chromosome paint (red) (images labelled A), with corresponding G-Banding chromosome analyses.

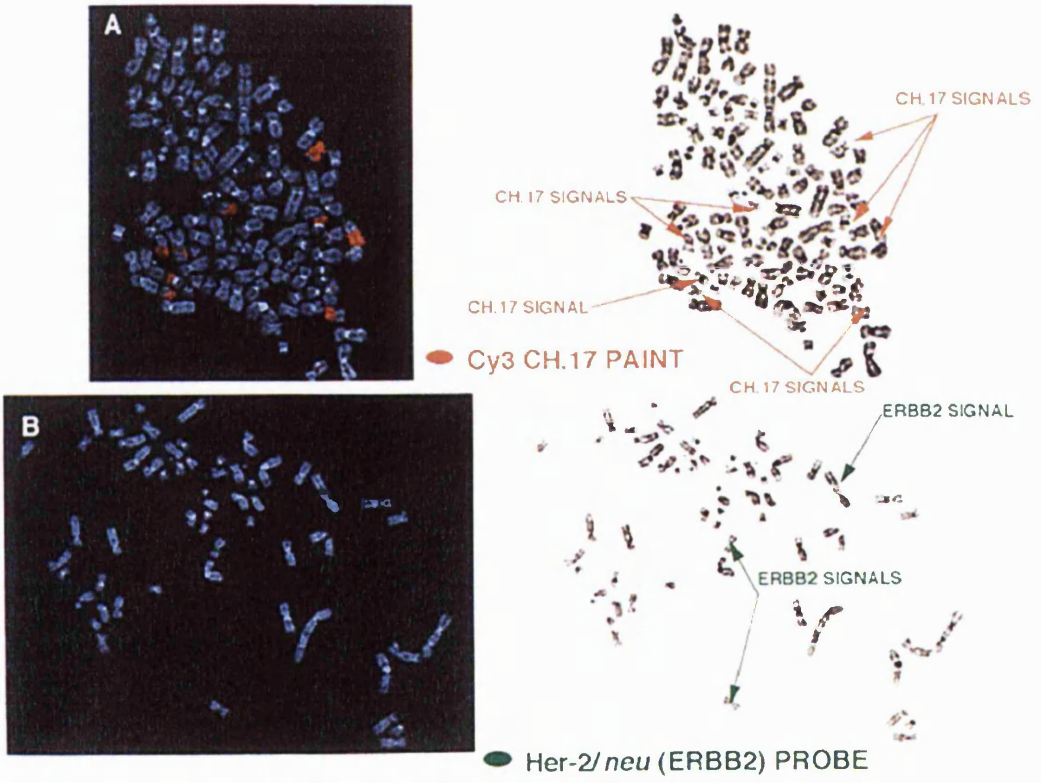


Figure 5.4: Metaphase chromosomes of MDA-MB-361 breast cancer cell line with Her-2/*neu* amplification and chromosome 17 alterations.

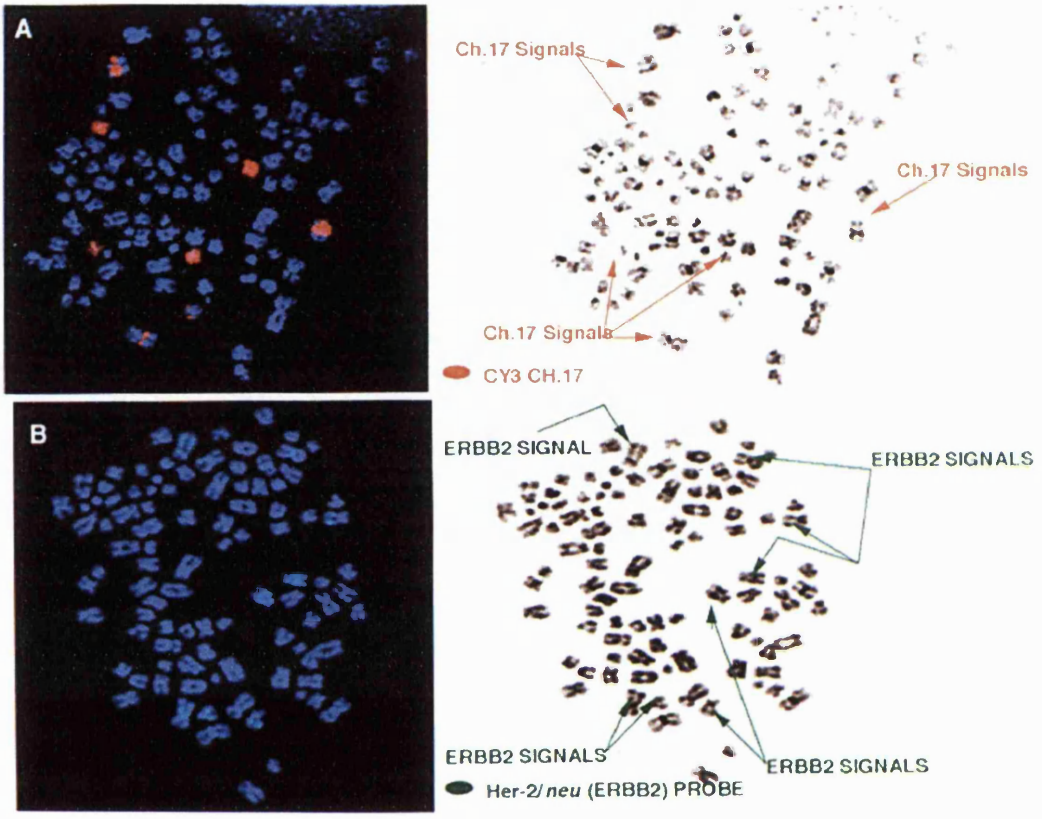


Figure 5.5: Metaphase chromosomes of BT-474 breast cancer cell line with Her-2/*neu* amplification and chromosome 17 alterations.

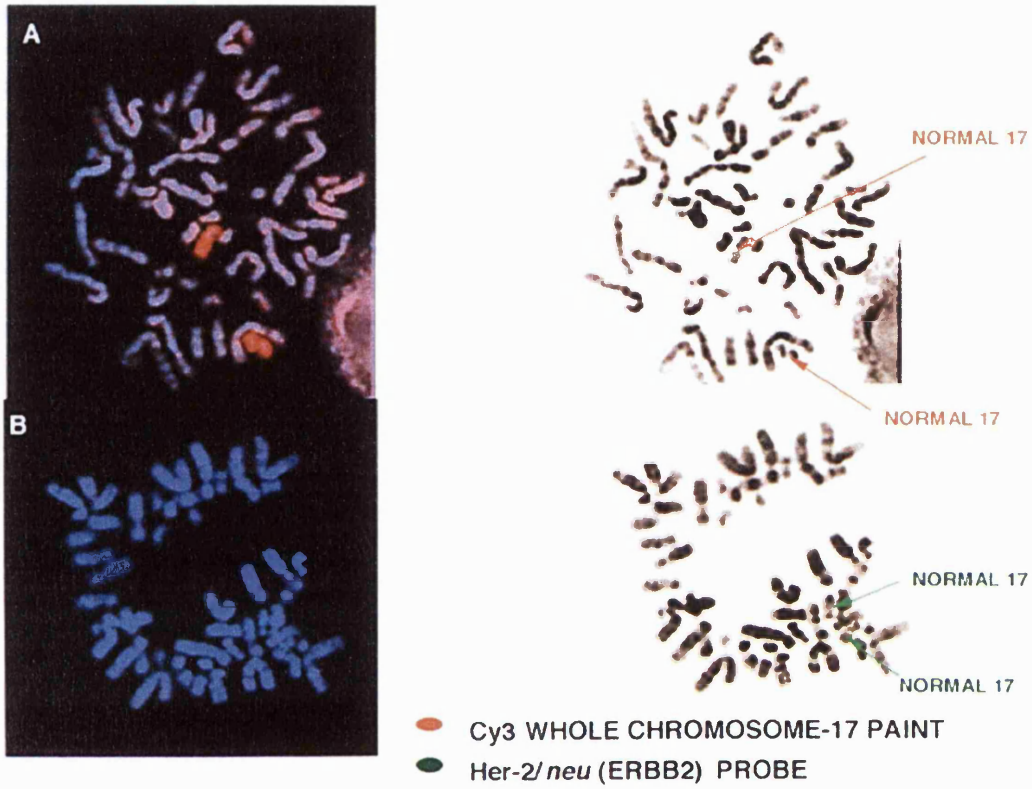


Figure 5.6: Metaphase chromosomes of a normal BM, illustrating 2 normal Her-2/*neu* signals located on chromosome 17.

The chromosomal distribution of Her-2/*neu* signals was different among the two cell lines with most of the clusters occurring on chromosomes other than 17 (Table 5.4).

Cell Line	No. of Her-2/*neu* Clusters		No. of Single Her-2/*neu* Signals	
	On Chr 17	On Other Chr	On Chr 17	On Other Chr
BT-474	2	7 or 8	3 or 4	2 or 3
MDA-MB-361	0	1	4	0

Table 5.4: Distribution of Her-2/*neu* signals on chromosome 17 and on other chromosomes in the two breast cancer cell lines.
Identification of chromosomes with Her-2/*neu* copies was based on DAPI-stained metaphase preparations and simultaneous dual-colour FISH with Her-2/*neu* and chromosome 17 whole chromosome probes.
Chr, chromosome(s).

The number of Her-2/*neu* copies in a typical cluster varied from 6 to 20 and the number of clusters per cell line varied from 1 to 10. The complex karyotype of these cells made the detailed determination of the sites of amplification difficult. For example, the MDA-MB-361 cell line contained two long marker chromosomes with three Her-2/*neu* clusters. Although these chromosomes reacted with a chromosome 17 centromeric probe, they did not stain at all with the respective whole chromosome paint. In addition to Her-2/*neu* clusters, the BT-474 cell line also contained single integrated Her-2/*neu* copies in chromosomes other than 17.

The results of metaphase FISH analysis of the two prostate Ca micrometastatic cell lines, revealed consistent chromosomal imbalances.

Chromosomal losses were frequently encountered on chromosome arm 8p (del(8)(p) [deletion of short arm of chromosome 8]), where the region of loss was assigned to 8p11 (Figure 5.7), and chromosome 7q (del(7)(q22) [long arm of chromosome 7]), assigned to region 7q22 (Figure 5.8), and may be indicative of the presence of tumour suppressor genes. Chromosomal gain occurring in the majority of metaphase preparations, involved chromosomal arm 7q (resulting in a trisomic population) (Figure 5.8).

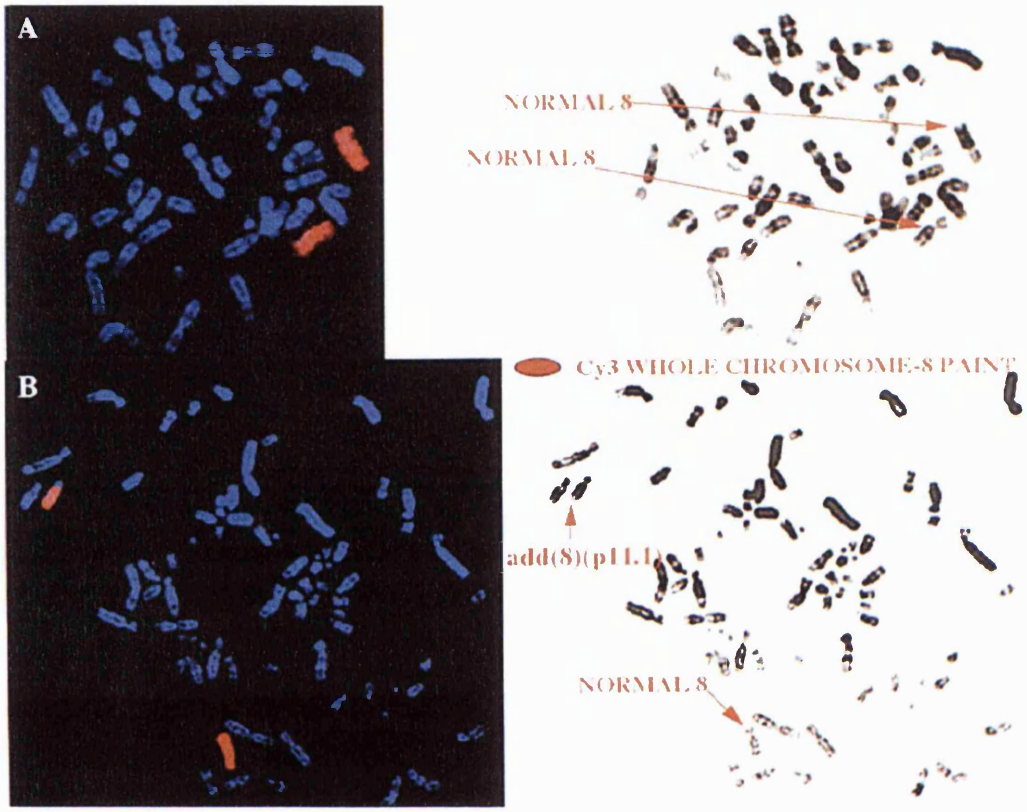


Figure 5.7: Metaphase FISH analysis of a micrometastatic prostate cancer cell line (PC-R1), illustrating chromosome 8p loss (B), compared to metaphase chromosomes from normal BM (A).

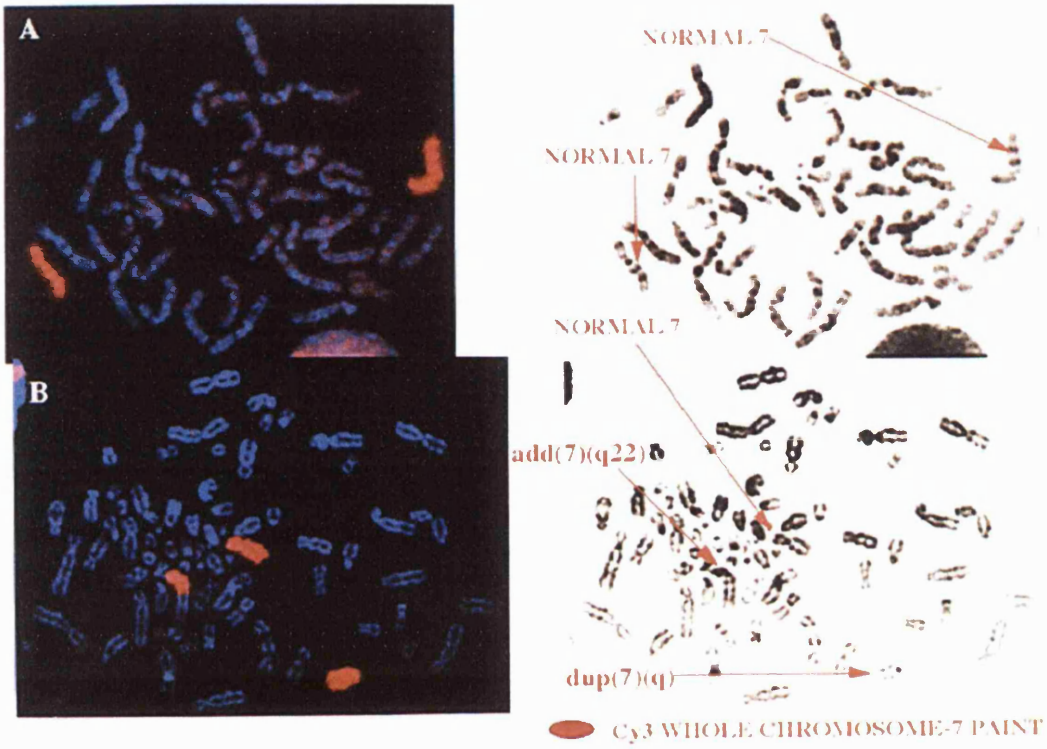


Figure 5.8: Metaphase FISH analysis of a micrometastatic prostate cancer cell line (PC-H1), illustrating chromosome 7 imbalance (B), compared to metaphase chromosomes from normal BM (A).

5.4 Discussion

Epithelial cells in the BM of cancer patients detected by Abs against CKs have prognostic significance in a range of such tumours and they may be a marker of tumour cell dissemination capacity (Cote *et al*, 1991; Lindemann *et al*, 1992; Schlimok *et al*, 1992; Jauch *et al*, 1996; Pantel *et al*, 1996). Although their ability to form metastases has been questioned (Mansi *et al*, 1989), characterisation of their phenotype and genetic profile may help to define their role in this phenomenon.

Biological analysis of disseminated tumour cells has been hampered by their low frequency. A method enabling characterisation of rare identified tumour cells would clearly improve research in this field. The basis of the presented sequential immunophenotyping and genotyping by FISH is a combination of directly alkaline phosphatase-labelled F_{ab} fragment of an anti-CK Ab, or the Cy3 immunofluorescent detection of an unconjugated IgG1 form of the same Ab, in combination with FISH using specific probes. The antigen signal is retained and there is no negative effect of the alkaline phosphatase complexes or Cy3-conjugated secondary Abs, on the FISH results. The main advantage of the described method is that genotyping can be successfully applied after immunophenotyping, thus ensuring analysis of the same individual disseminated tumour cell.

Primary chemotherapy alone, or combined with radiotherapy, is increasingly being applied to reduce the need for extensive surgery in the treatment of patients with BrCa (Bonadonna *et al*, 1990; Rilke *et al*, 1996). Breast carcinomas that overexpress Her-2/*neu* are more aggressive (De Potter *et al*, 1995), less responsive to CMF-containing adjuvant therapy regimens (Gusterson *et al*, 1992), may benefit from high-dose adjuvant chemotherapy

(Muss *et al*, 1994) and when steroid receptor positive (Pietras *et al*, 1995), may be unresponsive to tamoxifen (Hynes and Stern, 1994) or to tamoxifen with radiotherapy (Sjogren *et al*, 1998). Moreover, Press and collaborators (1997), have demonstrated that Her-2/*neu* is an independent predictor of poor clinical outcome and, more recently, the Toronto Breast Cancer Study Group has found that Her-2/*neu* amplification is an independent prognostic factor for risk of recurrence in axillary node negative BrCa (Andrulis *et al*, 1998). Recent clinical studies, in keeping with preclinical evidence (Harwerth *et al*, 1993; Baselga *et al*, 1996), have shown some benefits in stage IV Her-2/*neu* overexpressing breast cancers treated by recombinant humanised monoclonal (rhmAb) Her-2/*neu* (Baselga *et al*, 1996). In this approach, reliable information on Her-2/*neu* overexpression, in addition to other biological markers, will be important to obtain correlations with response rate.

The major advantage of FISH, as compared with other methods for quantitating Her-2/*neu* amplification, is that it allows measurement both of the average level of amplification in a tumour as well as the actual number and distribution of Her-2/*neu* genes in morphologically defined single cells, and allowing small abnormal populations to be identified. The method is rapid, nonradioactive, and requires very little tumour material. In BrCa cell lines with medium level (MDA-MB-361) or high level (BT-474) Her-2/*neu* amplification, FISH revealed an extensive cell to cell variation in gene copy number. However, such intratumour heterogeneity has often not been detected in immunohistochemical analyses of Her-2/*neu* overexpression (van de Vijver, 1988; Slamon *et al*, 1989; Kallioniemi *et al*, 1991), probably because the distinction between different degrees of overexpression is not possible. Previous reports based on Southern analysis have indicated that the level of

gene amplification is a better prognostic predictor in BrCa than the mere presence or absence of gene amplification (Slamon *et al*, 1989; Paterson *et al*, 1991), however, this method may miss low level abnormal populations.

FISH is a potential alternative to immunohistochemistry for assessment of Her-2/*neu* activation. The quantitative nature of immunohistochemical analysis is limited by the physicochemical properties of the immunoenzyme reaction and the subjective assessment of staining intensity. The detection of overexpression is also dependent on variation in the degree of tissue fixation and the specificity of the Abs used (Slamon *et al*, 1989; Kallioniemi *et al*, 1991). Western blotting, a potentially more quantitative assay of overexpression, is often confounded by the dilution effect caused by stromal proteins and has been shown to be an inaccurate method for Her-2/*neu* analysis (Slamon *et al*, 1989).

A unique property of FISH is that it allows evaluation of the pattern of amplification on the basis of spatial distribution of amplified gene copies in interphase nuclei and in metaphase chromosomes. Metaphase analysis of the BrCa cell lines indicated that amplified genes were always intrachromosomal but distributed between many different chromosomes. The appearance of amplified Her-2/*neu* copies as clusters in the interphase nuclei of the BrCa cell lines also suggested intrachromosomal location. Her-2/*neu* copies carried in extrachromosomal structures such as double minutes (DMs) and episomes are less likely to be clustered in interphase cells possibly because of their size. Cytogenetic studies of primary breast cancers have revealed a high prevalence of homogeneously staining regions but no DMs, suggesting the predominance of intrachromosomal gene amplification (Saint-Ruf *et al*, 1990).

The pattern of amplification as defined by the distribution of Her-2/*neu* signals in interphase nuclei may reflect the mechanism of gene amplification. According to the episome model of oncogene amplification, extrachromosomal amplification is an early step, whereas the integration of amplified genes into chromosomes occurs later during tumour progression (Wahl, 1989). This model is supported by the frequent cytogenetic findings of DMs in short-term cultures of various primary tumours as well as by the presence of episomal amplification of N-*myc* in neuroblastomas *in vivo* (Wahl, 1989; VanDevanter *et al*, 1990). As no evidence of extrachromosomal Her-2/*neu* genes was found in the BrCa cell lines, it is likely that extrachromosomal amplification, if it existed, must have occurred in the preclinical phase of tumour growth. Gene amplification in cell lines selected by increasing drug concentrations often first appears on a single chromosome carrying the original gene locus (Trask and Hamlin, 1989). There is no evidence of double-minute chromosomes as precursors of intrachromosomal structures. Initially, multiple copies of very large sequences may be amplified on a single chromosome, whereas later more condensed gene clusters are formed that may also translocate to other chromosomes (Trask and Hamlin, 1989; Smith *et al*, 1990). The end result is a pattern similar to that observed here in the highly amplified BrCa cell lines. The BrCa cell lines studied here have had considerable opportunity to evolve since the initial amplification events. Analysis of the Her-2/*neu* amplification pattern by FISH in PB- and BM-derived metastatic cells could further clarify the mechanism of Her-2/*neu* amplification in BrCa.

The finding of aneuploidy involving chromosome 17 in these BrCa cells may be significant for future research insofar as several cancer-related genes are known to reside on this chromosome. The tumour suppressor gene *nm23*,

BRCA1, the tumour suppressor gene involved in hereditary breast/ovarian cancer, has been mapped to 17q21 (Hall *et al*, 1990), and *TP53*, another tumour suppressor, is located at 17p13 (Isobe *et al*, 1986; McBride *et al*, 1986). As for chromosome 17 polysomy, it has been demonstrated that polysomy identified by FISH is related to DNA aneuploidy which in turn, in BrCa, is related to a poor prognosis (Gnant *et al*, 1993). Polysomy was expressed as chromosome 17 copy number per cell in the BrCa cell lines that were aneusomic for this chromosome. Because chromosome 17 copy number generally reflects the total DNA content (ploidy) of Her-2/*neu* amplified cells, Her-2/*neu*:chromosome 17 ratio is a measure of Her-2/*neu* "amplification" as traditionally characterised by Southern blotting analysis. Also, it has been demonstrated that much of the Her-2/*neu* amplification in BT-474 and MDA-MB-361 was not located on chromosome 17, thus, reducing the utility of the Her-2/*neu*:chromosome 17 ratio. Previous studies have revealed a significant correlation between loss of heterozygosity (LOH) in chromosome 17p and regional lymph node metastasis (Takita *et al*, 1992), and one of the important mechanisms of LOH is loss of chromosomal component of two homologues. Interphase FISH analyses of the BrCa cell lines are apparently discordant with the results of LOH analyses. Therefore, the uniparental multiplication of chromosome 17 after the event of loss in 17p of one homologue might result in polysomic changes of this chromosome. More extensive studies may clarify the potentially significant correlation between chromosome 17 aneusomy detectable by FISH and certain clinicopathologic phenotypes of BrCa.

By using metaphase FISH, consistent chromosome 7 and 8 tumour-specific cytogenetic aberrations were established in the micrometastatic cancer cell lines that had been established from BM micrometastases by

immortalisation with a cDNA coding for the SV40 large T antigen (Pantel *et al*, 1995; Putz *et al*, 1999). The cell lines were derived from two patients with prostate Ca (PC-R1 and PC-H1); neither patient had clinical signs of overt metastases. The current approach is the first attempt to utilise unique-sequence probes to analyse metaphase chromosomes *in situ* at the genetic level in micrometastatic cell lines. Although previous studies utilising centromere-specific chromosome probes successfully examined numerical alterations of specific chromosomes in prostatic interphase nuclei (Brothman *et al*, 1992; Macoska *et al*, 1993), they were unable to examine the presence or absence of region-specific amplifications or deletions involving portions, but not all, of the target chromosomes. This ability is important in order to investigate potential tumour suppressor gene sequence deletions or oncogene sequence amplifications, which often occur in the absence of changes in whole chromosome number.

Loss of 8p sequences may well be the most common genetic alteration in prostate Ca. This loss also appears to be an early event in prostatic tumourigenesis, because LOH at 8p was described in prostatic intraepithelial neoplasia, the putative precursor lesion of prostate Ca (Emmert-Buck *et al*, 1995; Saric *et al*, 1999). These, and previous data, strongly suggest the presence of a prostate tumour suppressor gene in this region of chromosome 8p. Several candidate genes have been studied at 8p, such as the *NKX3.1* gene located at 8p12-p21 (He *et al*, 1997; Brothman *et al*, 1999). This homeobox gene was highly expressed in an androgen-dependent LNCaP cell line after androgen stimulation, but undetectable in two androgen-independent prostate cell lines. Another potential candidate gene at 8p22 is the recently identified *FEZ1* gene, which codes for a leucine zipper protein the expression of

which is altered in many tumours, including prostate, oesophageal, and BrCa (Ishii *et al*, 1999). Concomitantly, evidence for increased copy number of sequences on the long arm of chromosome 8 have been observed in a subset of prostate tumours with loss of 8p sequences (Bova *et al*, 1993), suggesting the presence of an activated oncogene on this chromosome as well. The finding of a higher frequency of 8p loss compared with gain of 8q sequences suggests that the former alteration may precede the latter and that this latter event may be more important in tumour progression. In any case, the identification of the chromosome 8p genes involved in prostate carcinogenesis should provide important insight into this process.

Gain of chromosome 7, especially gain of 7q, may be associated with advanced disease. Gain of chromosome 7 is correlated with high local tumour stage and grade (Takahashi *et al*, 1994; Alers *et al*, 1997), whereas aneusomy of chromosome 7 is a potential marker for poor prognosis (Alcaraz *et al*, 1994; Takahashi *et al*, 1995). Gain of chromosomal regions 7p12-p21 and 7q11.3-q33 has been detected by comparative genomic hybridization (CGH) analysis in (lymph node) metastases (Cher *et al*, 1996). The *EGFR* proto-oncogene, a gene possibly involved in prostate Ca (Schwartz *et al*, 1999) and other human neoplasms is located on chromosome 7. Furthermore, the *MET* proto-oncogene, which maps to 7q31.1, is expressed in the majority of both primary tumours and metastases (Pisters *et al*, 1995). The gain of chromosome 7 in the cell lines PC-R1 and PC-H1, suggest that this chromosome may play an important role in progression and especially metastasis, and that this may be associated with aggressive tumour behaviour and a poor prognosis in patients with prostate Ca.

Chapter 6 Tumour Cell Enrichment and Interphase Cytogenetic Analyses

6.1 Introduction

Evidence suggests that primary cancers shed viable clonogenic cells into the circulation at an early stage (Fidler, 1973; Liotta *et al*, 1974); however, the natural history of these cells, their ability to establish metastases, and their impact on prognosis are unclear. For instance, circulating tumour cells have been detected by PCR in cancer patients with a good prognosis who are unlikely to develop metastatic disease (for example, in patients with no measurable serum PSA on hormonal therapy) (Johnson *et al*, 1995; Ghossein and Rosai, 1996). In addition, ICC has detected cancer cells in the BM in a proportion of patients with clinically localised disease (Pantel *et al*, 1993a; Pantel and Riethmüller, 1996). If tumour cell shedding is, in fact, an early event in tumourigenesis, it may even be possible to detect cancer cells in the bloodstream before the primary tumour is large enough to be detected by standard screening examinations.

In primary BrCa, the number of axillary nodes with tumour cell infiltration provides, at present, the strongest predictor of clinical outcome (Fisher *et al*, 1978). However, a substantial number of patients with negative lymph nodes at the time of surgery develop metastatic disease (Fisher, 1977) and additional measures to identify women at risk are needed. Thus, the detection of circulating tumour cells in the PB and/or the BM might provide important information for the prediction of disease progression. However, the value and usefulness of studying such associations, as a possible aid in therapeutic

decision making, depends on the availability of practical and specific detection methods.

Dissemination of tumour cells is usually occult and leads to recurrent, metastatic, disease in cancer patients who have undergone resection of their primary tumour. Including the detection of disseminated epithelial tumour cells in the staging of cancer patients would aid in determining the prognosis, in identifying patients in need of potentially curative treatment and help in monitoring therapeutic endeavours. Furthermore, methods for detection of tumour cells will be useful to identify contaminating tumour cells in autologous stem cell grafts prior to reinfusion.

Approximately 40 ml of PB, 10 ml BM aspirate and just 0.1% of the cells from a mobilised PBSC harvest, are required for estimation of tumour cell load. A tumour cell detection method should be sensitive enough to identify a single tumour cell in such samples, i.e. it should be possible to detect one tumour cell in about 2.5×10^7 nucleated PB cells, 1×10^8 BM cells and 2.5×10^7 PBSCs.

Several methods have been used for detection of epithelial tumour cells in mesenchymal cell samples such as blood, BM and lymph nodes, but most of them are limited by small analysis capacity. For microscopic evaluation cell samples of about 0.5×10^6 cells are cytocentrifuged onto slides and tumour cells are detected either by ICC or immunofluorescent staining (Redding *et al*, 1983; Schlimok *et al*, 1987; Salvadori *et al*, 1990; Pantel *et al*, 1993b). Screening of 2.5×10^7 nucleated blood cells would require preparation and analysis of numerous cytocentrifuge slides. The use of flow cytometry with sensitivities of 10^{-4} to 10^{-7} has been reported (Leslie *et al*, 1990; Gross *et al*, 1995; Simpson *et al*, 1995). However, to reach the required sensitivity, several hours were needed to analyse one sample, which is not acceptable when large

numbers of samples are to be analysed. Using RT-PCR for identification of tumour cell related genes (Datta *et al*, 1994; Burchill *et al*, 1995; Neumaier *et al*, 1995), it is possible to analyse RNA from more than 2.5 x 10^8 blood cells at once, but it appears to be difficult to establish standardised procedures which would provide high sensitivity and zero false-positive results (Datta *et al*, 1994).

To improve the sensitivity and specificity of epithelial tumour cell detection and to further characterise the tumour cells, larger quantities of MNCs should be examined. An alternative approach is to enrich tumour cells present from PB and BM by positive or negative selection before detection steps or further characterisation, as illustrated schematically in Figure 6.1. This enrichment is in addition to the standard density gradient procedure used to isolate the mononuclear cell fraction (Chaiwun *et al*, 1992; Pantel *et al*, 1994). Several selective enrichment methods are currently being tested. By the use of various density gradients and antibody-coupled magnetic particles, tumour cells (from cell lines) have been enriched by several orders of magnitude in model systems (Naume *et al*, 1997, 1998; Martin *et al*, 1998; Racila *et al*, 1998). A method has been developed for the positive enrichment of cancer cells mixed with PB using a MoAb against CK8/18 and paramagnetic nanoparticles (MACS) (Griwatz *et al*, 1995). Because of the intracellular location of CKs, this method requires fixation/permeabilisation of the cells before separation and excludes the possibility of functional studies of viable tumour cells after separation. Ross *et al* (1998a, 1999) have reported on the ability of a prototype avidin-biotin based tumour-enrichment column (TEC) to select BrCa cells from BM and PBSC specimens, following incubation of the breast-reactive TEC PAN-05 anti-epithelial cell biotinylated Ab. In cell-seeding experiments, the TEC assay was able to detect one seeded BrCa cell in 10^8 haematopoietic cells (Ross *et al*,

1998a). Log enrichment values ranged from 2.27 to 2.80 in seeded BM specimens and from 2.32 to 2.50 in seeded PBSC specimens (Ross *et al*, 1999). In patient specimens from BrCa patients with Stage II-IV disease, treated with a variety of clinical protocols, log enrichment values ranged from ≥0.9 to ≥1.8 (Ross *et al*, 1999). Tumour cells can be selected using beads coated with Abs against tumour-associated antigens, or alternatively normal blood cells in the preparation can be depleted by the use of beads coated with Abs against haematopoietic cell antigens. These selection strategies have the additional advantage that the tumour cells remain viable and can be used for additional studies, including the propagation of malignant cells *in vitro* (Rye *et al*, 1997). However, extensive evaluation by "ring experiments" is required to determine whether these enrichment techniques are superior to "standard" methods.

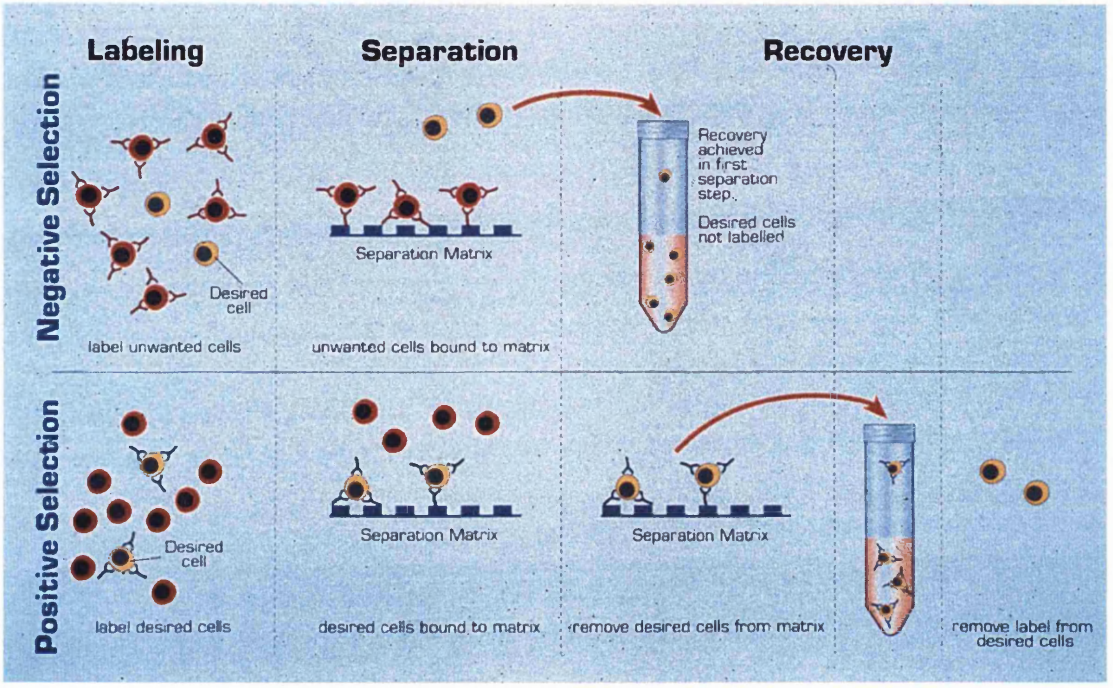


Figure 6.1: Schematic drawing comparing the principles of positive selection and negative depletion for the enrichment of epithelial tumour cells from mesenchymal cell samples.

As stated previously, an alternative approach for tumour cell enrichment in MNC samples is to use negative selection. Initial reports indicated that tumour cell enrichment through the removal of the majority of $CD45^+$ haematopoietic cells can give a product suitable for further analysis (Kremens *et al*, 1994). We have previously reported on the use of the StemSep™ procedure for enrichment of circulating epithelial tumour cells from samples of PB, BM and pleural effusions (Theocharous *et al*, 1999). Haematopoietic cells are cross-linked to magnetic particles using tetrameric Ab complexes comprised of two murine IgG_1 MoAbs, held in a tetrameric array by two rat anti-mouse MoAbs. One murine Ab recognises the cell surface antigen and the other recognises the dextran on the magnetic particle (Figure 6.2). A cocktail of tetrameric Ab complexes target CD2, CD14, CD16, CD19, CD36, CD38, CD41, CD45, CD66b and glycophorin-A on genetically stable, normal haematopoietic cell surfaces. The cell suspension is passed through a column placed in a magnet, and the labelled cells are retained while the epithelial tumour cells are eluted and collected in the flowthrough (Figure 6.3). The enriched, viable cells have not been labelled with Abs and are suitable for further analysis, including detection, utilising a range of assays, *ex vivo* culture and further functional as well as phenotypic characterisation. In cell-seeding experiments, the StemSep™ assay was able to detect one seeded tumour cell in 10^8 haematopoietic cells (Dr. Terry E. Thomas, personal communication).

It has been suggested that the number of micrometastatic cells in the BM reflects the peripheral tumour burden in each patient (Cote *et al*, 1991). However, the biological properties of the malignant cells, rather than their number, might determine the clinical outcome. Therefore, it is of interest to characterise such cells for biological markers associated with an aggressive

tumour phenotype. In most cases studied, including the data presented in Chapter 4, relatively few tumour cells were found in the clinical samples (Pantel *et al*, 1994; Naume *et al*, 1997), and the number of tumour cells available for further analysis was limited, requiring a sensitive method for their enrichment and characterisation.

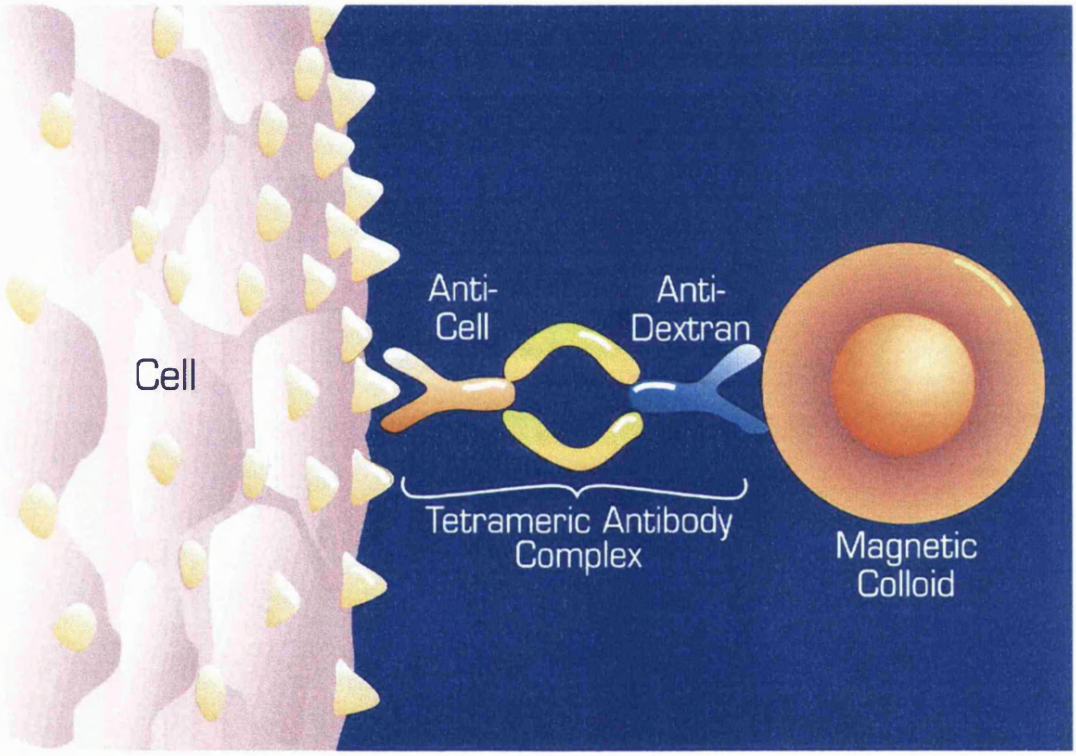


Figure 6.2: Schematic drawing of StemSep™ magnetic cell labelling.
(Courtesy of StemCell Technologies).

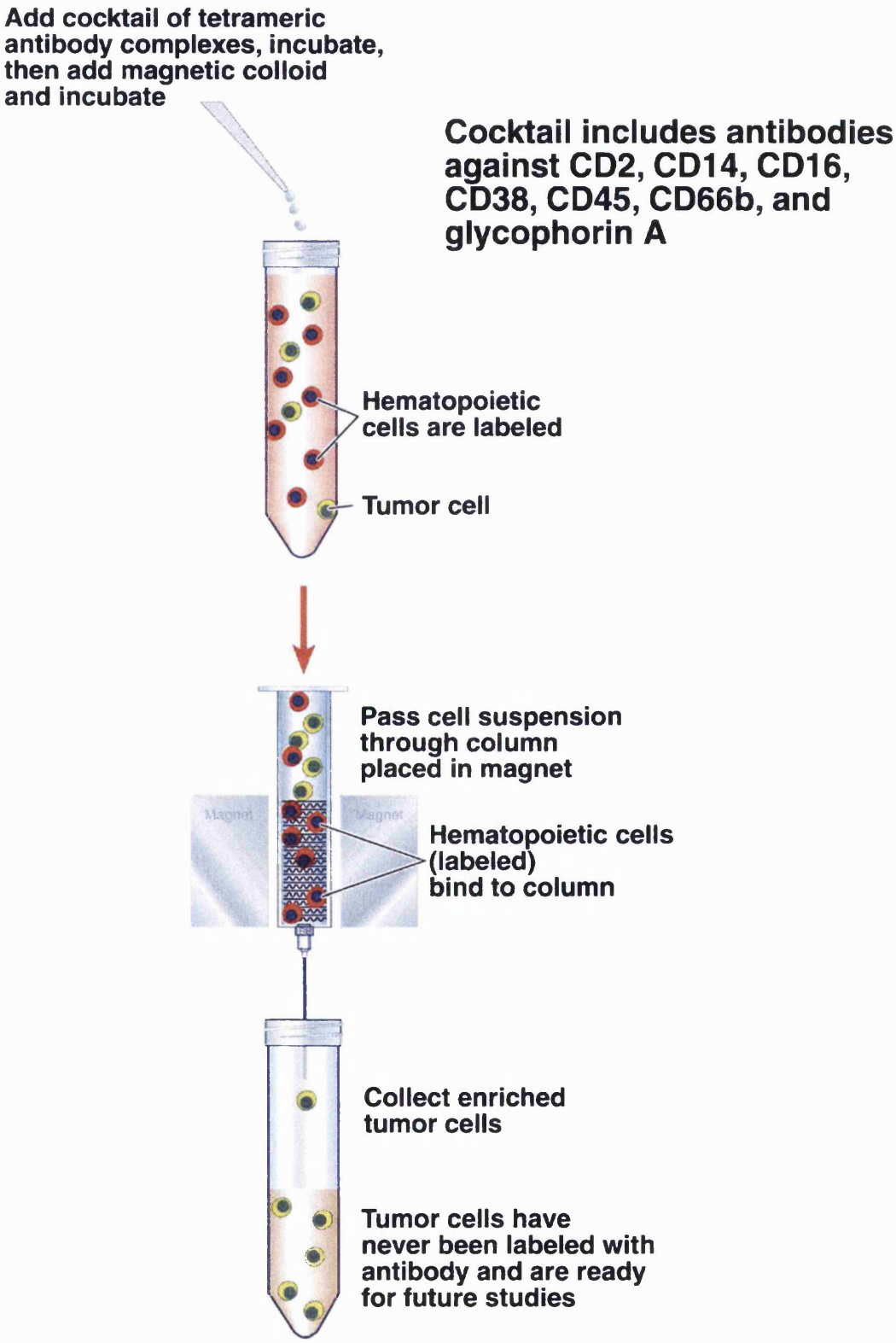


Figure 6.3: StemSep™ procedure for enriching circulating tumour cells.

(Courtesy of StemCell Technologies).

The purpose of this part of the study was to compare positive and negative separation techniques for the specific, rapid and efficient enrichment of viable disseminated cancer cells, from haematopoietic cell samples such as PB and BM, from patients with epithelial malignancies (BrCa and prostate Ca). These enrichment techniques were compared with the tumour cell detection efficiency of the standard Cell-Tak® Cell and Tissue Adhesive immunoassay.

Clinical studies including large numbers of patients with BrCa have shown that the Her-2/*neu* proto-oncogene is amplified and overexpressed in 20-30% of patients with invasive BrCa. Furthermore, it is associated with distant metastases in particular patient subgroups (Slamon *et al*, 1989). Notably, Her-2/*neu* amplification and overexpression, has been associated with a poor prognosis (Slamon *et al*, 1989; Borg *et al*, 1991; Press *et al*, 1997). As illustrated in Chapter 5, interphase-FISH can be used for detection of amplifications and other genetic alterations in tumour cells. The sensitivity of the method is high, and clonal variation among the cancer cells can be revealed. If high quality molecular marker probes are used, only a small number of cells are needed for analysis. A sensitive technique was established for the detection and amplification analysis of haematogeneously spreading Her-2/*neu*-positive epithelium-derived cells from the PB and BM of BrCa patients by combining enrichment and FISH.

Radical prostatectomy (RP) is an effective treatment for prostate Ca, with excellent survival rates if the cancer is localised (Epstein *et al*, 1993). However, approximately 30% of patients with clinically localised disease at the time of surgery will experience tumour recurrence. These patients presumably have occult micrometastatic dissemination that have seeded into the BM, which is undetectable by imaging modalities such as pelvic computed tomographic (CT)

scan, transrectal ultrasound, magnetic resonance imaging (MRI), and bone scan. Moreover, tumour cells appear to be released into the blood during RP, posing an additional risk for subsequent metastatic relapse (Eschwège *et al*, 1995). Thus, it is important to determine whether these epithelial tumour cells possess genomic or functional features consistent with tumour derivation and metastatic capability. The combined immunophenotype/genotype approach following tumour cell enrichment as described above, was used to detect and quantify epithelial cells in BM aspirates from patients with prostate Ca taken at the time of RP to assess the molecular cytogenetic features of the detected cells. Cells labelled immunofluorescently with Abs against CK heterodimers 8-18 and 8-19 were quantified in BM aspirates from 20 patients with prostate Ca. Aneusomy of chromosome 7 occurs in primary prostate tumour cells (Baretton *et al*, 1994; Jones *et al*, 1994; Visakorpi *et al*, 1994; Zitzelsberger *et al*, 1994) and is associated with aggressive disease and metastases (Qian *et al*, 1995). The relationship between the presence and frequency of epithelial cells in BM aspirates, clinical stage, and tumour grade is discussed.

6.2 Materials and Methods

6.2.1 Patients

Samples of BM aspirated from the posterior iliac crest and/or peripheral venous blood, obtained from 26 of the original 94 BrCa patients admitted to the Department of Clinical Oncology, were available for the enhanced detection of tumour cells by enrichment ± Her-2/*neu* characterisation by FISH. In the preliminary phase of the study, multiple samples of BM (5 aspirates) and/or PB (13 samples) were further analysed from 11 random, unselected patients with primary BrCa (3 adjuvant therapy patients during treatment with Dox./CMF or

with classical CMF and 8 patients with high-risk primary tumours who were undergoing primary medical chemotherapy [AC] before definitive surgery). This evaluated carcinoma-cell enrichment, prior to immuno-staining, and compared primary ICC data with ICC after positive selection or negative depletion of carcinoma cells. In addition, BM (13 aspirates [10 at the time of initial diagnosis and 3 at the time of definitive surgery]) and/or PB (18 samples [13 at the time of pre #1 cytotoxic chemotherapy, 1 pre #4, 2 pre #8, and 2 at the time of clinical relapse]) from 15 patients with primary non-metastatic BrCa (6 adjuvant therapy patients and 9 primary medical therapy patients), and 5 patients being treated for advanced metastatic disease, were further analysed to assess the isolation and characterisation of epithelium-derived Her-2/*neu* oncogene-positive carcinoma cells from mesenchymal tissue. As negative controls, 20 samples from normal volunteer donors without evidence of an epithelial malignancy were examined in parallel.

Bone marrow aspirates taken from the posterior iliac crest (uni- or bilaterally), were obtained from 20 prostate Ca patients at the time of RP, following admission to the University Department of Urology, at the Royal Free Hospital in London, after providing written informed consent. The investigations performed in this part of the study were approved by the institutional review board. The study population consisted of: (a) 10 patients with clinically organ-confined prostate Ca; (b) 7 patients with capsular penetration; and (c) 3 patients with advanced stage disease (lymph node metastases [pT4N+]), either newly diagnosed or with treatment failure. The control population consisted of patients with benign prostatic hypertrophy (BPH), patients without prostatic disease undergoing surgery and young healthy volunteers. Patients were categorised on the basis of disease extent using the tumour-node-metastasis

(TNM) system (Schroder *et al*, 1992): organ-confined tumour (pT2), pT2a (<1.5 cm or ≤ half of one lobe), pT2b (>1.5 cm or ≥half of one lobe), and pT2c (tumour involves both lobes); and local tumour extension beyond the prostate (pT3), pT3a (unilateral capsular tumour extension), pT3b (bilateral capsular tumour extension), and pT3c (tumour involves seminal vesicle[s]). Grading was evaluated using the Gleason grading system (Gleason, 1992).

6.2.2 Statistical Analysis

Differences in positivity rates were analysed by Fisher's exact test for contingency tables (for the comparison of three rates of positivity, the Chi-square test was used). The Mann-Whitney U test was used to assess the differences in the medians, differences between medians of independent samples with continuous variables were calculated from the *t* test [95% confidence interval (CI)]. Data from three groups were compared by the nonparametric Kruskal-Wallis test. A p value of less than 0.05 was considered to indicate a statistically significant difference. Two-tailed p values were used to assess the significance level. For statistical analysis, GraphPad Prism® software (San Diego, CA, USA) (version 2.01) was used.

6.3 Results

6.3.1 Specificity of the TEC Capture Ab for Positive Selection

The capture Ab used in the TEC assay is a pan-epithelial glycoprotein (PAN-05, $NeoR_x$, Seattle, WA) that reacts with a variety of epithelial cell-derived tumours. Immunostaining of 60 paraffin-embedded tumours from patients with breast, prostate, ovarian, colon and lung tumours showed specific localisation of positive immunostaining to the membranes of tumour cells. Interstitial tissues, inflammatory cells, and non-tumour cells remained unstained. Similar immunostaining of paraffin-embedded tissue samples from a variety of patients with non-epithelial cell-derived malignancies (non-Hodgkin's lymphoma, leiomyosarcoma) showed no reactivity with the PAN-05 Ab. Immunocytochemical staining of fresh normal donor BM and PBSC specimens showed no non-specific cross reactivity with the Ab (Dr. Amy A. Ross, personal communication, unpublished observations). For these reasons, PAN-05 was chosen as a suitable tumour-enrichment Ab.

6.3.2 Absolute Cell Numbers Processed

Previously reported cell-seeding experiments using the TEC with the CAMA BrCa cell line at concentrations ranging from $1:10^3$ to $1:10^8$ showed the percentage of tumour cell recovery ranged from a low of 15% to a high of 67%, with the mean percentage of tumour cell recovery at 36.2% (Ross *et al*, 1998b). Percentage of tumour cell recovery is a function of the total number of tumour cells seeded into the specimen. No significant differences were observed in percentage of tumour cell recovery in seeded normal donor BM, or seeded normal donor PBSC specimens (Dr. Amy A. Ross, personal communication, unpublished observations).

The mean number of haematopoietic cells per enrichment for 38 samples, BM (n = 25) and PB (n = 13), is presented below. Fresh BM, prior to processing, ranged from 5-15 ml in volume, with a median of 10 ml per sample. The mean number of cells pre-enrichment (post-Ficoll) was 41.2 ± 8.5 x 10^6, with a median of 34.0 x 10^6 per sample. The mean number of PB MNCs pre-enrichment was 21.8 ± 2.0 x 10^6, with a median of 21.8 x 10^6 per sample. In contrast, >99% of normal haematopoietic cells were depleted following tumour cell purification. The mean number of BM-derived nucleated cells post-enrichment was 93.4 ± 22.5 x 10^3, with a median of 55.9 x 10^3 per sample. In comparison, the mean number of PB-derived cells post-enrichment was 50.5 ± 14.5 x 10^3, with a median of 25.6 x 10^3 per sample. The overall cell viability was 95.5 ± 4.5% and 90.8 ± 7.4% for the pre- and post-enrichment fractions, respectively.

6.3.3 ICC Positive Staining in the Pre- and Post-Enrichment Fractions

Positive staining directed toward a common epitope of CK polypeptides, including the heterodimers CK8-18 and CK8-19 (MoAb A45-B/B3) was not detected in the enriched fractions of the 20 samples from healthy volunteer donors. Twenty three of thirty eight (60.5%) samples scored immunocytochemically positive using the standard Cell-Tak® Cell and Tissue Adhesive immunoassay without prior selection of epithelial tumour cells. 28/38 (73.7%) of the same samples were tumour cell positive after enrichment (p = 0.3289, Fisher's exact test). Four PB samples remained negative, even after tumour cell enrichment. These samples were taken pre #8 AC chemotherapy in one primary medical therapy patient at the time of definitive surgery, pre #4 CMF and pre #4 AC chemotherapy in one adjuvant and one primary medical

therapy patient, respectively, and pre #1 of adriamycin, taxotere chemotherapy from a primary medical therapy patient. Six BM aspirate samples remained negative following tumour cell enrichment. All samples were obtained from prostate Ca patients at the time of RP (4 with clinically organ-confined tumours, one showed local tumour extension beyond the prostate [capsular penetration], and one whilst receiving anti-androgen therapy). The detection rate in all BM samples was improved by tumour cell enrichment from 64% (16/25) to 76% (19/25), and in all PB samples from 53.8% (7/13) to 69.2% (9/13).

For the pre-enrichment fraction, the number of CK^+ cells ranged from 0 to 12 cells detected out of 2 x 10^6 haematopoietic cells per slide. The mean tumour cell count in the 38 samples investigated was 2.4 cells per 2 x 10^6 MNCs (median, 1.5 per 2 x 10^6), without enrichment. After processing the cells on the tumour enrichment devices, 28/38 (73.7%) samples contained CK^+ cells in the post-enrichment fraction. Examples of slides pre- and post-enrichment showing many CK^+ tumour cells, particularly in the post-enrichment fraction is shown in Figure 6.4. The number of CK^+ cells ranged from 0 to 368 cells detected out of 40-50 x 10^3 haematopoietic cells per slide. Moreover, the ability to detect absolute numbers of CK^+ cells was enhanced in the post-enrichment fraction over the pre-enrichment fraction (Figure 6.5). As shown in Figure 6.5, there was a significant difference ($p = 0.0048$) between the pre-enrichment and the post-enrichment fraction for the 38 samples evaluated. The number of CK^+ cells (mean $\pm$ standard error of the mean [SEM]) was 2.3 ± 0.5 and 32.6 ± 10.4 for the pre-enrichment and post-enrichment fractions, respectively, indicating an overall 2.7 log enrichment.

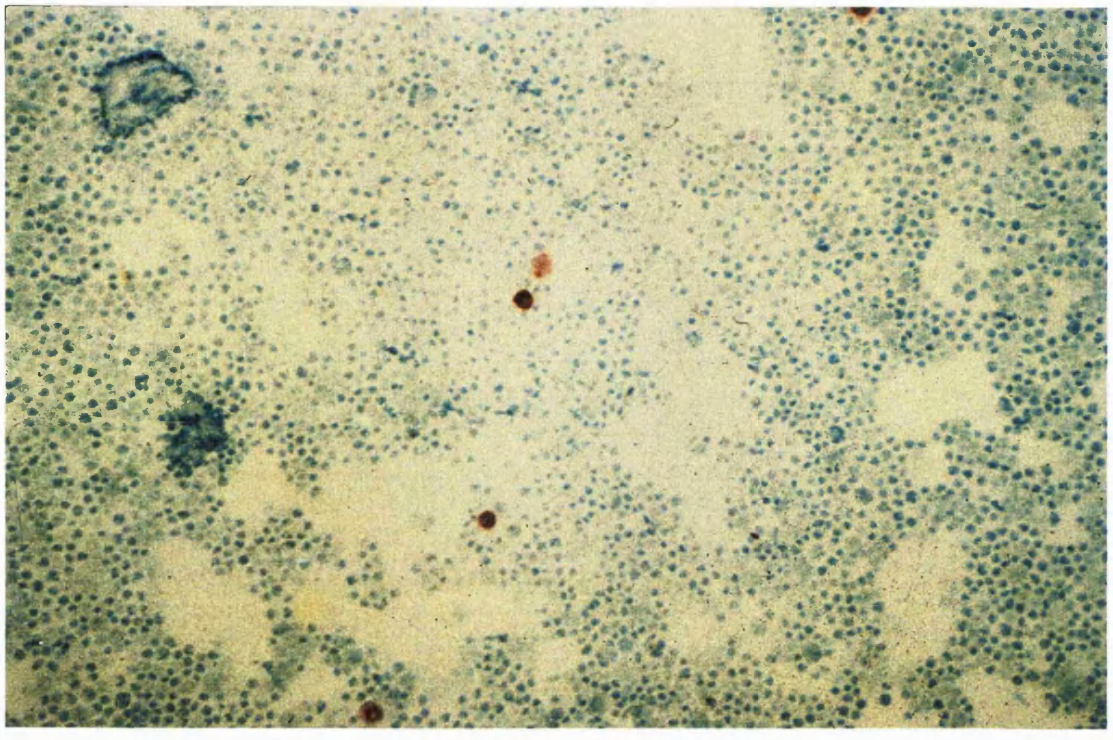


Figure 6.4A: Representative sample illustrating pre-enrichment.

Single CK^+ cells in BM (~10 ml of unprocessed sample, yielding ~50 x 10^6 MNCs [minimum number of cells recommended for processing on the TEC column]) of a patient with non-metastatic carcinoma of the breast. Cell-Tak® adhesive system preparation stained with MoAb A45-B/B3.

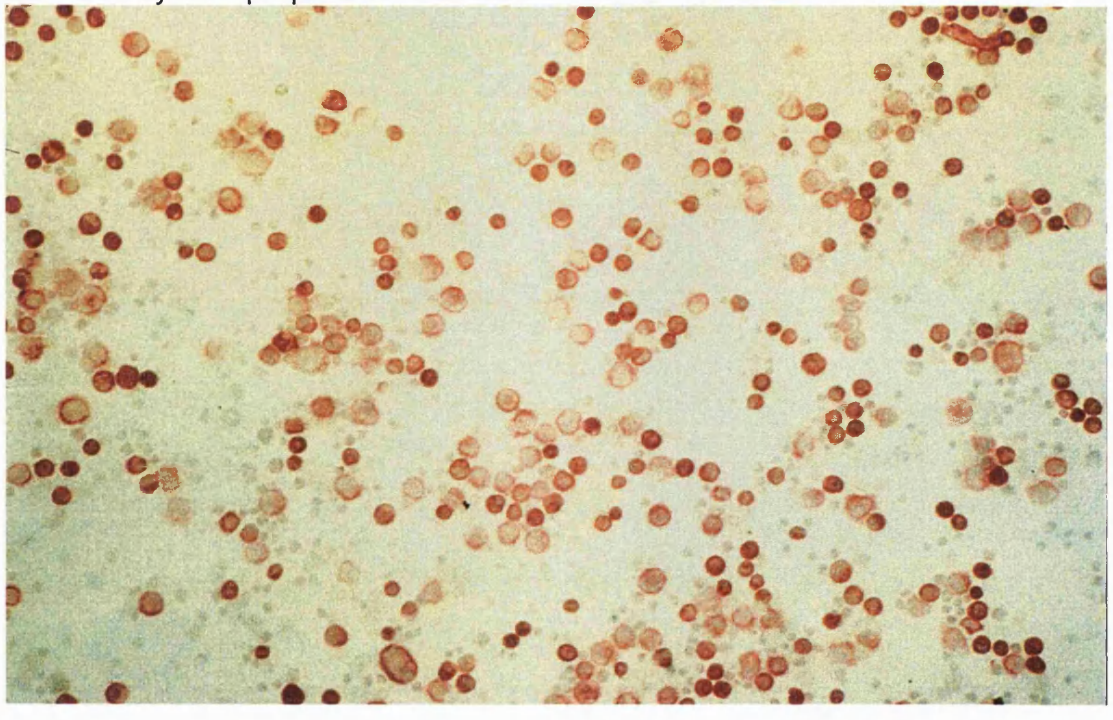


Figure 6.4B: Representative sample illustrating post-enrichment.

Enriched, adsorbed fraction of CK^+ cell population of clinical sample, following gentle agitation of column bed. Cell-Tak® adhesive system preparation stained with A45-B/B3 (red stain) while residual haematopoietic cells are unstained.

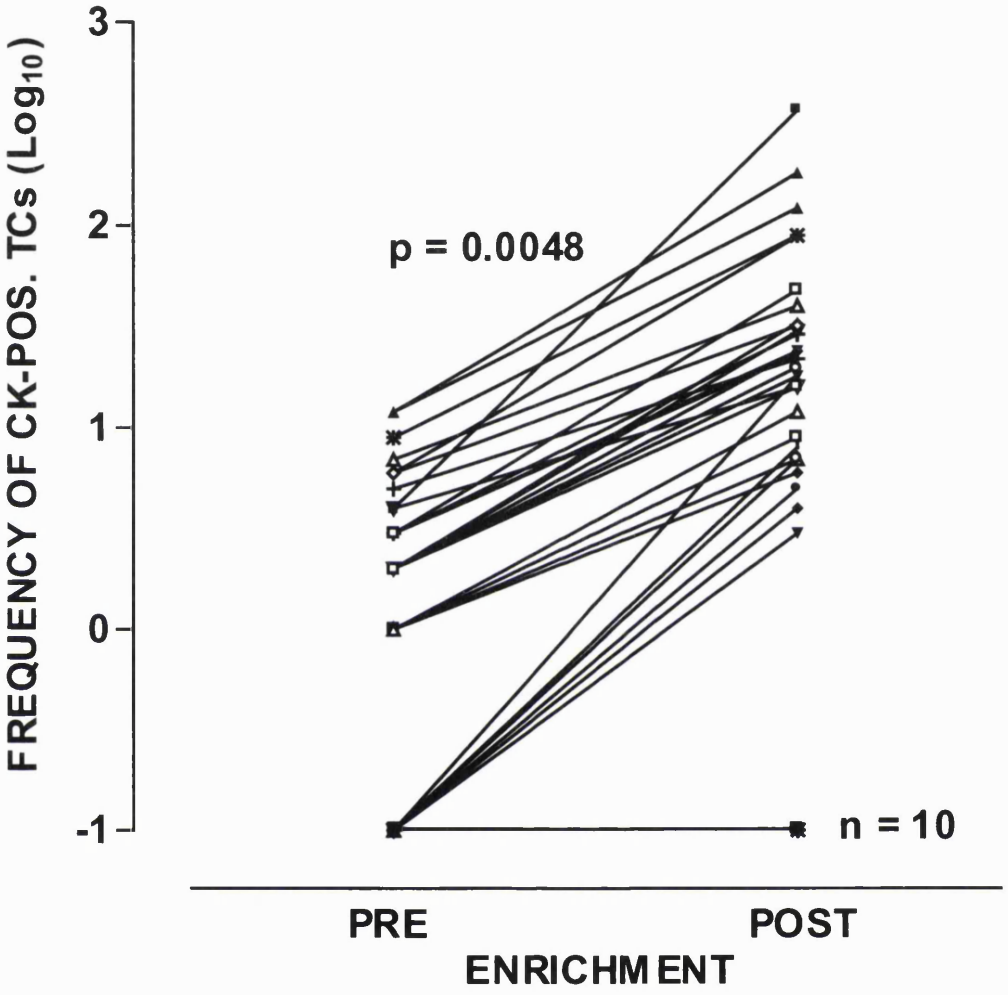


Figure 6.5: Enhanced detection of CK^+ cells in haematopoietic samples.
The results are shown for 11 BrCa and 20 prostate Ca patients, and 38 samples processed on tumour enrichment columns (25 BM and 13 PB).

Slightly enhanced detection was observed with BM samples in comparison with PB samples, in both the pre-enrichment and post-enrichment fraction, as shown in Figure 6.6. Furthermore, there was a significant difference between the BM and PB post-enrichment fractions ($p = 0.0453$).

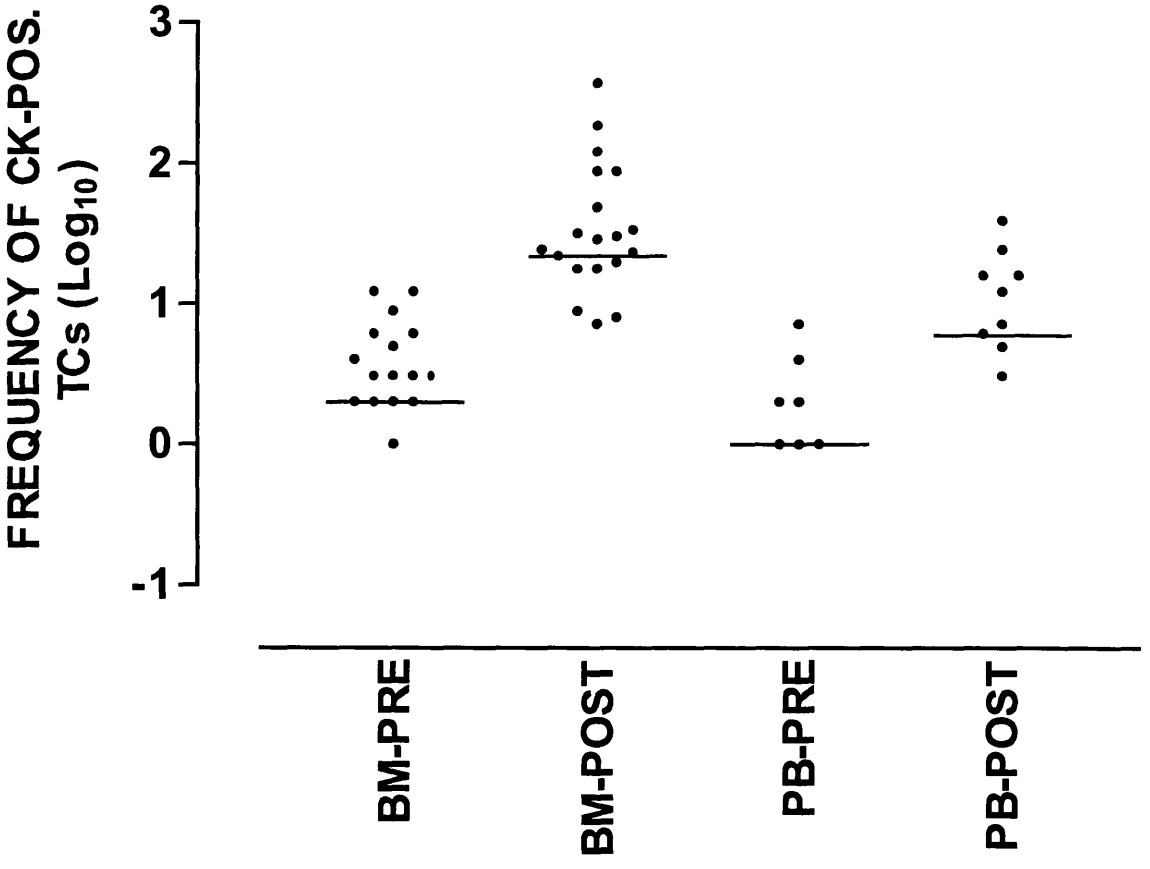


Figure 6.6: Scattergram showing tumour cell counts without selection versus tumour cell counts after enrichment, per sample.

Median + range of CK^+ cells detected for BM samples in comparison with PB samples for both the pre-enrichment (post-Ficoll) and post-enrichment fraction. There was a significant difference between the BM and PB post-enrichment fractions ($p = 0.0453$).

6.3.4 Isolation of Bone Marrow- and Blood-Borne Cytokeratin-Positive and Cytokeratin/Her-2/neu-Double-Positive Cells From Breast Cancer Patients

Bone marrow (13 aspirates [10 at the time of initial diagnosis and 3 at the time of definitive surgery]) and/or PB (18 samples [13 at the time of pre #1 cytotoxic chemotherapy, 1 pre #4, 2 pre #8, and 2 at the time of clinical relapse]) from 15 patients with primary non-metastatic BrCa (6 adjuvant therapy patients and 9 primary medical therapy patients), and 5 patients being treated for advanced metastatic disease, were also further analysed to assess the isolation and characterisation of epithelium-derived Her-2/*neu* oncogene-positive carcinoma cells from haematopoietic tissue (confirmed by FISH). In 9/13 (69.2%) BM and 17/24 (70.8%) PB samples, tumour cells were detected which carried amplification of the region 17q11.2-q12 (Her-2/*neu*); identified by the fluorescence pattern of their nuclei (double positive for CK and Her-2/*neu* [DB^+]). No cells that carried only a Her-2/*neu* aberration were found. All of the samples showed a clustered distribution, although a small number of cases (predominantly PB samples) also showed diffuse signals. As the level of gene amplification was sometimes heterogeneous among the nuclei of the same specimen, an average was calculated and the cases were then sub-classified on the basis of the results obtained in Chapter 5, from the BrCa cell lines with low level (MCF-7), medium level (MDA-MB-361), or high level (BT-474) Her-2/*neu* amplification (examples of fluorescent images are shown in Figure 6.7):

- Normal level of expression: 2 Her-2/*neu* signals per cell.
- Low level of amplification: 4-5 Her-2/*neu* signals.
- Medium level of amplification: 6-30 Her-2/*neu* signals.
- High level of amplification: more than 30 Her-2/*neu* signals.

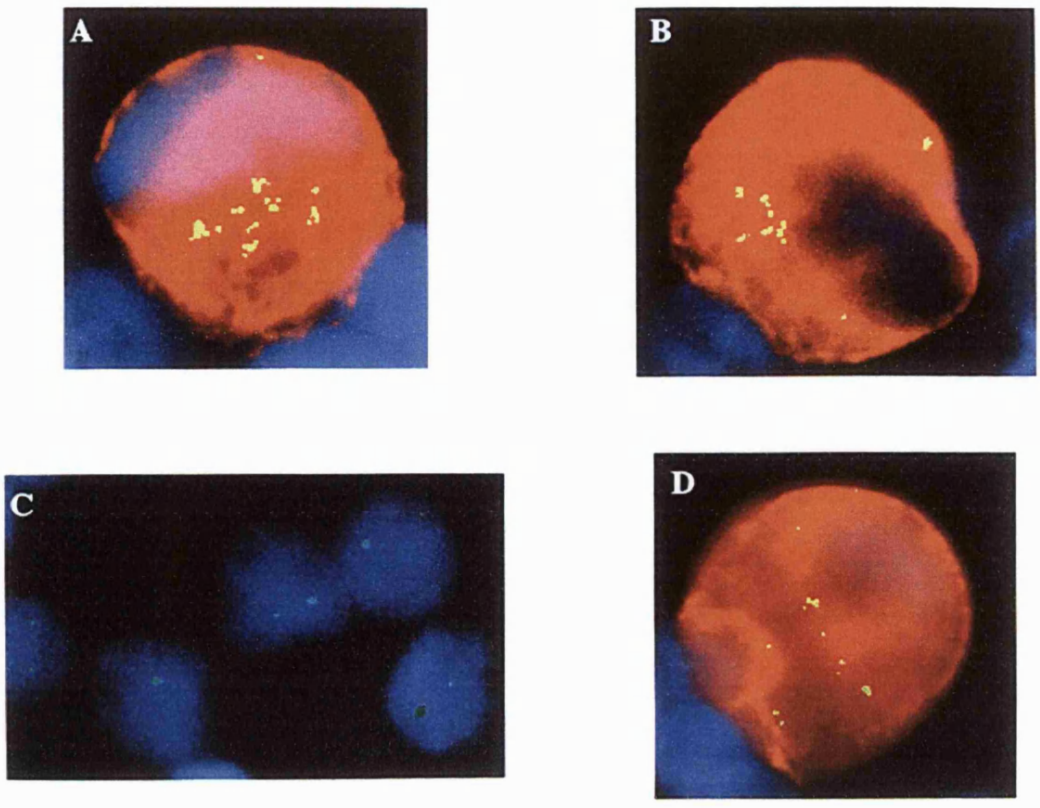


Figure 6.7: Combined fluorescent immunolabelling and interphase FISH, illustrating different molecular genetic mechanisms of Her-2/*neu* (c-*erb*-B2) proto-oncogene overexpression in DB^+ BrCa cell lines and clinical material.

A, CK^+ cell of BT-474 showing high level Her-2/*neu* intrachromosomal amplification signals, arranged as clusters in the nucleus. B, CK^+ cell of MDA-MB-361 showing medium level Her-2/*neu* intrachromosomal amplification signals, arranged as clusters in the nucleus. C, single CK-negative BM MNCs from a normal donor, showing two hybridization signals for Her-2/*neu* per cell. D, micrometastatic CK^+ epithelial cell in BM aspirate of patient V. M. showing medium level Her-2/*neu* extrachromosomal amplification, with signals dispersed within the nucleus.

The specificity of the assay was further confirmed in 15 healthy volunteers (laboratory staff) and 5 patients who were undergoing surgery for a variety of reasons. In none of these controls could CK^+/DB^+ cells/clusters be detected. The BrCa patients shown to have epithelial-derived cells, with phenotypes staining positively either for CK alone or double positive for CK and Her-2/*neu* are detailed in Table 6.1A and 6.1B, and the combined multiple BM/PB sample data are illustrated graphically in Figure 6.8.

Patient	PB Pre #1	BM1	Current Clinical Status
G. W.*	16 DB^+ (Low)	19 DB^+ (High)	Disease free
N. K.	24 DB^+ (Medium)	16 DB^+ (High)	Relapse
B. G.	20 CK^+ (Normal)	CK^-	Disease free
A. B.	16 DB^+ (Low)	14 DB^+ (High)	Relapse
K. A.	22 CK^+ (Normal)	CK^-	Disease free
N. P.	30 DB^+ (Low)	89 DB^+ (High)	Disease free
P. F.	12 CK^+ (Normal)	CK^-	Disease free
C. G.	6 CK^+ (Normal)	CK^-	Disease free
V. M.	29 DB^+ (Low)	122 DB^+ (Medium)	Tumour relapse & death
S. S.	16 DB^+ (Low)	182 DB^+ (High)	Tumour relapse & death

Table 6.1A: Combined marrow/blood-borne CK/Her-2/*neu* epithelial tumour cell status in both primary medical therapy and high-risk adjuvant patients.

Words in parentheses denote differing levels of Her-2/*neu* amplification as detailed above.

Numbers refer to the sum of cancer cells detected following tumour cell enrichment.

G. W.*: Patient treated with HDC and autologous (CK^-) PBSC transplant, as part of the Anglo Celtic Co-operative Oncology Group clinical trial.

Abbreviations: BM1, BM aspiration at the time of diagnostic surgery, pre-chemotherapy; PB Pre #1, PB sampling pre-cytotoxic therapy; DB^+, double positive carcinoma cell for CK and Her-2/*neu*; CK^+, cytokeratin-positive cell with normal Her-2/*neu* status; CK^-, no detectable tumour cells.

Patient	PB Tumour Cell Status	PB Her-2/*neu* Status
L. S. (M)	14 DB^+	High
H. G. (M)*	19 DB^+	High (Pre rhmAb‡)
H. G. (M)§	7 DB^+	Low (Post rhmAb‡)
B. N. (M)	9 DB^+	High
T. A. (M)	15 DB^+	High
B. G. (M)	10 DB^+	High
J. D. (NM)	16 CK^+ (Pre #1 CMF)	Normal
L. S. (NM)	12 CK^+ (Pre #1 CMF)	Normal
E. O. (NM)*	6 DB^+ (Pre #8 CMF)	Low
E. O. (NM)§	40 DB^+ (Relapse)	Medium
E. A. (NM)	16 DB^+ (Relapse)	Medium
E. R. (NM)	20 CK^+ (Pre #1 AC)	Normal

Table 6.1B: Peripheral blood-borne CK/Her-2/*neu* epithelial tumour cell status in additional metastatic and non-metastatic BrCa patients.

*§Denote multiple samples from the same patient during the course of therapy. Abbreviations: M, metastatic disease; NM, non-metastatic disease; CMF, cyclophosphamide, methotrexate, 5-fluorouracil; AC, adriamycin, cyclophosphamide; rhmAb, recombinant humanised monoclonal Her-2/*neu* antibody treatment.

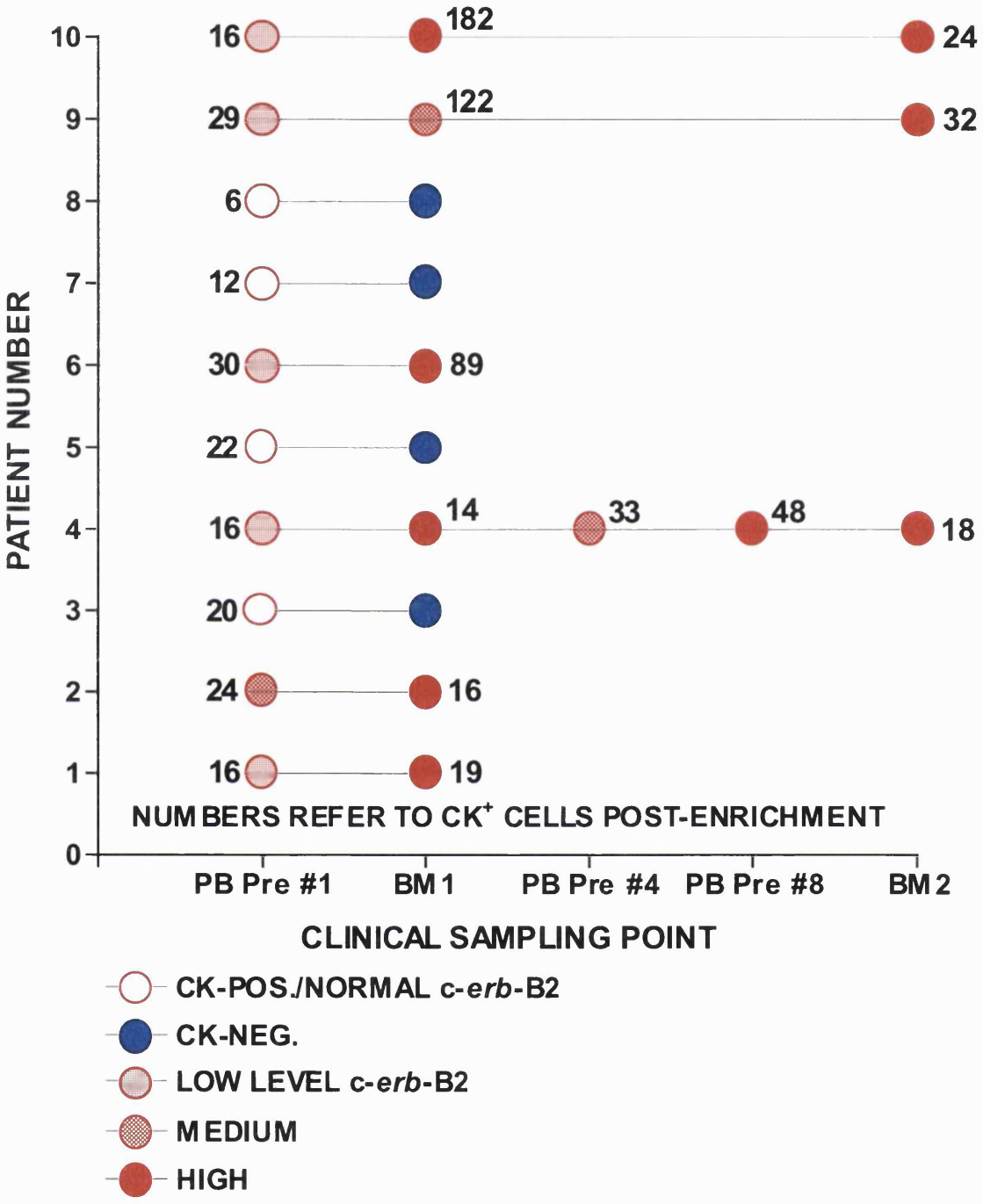


Figure 6.8: Her-2/*neu* overexpression enhances metastatic potential of blood-borne CK-pos. cells by induction of metastasis-associated properties.

Overexpression of the Her-2/*neu* gene can be correlated with both clinical status and poor clinical outcome (including tumour relapse and death), as illustrated in Tables 6.1A and 6.1B, conceivably by enhancing the intrinsic metastatic potential of human epithelial tumour cells. Based on these findings, it can be postulated that the occurrence of blood-borne DB^+ carcinoma cells lead to increased metastatic potential in human BrCa, which may result in more metastatic lesions, including dissemination to the BM microenvironment, as demonstrated by the clinical correlation found in 8/10 patients with localised non-metastatic stage II and III BrCa (Table 6.1A). Overexpression may also lead to increased resistance to certain chemotherapeutic agents, which may cause DB^+ tumour cells to have a poor response to therapy. The combined consequence is that patients with DB^+ tumour cells have a poor clinical outcome. Therefore, more aggressive therapy, including the use of recombinant humanised monoclonal Her-2/*neu* antibody treatment (trastuzumab [Herceptin™]), or HDC and PBSCT (patient G. W. who remains disease free despite the detection of epithelium-derived DB^+ cells in both the PB and BM compartments prior to dose intensification), might be beneficial to those patients with detectable DB^+ cancer cells.

6.3.5 Low Frequency Epithelial Cells, Enriched From Bone Marrow Aspirates of Prostate Carcinoma Patients

The control subjects consisted of 10 young healthy volunteers (8 male and 2 female), 13 surgical patients without evidence of prostatic disease, including 8 with other malignancies (e.g., bladder cancer or sarcoma), and 7 patients undergoing transurethral resection of the prostate for pathologically

confirmed BPH. No CK^+ cells were detected in any of the control samples tested.

The relation between the prevalence of CK^+ BM aspirates at the time of radical prostatectomy and tumour classification was evaluated. Sixty percent of BM aspirates (12 of 20 patients) contained CK^+ cells, detected by using the conventional Cell-Tak® Cell and Tissue Adhesive immunoassay. Cytokeratin-positive BM aspirates occurred in 3 of 10 patients (30%) with pT2N0 tumours, 6 of 7 patients (85.7%) with pT3N0 tumours, and 3 of 3 patients (100%) with pT4N+ tumours (χ^2 7.679, p = 0.0215).

The frequency of CK^+ cells in 12 CK^+ BM aspirates varied among prostate carcinoma patients. The frequency of CK^+ cells in BM specimens that contained at least 1 CK^+ cell ranged from 1-9 CK^+ cells (mean, 95% confidence interval (CI), [3.417, 1.997-4.836]) per 2 x 10^6 MNCs. The median CK^+ frequency per 2 x 10^6 MNCs screened in BM aspirates categorised according to tumour classification is summarised as follows: pT2N0, 0.0; pT3N0, 2.0; pT4N+, 6.0 (p = 0.0039; Kruskal-Wallis test [one-way ANOVA]).

6.3.6 Aneusomy Analyses of Tumour Cells in Bone Marrow Aspirates

Numerical abnormalities involving chromosome 7 in enriched CK^+ cells were measured using FISH. Chromosome 7 hybridization signals were quantified in CK^+ cells from 14 patients. Three BM aspirates had <10 CK^+ cells and 11 BM aspirates contained >10 CK^+ cells post-enrichment, some of which were present in clusters. Chromosome 7 signals were quantified in 90 CK^+ cells from patients with pT2N0 tumours, 133 CK^+ cells from patients with pT3N0 tumours, and 225 CK^+ cells from patients with pT4N+ tumours. Approximately 35% of CK^+ cells displayed >2 copies of chromosome 7 (trisomy 7 [3 copies

only]) in the patients with pT2N0 tumours, 55% of CK^+ cells in patients with pT3N0 tumours, and 80% of CK^+ cells in patients with pT4N+ tumours (χ^2 60.52, $p<0.0001$). Thus, it is apparent that the majority of CK^+ prostatic cancer cells are aneusomic, with a statistically significant increase in the incidence of trisomy 7 seen with more advanced disease. Molecular cytogenetic analyses of CK^+ cells present in the same BM aspirate revealed heterogeneous aneusomy patterns. In BM aspirates obtained from patients with clinically organ-confined prostate Ca that contained CK^+ cells, the identified cells displayed varying combinations of two, three, and four copies of chromosome 7. Thus, the cells do not appear to be cytogenetically homogeneous.

6.4 Discussion

This part of the study was undertaken to explore the potential of enrichment techniques for the isolation, detection and further characterisation of epithelial tumour cells contaminating BM and PB. In several types of cancer, an association has been noted between early relapse and the presence of tumour cells in the BM at the time of surgery (Cote *et al*, 1991; Mansi *et al*, 1991; Diel *et al*, 1996). In view of this, detection of circulating malignant cells would clearly be of value as a predictor of prognosis. However, it is not known whether all such tumour cells have a phenotype that enables them to establish distant metastases, or whether they are heterogeneous with regard to their metastatic capacity. Alternatively, they could be random cells, released from the primary tumour at surgery, but are cleared shortly afterwards. Therefore, it seems important to study micrometastatic cells for characteristics associated with poor prognosis.

The prognostic value of detecting epithelial tumour cells in mesenchymal cell samples, such as blood and marrow, for metastatic disease is dependent on the number of cells analysed. Finding a single tumour cell among 5×10^5 to 2×10^6 analysed BM cells has already been shown to be an independent predictor for a higher risk of recurrent (metastatic) disease. More significant correlations might be obtained if additional cells could be analysed, since the standard method may categorise some patients with occult tumour cells as false negative. However, the manual screening of more than 2×10^6 cells per patient by standard ICC or even flow cytometry is not practical. To enable the analysis a higher number of haematopoietic cells from patients with cancer, for the presence of disseminated tumour cells, it is necessary to employ a highly sensitive and specific method of detection. The above results show that both

positive selection and negative depletion can be efficiently used as enrichment steps before ICC staining. Consequently, these techniques make rapid examination of large numbers of MNC possible. In addition, the examination of a restricted number of MNC can lead to a positive diagnosis based on ICC detection of a single cell reactive with an anti-epithelial MoAb. This is supported by the observation that more than half of the early stage cancer patients have been shown to have only 1 or 2 tumour cells detectable after ICC analysis of direct cytospin preparations of 2×10^6 BM MNC (Pantel *et al*, 1994). The use of enrichment was evaluated in combination with ICC in the detection of CK^+ tumour cells in samples obtained from women with locally-advanced, or metastatic BrCa, and from patients with prostate Ca taken at the time of radical prostatectomy. A total of 38 samples were analysed both by ICC alone and by enrichment followed by ICC. With ICC alone, tumour cells were detected in 23 (60.5%) of 38 samples, with a range of 1 to 12 CK^+ cells per 2×10^6 haematopoietic cells screened. The rate of detection rose to 73.7% (28/38 samples) ($p = 0.3289$) when enrichment was used prior to ICC. The number of CK^+ cells ranged from 3 to 368 cells ($p = 0.0048$) detected out of 40-50 $\times 10^3$ haematopoietic cells per slide remaining after selection. With the described enrichment techniques, it was possible to examine 10-fold to 20-fold more cells, resulting in the same number of slide preparations for tumour cell diagnosis as with the standard ICC technique. Accordingly, the enrichment methods provide a detection efficacy (recovery) of 60%-90% of the tumour cells as compared to the standard Cell-Tak® Cell and Tissue Adhesive positive immunoassay result. This is higher than that reported by Griwatz *et al* (1995), who used the MACS system for positive tumour cell enrichment. In addition, the positive enrichment technique yields a highly enriched tumour cell product (mean 2.5 log enrichment

[range, 2.1-3.4 log]). Tumour cell seeding experiments have shown that the TEC is capable of isolating one tumour cell in 10^8 BM and/or PBSC product (Ross *et al*, 1998a, 1999). This enhanced tumour cell capture ability is probably due to the fact that the capture Ab used in the TEC system (PAN-05 anti-epithelial-cell biotinylated Ab) has been shown to be highly reactive with tumour cells of epithelial origin and non-reactive with cells of haematopoietic origin (Ross *et al*, 1998a, 1998b, 1999). Thus, the TEC simultaneously enriches tumour cells and depletes haematopoietic cells from the test specimen. Another advantage of this positive enrichment technique over the previously reported MACS separation through CK8/18 (Griwatz *et al*, 1995) is that isolation of tumour cells through surface Ag instead of CK Ag does not require pre-fixation of the cells. Functional studies of the isolated tumour cells are, therefore, possible. On the other hand, surface epithelium antigens may be more heterogeneously expressed than CK, with the possible consequence of enrichment failures when tumour cells are picked up through such antigens. However, screening of patient samples showed no tumour cell detection failure as compared with the standard ICC. Screening of larger patient series, however, may reveal tumour cell Ag heterogeneity. Thus, a cocktail of biotinylated Abs should be considered for the avidin-biotin based enrichment procedure. Indeed, several antigens on tumour cells are heterogeneously expressed (Schlimok *et al*, 1990; Pantel *et al*, 1991, 1993a). Potential clinical applications for this particular methodology may include purging of harvested transplant products and assessing the efficacy of various purging techniques, prior to autologous stem cell reinfusion.

The negative depletion technique detects tumour cells at a frequency comparable to the positive enrichment technique ($<2 \times 10^{-7}$). The efficacy of

tumour cell enrichment by the described negative depletion technique is in agreement with that reported by Kremens *et al* (1994), where neuroectodermal cell lines were enriched by depletion of haematopoietic cells. The purity of tumour cells by this technique, however, is slightly lower than that seen with positive enrichment. Hence, positive and negative selection may have different advantages. The more purified positive enrichment product should be suitable for further characterisation or diagnostic procedures based on molecular approaches, such as PCR. Reverse transcriptase PCR-based detection of CK^+ cells in BM or PB from BrCa patients has previously been reported. However, CK amplification has also been detected in normal haematopoietic cells using this technique (Traweek *et al*, 1993; Krismann *et al*, 1995; Neumaier *et al*, 1995), supporting the need for a more highly enriched tumour cell product for the PCR analysis (Hardingham *et al*, 1995). The increase in purity by positive selection would give higher discriminative strength to the PCR method, as fewer normal haematopoietic cells would be present to interfere with the analysis. The negative depletion technique leaves the tumour cells unlabeled and unaltered. This is an advantage if further biologic characterisation of the tumour cells by double labelling (e.g. FISH) or immunofluorescent staining is performed. Another possible advantage of the negative depletion technique is its possible use as a tumour cell enrichment tool independent of the tumour cell characteristics or the type of carcinoma.

The occurrence of DB^+ tumour cells (double positive for CK and Her-2/*neu*) would be a prerequisite for advanced BrCa, as compared to localised disease, if one assumes that those cells are likely to be the metastasis-forming entities. Moreover, based on the above observations, one could hypothesise that overexpression of the Her-2/*neu* proto-oncogene may characterise an

aggressive subset of PB-derived micrometastatic BrCa "stem cells", and such overexpression could be correlated with poor clinical outcome. This view is strongly supported by Pantel *et al* (1993a), who showed that BrCa patients with distant metastases (M_1) had BM-lodging epithelium-derived cells co-expressing the Her-2/*neu* protein. In molecular terms, the concept of DB^+ carcinoma cells appearing in the PB and forming BM micrometastasis is further supported by the fact that metastatic deposits derived from a primary BrCa did not lose the ability to express Her-2/*neu* (Niehans *et al*, 1993).

Tumour cell invasion of basement membranes is a crucial event in the complex multi-step process that leads to metastasis. It has been hypothesised that the invasion process consists of three steps: (a) tumour cell adhesion to endothelial cells and attachment to the matrix; (b) tumour cell secretion of hydrolytic enzymes (matrix metalloproteinases, [including collagenase IV]), which can locally degrade the matrix; and (c) tumour cell locomotion into the region of the matrix modified by proteolysis (Nicolson, 1988, 1989). Overexpression of the normal Her-2/*neu* gene may enhance the intrinsic metastatic/invasion-associated properties of human PB-derived BrCa cells by increasing cell adhesion, cell motility, and secretion of membrane-degrading enzymes. Transfection of an activated Ha-*ras* in OVCAR-3 human ovarian cancer cells was shown to increase the 92 kDa collagenase IV activity, and this requires multiple transcription factor binding sites in the 92 kDa collagenase IV promoter, including a PEA3/*ets* motif, AP-1 sites, a NF-κB consensus sequence, and a GT box (Gum *et al*, 1996). Interestingly, overexpression of the Her-2/*neu* receptor may lead to increased tyrosine kinase activity, which is required for association with and tyrosine phosphorylation of Shc (Xie *et al*, 1995). Phosphorylation of Shc in turn leads to binding to Grb2/Sos and triggers

signal transduction through the *ras-raf*-MAP kinase pathway. Therefore, it can be hypothesised that *ras* activation may also be involved in the increased collagenase IV activity and enhanced metastatic potential induced by Her-2/*neu*. In addition, it has recently been found that overexpression of the Her-2/*neu* gene in BrCa cells confers increased resistance to the chemotherapeutic drug Taxol via *mdr-1*-independent mechanisms (Yu *et al*, 1996). Therefore, different treatment strategies might be appropriate in those patients whose PB cancer cells express high levels of Her-2/*neu*.

In summary, the finding of Her-2/*neu* expression on blood-borne (and consequentially on marrow-borne) epithelium-derived cells supports the conclusion that metastasis is not a random process but might result from the preferential release, survival and growth of subpopulations of cells such as those that overexpress Her-2/*neu*, a proto-oncogene that exists within the parent tumour (Fidler, 1990). Furthermore, clinical data from the international (Ludwig) Breast Cancer Study Group (Gusterson *et al*, 1992) trial support this observation. Overexpression of Her-2/*neu*, even on a focal basis, in cancer tissues has been shown to correlate with disease poorly responsive to adjuvant chemotherapy. Yokota *et al* (1986) have reported amplification of Her-2/*neu* in a metastatic tumour while it was not detectable in the primary breast tumour. As mentioned above, Niehans *et al* (1993) and Pantel *et al* (1993) have found that Her-2/*neu*-positive cells from BrCa patients lodge in the BM or spread to distant organs. The results presented here complement the findings of Niehans *et al* (1993), Pantel *et al* (1993) and Yokota *et al* (1986). From the above preliminary data, it can be concluded that blood-borne epithelium-derived Her-2/*neu*-positive cancer cells are possibly the connecting link between a primary tumour and the distant, including BM, metastases.

It is assumed that assays for micrometastases which detect rare epithelial cells in BM aspirates from cancer patients are measuring disseminated cells that have seeded from the primary tumour. Molecular approaches were used to determine whether CK^+ epithelial cells in BM aspirates, taken at the time of radical prostatectomy, from patients with prostate Ca exhibited features consistent with the tumour origin (i.e., cytogenetically aberrant).

Epithelial cells were present in BM aspirates in 60% of patients with prostate Ca. There was a statistically significant ($p = 0.0215$) finding of a higher prevalence of CK^+ BM aspirates from patients with pT4N+ tumours as compared to patients with pT2N0 and pT3N0 tumours. The prevalence of CK^+ specimens in this series is higher compared to that reported in other studies (Bretton *et al*, 1994; Oberneder *et al*, 1994; Pantel *et al*, 1995b, 1996). This may be attributed to the improved sensitivity of the Cell-Tak® Cell and Tissue Adhesive immunoassay, compared to conventional cytospin preparations, where increased cell losses may occur during the process of cytocentrifugation. For example, Oberneder *et al* (1994) detected CK^+ cells in 33% of patients with prostate Ca. Pantel *et al* (1995b) found CK^+ cells in 54% of Stage C patients by harvesting BM aspirations from ≥2 sites. Bretton *et al* (1994) found that CK^+ cells were present in BM from 30% of patients with varying stages of prostate carcinoma.

Molecular cytogenetic analysis of the enriched CK^+ population in BM aspirates from patients with prostate Ca was carried out. Gain of chromosome 7 more frequently occurs in metastatic tumours than primary prostate tumours (Baretton *et al*, 1994; Takahashi *et al*, 1994, 1995) and may be associated with an increased risk of developing distant metastases. Cytokeratin-positive cells

that disseminate to BM were evaluated for chromosome 7 aneusomy by hybridising the CK^+ cells with a chromosome 7 pericentromeric DNA probe. The number of hybridization signals in CK^+ interphase cells was considered to be proportional to chromosome copy number. The data demonstrate that approximately 35% of CK^+ cells in BM aspirates displayed gain of chromosome 7 (trisomy 7) in the patients with pT2N0 tumours, 55% of CK^+ cells in patients with pT3N0 tumours, and 80% of CK^+ cells in patients with pT4N+ tumours ($p<0.0001$). The finding that the majority of CK^+ cells in BM aspirates from prostate Ca patients are cytogenetically aberrant are consistent with CK^+ cells originating from a primary tumour, even in enriched fractions containing <10 CK^+ cells.

Heterogeneity in the aneusomy pattern among CK^+ cells within individual patients with clinically organ-confined prostate Ca was observed. These data may suggest that multiple, cytogenetically distinct populations of tumour cells, have seeded into the BM or that the genomic instability associated with tumour cells may alter the aneusomy features of the seeded cells (in comparison to BrCa patients with Her-2/*neu* overexpression). However, in patients with metastatic disease, the CK^+ cells uniformly showed the same hybridization pattern, which may indicate seeding of a clonal tumour population. Analysis of additional cells would be required to confirm the possibility of molecular heterogeneity and/or genomic evolution among CK^+ cells in BM aspirates. However, it still is unclear whether CK^+ cells in BM aspirates contribute to the generation of metastases. According to Paget's seed and soil hypothesis (1889), only a minority of disseminated tumour cells are able to proliferate at distant sites. Increased understanding of the molecular cytogenetic changes and functional features associated with metastatic development will facilitate

assessment of the metastatic potential of low frequency epithelial tumour cells in BM aspirates and PB samples.

Chapter 7 General Discussion

Despite the progress made in clinical oncology in recent decades, recurrent disease is frequent and often manifest as metastatic recurrence. The detection of residual cancer is a marker of failed disease eradication, and early detection provides the possibility to modify the treatment. Many tumours remain clinically occult until they are significantly advanced. The reasons for this are varied. For example, the remote anatomical location of the pancreas makes it unlikely that pancreatic cancer will be detected before it has invaded neighbouring tissues. Although the breast is anatomically accessible, breast cancer undergoes early metastasis; consequently, 12-37% of small (<1 cm) mammographically detected breast cancers have already metastasised at diagnosis (Wilhelm *et al*, 1991; Chadha *et al*, 1994). Even in prostate Ca, in which prostate-specific antigen can be quantified in the serum, there is a substantial percentage of patients with elevated PSA in whom the diagnosis remains uncertain and *vice versa* (i.e., definitive diagnosis with normal PSA). These are examples of a need for improved detection techniques for cancer.

Screening for Cancer

Much progress has been made in cancer screening over the past decade, but more needs to be done if screening is to make a major impact on cancer mortality. The three diseases in which the greatest advance in screening have been made are cervical, breast, and colorectal cancer. Since screening can only identify individuals in need of treatment, it alone cannot affect the course of cancer in the absence of effective treatment. Screening tests can be divided according to the type of abnormality they aim to detect.

Many tests are aimed at early detection. Such tests may reduce the mortality and morbidity associated with advanced disease. Other screening modalities aim to detect pre-cancerous lesions. The classic example of this is cervical cytology, and sigmoidoscopy shares this goal. Here the aim is to detect precursor lesions before they become malignant, so excision benefit can reach close to 100%. In this setting a major problem is knowing which lesions are likely to become malignant if left untreated, so as to avoid over-treatment of benign lesions. A third form of screening, which is increasingly being used is genetic risk testing. This field will develop as more cancer associated genes are found, and tests for mutations become better automated. Genetic screening will involve families, require intensive follow-up of individuals testing positive, and the use of chemopreventive agents or prophylactic surgical removal of the organs at risk. This is already happening in BrCa where women with a family history are being offered prophylactic radical mastectomy. Ethical and socioeconomic issues are at the forefront in this area but are beyond the scope of this work.

Cervical Cancer

The universally accepted screening method for preventing cervical cancer is cytological examination of cervical scrapes which have been smeared onto slides and stained by Papanicolou's method. Following organised screening programmes in the Nordic countries during the 1960s, by the mid-1970s large reductions in incidence and mortality were seen (Läärä *et al*, 1987). In Britain, mortality reductions have only been apparent in the last few years (Sasieni *et al*, 1995). However, these are now substantial and are continuing at a rate of reduction in mortality of 7% per year. Screening programmes have

been less well organised in many other countries and the benefits in terms of national mortality trends have been less clear.

Successful as it has been, screening by cytology is not without its problems. It is labour intensive and requires highly skilled and somewhat subjective judgements. The costs and infrastructure required for cytology screening are still beyond the reach of the most needy countries, and even in the developed world this is expensive.

New technologies are being developed which, although initially expensive, may ultimately make it possible to undertake screening at lower cost and with less technical expertise. Work on automating the reading of smears has been underway for over 30 years (Banda-Gamboa *et al*, 1992) and a range of computer-assisted systems are now beginning to become available.

Use of liquid based cytology is also likely to lead to improvements in results. With liquid cytology the cells are plated out as a thin-monolayer, which is ideally suited to automated reading, as many of the problems which have plagued automated reading, such as drying artefacts, clumps of cells and obscuring blood and mucus, are removed.

Stains for new targets may also revolutionise cytology. The recent understanding of the regulatory proteins of the cell cycle offer a potential target for screening. Abnormal cells are proliferating, while normal squamous cells are "end" cells and no longer express many of the proteins found in cycling cells. Initial results using Cdc6 or Mcm5 (Williams *et al*, 1998), are encouraging.

Another, more fully developed approach, is to screen for high risk strains of the human papillomavirus (HPV_{hr}). This virus is found in over 95% of all cervix cancers and has been clearly established as the primary causal agent.

HPV_{hr} can only be reliably detected by DNA-based tests, and morphological changes on cytology or histology more often detect HPV 6 and other low risk types which produce benign lesions. Early tests for HPV DNA had both sensitivity and specificity problems, but reproducible results are now being achieved with the newer tests.

Definitive studies have yet to be completed but a number of studies have shown very promising results using consensus primer PCR or the Hybrid Capture microtitre assay (Cuzick *et al*, 1995; Schneider *et al*, 1996). Sensitivities for cervical intraepithelial neoplasia (CIN) 2/3 are almost always higher than for cytology and specificities are similar to that for borderline smears. Overall these studies suggest that adding HPV_{hr} testing to primary screening could increase the early detection rate of high grade CIN by 30-100%. This may both reduce the incidence of cancer, and allow the screening interval to be increased to 5 years or longer, especially in women over the age of 50 years who have never had an abnormal smear.

Persistence is the key attribute of HPV_{hr} infections related to high grade disease. This can only be directly verified by repeated testing using current assays, but transient infections lead to a large number of "false-positive" results, especially in younger women. Restricting HPV testing to women over the age of 30 or 35 years (at least for primary screening) substantially reduces the false-positive rate. Viral load is also important. Quantitative assays with thresholds for positivity of approximately 10^4 HPV copies in a smear give much better specificity and little loss of sensitivity for high grade CIN (Cuzick *et al*, 1992).

Additionally, HPV_{hr} testing offers scope for better detection of incomplete excision and residual disease in women who have been treated for CIN. Several reports suggest that the persistence of HPV positivity after treatment is

an accurate method of assessing treatment failures and this could be used to safely return negative women to positive screening after a single follow-up (Elfgren *et al*, 1996).

Breast Cancer

Mammographic Screening

The most fully investigated modality for early detection of BrCa is mammography. This method is aimed at early detection of invasive cancer and so is limited by the fact that this may still be too late to affect survival. As a result, only moderate benefits of the order of 20-30% mortality reduction can be expected from this approach. Eight randomised prospective trials have addressed the impact of mammographic screening on BrCa mortality. These are the Health Insurance Plan (HIP), Swedish Two County, Gothenburg, Stockholm, Malmo, Edinburgh, the Canadian National Breast Screening Study I (NBSS I), and the NBSS II (Fletcher *et al*, 1993). The impact of screening differs between younger and older women. For women over age 50 at the start of the screening trials, a significant benefit is seen after 7-9 years of follow-up, and longer follow-up does not change the magnitude of this benefit. In contrast, for women below age 50 at the start of the screening trials, the impact of screening emerges gradually, with a significant reduction in mortality appearing after about 12 years of follow-up. At least two possible explanations have been proposed to account for this difference (Fletcher, 1997). Compared with older women, cancer in younger women may be detected earlier in the natural history of the disease as a result of screening. Thus, a reduction in BrCa mortality may take longer to appear. Alternatively, perhaps screening is not effective in younger women. Therefore, the delayed benefit of screening women below age

50 at entry into the clinical trials may be the result of these women undergoing annual screening beyond age 50. This "age creep" effect was studied by deKonig *et al* (1995) using a computer simulation model. Their model suggested that most of the reduction in BrCa mortality for women between the ages of 40 and 49 at the start of the screening trials was, indeed, the result of screening these women after they passed their 50th birthday.

Despite the controversy over the effectiveness of mammographic screening for women aged 40 to 49, at least four potentially harmful consequences of this particular type of screening exist:

- Lead time — Some women are given advanced notice of a cancer diagnosis without tangible gain.
- Radiation exposure — May increase the risk of BrCa, particularly in women who carry the gene for ataxia-telangiectasia.
- False-positives — Leads to unnecessary breast biopsies.
- Overdiagnosis — There are emotional/financial consequences of being falsely labelled as a cancer patient.

There appears to be limited scope for further improvements in mammography although digital mammographic and computer aided techniques may increase sensitivity. The more widespread use of hormone replacement therapy, which increases the radiographical density of the breast, may even erode some of the benefits previously seen.

Screening by Breast Self-examination

Breast self-examination is widely promoted as a means of reducing BrCa mortality. However, as yet there is little evidence to support its efficacy. Only two randomised trials have examined the effect of screening by self-

examination on BrCa mortality. In 1992, Semiglazov *et al* reported the preliminary results of the World Health Organisation (WHO)/Russian trial. The number of breast cancers detected in the two groups (half randomised to receive self-examination training, and the other half received usual care) were nearly identical, and there was no significant difference in mortality. Another breast self-examination trial was initiated in Shanghai, China (Thomas *et al*, 1997). Screening mammography was not available. After 5 years, the number of BrCa cases and BrCa mortality were nearly identical in the two groups. The St. Petersburg and Shanghai trials have only reported early results, and longer follow-up may eventually show that screening by self-examination affords a mortality benefit. Nonetheless, it is surprising that a downstaging of tumours was not observed in either trial as a result of screening by self-examination. Of note, both trials showed that the incidence of unnecessary biopsies increases with breast self-examination.

Screening by Physical Examination

In contrast to screening by breast self-examination, trained personnel are required to carry out screening by physical examination (PE). Unfortunately, to date no randomised clinical trials have compared screening by PE with no screening. Screening by PE may carry fewer hazards than screening by mammography (less lead time, no radiation exposure, fewer false-positives, and less overdiagnosis).

Colorectal Cancer

The screening method that has received the most attention for colorectal cancer is the faecal occult blood test. The objective of the test is to detect

cancers at an early stage when they are still treatable, and the very good survival of Dukes' stage A cancers (>90% at 5 years) compared with colorectal cancer overall (approximately 30% at 5 years) suggests this could be successful.

In one study (Mandel *et al*, 1993) a mortality reduction of 33% for colorectal cancer was found for annual faecal occult blood testing. In that trial, rehydrated tests were used which increased the positivity rate to almost 10%, leading to many unnecessary colonoscopies. Rehydration improves sensitivity but at the expense of a large number of false-positive tests.

Another approach to colorectal cancer screening is based on the work of Morson (1976) who proposed that most cancers arise from pre-existing adenomas. Adenomas are pre-cancerous growths which occur throughout the large bowel and have the same sub-site distribution as cancers, but occur earlier in life. An approach based specifically on this idea is to use sigmoidoscopy as a screening tool. This is far less expensive or traumatic than complete colonoscopy and approximately 60% of colorectal cancers occur in the region accessible by this instrument. Evidence is mounting for its efficacy. Selby and colleagues (1992) have shown, in a case-control study in high-risk families, that mortality due to cancers within the limit of the sigmoidoscope was reduced by 60% for at least 10 years and similar results have been reported in another smaller study (Newcomb *et al*, 1992). A very large study looking at the effect of any endoscopic procedure on colorectal cancer has also shown a large benefit that does not diminish over a 10-year period (Müller and Sonnenberg, 1995). Several other studies have suggested that endoscopic surveillance of the bowel greatly reduces colon cancer rates, but a direct demonstration that infrequent screening by sigmoidoscopy will reduce mortality requires a large

randomised trial. Such a trial, proposed by Atkin and colleagues (1993), is based on the promise that most of the benefit of sigmoidoscopy will accrue from a single screening test and only a small group of individuals (approximately 5%) would need colonoscopy and further surveillance.

Prostate Cancer

The problem of how to discriminate aggressive prostate cancer with lethal potential from indolent cancers which are likely to remain asymptomatic for the remainder of the patient's lifetime remains unsolved. Nevertheless, prostate cancer is a significant public health problem and an obvious target for screening. Digital rectal examinations have been used for many years and still have a role in screening, but are subjective and lack sensitivity (Bentvelsen and Schroder, 1993). Prostate specific antigen (PSA) testing has good sensitivity, especially when used at a 4μg/ml cut-off. However, ~~specificity is less good~~ THE ASSAY IS LESS SPECIFIC, and the test measures tumour volume but not aggressiveness, and so does not distinguish benign prostatic hypertrophy from invasive cancer. Age-specific cut-offs have been suggested to improve specificity, but there is still substantial overlap between normals and those with cancer. Transrectal ultrasound is too invasive and expensive to use as a primary screening test, but may have a role as a secondary test in individuals with slightly elevated PSA level. Further markers of tumour aggressiveness, either measured in serum or needle biopsy specimens, are needed to determine which patients are in need of curative treatment. This is urgently needed since radical surgery carries a high morbidity, often leading to impotence and/or incontinence.

Concluding Discussion and Future Studies

In 1869, Ashworth described cells in the blood that resembled those observed in the tumour at *post-mortem*. The metastatic process consists of a sequence of events in which tumour cells, in the primary site, erode the endothelial basement membrane, penetrate a blood vessel, become motile, and spread to distant sites by blood or lymph. New parameters need to be defined that better identify those patients at the greatest risk of relapse, because this would provide information critical to the subsequent management. Detection of the earliest manifestations of tumour dissemination a promising approach that should improve risk assessment and the identification of those patients who would benefit from adjuvant therapy. Since the aforementioned screening approaches cannot be used to monitor efficacy of chemotherapy during treatment, increasing the number of tools for detecting micrometastases is likely to impact on overall mortality.

During the last 10 years, new immunological and molecular analytical procedures have been developed to identify and characterise minimal residual cancer. The results have changed concepts of the frequency and significance of tumour cell dissemination. Minimal residual cancer is associated, in general, with a poor prognosis. However, there is considerable evidence that it may be transient and thus would not preclude a sustained remission of the patient without further treatment (Moss *et al*, 1991). The improved sensitivity of the novel cell adhesive technology developed during the preliminary phase of the research programme, in comparison to conventional cytocentrifugation preparations, is capable of reliably and reproducibly detecting one CK^+ tumour cell in 2×10^6 normal haematopoietic MNCs. The most important evaluation of

this assay is to screen for early detection of carcinomas, particularly in high risk patients, e.g., those with BRCA mutations.

The advantage of ICC compared to either PCR or conventional flow-cytometry, is the potential to examine the detailed cytomorphology of the individual cells detected and thus characterise them further. To date, the encouraging results using improved assays from studies on the prognostic relevance of disseminated tumour cells in BM should be standardised, categorised, and incorporated into the staging nomenclature of the International Union Against Cancer. As part of the pathological assessment process, additional tumour-staging information could be provided by including micrometastases in the TNM classification system.

Not all of the cells that circulate in the PB might have the capability to attach and proliferate in distant sites. Nevertheless, the finding of haematogenous dissemination in patients with clinically organ-confined tumours suggests that haematogenous dissemination is not a late event in the natural history of the disease. It also suggests that lymphatic spread is an associated, but not essential, step in the dissemination of the disease. The risk of relapse among the patients with node-negative cancer who have BM micrometastases may be sufficiently high to warrant the administration of adjuvant chemotherapy. The above findings support the view of Fisher and colleagues (1981), who maintained that different pathways of tumour cell dissemination cause distinct patterns of metastasis. Analysis of the different metastatic routes that independently predict clinical relapse may provide complementary prognostic information. Persistent seeding of tumour cells in the circulation is likely to be associated with adverse clinical outcomes for epithelial cancers in all stages. The results add to the data from investigations restricted to the BM

compartment, that ICC for CK can specifically detect circulating tumour cells in not only metastatic, but also localised epithelial cancers.

Because of their accessibility, PB and BM samples are obvious candidates for monitoring minimal residual cancer at the sub-clinical stage. Periodic examination of these mesenchymal tissues during therapy has indicated whether the therapeutic approach being used was effective. The pan-CK A45-B/B3 Ab conjugated with alkaline phosphatase or with a fluorescent dye was chosen for identifying cells of epithelial origin. The cytochemical and fluorescent signals from the CK Ab were both specific and very intense, making it easy to quantify the positive epithelial cell numbers. Aneuploidy of the chromosomes examined, compared with gene copy number, served as an indicator of a malignant clone when it was correlated with the above combination staining method. The development of specific and sensitive ICC as well as molecular cytogenetic tools, have extended the knowledge of the frequency and type of distribution of disseminated neoplastic cells in patients with epithelial malignancies. This emphasises that the presence of these cells, particularly in the early stages of the disease, might have a significant impact on prognosis. In the future, the earlier detection of micrometastases could mean that treatment is initiated earlier when tumour volume is small and, more importantly, at a time when a genetically unstable tumour is at an earlier stage in its evolution. This in turn suggests that early-stage patients with disseminated cancer cells might particularly benefit from adjuvant therapy modalities, for example with drugs that eliminate both cycling and dormant cancer cells. In the future, monitoring the dynamic changes in the residual tumour cell burden during adjuvant systemic therapies will become an important application of the detection assays.

A major challenge is to identify the underlying molecular changes that switch cells to a metastatic state, with the ultimate aim being to devise treatments that inhibit metastasis. The outlined current strategies for detection and characterisation of cancer micrometastasis might help to design and control new therapeutic strategies for prevention of metastatic relapse in patients with operable primary carcinomas. Minimal residual disease offers the advantage of a small burden of dispersed tumour cells which are more accessible to intravenously applied drugs than gross metastasis. In view of the heterogeneous nature of micrometastatic cells in PB and BM, therapies that are also directed against highly genetically aberrant tumour cells (for example high level Her-2/*neu* amplification), such as antibody-based immunotherapy, might be complementary to chemotherapy.

It is widely accepted that metastasis is not a random, but a highly selective, process that favours the survival and growth of a few subpopulations of cells that pre-exist within the parent neoplasm (Fidler, 1990). As discussed by Liotta *et al* (1991), estimating the size of the metastatic subpopulation has clinical significance, as a prognostic assay based on a sample of the primary tumour would be highly inaccurate if the aggressive subpopulation was only a small proportion of the total number of tumour cells. In the present study, both single and clustered cells positive for CK/Her-2/*neu* were isolated from the PB and BM of BrCa patients. It would be interesting to further investigate the metastatic behaviour of these cells, especially in the step of extravasation, which follows the tumour cells' passage through the bloodstream. An *in vitro* model can be designed for the venule wall that consists of an endothelial monolayer growing on porous membranes pre-coated with extracellular matrix basement membrane. The transendothelial penetration by BrCa cells followed

The BrCa data presented above, have indicated that a proportion of patients present with tumour cells in BM and PB after adjuvant chemotherapy, and that these resistant tumour cells may have particular prognostic relevance. This supports the possibility for immunological monitoring of disseminated cells for individual patients, and routine testing for rare tumour cells by enrichment would be desirable to improve the detection and further characterisation over the course of longitudinal studies. In therapeutic studies, long-term follow-up is required to establish whether the therapy-associated reduction in individual disseminated cells is associated with improved prognosis. It would also be of interest to test blood from patients in clinical remission many years after therapy of a breast or prostate carcinoma to determine whether any circulating tumour cells remained. There is increasing evidence from clinical observations, experimental models of dormancy, and the findings of circulating tumour cells in patients with clinically organ-confined disease, as reported here, suggesting that many cancers are systemic diseases which are not cured by present day therapy.

Thus far, the biology of circulating and BM micrometastases has remained poorly understood. This lack of knowledge is equally important in patients that remain free of cancer despite the presence of distant tumour cells at the time of diagnosis, and who represent a particular paradox. The present results indicate that CK^+ micrometastatic tumour cells represent a selected population of cancer cells which still express a considerable degree of genetic heterogeneity. With the continuing development of new techniques like single-cell PCR and the *in vitro* expansion of micrometastatic cells (Putz *et al*, 1999), it will become possible to further determine the intrinsic genotypic characteristic features of these cells.

by invasion of the underlying basement membrane can be examined. The *in vitro* model could be evaluated by using the BrCa cell lines expressing different levels of Her-2/*neu*. Cells could then be tested following purification from PB and BM (to further confirm the intrinsic metastatic/invasion-associated properties of PB-derived micrometastatic BrCa "stem cells", and homing to the BM microenvironment), and possibly from disaggregated surgical breast tissue to explore the invasion capacity of cells from benign and malignant breast tissues and the role of Her-2/*neu* in their invasive potential. Further ICC analysis of the invasive cell populations with high locomotive capability, may reveal the expression of proteins that are likely to be involved in the metastatic invasion process (for example matrix metalloproteinase MMP-2, CD44, and integrins $\alpha v\beta 3$ and $\alpha 6$) (Liotta *et al*, 1991; Stetler-Stevenson *et al*, 1993; Brooks *et al*, 1996; Imhof *et al*, 1996; Sy *et al*, 1996).

The PB-derived DB^+ cells identified in the current study (assumed to be the prime candidates for precursor metastasis-forming entities), indicate an important functional role of Her-2/*neu* in the metastatic steps that require cell migration, and that overamplification is sufficient to induce a highly motile cellular phenotype. It was reported that a critical level of $p185^{Her\text{-}2/neu}$ seems to be necessary to achieve transformation (DiFiore *et al*, 1990), which can be explained by a model in which there is an equilibrium between monomeric and dimeric forms of Her-2/*neu* (Di Marco *et al*, 1990). As the quantity of $p185^{Her\text{-}2/neu}$ increases by overexpression, the equilibrium is shifted to the dimeric state resulting in constitutive activation of the tyrosine kinase and inappropriate cellular signalling, subsequently leading to a locomotive phenotype (Di Marco *et al*, 1990).

As mentioned above, metastasis results from the preferential survival and growth of a few subpopulations of cells (Fidler, 1990). Clinical data support this view (Gusterson *et al*, 1992). Overexpression of Her-2/*neu* even on a focal basis in cancer tissues has been taken as indicating patients who are poorly responsive to adjuvant chemotherapy (Gusterson *et al*, 1992). The detailed Her-2/*neu* phenotypic characterisation of the metastatic subpopulations, as developed in this study, could be essential for devising new therapeutic approaches toward the Her-2 signalling cascade. These approaches would include the use of trastuzumab (Herceptin™), ansamycins which produce a rapid reduction in the level of Her-2 expression in cell lines that overexpress Her-2 (Zheng *et al*, 2000), antisense approaches (Roh *et al*, 1999), and novel antibodies directed toward the dimerisation of Her-2 and conjoiners (Agus *et al*, 2000). The applicability in studying other epithelial malignancies that disseminate to BM and the contribution of additional, individual genes to metastasis would also be possible. For instance, Signoretti *et al* (2000), have recently shown that progression of prostate Ca toward androgen independence is characterised by a gradual increase in Her-2/*neu* expression by the tumour cells. This observation is analogous to the statistically significant increase in the incidence of trisomy 7 (possibly through c-*met* proto-oncogene amplification which maps to chromosome 7q31.1), being observed with more advanced prostate Ca, in the present study. The authors proposed that Her-2/*neu* may function by initially permitting prostate Ca cell survival in an androgen-depleted environment. The authors also speculated that, over time, reactivation of proliferation occurs in Her-2/*neu*-positive cells, probably in association with additional genetic events. Her-2/*neu* targeting in advanced androgen-independent prostate Ca is, therefore, justified. More important, the authors

suggest that the combination of androgen ablation and Her-2/*neu* targeting could be effective in androgen-dependent tumours.

In addition to Her-2/*neu*, Clark *et al* (2000) recently described the use of high-density DNA microarrays to identify several genes that are selectively upregulated in metastatic mouse and human melanoma cells compared with their non-metastatic counterparts. Remarkably, they found that overexpression of one of these genes (*RhoC*), alone stimulated metastasis. Bittner *et al* (2000) have also used microarrays to compare different subgroups of human melanoma, and found a distinct pattern of gene expression in highly invasive melanoma cells. It was perhaps not surprising to see no unique gene-expression 'fingerprint' for metastatic melanomas: Bittner *et al* (2000) showed that the gene-expression profiles of different subgroups of human melanoma vary greatly. What was significant was that three of the genes identified by Clark *et al* (2000) (those encoding fibronectin, thymosin β4 and RhoC) showed increased expression in all human and mouse melanoma-derived metastases. Increased fibronectin expression also correlated with higher invasive capacity in the study of Bittner *et al* (2000) (which did not, however, include RhoC or thymosin β4). As stated above, fibronectin is a component of the extracellular matrix and promotes the migration of several cell types, including melanoma cells (Silletti *et al*, 1998). The production of fibronectin by melanoma cells might allow them to lay down their own promigratory matrix. Thymosin β4 binds monomeric actin, a component of the cytoskeleton, and may act as an actin buffer, preventing spontaneous polymerisation of actin monomers into filaments but supplying a pool of actin monomer when the cell needs filaments (Chen *et al*, 2000), and this is likely to relate to the need for cells to migrate. RhoC is a member of the Rho family of small GTP-hydrolysing proteins, several of which

are known to regulate cell migration (Hall, 1998); RhoC is highly related to RhoA and RhoB. Unlike the *Ras* genes, the three *Rho* genes have not yet been found to be mutated in human cancers, but they can contribute to cell transformation to a cancerous state, motility and invasion, at least under experimental conditions (Itoh *et al*, 1999). Although the expression of *RhoA* and *RhoB* appeared not to be altered in metastatic melanoma cells, induced overexpression of *RhoC in vitro* in the poorly metastatic human melanoma cell line induced cells to become highly metastatic (Clark *et al*, 2000). The approaches of Clark *et al* (2000) and by Bittner *et al* (2000) provides an unbiased method by which to pinpoint important, and potentially new, contributions to cancer, in comparison to the work on Her-2/*neu* as illustrated in the present study. It should now be possible to work back from transcriptional changes to identify the one or more key genetic or epigenetic events that induce metastasis.

The Her-2/*neu* data (and to a certain degree the trisomy 7 data) generated in this study, suggests that cells carrying this aberration may have been selected during the process of occult tumour cell dissemination. The presence of such a micrometastasis-specific anomaly supports the model of clonal metastasis, which implicates that one tumour cell clone disseminates because of a decisive event discriminating it from the other primary tumour cells. After its arrival at the secondary site (e.g., BM), this clone might need to undergo further genomic change to evolve into an overt metastasis. With reference to a hypothesis for the metastasis pathway, the formation of tumour cell clusters as observed in certain patients in this study, may be the result of clonal growth of circulating cancer cells in the blood. The clusters are an indication that, once the circulating cancer cells obtain the ability to survive in

the patients' blood, they proliferate through mitotic division and clonal growth to form a circulating cancer cell cluster: a circulating microtumour. This tumour could be arrested in the small veins or capillaries and may continue to grow as it is supplied with sufficient nutrients available from within the blood vessels. Growth would enable the microtumour to exert pressure on the inside of the vessel. A photomicrograph by Greene (1965), provides evidence of a cancer cell or possibly a cancer cell cluster that has arrested in a distended capillary at the junction of ectoderm and mesoderm (Figure 7.1).

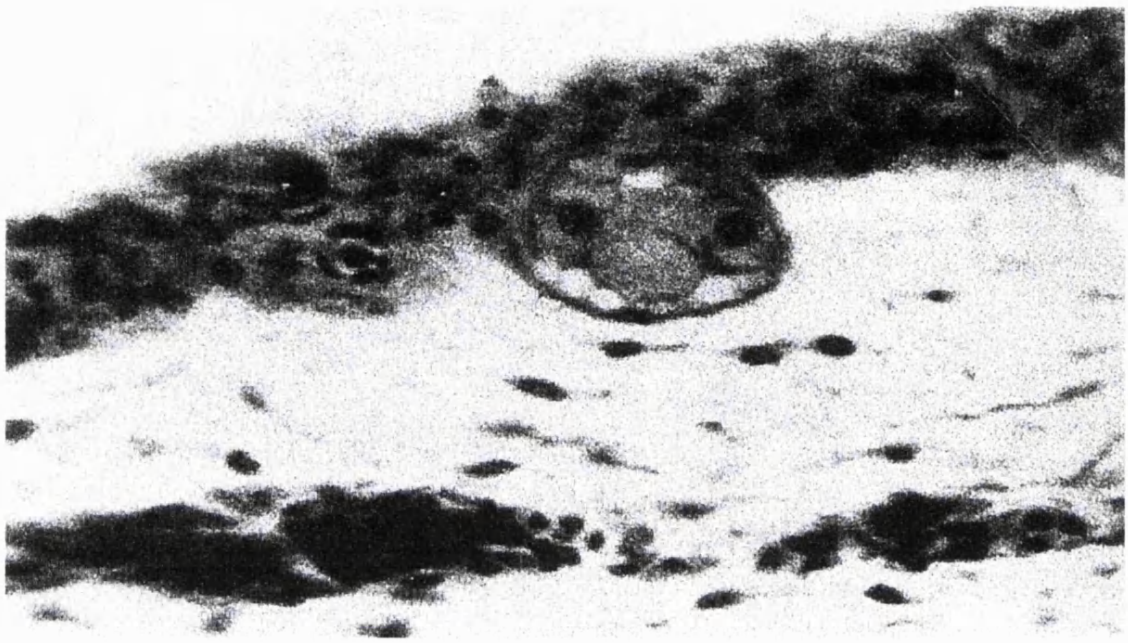


Figure 7.1: Photomicrograph illustrating the intravascular growth process of the circulating cancer cell after it has arrested in the capillary.
(Figure taken from Greene, 1965). (COPYRIGHT APPLIED FOR).

This photomicrograph may illustrate part of the intravascular growth process wherein the circulating cancer cell has arrested in the vessel and undergone subsequent divisions that result in pressure being exerted on the walls of the invaded vessel. The photomicrograph exhibits a thickened capillary wall and prominent endothelial nuclei. The invasive abilities of and the pressure exerted by these cancer cells potentially may damage the vessel. In time, the cancer cells may have the opportunity to extravasate and grow around the vessel in a multidirectional manner. Angiogenic factors may be secreted from the cancer cells as the tumour enlarges and may induce the invaded vessel endothelium to proliferate and form new vessels to vascularise the tumour tissue.

In conclusion, the data generated here, contribute to an increasing body of evidence demonstrating that detection and characterisation of tumour cells disseminated in PB or BM can provide clinically important data that are of value for tumour staging and for prognostication which can identify surrogate markers for early assessment of the effectiveness of adjuvant therapy. Thus, these data would have a substantial influence on future oncologic diagnosis and treatment. At the very least, examination for occult metastases should be incorporated into future clinical trials to evaluate treatment. In the future, adjuvant therapy, specifically tailored to the disease in subgroups of individual patients with residual disease, may improve cancer control.

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Publications

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Immunocytochemical Detection of Breast Cancer Cells: A Comparison of Three Attachment Factors

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ABSTRACT

The evaluation of contaminating breast cancer cells in hematopoietic grafts is of considerable importance for monitoring the efficiency of purging procedures. We report a comparison of three systems for the in vitro detection and enumeration of metastatic breast cancer cells. Breast cancer cells from established cell lines were mixed with Daudi cells at dilutions ranging from 1:10 to 1:1,000,000, and a predetermined number were fixed in defined areas on microscope slides coated with one of the following attachment factors: (i) Cell-Tak® Cell and Tissue Adhesive, (ii) 0.1% solution of Poly-L-Lysine, or (iii) Cel-Line HTC Super Cured® slides. We employed a specificity-proven pancytokeratin antibody (A45-B/B3) and the alkaline phosphatase-antialkaline phosphatase (APAAP) staining technique. In multiple experiments, one breast cancer cell in 1,000,000 Daudi cells could reliably be detected in the Cell-Tak and Cel-Line systems and 1 in 100,000 with the Poly-L-Lysine system. The observed number of seeded cells showed a highly significant correlation with the number of cells seeded ($p < 0.0001$ in all cases). Finally, we used the Cell-Tak method to evaluate clinical material from various sources: from patients with primary carcinomas of the breast, prechemotherapy, and during various chemotherapeutic regimens, as well as from patients with metastatic disease. The system consistently detected tumor cells in bone marrow samples from these patients. All peripheral blood samples from patients with metastatic disease tested positive at incidences ranging from 5 to 19/10^6 peripheral blood mononuclear cells. This is a simple and reliable technique that allows rapid screening of large cell numbers with high resolution of positive cells.

INTRODUCTION

HIGH-DOSE CHEMOTHERAPY (HDC) followed by autologous transplantation of peripheral blood progenitor cells (PBPCT) is being used increasingly to treat high-risk adjuvant or metastatic breast cancer (1). Furthermore, it is believed that PBPC collections are less likely than BM harvest products to be contaminated with circulating tumor cells.

In an elegant study, Brugger et al. (2) measured cytokeratin (CK)-positive epithelial cells in serial peripheral blood (PB) specimens in 7 newly diagnosed breast cancer patients. These individuals had stage IV disease and had received conventional chemotherapy and granulocyte colony-stimulating factor (G-CSF) for mobilization of progenitor cells from the bone marrow (BM) into the PB. They reported concomitant mobilization of tumor cells. This report of mobilization of tumor cells after chemotherapy and hematopoietic growth factor treatment suggests that the transplanted material may reexpose the patient to metastatic disease. The assay was performed with anti-CK antibodies and an epithelial cell antibody (HEA125). In some cases, plasma cells were stained weakly by HEA125.

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Although the full extent of clinical benefit of dose-escalated therapy and PBPC rescue has not been determined, evidence is growing that infused tumor cells may be detrimental. Some evidence is provided by the poor clinical outcome of lymphoma patients with postpurge marrows positive for tumor by polymerase chain reaction (PCR) analysis (3). There is now adequate evidence to justify the development of techniques for purging cancer cells from contaminated BM harvests before reinfusion (4).

There is little information about the clinical significance of tumor contamination for peripheral stem cell transplantation. In neuroblastoma, PBPC harvests have been shown to contain contaminating tumor cells irrespective of marrow involvement (5). Furthermore, in studies of children with neuroblastoma, the presence of malignant cells in the blood during therapy was significantly associated with relapse of disease (6). Similarly, in one other study in patients with breast cancer, tumor-contaminated PBSC collections resulted in a poor clinical outcome (7). Differences may be related to the clonogenic potential of metastatic cells. However, other data indicate that CK-positive micrometastatic breast cancer cells in the BM carry with them an oncogenic potential. Furthermore, the majority of these cells appear to be in a dormant state of cell growth (8).

It is, thus, vital to establish a sensitive method for detecting tumor cells in BM or PBSC collections before transplantation. Detection and enumeration of rare breast cancer cells in blood or BM may also be helpful in determining prognosis and directing appropriate therapy.

The goal of the current study was to establish a sensitive and reproducible immunocytochemical (ICC) assay capable of detecting very low levels of tumor cells by attachment to defined areas on microscope slides coated with various adhesive factors. Principally, we wished to optimize the distribution of the cells into a single cell layer and to ensure minimal cell loss during processing so that the number of positive events could be related directly to the number of cells fixed onto the slides. We employed a specificity-proven pan-CK antibody (A45-B/B3) and the alkaline phosphatase-antialkaline phosphatase (APAAP) staining technique in the absence of counterstaining to optimize the signal/noise ratio. No studies are yet available to compare the sensitivity and specificity of this method with PCR.

MATERIALS AND METHODS

Tumor cell lines

Cells of the human breast cancer cell lines MDA-MB-361, BT-474, and MCF-7 were used [all from American Type Culture Collection (ATCC), Rockville, MD]. MDA-MB-361 cell line was maintained in culture in Leibovitz's L-15 medium with 15% fetal bovine serum (FBS), BT-474 in RPMI-1640 medium with 10% FBS, and MCF-7 in Eagle's minimum essential medium (MEM) with nonessential amino acids and sodium pyruvate with 10% FBS. The Daudi B lymphoblastoid cell line was propagated in RPMI-1640 medium with 20% FBS and was subcultured biweekly.

All culture media were supplemented with 2 mM L-glutamine, penicillin (100 μg/ml), and streptomycin (100 μg/ml). Cells were maintained at 37°C with 5% CO_2 in a humidified atmosphere. Culture media and supplements were from GIBCO (Paisley, Scotland.)

The adherent growing cells were also subcultured biweekly and harvested by detaching the cells from the tissue culture flask by exposure to a 1 × solution of trypsin/EDTA for 5–10 min at 37°C. Cells were then washed in the appropriate medium, and viable cells were counted by trypan blue exclusion test.

Preparation of specimens for analysis

Breast cancer cells were isolated by trypsinization, washed twice in phosphate-buffered saline (PBS) (as were Daudi suspension culture cells), and resuspended at 5×10^6/ml in PBS. Daudi cell samples processed as described were divided into several tubes and mixed with different concentrations of breast cancer cells at dilutions of 1:10, 1:100, 1:1,000, 1:10,000, 1:100,000, and 1:1,000,000. One hundred microliters of each cell suspension (5×10^5 cells) was attached by sedimentation on defined areas (spots) (1.4 cm in diameter) on microscope slides coated with one of three adhesive factors. Triplicate samples of 2×10^6 cells were immunostained with monoclonal antibody (mAb) A45-B/B3.

Attachment factors

Prior to use, all microscope slides were cleaned with 1% HCl in 70% ethanol, and coated with one of the following attachment factors: (i) Cell-Tak® Cell and Tissue Adhesive (Universal Biologicals Ltd., London, U.K.), (ii) 0.1% solution of Poly-L-Lysine (Sigma, Poole, UK), or (iii) Cel-Line HTC Super Cured® slides (Cel-Line Associates, Newfield, NJ).

Cell Tak. Cell-Tak is a formulation of polyphenolic proteins from the anchoring glue secreted by the marine mussel, *Mytilus edulis* (9). Glass microscope slides were cleaned, and after drying, defined areas were covered with a freshly prepared solution of Cell-Tak, 5.0 μg/cm^2 of surface area [determined after a preliminary dose-response experiment to demonstrate optimal density (data not shown)] in 0.1M $NaHCO_3$, pH 8.0, or 0.1 M HEPES buffer, pH 8.0. The adhesive is provided in a dilute acetic acid solution and is poorly soluble around neutral pH,

causing it to be adsorbed to the slide. After 20 min at ambient temperature, the slides were rinsed in sterile water. Cells (5×10^5) were attached on each spot. After a 30 min incubation period, the slides were gently rinsed with serum-free medium to remove dead cells and cellular debris. Subsequently, slides were air-dried for 12–24 h and stored at −70°C until used.

Poly-L-Lysine. The principle behind this method is that the polycationic polylysine molecules adsorb strongly to glass surfaces, leaving cationic sites that combine with the anionic sites on cell surfaces. Working diluted Poly-L-Lysine solution was prepared by diluting Poly-L-Lysine solution 1:10 with deionized water before coating defined areas on clean glass microscope slides. Surfaces were prepared by covering them briefly (5 min) with a 0.1% solution of Poly-L-Lysine in water. The slides were then washed sequentially with running water and serum-free medium. They were dried in a 60°C oven for 1 h or at ambient temperature overnight and either stored at 4°C or used immediately. Again, after a 30 min incubation period with the cell suspension, they were gently rinsed with serum-free medium, air-dried for 12–24 h, and if not stained immediately, were stored at −70°C until use.

Cel-Line. To perform the ICC assay within this system, 5×10^6 cells/ml were seeded into the wells (5×10^5 cells per well) of specially designed, Cel-Line HTC® (heavy polytetrafluoroethylene) (Teflon®)-coated Super Cured slides (14-mm wells), by sedimentation. The slides were air-dried and, if not stained immediately, were stored at −70°C until use.

Collection of clinical specimens

Venous blood samples were obtained from central venous catheters or peripheral veins. Twenty milliliters of blood was collected in heparinized tubes before processing. Three to five milliliters of BM aspirate containing a minimum of 1×10^7 cells/ml obtained from the upper iliac crest were collected in sterile sodium heparin tubes for ICC assay analysis. PBSC aphereses and BM harvest collections were performed at the Royal Free Hospital. Representative aliquots containing at least 1×10^7 cells/ml from each pheresis/BM harvest session were similarly processed for ICC assay analysis.

Processing of clinical specimens for ICC analysis

Clinical samples were diluted with PBS (without Ca^{2+}/Mg^{2+}, pH 7.4) (GIBCO). Diluted samples were layered over Ficoll-Hypaque (Nycomed Pharma, Oslo, Norway) and then subjected to density-gradient centrifugation at 900*g* for 30 min. The light-density mononuclear cell (MNC) fraction was resuspended and washed twice in PBS (500*g* for 10 min), tested for viability (trypan blue exclusion), counted, and resuspended in PBS at a dilution of nucleated cells of 5×10^6/ml. Cryopreserved BM isolates were rapidly thawed in a 37°C waterbath, incubated with 100 μl of deoxyribonuclease I (Sigma, Poole, Dorset) at 2000 Kunitz U/ml for 5 min at 37°C to prevent clotting, and washed twice with serum-free RPMI-1640 medium supplemented with heparin at 1500 rpm for 10 min. Again, MNC were resuspended in PBS, tested for viability, counted, and resuspended in PBS at a cell dilution of 5×10^6/ml.

Preparation of clinical specimen adhesion slides

The Cell-Tak adhesive system was used to test the validity of our ICC assay. Isolated MNC were attached to Cell-Tak-coated adhesion slides consisting of three spots, each 1.4 cm in diameter. Routinely, two slides with a total of six spots were examined for each patient and for each time point. One hundred microliters of the cell suspension was attached on each spot (5×10^5 cells/spot). After a 30 min incubation period, the slides were gently rinsed with serum-free medium to remove dead cells and cellular debris. Subsequently, cells were air-dried for 12–24 h and stored at −70°C until use.

Immunocytochemistry

mAb A45-B/B3 (IgG_1), which detects a common epitope on a variety of CK components, including CK8, 18, and 19 (kindly provided by Dr. E. Felber, Micromet GmbH, Munich, Germany) (10), was used as the reference antibody for tumor cell detection in Daudi cell preparations and clinical specimens. The mAb was used at optimal concentrations ranging from 1 to 10 μg/ml. Appropriate dilutions of mouse myeloma proteins served as IgG_1 isotype control (Serotec, Oxford, U.K.).

The antibody reaction was developed with the alkaline phosphatase-antialkaline phosphatase (APAAP) technique combined with the new fuchsin stain (Sigma, Poole, U.K.) for visualizing antibody binding (11). Briefly, the immunostaining was performed after preincubation with normal serum to block nonspecific binding. After incubation with the primary antibody, a polyvalent rabbit antimouse Ig antiserum (Dako Ltd, High Wycombe, U,K.) and preformed complexes of alkaline phosphatase and monoclonal antialkaline phosphatase antibodies (Dako) were used at the dilutions recommended by the manufacturer (Dakopatts). To stop the enhancement reaction, the slides were rinsed in distilled water. Using the APAAP staining technique, we observed a strong color reaction without a background reaction. Cells containing CK components were stained bright red. Counterstaining could, therefore, be omitted, which allowed a faster screening for stained cells occurring at low frequencies. Each immunostaining assay contained a neg-

ative as well as a positive control. Immunostained as described, one spot incubated with nonimmune mouse serum served as a negative control, and one spot with the human breast cancer cell line MCF-7 served as a positive control. In addition, 9 normal BM and 6 normal PB samples as well as numerous samples from patients with hematologic malignancies incubated with the breast-reactive mAb served as additional negative controls. Nonreactive normal hematopoietic cells on the patient preparations served as additional internal negative controls for APAAP staining. After seeding into the Daudi cell line, at each dilution, 12 spots with at least 6×10^6 cells in total were analyzed for the presence of breast cancer cells. Four spots with at least 2×10^6 total cells were analyzed for the presence of tumor cells in clinical specimens using a standard microscope. The total numbers of either epithelial or Daudi cells present on control slides on each spot within each adhesive system were calculated by counting the cell number within 20 quadrants out of 100 total quadrants five times from different fields using a graticule within the ocular. The total number of MNC isolated from clinical samples present on each spot within the Cell-Tak adhesive system were also calculated using this methodology (data not shown).

Statistical analysis

χ^2 tests were used to compare the observed number of breast cancer cells with the number of cells seeded in all three adhesive systems. Results were expressed as percentage ± percentage coefficient of variance (CV). χ^2 tests were also used to compare the total number of either breast cancer or Daudi cells present on control slides on each spot within each adhesive system with the expected total number.

RESULTS

Quantitation of breast cancer cells in Daudi cells using ICC

Cultured breast cancer cells were added to Daudi cell suspensions to create final concentrations of between 1 and 1000 per 1,000,000 Daudi cells. These mixtures were then stained and analyzed as described, and the number of tumor cells (observed) was plotted against the number seeded (expected) for each adhesive system investigated. Highly significant correlations of expected versus observed values were found. Linear regression analyses were performed for all three adhesive systems, where the ranges examined were 0.0001%–0.1% (approximately three orders of magnitude), indicating strong correlation between expected and measured numbers of tumor cells ($p < 0.0001$) present in all test systems. Intraassay reproducibility was excellent for each system (Fig. 1A, B, C) (median CV: Cell-Tak, 8.81%, Cel-Line, 7.47%, and Poly-L-Lysine, 9.72%). All test samples were retested on four occasions to assess interassay reproducibility (Fig. 1D), which was also well within acceptable limits for a routine clinical assay (median CV: Cell-Tak, 2.42%, Cel-Line, 6.84%, and Poly-L-Lysine, 6.83%). There were no significant differences between the observed and expected numbers of breast cancer cells in this system down to the $1{:}10^5$ dilution, irrespective of the adhesive used (Table 1). Below the $1{:}10^5$ dilution, the Cell-Tak and Cel-Line slides continued to show significant correlation between expected and observed numbers and no significant difference between the number of breast cancer cells detected and the number seeded. In contrast, the Poly-L-Lysine slides showed a significant difference between observed and expected numbers (χ^2 42.2, $p < 0.10$) at the $1{:}10^6$ dilution. Despite this, the overall correlation for Poly-L-Lysine slides remained highly significant ($p < 0.0001$).

Graticule data

After calculating the total number of either epithelial or Daudi cells present on each spot within each adhesive system by counting the cell number within quadrants, no significant differences ($p > 0.1$) were seen between the number of cells applied and the number retained after processing (Table 2). Thus, it was shown that significant cell numbers were not lost after completion of the ICC staining method. This was also found to be the case after calculating the total number of MNC from clinical material studied present on each spot within the Cell-Tak system (data not shown).

Immunocytochemical and additional morphologic analyses also showed that cells from the epithelial tumor cell lines were larger than Daudi cells, which are comparable to PB MNC. This size differential was also noted between circulating breast tumor cells and MNC in the clinical samples studied.

Detection of tumor cells in clinical specimens

The Cell-Tak adhesive system was used to examine clinical specimens from patients with breast cancer—both from patients undergoing adjuvant treatment prechemotherapy and postchemotherapy and from patients with metastatic disease. We also included previously cryopreserved samples of BM harvests from four breast cancer patients with metastatic disease.

Detection of circulating breast cancer cells

The incidence of CK-positive cells in clinical PB samples was higher in patients with metastatic disease than

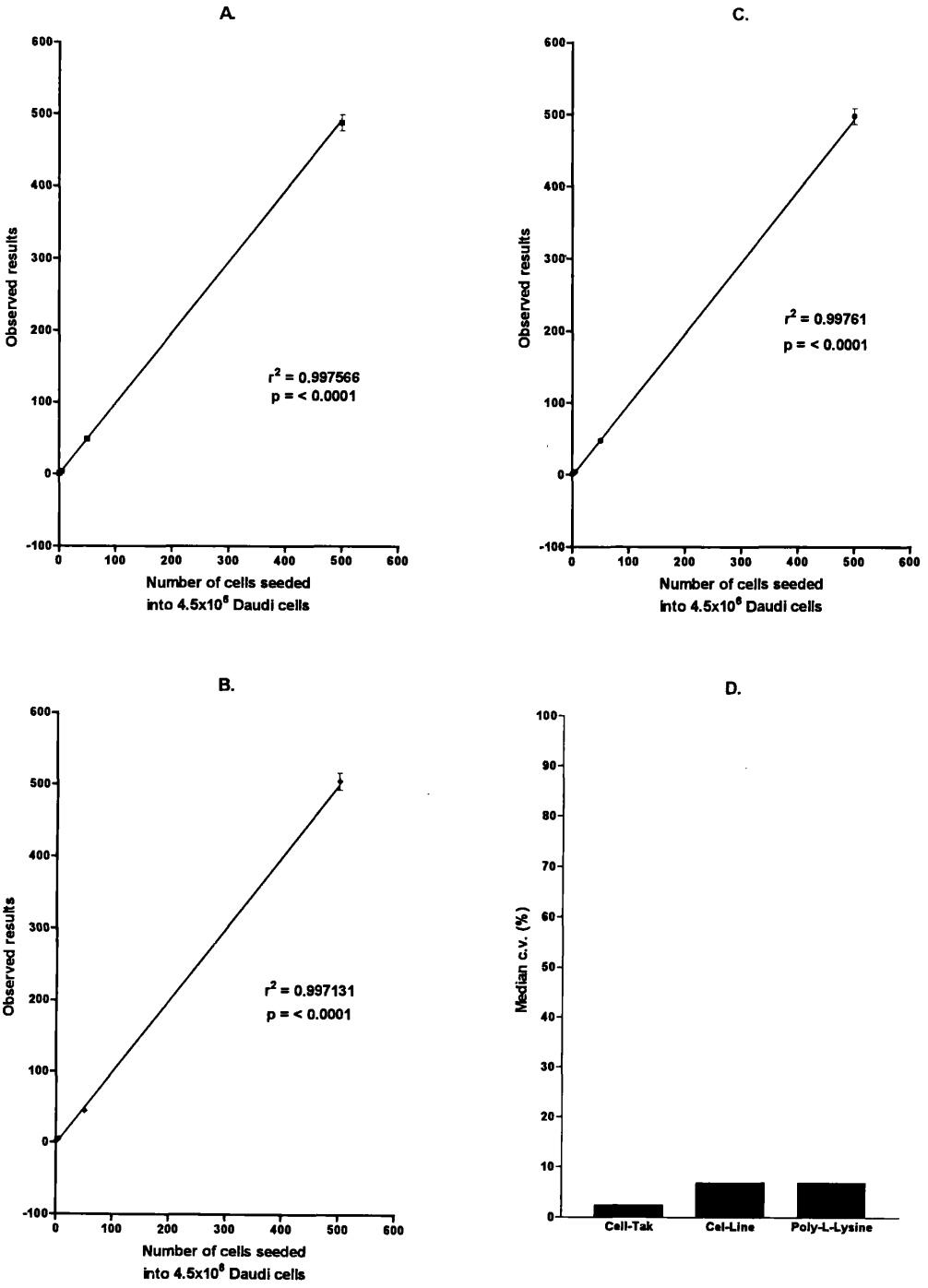

FIG. 1. Comparison of adhesion factors in the detection of seeded breast cancer cell lines into Daudi cells. The observed number of breast cancer cells is plotted against the number of cells seeded to illustrate intraassay variation for each adhesive system. (A) Cell-Tak Cell and tissue adhesive; (B) 0.1% solution of Poly-L-Lysine, (C) Cel-Line HTC Super Cured slides, and (D) direct interassay comparison within each adhesive system.

in patients without overt metastatic disease, although the small number of samples in this preliminary analysis precluded statistical evaluation. Circulating tumor cells were detected by APAAP staining in 6 of 6 blood specimens drawn from patients with metastatic disease. The concentration of tumor cells ranged from 10 to 38 per 2×10^6 MNC (median 22.5 cells). Six of 14 specimens obtained from patients without overt metastatic disease were positive for breast cancer cells (range 1–5 per 2×10^6 MNC, median 2 cells). In 2 of these subjects, repeat samples drawn after chemotherapy tested negative.

TABLE 1. DETECTION OF BREAST CANCER CELL LINES IN DAUDI CELLS USING ICC IN ALL THREE ADHESIVE SYSTEMS[a]

Slide coating	*% Tumor cell dilution*	*% Positive detected Tumor cells* $\bar{x} \pm$ *%CV*
Cell-Tak	0.1	0.1001 ± 0.0039
	0.01	0.0098 ± 0.0009
	0.001	0.0009 ± 0.0003
	0.0001	0.00008
Poly-L-Lysine	0.1	0.1005 ± 0.0047
	0.01	0.0097 ± 0.0010
	0.001	0.0010 ± 0.0002
	0.0001	0.00017
Cel-Line slides	0.1	0.1000 ± 0.0035
	0.01	0.0095 ± 0.0011
	0.001	0.0010 ± 0.0002
	0.0001	0.00010

[a]Triplicate samples of identical sizes (2×10^6 cells) at each dilution were stained with mAb A45-B/B3 using the APAAP technique.

Comparison of tumor involvement in BM and PBSC collections

Tumor cell involvement detected by ICC occurred less frequently in PBSC than in BM. Two PBSC collections were obtained from each of 3 patients without overt metastatic disease. One of these tested positive for circulating tumor cells detected by ICC. In contrast, tumor cells were detected in all BM collections obtained from 6 patients (4 metastatic, 2 nonmetastatic). The concentration of tumor cells detected in the immunocytochemically positive PBSC collection was 6 per 2×10^6 MNC. In contrast, the concentration of tumor cells in the immunocytochemically positive BM specimens ranged from 1 to 62 per 2×10^6 MNC, with a median of 13 cells (Fig. 2), ranging from 0.00005 to 0.0031% of total MNC. This represented a total tumor burden in the reinfused BM of $0.62–5.94 \times 10^4$ cells.

Diagnostic BM aspirates from 2 of 3 patients with nonmetastatic disease contained immunocytochemically detectable tumor cells. The first of the 2 positive aspirate samples was taken steady-state prechemotherapy. Ultimately, immunocytochemically negative PBSC collections were obtained from this patient. The second positive sample was taken from a patient 21 days post-ABMT. The negative BM aspirate sample was again steady-state prechemotherapy. However, the BM collection from this patient was immunocytochemically positive for tumor cells.

DISCUSSION

Several methods for detection of low-level tumor contamination have been reported, each with advantages and disadvantages. In a study by Schlimok et al. (12), who compared immunocytochemistry, conventional cytology, and histology, immunocytochemistry was clearly superior in detecting breast cancer cells in BM of patients without evidence of metastases. Studies of BM samples spiked with cancer cells from cell lines (13), followed by immunofluorescent staining and microscopic evaluation, have reported sensitivities of detection of 1/100,000 normal BM cells. Furthermore, others have reported a comparison of morphologic, flow-cytometric, and immunocytochemical techniques in which the immunocytochemical method was most sensitive, detecting 1 tumor cell in up to 400,000 BM cells (14). Cote et al. (15), using bone marrow aspirates from breast cancer patients, identified 1 positive cell among 50,000–100,000 hematopoietic cells. In a more recent study, a flow-cytometric method was shown to have a sensitivity of detecting 1 tumor cell in 200,000 MNC (16). The application of PCR in breast cancer tumor detection is limited because of the lack of availability of relevant molecular markers. Only recently has PCR been applied to the detection of occult tumor micrometastases in solid tumor

TABLE 2. GRATICULE DATA ILLUSTRATING THAT SIGNIFICANT CELL NUMBERS WERE NOT LOST AFTER ICC STAINING METHOD FROM CONTROL SLIDES IN BOTH BREAST CANCER AND DAUDI CELL LINES IN ALL THREE ADHESIVE SYSTEMS

Slide coating	*Cell type*	*Total No. of cells applied* ($\times 10^5$)	*Total No. of cells observed* ($\times 10^5$)	χ^2	*p*
Cell-Tak	Tumor	5.00	4.65	2.169	>0.50
Poly-L-Lysine	Tumor	5.00	4.53	2.093	>0.50
Cel-Line slides	Tumor	5.00	4.68	2.155	>0.50
Cell-Tak	Daudi	5.00	5.51	3.292	>0.50
Poly-L-Lysine	Daudi	5.00	5.74	3.931	>0.1
Cel-Line slides	Daudi	5.00	5.22	1.703	>0.50

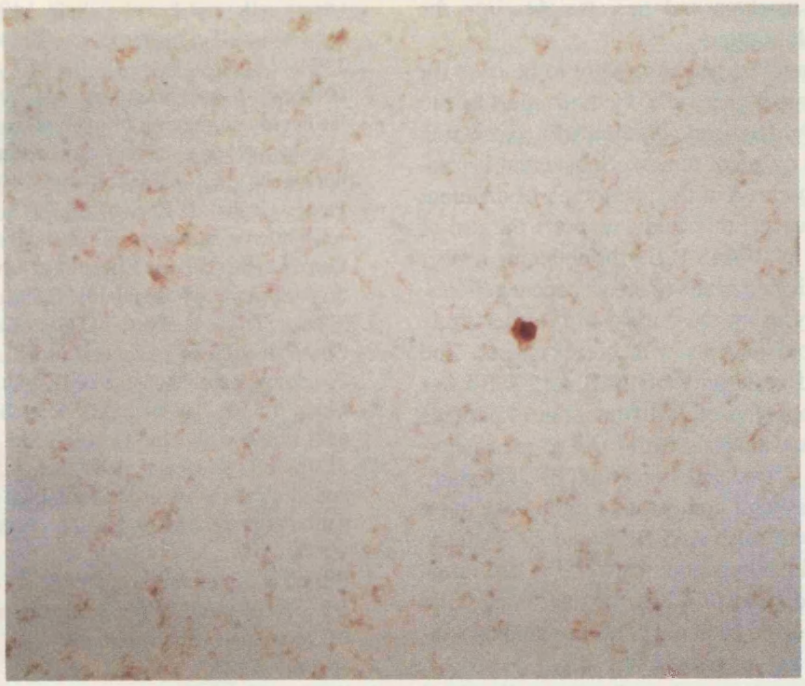

FIG. 2. Single cytokeratin-positive cell in bone marrow of a breast cancer patient. Cell-Tak adhesive system preparation stained with mAb A45-B/B3 using the APAAP technique (red stain). Hematopoietic cells are unstained.

malignancies (17). The sensitivity and specificity in large studies involving patients with solid malignancies have not yet been evaluated. Furthermore, Krismann et al. (18), in a study involving the detection of hematogenous lung cancer dissemination, concluded that reverse transcriptase-PCR (RT-PCR) analysis of CK19 mRNA expression in PB MNC gave rise to a significant number of false-positive results on the basis of nontissue-specific, constitutive low-level (illegitimate) transcription. Analogous to this study, similar restraints may hold true for breast and prostate cancer patients for whom CK19 RT-PCR has been suggested as a rapid, sensitive, and highly specific method for detection of systemic tumor spread.

A sensitive and reproducible method for detection and enumeration of epithelial tumor cells requires rigorous evaluation in model systems before application in the clinical setting to ensure both reliable and accurate results.

The routine immunocytochemical analysis of BM from patients with breast cancer can present some problems. In most previous studies, immunocytochemical techniques have been used that require examination of BM spread over several slides (12,15). Multiple marrow samples present problems for the patient and also for repeat sampling. The addition of predetermined numbers of cells to defined areas of coated adhesive microscope slides allowed cells to sediment, spread, and attach and led to better distribution of cells and, thus, easier analysis. As a consequence, a larger number of cells could be applied per slide. The adhesive nature of the test surfaces prevented significant cell loss during extensive liquid incubation and washing procedures. Our data illustrate that in multiple seeding experiments performed, one breast cancer cell in 1,000,000 Daudi cells could reliably and reproducibly be detected in the Cell-Tak and Cel-Line systems, and 1 in 100,000 could be detected with the Poly-L-Lysine system. The HTC Super Cured slides also kept samples from cross-contaminating and increased the capacity within each well. They also offered enhanced cell attachment and spreading capabilities within the well. Furthermore, up to 2×10^6 cells can be readily screened from clinical material obtained, without the need to examine multiple cytospin preparations, and we demonstrate the feasibility of ultrahigh sensitivity detection of tumor cells in PB and hematopoietic samples.

Subsequent morphologic confirmation of the malignant nature of CK-positive cells can be done by counterstaining of frozen slides prepared at the time of sample processing or by the application of fluorescence in situ hybridization (FISH) technology. FISH would facilitate the further biologic characterization of contaminating/disseminated tumor cells with respect to their metastatic potential and may provide a powerful approach that can be used to detect overamplification of

various prognostic markers and new insights into the course of an individual tumor.

If immunocytochemical techniques are to be used for the detection of breast cancer cells, methods must be developed to deal with heterogeneity of antigen expression within and between different tumors. Substantial heterogeneity has been observed in the phenotype of different breast cancers. However, this study supports the use of the broad-spectrum mAb A45-B/B3 for detecting cancer cells in hematopoietic cell grafts of breast cancer patients, and we are currently using these assays to further study clinical material from patients with breast cancer. The high specificity and sensitivity of mAb A45-B/B3 has been supported by analysis of BM from control patients and by double-marker analysis with mAb to mesenchymal marker proteins (CD45 and vimentin) (19). The study also addressed the issue of comparative immunostaining of BM specimens with mAb A45-B/B3 and CK2 (to the epithelial cytokeratin component 18, CK18) and indicated that downregulation of CK18 in micrometastatic carcinoma cells occurred in about 50% of samples analyzed, regardless of the primary tumor origin.

Correlation between the number of tumor cells added and the number of cells detected by ICC has been shown to be linear over a range of several logs in all three adhesive systems. This technique should allow quantitation of tumor cells in BM samples, and it may also prove useful in monitoring the response of micrometastatic disease to cytotoxic or hormonal therapy. In addition, screening for tumor cells by ICC should allow a more rational selection of candidates for autologous stem cell transplantation. The excellent sensitivity and specificity of the ICC method supports its introduction into tumor staging classifications. Results obtained by this method may then serve as a standard for alternative approaches to substantiate the claim of increased sensitivity over immunocytology applying mAb to CK.

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The Oncologist
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"Less is More": The Role of Purging in Hematopoietic Stem Cell Transplantation

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INTRODUCTION

The epithet "less is more" is usually applied to the essentials of good design, but it might be equally true of autologous blood or marrow transplantation. Ever since autologous marrow transplantation was first used to reconstitute recipients of high-dose chemotherapy or radiotherapy, there has been much discussion about the relative contribution of residual tumor cells in the graft to the occurrence of subsequent relapse. It was not until the early 1990s that this risk was finally confirmed by the use of gene marking [1]. A retroviral vector was used to mark a proportion of the autologous remission bone marrow from patients with acute myeloid leukemia (AML) before marrow infusion after high-dose therapy. Two recipients relapsed and both had leukemic blasts with the marker, the neomycin-resistance gene.

As the safety of autologous hematopoietic stem cell transplantation has increased, the use of high-dose therapy followed by stem cell "rescue" is becoming more widespread. The malignancies treated in this way include leukemia, lymphoma, myeloma, neuroblastoma, breast cancer, and ovarian tumors. In each of these conditions a number of important questions should be addressed: Can we identify and quantitate tumor cells in the grafts and establish their oncogenic potential? If so, how best can we remove them? Can they be removed without compromising the graft, and will such purging produce a clinically significant reduction in relapse risk? Finally, will the procedure be cost-effective?

QUANTIFICATION AND CHARACTERIZATION OF CONTAMINATION

Contaminating tumor cells are inadvertently harvested along with hematopoietic stem cells and subsequently infused into patients undergoing high-dose therapy and transplantation. Such cells may be responsible for a proportion of relapses, but the extent of this clinical problem is still unknown and is likely to be determined by the frequency or number and biological properties of tumor cells in different types of hematopoietic harvests (marrow versus non-mobilized blood versus mobilized blood).

Several methods for detection of low-level tumor contamination have been reported. Generally, hematopoietic and immune cell tumors are detected using molecular techniques, and solid tumor micrometastases are detected by immunocytochemical assays (Table 1).

Table 1. Methods of detecting low-level tumor contamination

Tumor type or disease	Method	Reference
Acute lymphocytic leukemia	Specific chromosomal translocations; Identifiable immunoglobulin gene translocation; T-cell receptor gene translocation	[2]
Acute myeloid leukemia	Specific chromosomal translocations; Cross-lineage rearrangements of immunoglobulin; Gene translocation; T cell receptor gene translocation; Immunological marker analysis	[3]
Chronic myelogenous leukemia	Breakpoint region of Philadelphia chromosome; FISH to mark fusion of *bcr* and *abl*	[4]
Lymphoma	bcl2 translocations	[5]
Solid tumors	Presence of epithelial cells in marrow	[6]

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Peripheral blood stem cell collections and bone marrow from patients with breast cancer have been evaluated for the presence of tumor cells using sensitive immunocytochemical techniques. It is believed that peripheral blood progenitor cell collections are less likely than bone marrow harvests to be contaminated with tumor cells [7]. Tumor cells and progenitor cells both are mobilized after chemotherapy and administration of hematopoietic growth factors (granulocyte colony-stimulating factor) [8]. Several investigations have demonstrated tumor cell contamination of 10% to 30% of leukapheresis products used for transplants [7], and, in patients with neuroblastoma, stem cell harvests have been shown to contain contaminating tumor cells irrespective of marrow involvement [9]. In other studies in patients with breast cancer or lymphoma, tumor-contaminated peripheral blood stem cell collections resulted in a poor clinical outcome [10, 11].

The sensitivity of immunocytochemical methods for epithelial tumor cell detection is limited by the number of cells examined [12]. The 95% confidence limit of sensitivity is approximately one tumor cell in 10^5 nucleated cells for 3×10^5 cells evaluated. The time-consuming aspects of this assay might be solved by automated image analysis. The application of polymerase chain reaction (PCR) in breast cancer tumor detection is limited because of the lack of relevant molecular markers. Only recently has PCR been applied to the detection of occult tumor micrometastases in solid tumor malignancies [13], but the sensitivity and specificity in large studies involving patients with solid malignancies have not yet been evaluated. Reverse-transcription-PCR produced a significant number of false-positive results in detection of hematogenous lung cancer dissemination [14]. Although the false-positive rate can be decreased by decreasing the number of PCR cycles and by using fewer cells, this limits the sensitivity of the method. The likelihood of detecting legitimate epithelial tumor cells can be increased by including other cytokeratins or other genes, increasing the workload and cost, as well as the risk of inappropriate results because of tumor cell heterogeneity. This approach may not become routinely feasible until technical development of automated gene detection equipment, similar to that being applied in mutation analysis, becomes available.

It is likely that technological developments in automated detection of genes, image analysis, and hematopoietic stem cell harvest manipulations will have a major impact on this field. There may be a lack of coincidence between molecular detection of tumor cells and their clonal growth in culture, which may have implications for their clinical significance. These discrepancies may be associated with specific gene defects that confer aggressiveness and treatment resistance on the tumor cells. Molecular characterization would facilitate the further biological properties of contaminating tumor cells and may provide a powerful approach that can be used to detect over-amplification of various prognostic markers, as well as new insights into the course of an individual tumor. This may be as important as a determinant of time to treatment failure than the number of tumor cells inadvertently infused. This is an area of active research and we have recently developed methods for simultaneous immunocytochemistry and interphase FISH for the detection of chromosomal abnormalities and/or oncogene over-amplifications in cytokeratin-positive epithelial tumor cells in hematopoietic stem cell products (Fig. 1). It may prove necessary to evaluate multiple genes to confirm the nature of the tumor cell being detected, requiring the development of novel reagents and methods to detect specific genes and gene mutations.

Methods of Tumor Purging

The treatment of residual disease in patients after chemotherapy or transplantation is often referred to as tumor purging, but here we will concentrate on the strategies used to remove contaminating tumor cells from hematopoietic stem cell grafts.

In chemotherapy-responsive tumors, research has focused on designing additional treatments to purge autologous stem cell products. Such approaches have met with variable success, a predictable outcome given the highly heterogeneous nature of the malignancies studied. The intensity of chemotherapeutic purging strategies is limited in all cases by the toxicity of the drug on the hematopoietic stem cell in the graft. Analysis of registry data from the European Blood and Marrow Transplant group (EBMT) of 270 recipients of purged versus 224 recipients of non-purged grafts [15] showed no detrimental effect on engraftment. This analysis included a broad cross-section of chemotherapeutic regimens and suggested that hematopoietic stem cells are relatively resistant to these agents. The study did not show any benefit of tumor purging with respect to progression-free or overall survival. This may have been due to residual chemoresistant tumor cells within the graft, within the patient, or both. More intensive purging chemotherapy of hematopoietic stem cell grafts may be beneficial in some cases, but eventually this will reach the threshold of toxicity for the stem cell. Efforts have been made to increase this threshold by introducing the multidrug resistance gene into human hematopoietic stem cells.

Immunological approaches to ex vivo purging have been used for a number of years and have involved cytokines [16], cellular strategies [17], and specific monoclonal antibodies [18-20]. These approaches are effective in leukemia resistant to chemotherapy [21]. The first clinical use of monoclonal antibodies was for the ex vivo purging of T cells from allogeneic bone marrow grafts [22] and was

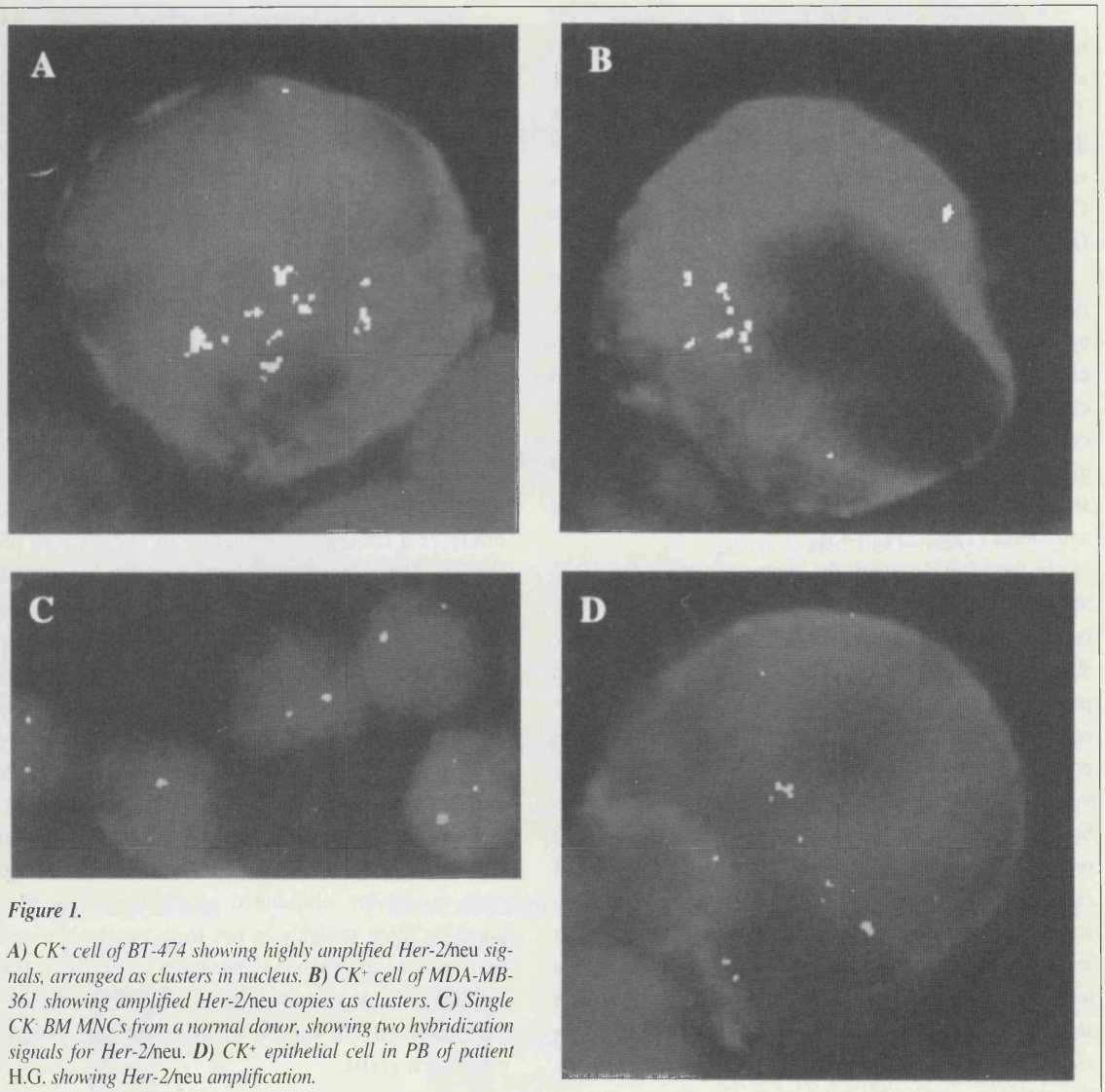

Figure 1.

*A) CK⁺ cell of BT-474 showing highly amplified Her-2/*neu *signals, arranged as clusters in nucleus.* ***B)*** *CK⁺ cell of MDA-MB-361 showing amplified Her-2/*neu *copies as clusters.* ***C)*** *Single CK⁻ BM MNCs from a normal donor, showing two hybridization signals for Her-2/*neu. ***D)*** *CK⁺ epithelial cell in PB of patient* H.G. *showing Her-2/*neu *amplification.*

followed by the use of monoclonal antibody and complement to deplete residual ALL cells from autologous bone marrow in four patients [23]. The treatment was safe and allowed normal engraftment in all patients.

Leukemias and lymphomas have proved good targets for ex vivo purging, since lineage and differentiation stage-specific antigens are known and high-dose therapy followed by bone marrow transplantation is an established treatment. Toxin-conjugated monoclonal antibodies, or non-conjugated monoclonal antibodies with additional complement, have been used in AML [24], ALL [18, 25], lymphoma [26], and myeloma [27, 28]. These monoclonal antibodies are targeted at differentiation antigens or, in the case of some B-cell malignancies, against surface immunoglobulin molecules, both of which are absent on normal hematopoietic stem cells [29, 30]. As autologous hematopoietic stem cell "rescue" after high-dose therapy becomes more commonly used in the management of solid tumors, there is a need for suitable purging strategies. Immunotoxin-conjugated monoclonal antibodies have been shown to selectively kill breast cancer cells in autologous graft material [31], although this awaits clinical application.

Apart from targeting toxin-mediated or complement-mediated lysis of tumor cells within grafts, the flexibility of monoclonal antibody technology can also enable physical separation of the normal and residual tumor cells. Monoclonal antibodies can be bound to solid phases such as plastic tissue-culture flasks or magnetic/paramagnetic beads. Positive selection describes retention and use of the selected cells of interest, also called "enrichment." Negative selection describes selection and disposal of the sorted cells, also called "depletion."

A major advance in the field of tumor purging was made with the demonstration that hematopoietic engraftment potential resides within the CD34+ cell subset [32]. Thus, instead of tumor-specific depletion strategies, in the majority of cases it has become possible to positively select normal cells. Since tumor cells rarely express CD34, the end product should, at least in theory, be free from contaminating tumor cells.

In vitro sterile cell sorting has been a common research tool for decades, but the difficulties in scaling-up to clinical ex vivo stem cell selection cannot be overemphasized. Clinical-grade monoclonal antibodies and closed system devices are required and evaluation protocols have had to be devised. Currently, five methodologies have completed at least phase I clinical trials, and all are based upon one or more anti-CD34 monoclonal antibodies (Table 2) [33-43].

In the clinical setting, the choice of device for CD34 selection for indirect tumor purging is a matter of personal opinion and circumstance, since comparative trials have shown them to be broadly comparable in terms of yield and purity. Direct comparison between trials is difficult, as the enumeration of CD34+ cells within hematopoietic stem cell products is not standardized and there is extreme interassay variability [44]. Despite limitations, many useful studies have been and are being performed, and it is likely that the only true test of purging efficacy can come from studies of clinical outcome. It is improbable that we can ever produce an entirely tumor-free autologous graft from patients with malignant diseases of the bone marrow or with tumors that metastasize to the marrow. In addition to positive selection strategies, immunomagnetic separation methods have been used to deplete tumor cells from grafts [45-48], and interest in this approach is increasing as tumor-specific determinants are identified in solid tumors.

Table 2. Cell sorting devices for clinical application

Device (company)	Methodology	Reference
Ceprate (CellPro) (Bothell, WA)	Biotinylated anti-CD34 mAbs and avidin	[33-36]
Isolex (Baxter) (Round Lake, IL)	Magnetic ferrous beads coupled to specific mAbs	[37]
FACS (Systemix) (Palo Alto, CA)	Sorting of electrostatically charged fluid droplets containing fluorochrome-labeled cells	[38, 39]
Cellector (AIS, Inc.) (Santa Clara, CA)	Panning; binding of Fc portion of mAb to flask with subsequent binding of cells	[40, 41]
MACS (Amcell) (Sunnyvale, CA)	mAbs conjugated with paramagnetic microparticles	[42, 43]

mAbs = monoclonal antibodies.

Many groups believe that tumor purging can be improved with multistep strategies (Fig. 2). Methods are being developed for the positive selection of epithelial tumor cells on the basis of specific surface-antigen expression that will be used as a second-stage depletion strategy after initial positive selection of CD34+ cells. Also, a system using immunoaffinity selection of CD34+ cells followed by FACS sorting for HLA-DR expression was able to produce bcr/abl positive stem cell products for more than 80% of donors with chronic myeloid leukemia in early chronic phase [49].

Double-sorting strategies are time consuming and expensive and will always reduce the yield of stem cells. An alternative approach is CD34+ cell selection with subsequent ex vivo expansion. This has a number of attractions, especially that a small harvest volume produces a consequent reduction in the absolute tumor burden. The culture conditions in which hematopoietic stem and progenitor cells can be expanded in vitro do not support the growth of epithelial tumor cells, thus producing specific purging. This may not be the case, however, with many hematological tumors. It has been shown that residual myeloma cells can survive ex vivo expansion [50], although more encouraging results have been seen in lymphoma [51]. Initial concerns about the potential loss of repopulating pluripotent hematopoietic stem cells in ex vivo culture and the risk of subsequent graft failure are unfounded as the high-dose therapy given to these patients is not truly myeloablative and such sorted and expanded cells have safely reconstituted patients with breast cancer [52]. Large randomized clinical trials will be needed to determine the benefit of using such grafts.

Conclusion

There is no doubt that our abilities to both accurately enumerate tumor cells in autologous hematopoietic stem cell products and to purge them to below detectable levels are improving and will continue to do so. However, the value of tumor purging is still debated. While one study [1] showed that tumor cells in relapsed patients can be shown to be derived from the infused graft, it did not demonstrate that this was the only source of tumor cells and could not prove that efficient purging would have prevented relapse in either of the two cases.

It is likely that the benefit of purging will be dependent upon the tumor type and stage of disease, as well as the degree of purging possible in the individual case. To date, most trials in which progression-free and overall

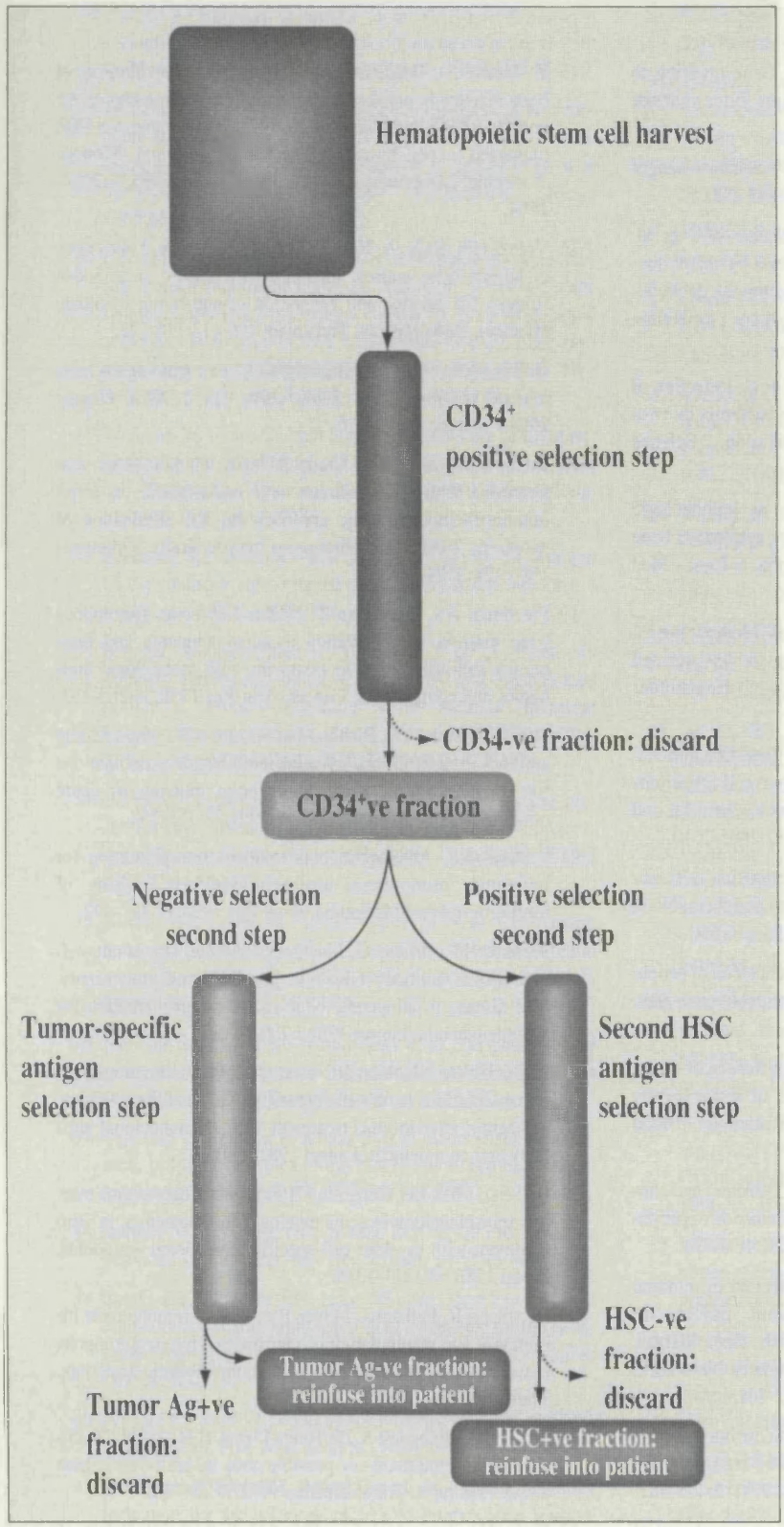

Figure 2.

survival have been evaluable have been in hematological malignancies. A six-year single center study showed no benefit of purging in patients with non-Hodgkin's lymphoma [53], nor did a large study from EBMT [15]. Registry data from the American Bone Marrow Transplant Registry (ABMTR) [54] showed an outcome which was worse in patients with AML who received syngeneic grafts from identical siblings than in those who received autologous non-purged grafts. Although one must be cautious in comparing recipients of autologous transplants with those receiving allogeneic grafts, these transplants were from genetically identical siblings and might be considered analogous to the ultimately "purged" autologous graft. Given these results, we might conclude that there is no role for purging in autologous transplantation, but this ignores the heterogeneity of different tumors and the fact that techniques for treatment of in vivo residual disease are improving.

Irrespective of the contribution of residual tumor cells in vivo to disease relapse, the infusion of tumor cells into an immunocompromised host is undesirable and purging strategies should be optimized. Whether such approaches are cost-effective will depend upon their concomitant morbidity, together with effects on progression-free and overall survival. The technology of stem cell graft engineering is expanding rapidly; the challenge is to design and conduct clinical trials to properly evaluate these new techniques.

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Standardization of the immunocytochemical detection of cancer cells in BM and blood: I. establishment of objective criteria for the evaluation of immunostained cells

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Background

Detection of isolated tumor cells (TC) in BM from carcinoma patients can predict future relapse. Various molecular and immunocytochemical (ICC) methods have been used to detect these cells, which are present at extremely low frequencies of 10^{-5}–10^{-6}. The specificity and sensitivity of these techniques may vary widely. In 1996, a European ISHAGE Working Group was founded to standardize and optimize procedures used for the detection of minimal residual disease. We have attempted to develop objective criteria for the evaluation of immunocytochemically identifiable cancer cells.

Methods

An interlaboratory ring experiment was performed, to compare the screening and detection of micrometastasis-positive events between different laboratories. The discrepant results induced us to establish a common consensus on morphological criteria applicable to the identification of immunostained micrometastatic TC.

Results

Based on this consensus evaluation, we propose a classification of stained elements into three groups: (1) 'TC's show pathognomonic signs of epithelial TC-nature, as defined by a clearly enlarged nucleus or clusters of ≥ 2 immunopositive cells. (2) 'Probable TC's represent morphological overlap between hematopoietic cells (HC) and TC which lack pathognomonic signs of TC-nature, but do not exhibit clear morphological features of HC. These cells are considered as TC if control staining with an isotype-specific, unrelated Ab is negative. (3) 'TC-negative' cells are defined as 'false positive' HC, skin squamous epithelial cells and artefacts.

Discussion

The proposed classification of immunostained events is a first step towards the development of standardized immunocytochemical assays for the detection of occult micrometastatic TC in BM or blood.

Keywords

micrometastasis, bone marrow, immunocytochemistry, morphology, standardization, classification, carcinoma, neoplasm metastasis.

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Introduction

The detection of rare epithelial tumor cells in mononuclear cell (MNC) fractions obtained from BM aspirates, peripheral blood (PB) samples or leukapheresis products by an immunocytochemical (ICC) method requires high specificity and sensitivity of the read-out system. The results of ICC staining are influenced by:

- Variability in the expression and distribution of the targeted epithelial antigen.
- Cross-reactivity of the applied monoclonal antibody (MAb) with related epitopes present on hematopoietic cells.
- Interactions with Fc-receptor-bearing leukocytes.
- The occasional illegitimate expression of epithelial or tumor-associated Ag in normal hematopoietic cells (HC).
- The specificity of the visualization step/substrate reaction of the ICC assay.
- The consistency of the screening process.

As several reports have shown that false positive events can occur, the mere detection of an immunostained event might not be sufficient to conclude that a tumor cell is present in the specimen [1–4]. Although the use of negative controls (e.g. unrelated isotype control Abs) increase the diagnostic accuracy of ICC assays, both the illegitimate expression of the targeted epithelial Ag in HC and the cross-reactivity of the applied MAb might result in a specific positive reaction in non-epithelial cells. Some patient samples may possess both unspecifically-stained hematopoietic cells, as well as true positive epithelial tumor cells [3]. These patient samples would be categorized as tumor cell-negative, or not evaluable, if immunostained cells were also detected in the negative controls. Therefore, in addition to the evaluation of immunopositivity, an examination of the morphology of individual immunopositive cells should add important information about the nature of these cells.

The European ISHAGE Working Group for Standardization of Tumor Cell Detection was founded to meet the requirements for standardization and quality assurance of procedures used for the detection of cancer micrometastases in BM, blood and leukapheresis products. The group has the following aims:

- Develop and publish a consensus on the read-out of immunocytochemical assays (i.e. classify stained events as true-positives, artefacts and questionables).
- Evaluate the specificity and sensitivity of different MAbs against cytokeratins and mucins using the same Ab-preparations and protocols on BM samples from control and cancer patients.
- Standardize pre-analytical variables, such as the site and quality of BM aspirates and the preparation of slides (e.g. cytospins versus adhesive slides).
- Evaluate different tumor-cell enrichment devices for positive and negative enrichment strategies.
- Evaluate different automated scanning microscopes.
- Evaluate new detection methods (e.g. reverse transcriptase-PCR (RT-PCR), flow cytometry).

During 1997 a 'ring experiment' was performed by the European Working Group to assess the coherence between different observers, with regards to both the screening itself and to the morphological evaluation of ICC-detected cells. This paper is the result of a collaboration between seven European groups and it should form the basis for standardization of the evaluation of immunostained micrometastatic carcinoma cells in BM and blood.

Materials and methods

Collection of BM

After informed consent, $\leqslant 40$ ml BM was aspirated from anterior and posterior iliac crest, bilaterally, from breast-carcinoma patients at the time of surgery and from posterior iliac crest, unilaterally, from non-carcinoma BM donors. All aspirations were performed into heparinized tubes.

Processing of BM mononuclear cell samples

The BM aspirates were diluted 1:1 in PBS (Gibco, Life Technologies, Roskilde, Denmark) and separated by density centrifugation through Lymphoprep (Nycomed, Oslo, Norway). MNC were collected from the interphase layer, washed twice in 10% FCS (Biological Industries, Kibbutz Beit Haemek, Israel) in PBS (Gibco) and resuspended to 1×10^6 cells/ml. The cells were then centrifuged down to polylysine-coated glass slides in a Hettich cytocentrifuge (Tutlingen, Germany) (0.5×10^6 MNC/spot). The slides were air-dried overnight and stored at -80°C until immunostaining was performed.

Immunocytochemical staining: APAAP technique

The cytospins were fixed 10 min in acetone, prior to 30 min incubation (moist chamber) with the AE1 and the AE3

pancytokeratin MAbs (Sanbio, Uden, The Netherlands) (IgG_1, 1.1 μg/mL of each). No additional fixative was applied prior to the incubation with A45-B/B3 MAb (Micromet, Munich, Germany) used in the ring experiment.

Specimens used for negative controls were incubated with mouse myeloma Ig (IgG_1) (Bionetics, Maryland, USA) or mouse anti-FITC (IgG_1) (Mikromet) instead of AE1/AE3, in the same concentration. Then followed 30 min incubation with polyvalent rabbit anti-mouse Ab (DAKO, Code no. Z 259, 1:25 dilution) and, subsequently, preformed complexes of AP/monoclonal mouse anti-AP (APAAP-complex) (DAKO, Code no. D 651, diluted 1:25 to 1:50). The slides were washed twice in tris-HCl (5 min) between each incubation. The colour reaction was developed by 10 min incubation with New Fuchsin solution (0.26%) (Aldrich Chemical Company, Milwaukee, Wisconsin), containing 0.65% (w/v) naphtol-AS-BI phosphate and 0.45% (w/v) levamisole (Sigma, St Louis, MO). Some of the slides were counter-stained with hematoxylin for 1 min to visualize nuclear morphology. Four slides were prepared for each cytokeratin staining and four additional slides were prepared for negative control. The slides were mounted in Kaiser's glycerine–gelatine (Chroma Gesellschaft Gmbh, Germany).

Immunocytochemical detection of positive cells

The slides used for the description of morphological criteria were evaluated by light microscopy by a pathologist. By means of an 'England Finder' (Graticules Ltd, Tonbridge, Kent, UK) the coordinates of positive events were noted, facilitating later reidentification of detected cells. The morphological evaluation and classification of immunopositive cells were judged by all laboratories and consensus obtained.

Results

European ring experiment results

In order to analyze interlaboratory variation in the screening efficiency and read-out of micrometastasis by ICC, cytospins containing BM MNC from 18 carcinoma patients and from seven non-carcinoma donors were circulated among seven European laboratories working in the field of micrometastasis. The participants screened and evaluated the cytospins in a blinded fashion, independently of each other. Their individual diagnoses are shown in Tables 1, 2 and 3. Considerable differences were revealed, both in the number of cells scored positive in each sample and in the fraction of positive and negative samples. Twelve clinical marrow samples from carcinoma patients, judged positive by the reference group were distributed (Table 1). Only three were scored positive by all other centers that evaluated them and one was scored negative by all the others. For the remaining, both positive and negative results were obtained. Of six BM samples from carcinoma patients, judged negative by the reference group (Table 2), two marrows were concluded positive by two or three of the others, among which two had chosen the diagnosis 'TC?' on one of the specimens. Seven BM samples from non-carcinoma donors (Table 3) were evaluated as negative by the reference group. Most centers confirmed the negative findings; only four groups diagnosed 1–2 tumor cells out of a total of 48 evaluations.

The observed discrepancies between the different groups may, in part, be caused by variations in the precision of the screening process itself. However, the subsequent discussion in the Working Group revealed that the main reason for this inconsistency was a considerable variation in the read-out, i.e. the conclusive evaluation of individual cells. A consensus on classification of stained events as true/false-positives, questionables and artefacts is therefore urgently needed.

Morphology of immunostained cells

To standardize the evaluation of immunopositive events, a detailed examination of the characteristics of micrometastases and false-positive HC was performed. Figures 1 and 2 show photomicrographs of a morphological spectrum of tumor cells (TC) detected in patients with high micrometastatic load and positive cells detected in negative-control specimens (from various sources). A standard APAAP ICC protocol, with anti-cytokeratin MAbs and isotype-matched negative-control MAbs has been used, as described above. There is a large variation in the morphology among the immunopositive cells of both epithelial and hematopoietic origin. In Figure 1, typical morphological differences between TC (left picture of each figure letter) and false positive HC (right picture of each figure letter) are presented. Figure 2 shows examples of occasional morphological similarities between selected TC (left pictures) and negative control-positive cells (right pictures). Clusters of two or more positive cells represent a pathognomonic criterion for epithelial cell nature (Figure 1a,c, left). The size of micrometastatic breast-carcinoma cells may vary from similar to, to >3 times the size of the surrounding HC. The TC nuclei are typically enlarged, with irregularly fine-

Table 1. Detection of tumor cells in BM samples from carcinoma patients judged as cytokeratin-positive by the reference group[1]

Cytospin no.	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Ref. group
25.1/25.2	0	4 +[2]	0	n.d.[3]	n.d.	9 +	2 +
30.1/30.2	0	2? +[4], 1 +	0	0	n.d.	n.d.	1 +
36.1/36.2	n.d.	0	0	0	n.d.	n.d.	1 +
39.1/39.2	n.d.	2 +	2 +	n.d.	n.d.	3 +	2 +
40.1/40.2	n.d.	n.d.	0	n.d.	n.d.	2 +	1 +
42.1/42.2	n.d.	n.d.	1 +	n.d.	n.d.	16 +	1 +
26.1/26.2	1? +	0	0	n.d.	n.d.	4 +	7 +
28.1/28.2	0	0	1 +	0	n.d.	n.d.	3 +
33.1/33.2	1407 +	n.d.	1049 +	1200 +	n.d.	n.d.	600 +
35.1/35.2	n.d.	3 +	0	6 +	n.d.	n.d.	7 +
37.1/37.2	n.d.	4 +	0	n.d.	n.d.	1 +	4 +
41.1/41.2	n.d.	n.d.	0	n.d.	n.d.	1 +	3 +

[1] *ICC analysis of cytospins containing a total of 1 × 10⁶ MNC from each BM sample were analyzed by the indicated laboratories. The slides were incubated with the anti-cytokeratin MAb A45-B/B3 and immunopositive cells visualized by APAAP/New Fuchsin. Then the cytospins were screened by light microscopy for immunopositive cells and the detected number of tumor cells was registered.*

[2] *Number of tumor cells detected.*

[3] *n.d. = not done (i.e. not analyzed by that particular laboratory).*

[4] *? indicates a questionable tumor cell.*

Table 2. Detection of tumor cells in BM samples from carcinoma patients judged as cytokeratin-negative by the reference group[1]

Cytospin no.	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Ref. group
27.1/27.2	1? +[2]	1 +[3]	0	n.d.	n.d.	1? +	0
29.1/29.2	0	0	0	0	n.d.	n.d.	0
31.1/31.2	1 +	n.d.	0	1 +	n.d.	n.d.	0
32.1/32.2	0	n.d.	0	0	n.d.	n.d.	0
34.1/34.2	n.d.	0	0	0	n.d.	n.d.	0
38.1/38.2	n.d.	0	0	n.d.	n.d.	0	0

[1] *ICC analysis of cytospins containing a total of 1 × 10⁶ MNC from each BM sample were analyzed by the indicated laboratories. The slides were incubated with the anti-cytokeratin MAb A45-B/B3 and immunopositive cells visualized by APAAP/New Fuchsin. The cytospins were screened by light microscopy for immunopositive cells and the number of tumor cells was registered.*

[2] *? indicates a questionable tumor cell.*

[3] *Number of tumor cells detected.*

stippled chromatin (Figure 1c,e,g, left). They may have an irregular nuclear membrane (not shown). On the other hand, TC nuclei may appear anonymous and not clearly different from those of surrounding HC (Figure 2a, left). If nucleoli are present, they are often inconspicuous (Figure 1d, left).

The cytoplasmic staining can be strong and evenly distributed in TC (Figure 1f, left). More often, however, the TC-staining pattern is heterogeneous, with asymmetrically distributed strongly and weakly-stained parts (Figure 1a,b, g, left). TC often have a ring-like perinuclear condensation of color, with reduced staining intensity towards the periphery (Figure 1a,g,k, left). Furthermore, some TC may show irregular granular (Figure 1c, left), or coarse filamentous cytokeratin staining (not shown). Irregular condensations of the color may be a result of TC degeneration (Figure 1h, left).

Table 3. Detection of tumor cells in BM samples from non-carcinoma donors[1]

Cytospin no.	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Ref. group
3.1/3.2	0	0	0	0	0	0	0
5.1/5.2	0	0	0	0	0	0	0
7.1/7.2	n.d.	2 +[2]	0	0	1 +	0	0
12.1/12.2	0	0	0	0	0	0	0
14.1/14.2	0	0	0	1 +	0	1 +	0
16.1/16.2	0	0	0	0	0	0	0
21.1/21.2	0	0	0	0	0	0	0

[1] *ICC analysis of cytospins containing a total of 1 × 10⁶ MNC from each BM sample were analyzed by the indicated laboratories. The slides were incubated with the anti-cytokeratin MAb A45-B/B3 and immunopositive cells visualized by APAAP/New Fuchsin. The cytospins were screened by light microscopy for immunopositive cells and the detected number of tumor cells was registered.*

[2] *Number of tumor cells detected.*

In false-positive HC the cytoplasmic color tend to have an even, regular distribution (Figure 1g, right). Others exhibit a pale juxtanuclear area, possibly representing the Golgi apparatus in plasma cells (Figure 1a, right). Many HC have a microvacuolar cytoplasm (Figure 2d, right), whereas some only possess one or a few tiny, characteristic pin-point vacuoles (Figure 1c,d,e,i, right). Some HC possess regular cytoplasmic granules (Figure 1h, right). Breast-carcinoma cells may present one, or a few larger vacuoles (Figure 1i, left) as cytologists often find them in ordinary metastases from breast carcinoma of the lobular type. Even though false-positive HC may have strong cytoplasmic staining, the color covers their nucleus less frequently than does the color of epithelial cells (Figure 1f,i). The cytoplasmic border is usually sharp, or slightly irregular, in both TC and HC. However, in several HC the border possesses one or a few characteristic pouches (Figure 1j, right), or regular cytoplasmic snouts (Figure 1k, right).

As shown in Figure 2, occasionally TC (left) and rare false-positive HC (right) may have morphological similarities. The TC nucleus is not always obviously enlarged and it may have an anonymous chromatin texture (Figure 2a, left). The cytoplasmic staining may be as strong in HC as in TC (Figure 2b,e,f) and some TC show very weak staining (Figure 2c, left). A regularly microvacuolated cytoplasm usually indicates HC nature (Figure 2d, right), but multiple defects/holes in a degenerated TC cytoplasm (Figure 2d, left) may simulate microvacuoles. As mentioned, breast carcinoma TC may possess larger vacuoles (Figure 1i, left). These vacuoles should not be confused with pin-point vacuoles in HC. Representing adenocarcinoma cells, the TC often have an eccentrically localized nucleus (Figure 1j, 2e, left). This is also the case for several false-positive HC (Figure 2e, right), indicative of a plasma cell phenotype. The cytoplasmic staining in TC does not necessarily cover the nucleus (Figure 2a,e, left) and the false-positive HC staining sometimes does, at least partially (Figure 2d,f, right).

In Figure 3, examples of various other stained elements are presented. Surface epithelial squamous cells from the skin of the laboratory staff may contaminate microscopic slides. They are usually easy to recognize (Figure 3a, left), but small and rounded fragments of them may occasionally be difficult to interpret (Figure 3a, right). Deposits of red material with a central clear area (Figure 3b, left), or bubbles (Figure 3b, right), may imitate real cells. In some instances artefacts and partially destroyed immunostained cells are difficult to differentiate (Figure 3c,d,e,f).

Negative controls

The morphological overlap between TC and false-positive HC emphasizes the need for isotype-matched negative controls, in addition to the morphological evaluation. The number of cells in the negative controls should always equal the number in the diagnostic specimens. If positive cells are present both in the negative control and in the specific test, their morphology should be carefully evaluated and compared. If tumor-like positive cells are present in the

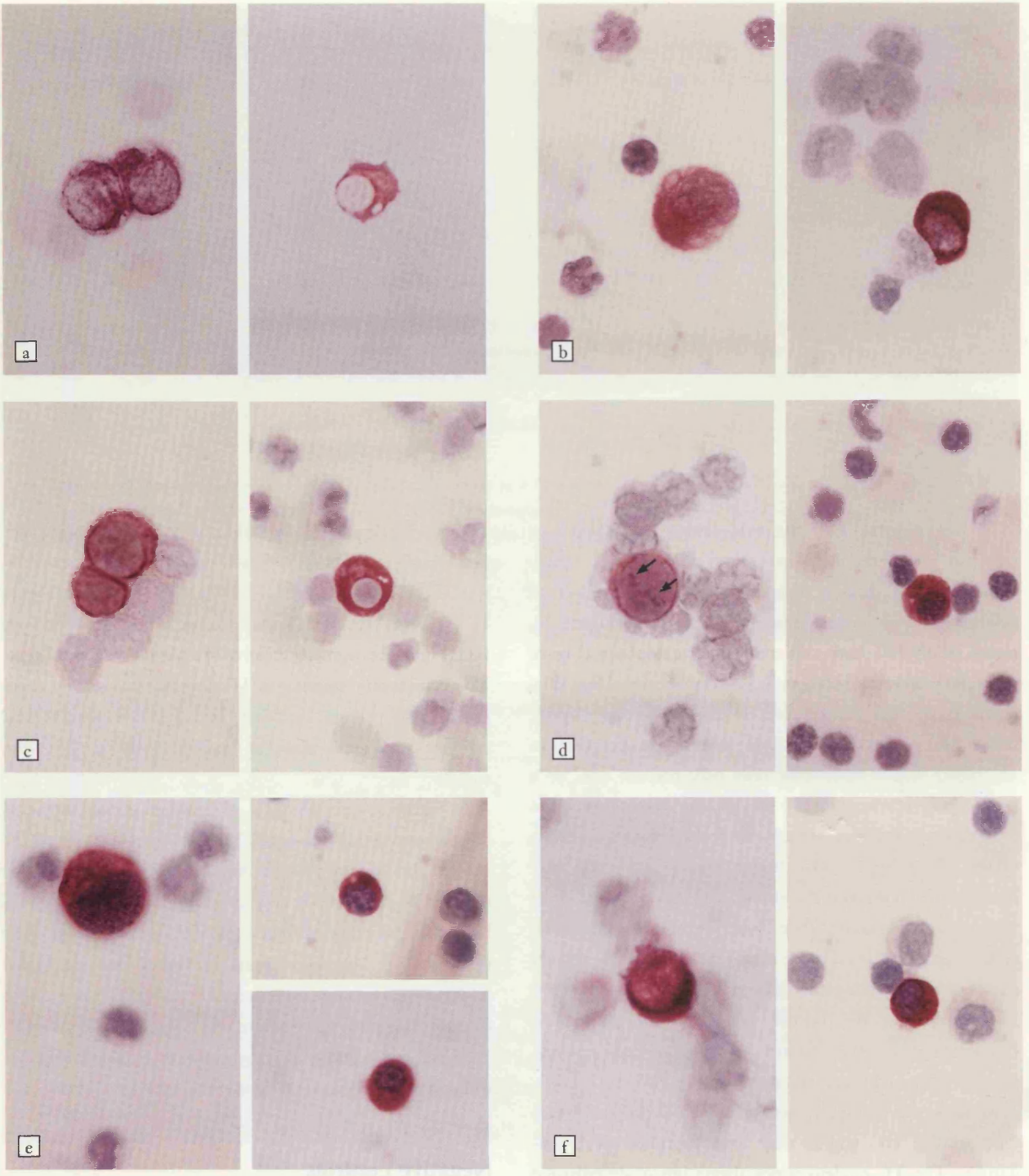
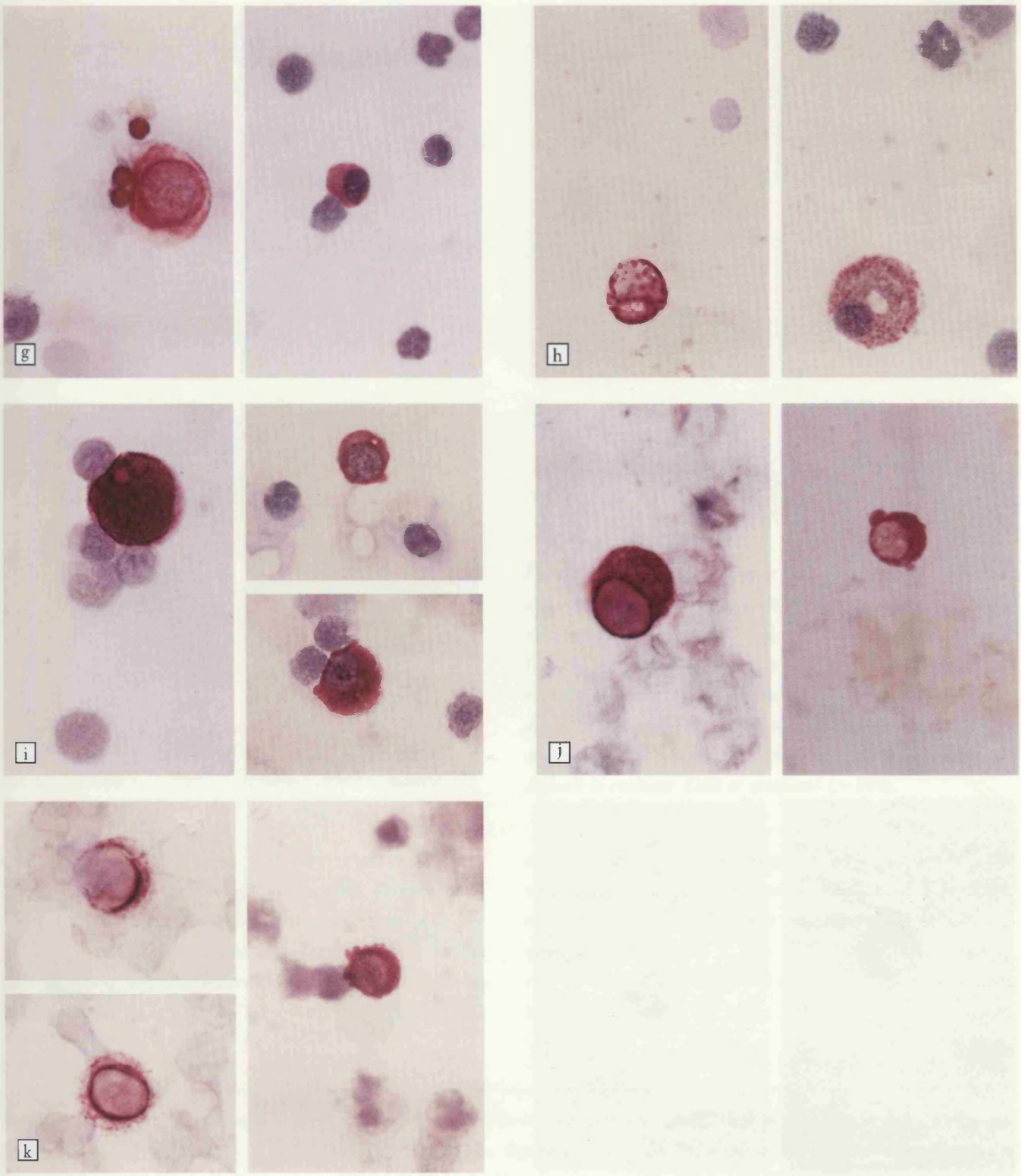


Figure 1. *Morphological differences between tumor cells (TC) and false-positive hematopoietic cells (HC). For each figure letter the* **left** *pictures show TC stained with AE1-AE3, APAAP and the* **right** *pictures show HC stained with mouse myleoma IgG or anti-FITC IgG, APAAP, or alkaline phosphatase only.* **(a)** *Cell clusters. Left: two or more cells in a cluster is a pathognomonic sign of epithelial nature. HC (right) occur as single cells.* **(b)** *Nuclear size. Left: markedly enlarged nucleus (size never observed in HC; megakaryocytes excepted) is also pathognomonic for TC. Right: same nuclear size as the surrounding HC.* **(c,d,e)** *Nuclear texture. Left: TC with irregularly fine-stippled chromatin (c,e). Nucleoli, if present, are usually small (d, see arrows). Right: HC with no discernible chromatin (c) or regularly dispersed chromatin as surrounding HC (d), or regular coarse chromatin indicating plasma cell nucleus (e). Lower cell of right picture e shows a typical plasma cell.* **(f,g,h,i)** *Cytoplasmic staining. Left: TC with cytoplasmic color*



partially covering the nucleus (f,i). Right: HC with cytoplasmic color not covering the nucleus. Left g: TC with cytoplasmic staining unevenly distributed with strongly and weakly stained parts and perinuclear color condensation. Right f,g: Homogeneous cytoplasmic staining in HC. Left h: Irregular color condensations/strong granules with varying sizes in TC. Right h: Regular granules with moderate color intensity in HC. Left i: A few large cytoplasmic vacuoles, typical of breast carcinoma metastasis. Right i: HC with regular microvacuolar cytoplasm (lower picture) or characteristic pin-point vacuole (upper picture). **(j,k)** *Cytoplasmic border. Left: TC with sharp (j) or slightly irregular (k) border. Right: Characteristic pouches on HC surface (j) or several cytoplasmic snouts (k). (× 600).*

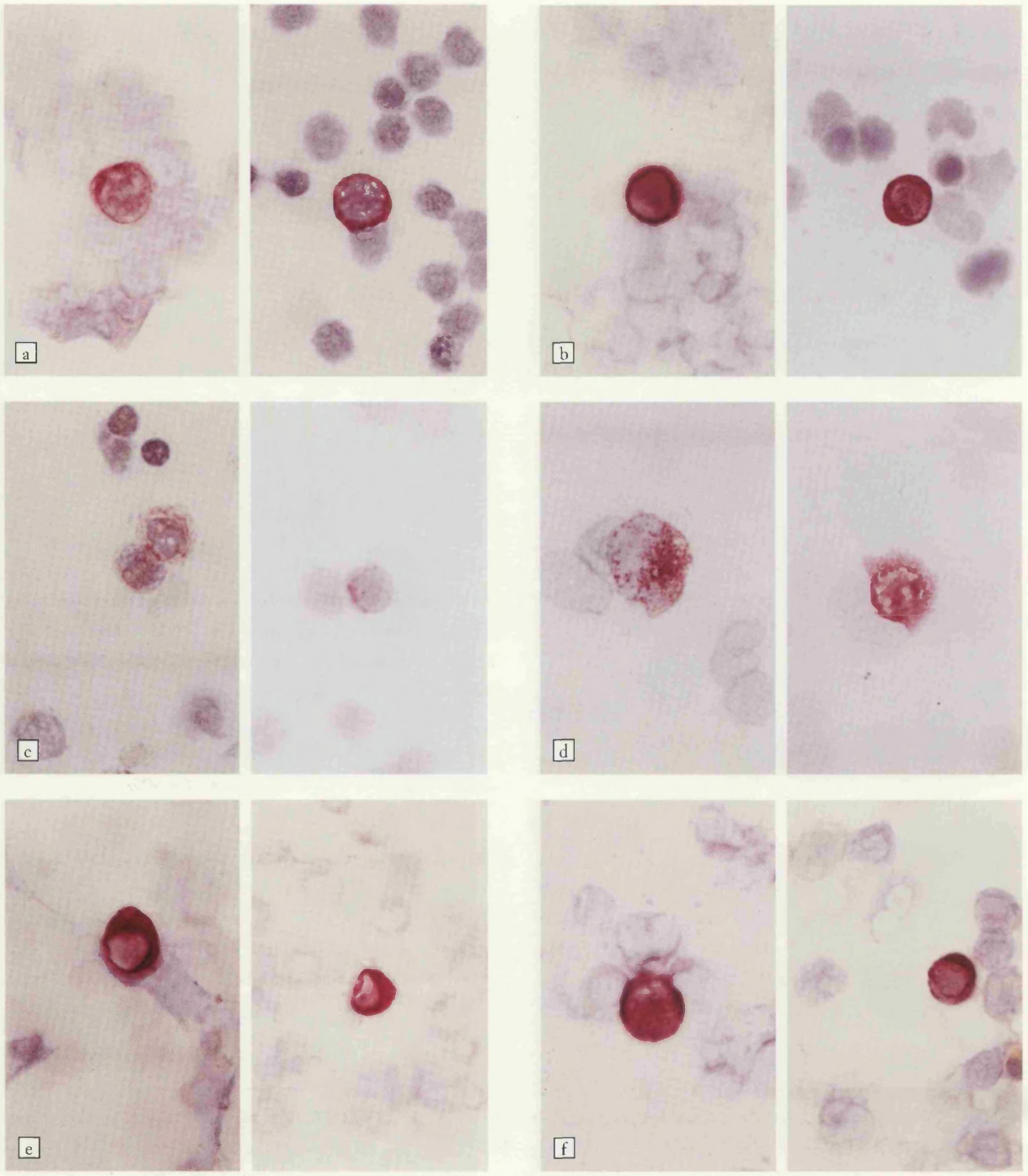


Figure 2.

negative control, no positive diagnosis can be concluded for that patient sample.

Classification of positive events

Based on the cells presented in Figures 1–3, we propose a classification of immunostained events into three groups: 'TC', 'probable TC' and 'TC-negative'. The criteria for this classification are summarized in Table 4. Cell clusters and immunostained cells with clearly enlarged or atypical nuclei are classified as 'TC' (Figure 1, all left pictures, Figure 2b,c,e,f, left). 'Probable TC' (or 'TC?') have no recognizable HC characteristics, but lack pathognomonic

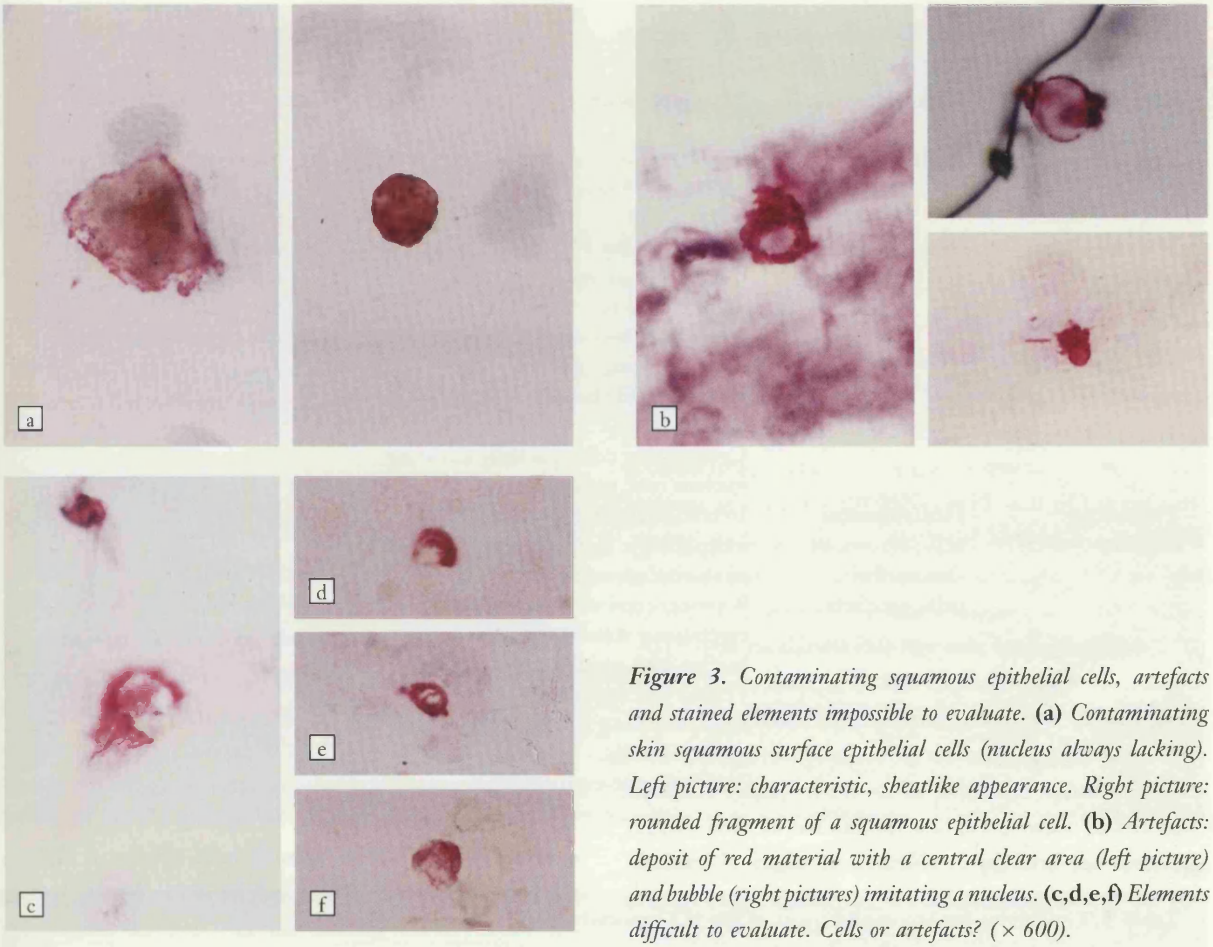


Figure 3. *Contaminating squamous epithelial cells, artefacts and stained elements impossible to evaluate.* **(a)** *Contaminating skin squamous surface epithelial cells (nucleus always lacking). Left picture: characteristic, sheatlike appearance. Right picture: rounded fragment of a squamous epithelial cell.* **(b)** *Artefacts: deposit of red material with a central clear area (left picture) and bubble (right pictures) imitating a nucleus.* **(c,d,e,f)** *Elements difficult to evaluate. Cells or artefacts? (× 600).*

signs of TC nature. Cells with this morphology are occasionally observed in negative controls (Figure 2a,b, e,f, right). A considerable proportion of these cells are probably true TC, if no similar cells are present in the corresponding negative control. We therefore propose to consider these cells also as TC-positive. Cells with HC characteristics, contaminating squamous epithelial cells and artefacts are classified as TC negative. The major characteristics for HC are: regular, symmetrical appearance, often with a low N/C ratio (Figure 1c,d,h, right), evenly-stained cytoplasm of weak or moderate intensity (Figure 1g, right), microvacuolar cytoplasm

Figure 2. (opposite) *Examples of occasional morphological similarities between selected TC (***left** *picture of each figure letter) and false-positive HC (***right** *picture of each letter).* **(a)** *Nuclear size. Example of TC with HC-sized nucleus and anonymous chromatin texture (left picture). Example of anonymous HC (right picture). (Cytoplasmic staining in TC is, however, more irregularly distributed.)* **(b)** *Strong cytoplasmic staining. Example of strong cytoplasmic staining in both TC and HC. The HC (right picture) has a small nucleus, but its fine-stippled chromatin resembles a TC nucleus. (Somewhat larger nucleus and perinuclear color condensation, however, in the TC to the left.)* **(c)** *Weak cytoplasmic staining. Example of weak cytoplasmic staining in both TC (left picture) and HC (right picture). (The two-cell cluster reveals the true epithelial nature of the cells to the left. The sparse red color also covers the TC nuclei.)* **(d)** *Microvacuoles/cytoplasmic defects. A degenerated TC (left picture) with defects in the cytoplasmic staining resembling microvacuoles in HC (right picture).* **(e)** *Similar TC and HC both with strong cytoplasmic staining not covering the nucleus. Eccentric nuclei. (The true TC to the left, however, has larger nucleus.)* **(f)** *Similar TC and HC both with strong cytoplasmic staining partially covering the nucleus. (Larger nucleus and more nuclear covering, however, in the true TC to the left.) (× 600).*

Table 4. Categorization of immunostained cells

Result	Cell-type	Characteristics	Comment
Tumor cell positive	Tumor cell ('TC')	Typical tumor cell morphology. Cell cluster, obviously enlarged or atypical nucleus.	This morphology is never found in false-positive HC.
Tumor cell positive (?)	Probable tumor cell ('TC?')	No convincing HC characteristics (see below), but morphology occasionally seen in false positive HC. Irregularly distributed moderate to strong staining (not mandatory). Fine-stippled chromatin (not mandatory). Cytoplasmic color partially covering nucleus (not mandatory).	Classified as 'tumor cell-positive' only if no 'TC?' are present in the corresponding negative control specimens.
Tumor cell negative	Hematopoietic cells, squamous skin epithelial cells, artefacts	Immunostained cells with HC-like morphology: microvacuolar cytoplasm, pin-point vacuoles, weak homogeneous staining, nucleus resembling surrounding HC, cell border with regular pouches. Plasma cell appearance. Contaminating squamous skin epithelial cells. Artefacts, not-evaluable elements.	

Table 5. Guidelines for accomplishment of the ICC procedure and analysis

Procedures	Specifications	Comments
1 Prepare MNC-containing slides	A total of $\geqslant 4 \times 10^6$ MNC	
2 Specific ICC analysis with pan-epithelial MAb(s)	1/2 of the specimen	
3 Isotype-specific negative control staining	1/2 of the specimen	
4 Prepare positive controls	BM MNC spiked with carcinoma cells	Keep positive control and patient slides apart during the staining procedure
5 Evaluation of the technical quality of the slides	Number of cells, overall preservation of cell morphology, staining intensity of positive controls	Easiest with nuclear counterstaining. If unsatisfactory technical quality, new slides have to be processed
6 Immunological screening	All stained cells are registered	Easiest without nuclear counterstaining
7 Morphological review	All immunopositive events are evaluated	See Table 4 and Figures 1–3. Nuclear counterstaining is advantageous
8 Conclusion	Tumor cell positive or negative?	

Table 6. Criteria for an optimal tumor-cell detection method

Specificity
Sensitivity
Reproducibility
Robustness
Objective read-out
Potential for automated analysis
Quantitation of tumor load
Characterization of tumor cells
Proven clinical significance

(Figure 1i, lower cell of right picture, Figure 2d, right), pin-point vacuoles (Figure 1c,d,e,i, right) and nucleus with size and texture identical to those of the surrounding HC. Regular pouches on the cell surface are typical of false positives (Figure 1j, right) and probably are smaller snouts with smooth contour (Figure 1i,k, right), also indicative of HC nature. Plasma-cell is suspected when chromatin condensations are coarse, but regular (Figure 1e, right). In the absence of pathognomonic signs of TC or HC a reliable identification of the cells requires a mutual consideration of the nuclear size and texture, the cytoplasmic staining and the cellular border, compared with the surrounding HC.

Discussion

In the international studies published so far, an array of different methods have been used in the immunocytochemical and molecular detection of breast-cancer micrometastases [5–10]. The specificity and sensitivity of these methods vary markedly. So far, no general consensus has been established to standardize the laboratory protocols for micrometastatic detection. The presented results of the first inter-laboratory ring experiment have illustrated the urgent need for a consensus on morphological criteria for micrometastatic TC diagnosis. Inter-laboratory discrepancy may be caused both by differences in screening and by variable morphological criteria for tumor-cell diagnosis. With this publication we have initiated a standardization process and propose a common classification of stained elements into three groups (Table 4): 'TC', 'probable TC' and 'TC-negative'. 'TC's are morphologically obvious tumor cells. 'Probable TC's represent a morphological overlap to HC. Within BM MNC from breast-cancer patients the presence of 'TC' or 'probable TC' are both positively correlated, both with T-stage and with presence of axillary lymph-node metastases (B. Naume, unpublished observations). We propose to consider these two categories of cells as 'micrometastasis-positive', on condition that similar cells are not found in the corresponding negative-control specimens. False-positive HC, contaminating squamous epithelial cells and artefacts are classified as 'TC-negative' elements.

The ICC procedure is at present performed differently in many laboratories [7]. We suggest the guidelines listed in Table 5 as an inter-laboratory standard for preparation, processing and evaluation of micrometastases. The analysis requires at least 4×10^6 MNC, with half of the cells incubated with specific pan-epithelial MAb(s) and the other half with isotype control MAb. Adequate ICC staining should be controlled by slides/samples with BM MNC spiked with carcinoma cell-line cells (positive control). To avoid TC contamination it is important to keep positive-control specimens separate from diagnostic patient specimens (in separate jars) during the staining procedure. The technical quality of the slides should be satisfactory, with regards both to cell number and preservation of cell morphology. It might be desirable to perform the screening process itself, identifying candidate cells, prior to nuclear counter-staining; the cells appearing colored against an unstained background. If visualization of nuclear morphology of detected cells is required, the hematoxylin staining may then be performed after removal of cover-slips from the selected immunostained slides.

An optimal TC detection method has to fulfill specific criteria (Table 6). This report is a step towards the fulfillment of several of these criteria. The highest specificity and sensitivity should be assured. Several groups have published strategies to further increase the sensitivity of TC detection [11–17]. At present we do not know what implications an increase in sensitivity will have for the reproducibility and clinical specificity of such tests. However, for the quantification and characterization of TC, enrichment strategies are probably needed. The TC detection method should be reproducible and robust, and the read-out of detected cells objective. There already exist a few automated analysis systems applicable to ICC-stained cytospins, which should, in the future, increase the objectivity, reduce the labour of screening and bring the ICC method closer to routine clinical use.

In the future, other methods for detection of epithelial

cells, such as flow cytometry, RT-PCR-based methods and double immunostaining techniques might replace ICC as the 'gold standard'. However, the new methods also require a standard technique for direct comparison in relation to specific clinical data. Therefore, the efforts made to standardize ICC tumor-cell detection are of utmost importance, both for today's clinical testing and for the establishment of new techniques.

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