MOLECULAR ASPECTS OF

MULTIPLE MYELOMA

MOLECULAIRE ASPEKTEN VAN HET

MULTIPEL MYELOOM

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Voor mijn moeder

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VOORWOORD

Kanker wordt gekenmerkt door de ongeremde groei van kwaadaardige cellen. Het is algemeen aanvaard dat de basis van kanker ligt in de beschadiging van het DNA in de cel. Dit bevat alle erfelijke informatie die nodig is voor de ontwikkeling en instandhouding van een organisme. Dus ook de informatie voor celdeling en allerlei regulatie systemen. Regulatie systemen zijn noodzakelijk om alle funkties die een cel behoort uit te oefenen, in goede banen te leiden. Als er in het DNA veranderingen optreden door beschadiging, bijvoorbeeld door een virusinfektie, radioaktieve straling, of langdurige blootstelling aan toxische stoffen, is het mogelijk dat de cel een kankercel wordt. Dat wil zeggen dat deze zich ongeremd gaat vermenigvuldigen en niet meer luistert naar signalen van buitenaf om hem in toom te houden. Gebeurt dit met cellen van ons afweersysteem, dat verantwoordelijk is voor de bescherming van ons lichaam tegen infecties, dan kan zich een leukemie ("bloedkanker") of een maligne lymfoom ("lymfklierkanker") ontwikkelen.

Men kan verschillende soorten leukemieën onderscheiden aan de hand van het type cel dat erbij betrokken is en de snelheid waarmee de ziekte voortschrijdt. Bijvoorbeeld een B-CLL, wat betekent chronische lymfatische leukemie van het B-cel type (een cel die antilichamen kan maken) en een T-ALL, dat is een acute lymfatische leukemie van het T-cel type (een cel die de B-cel kan helpen en die zelf virus geïnfekteerde cellen of kankercellen kan opruimen). Weer een andere soort kanker van het immuunsysteem is het multipel myeloom, een kanker van plasmacellen. Deze vorm van kanker komt voornamelijk voor bij oudere mensen.

Plasmacellen zijn de meest uitgerijpte cellen van de B-lymfocyten reeks. Zij zijn gespecialiseerd in de produktie van antilichamen. Bij patiënten met een vergevorderd stadium van het multipel myeloom worden veel plasmacellen (soms wel 90%) gevonden in het beenmerg, terwijl er normaal slechts 1-5% plasmacellen in het beenmerg voorkomen. Dit veroorzaakt problemen doordat zij de andere beenmergcellen verdringen en grote hoeveelheden antilichamen produceren. Tesamen kan dat aanleiding geven tot nierstoornissen, bloedarmoede, algehele malaise en infekties. Het meest kenmerkende van het multipel myeloom is de aanwezigheid van een paraproteïne (dit zijn de antilichamen gemaakt door de kwaadaardige plasmacellen) in het bloed en het optreden van botafbraak. Dit laatste wordt veroorzaakt door de produktie van botafbrekende stoffen door de plasmacellen en/of andere celtypen in het beenmerg. Patiënten met een multipel myeloom hebben dan ook relatief vaak last van botpijn en botbreuk.

De diagnose multipel myeloom is gemakkelijk te stellen als de ziekte al in een vergevorderd stadium is. Men vindt dan een paraproteïne in het bloed en veel plasmacellen in het beenmerg. Bij een multipel myeloom in een vroeg stadium is de

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diagnose echter moeilijk. Het aantal plasmacellen in het beenmerg is dan slechts in geringe mate verhoogd en er is nog geen botafbraak. Er is dan moeilijk onderscheid te maken tussen het multipel myeloom en benigne monoclonale gammapathie (BMG), wat een goedaardige aandoening is. Deze laatste vorm behoeft niet behandeld te worden.

Mensen met BMG hebben geen klachten en worden eigenlijk bij toeval ontdekt. Het kan echter ook zijn dat het een vroeg stadium van een multipel myeloom is, en dan is het zaak om zo vroeg mogelijk te behandelen. De vooruitzichten op genezing bij een multipel myeloom zijn namelijk zeer slecht als behandeling lang op zich laat wachten. Hoe eerder men kan behandelen, hoe beter het is. Echter, de behandeling op zichzelf is zwaar. Er wordt vooral behandeld met cytostatica en soms wordt been-mergtransplantatie toegepast. Het is dus belangrijk om onderscheid te kunnen maken tussen BMG en een beginnend multipel myeloom om zo de juiste behandeling te kunnen geven. Bovendien is het belangrijk om achter het mechanisme van het kankerproces te komen. Alle therapieën zijn tot nog toe gericht op het verwijderen van zoveel mogelijk kankercellen en het bestrijden van de symptomen. Als men eenmaal de oorzaak kent kan er misschien een therapie gevonden worden die deze oorzaak wegneemt zodat preventie en volledige genezing beter mogelijk worden. Dit is echter nog toekomstmuziek.

Het doel van dit onderzoek was om verschillen op te sporen tussen de kwaadaardige plasmacellen en normale plasmacellen. Dit is op verschillende niveaus onderzocht. Op DNA-niveau werd dit gedaan met behulp van transfectie studies waarbij het DNA uit multipel myeloomcellen werd overgebracht in een cellijn. Op deze manier is men in staat om oncogenen (genen die kanker kunnen veroorzaken) op te sporen. Ook werd het DNA van de tumorcellen onderzocht op de aanwezigheid van reeds bekende oncogenen. Op RNA-niveau werd onderzoek gedaan om de expressie van oncogenen te bestuderen. De RNA-expressie studies bij multipel myeloom patiënten zijn echter moeilijk doordat er nogal eens maar een beperkt aantal cellen voorhanden is. Bovendien bestaat het celmonster vaak slechts voor een klein deel uit tumorcellen. Een gevoelige methode voor RNA-expressie onderzoek is de RNA *in situ* hybridisatie. Met deze techniek is het mogelijk om het RNA in afzonderlijke cellen zichtbaar te maken zonder dat de cel kapot gaat, zodat bepaald kan worden in welk celtype het betreffende RNA is gevormd. Deze techniek is door ons geschikt gemaakt voor RNA-expressie onderzoek van het multipel myeloom.

1. INTRODUCTION

1.1. General features of multiple myeloma

Multiple myeloma (MM) is the most common manifestation of plasma cell malignancy accounting for about 1 per cent of all malignant diseases and slightly more than 10 per cent of the hematological malignancies (Blattner, 1980). The incidence of MM varies from 1 to 82 per 100.000 in different national and racial groups (Segi, 1977). It is predominantly a disease of the elderly. The mean age at diagnosis is around 60 years (Kyle, 1975). Peak incidence occurs between 75 and 80 years. Only 2 per cent of all cases appear in persons below the age of 20 years (Rubenstein, 1949; Howel and Alexanian, 1976).

The onset of MM is usually insidious and there are often multiple symptoms at presentation. Bone pain is the major symptom in about 60 per cent of cases. Symptoms of anaemia and uraemia account for perhaps a further 20 per cent, infection another 10 per cent and the final 10 per cent includes less common presentations such as hyperviscosity, amyloidosis, spinal cord compression, acute hypercalcaemia, effects of extraosseous tumor and a selection of miscellaneous rarities (Fudenberg and Virella, 1980).

More than 50 per cent of the patients respond to therapy with a more than 75 per cent reduction in total body myeloma cell mass (Durie and Salmon, 1982). They have a rapid improvement in bone pain and reversal of symptoms of hypercalcaemia. Bone lesions rarely heal (Rodriguez et al., 1972), depressed levels of normal immuno-globulins (Ig) rarely improve and complete disappearance either of paraprotein or the monoclonal plasma cell infiltration of the bone marrow (BM) is unusual.

Most patients with good initial response remain in clinical "remission" for an average of 3 years. Unfortunately, reduction of tumor burden by therapy (generally by alkylating agents) rarely exceeds 90 per cent and the large number of residual tumor cells leads to eventual relapse in virtually all patients. Such relapses are less sensitive to treatment with alkylating agents. The final stages of MM are characterized by progressive growth of drug resistant tumor cells, increasing bone pain, hypercal-caemia, and pathological features.

A correct diagnosis is not difficult to reach when (1) a monoclonal paraprotein is present in serum, urine or both; (2) myeloma cell infiltration is present in the BM; and (3) characteristic radiological bone lesions are observed. Diagnostic difficulties arise when a paraprotein is detected without the presence of other diagnostic features. There is a border region between malignant and benign plasma cell proliferations, where it may be impossible to reach an early and correct diagnosis and where only the subsequent clinical course can reveal the true nature of the process. Therefore it is most important to develop diagnostic tools to discriminate malignant from benign monoclonal gammapathies (BMG). Incorrect interpretation may result in an unneces-sary and even harmfull treatment with cytostatics of patients with BMG or immuno-deficiency. On the other hand, incorrect interpretation can lead to the discharge of

patients with a true B-cell malignancy to whom an appropriate therapy should be given (Radl, 1982). Consequently, every patient without typical signs of MM is subjected to repeated and expensive control investigations.

1.2. Cell biological aspects of multiple myeloma

1.2.1. Myeloma cells

MM is characterized by a high proportion of plasma cells in the BM and lytic bone lesions. The malignant proliferation of plasma cells predominantly takes place in the medullary cavity of the axial skeleton. Plasma cell infiltration of other sites is mainly a phenomenon seen late in the disease and is not always present (Kapadia, 1980).

Plasma cells represent the terminal differentiation stage of B-cell development. Their main function is antibody production and secretion. The mature plasma cell has an eccentric nucleus with a large amount of basophilic cytoplasm, due presumably to the abundant RNA required for protein synthesis. Plasma cells are seldomly seen in the circulation (< 0.1% of the lymphocytes) and are normally restricted to the BM and the secondary lymphoid organs (e.g. spleen and lymph nodes). In MM the percentage of plasma cells in the BM is at least 10% and can even reach values of 90% (Kyle, 1985). The malignant plasma cell phenotype is moderately heterogeneous among different patients. One can distinguish between plasmacytic, lymphoid and plasma-blastic variants with progressively increasing labeling index and adverse prognosis (Greipp and Kyle, 1983; Greipp et al., 1985). The majority of tumor cells are aneuploid with a differentiated B-cell phenotype but with subpopulations that also express early B (CALLA), possibly T, and even myelomonocytic features (M5, My7) (Durie et al., 1985, 1989; Epstein et al., 1988; Grogan et al., 1989).

In vitro culture of myeloma cells is difficult probably due to the low proliferation rate of plasma cells (Barlogie et al., 1985). In some cases cell lines could be established, mostly from patients in which the disease was already in an advanced stage (Latreille et al., 1982; Durie et al., 1985; Katagiri et al., 1985; Gazdar et al., 1986; Jernberg et al., 1987). With the outcome of the large scale production of recombinant growth factors like interleukins, more and more progress is being made in unraveling the requirements for plasma cell growth *in vitro* (see Section 1.2.3).

Although the malignant cell type in MM is the plasma cell, the origin of this malignancy is considered to take place in a less differentiated precursor cell. B-cells have been observed in the peripheral blood, which share the same idiotype as the circulating myeloma Ig using immunofluorescence studies (Mellstedt et al., 1974; Van Acker et al., 1979; Schedel et al., 1980; Bast et al., 1982; Bagg et al., 1989). By means of this idiotypic typing, pre-B cells were traced as the earliest precursor cell-type of MM (Kubagawa et al., 1979; Epstein et al., 1988).

B lymphocytes belonging to the myeloma clone could be detected by Southern blot analysis, using probes specific for Ig genes (see also Section 1.2.2). With this technique it was possible to demonstrate clonal gene rearrangements in peripheral blood lymphocytes identical to the gene rearrangements observed in the BM

(Berenson et al., 1987; Chiu et al., 1989, Van Riet et al., 1989). However, these findings may be due to contamination of the peripheral blood by myeloma cells as stated by Shimizu et al. (1980) and Clofent et al. (1989). To date there is no definite answer to the question in which differentiation stage of B-cell development the oncogenic event takes place.

1.2.2. Myeloma immunoglobulins

The most prominent feature of MM is the presence of a so called paraprotein or M-component in the serum or urine due to the abnormal production of Ig by the myeloma cells in the BM. Paraproteins of the IgG isotype are more frequently found in MM than those of the IgA, IgD or IgE isotype; in some cases only kappa (κ) or lambda (λ) light chains can be detected (Radl, 1985). Due to their relatively high concentration in serum, paraproteins can be isolated relatively easy. Therefore, much of our knowledge about Ig is derived from research of myeloma proteins.

Antibodies produced by a single plasma cell are of one specificity and Ig class. They are composed of heavy (IgH) and light chains (IgL). Like other cellular proteins, Ig molecules are synthesized as a result of transcription of specific DNA segments.

The IgH chains are encoded by chromosome 14, the IgL-κ chains by chromosome 2 and the IgL-λ chains on chromosome 22 (Croce et al., 1979; Shander et al., 1980; Erikson et al., 1981). IgH chain genes consist of variable (V), diversity (D), joining (J) and constant (C) gene segements. The IgL chain genes consist only of V, J and C gene segments. By recombining these gene segments, functional Ig genes are generated (Rosen et al., 1986; Tonegawa, 1983; Yancopoulos and Alt, 1986). For every antibody specificity, this recombination or rearrangement process is unique and therefore can serve as a tumor specific marker in B-cell malignancy. By means of the Southern blot technique using Ig-gene specific probes, it is possible to detect these rearrangements in cell samples in which the monoclonal cell population constitutes only 1% of the mononuclear cells (reviewed by Griesser et al., 1989).

In MM, Ig gene rearrangements could be detected in proportion to the degree of marrow plasmacytosis in most cases (Berenson et al., 1987; Chiu et al., 1989; Clofent et al., 1989) indicating the monoclonality of the disease. Although in some patients, with sufficient numbers of plasma cells in the bone marrow, this monoclonality could not be detected by Southern blot analysis (Humphries and Williams, 1989; K. Thielemans, personal communication). Ig rearrangement studies can resolve the question whether the idiotypically positive B cells found in the peripheral blood of some MM patients are indeed cells that belong to the malignant clone or just cells that adsorbed the paraprotein. Since purification of B cells from the blood does not usually lead to a 100% pure B cell population, plasma cells may still be present, and may account for the contradicting results obtained with this technique (Shimizu et al., 1980; Berenson et al., 1987; Chiu et al., 1989; Clofent et al., 1989).

1.2.3. Cytokines and multiple myeloma

In addition to Ig, myeloma cells can produce several cytokines. Many efforts

have been undertaken to determine the factor that is responsible for the bone lytic lesions which are so characteristic for MM. The first evidence for such a factor came from myeloma BM cell cultures. The supernatant of short-term BM cultures was found to contain a stimulator of bone resorption that was similar to osteoclast activating factor (OAF), a cytokine that was produced by human peripheral leukocytes when cultured with antigens or mitogens (Mundy et al., 1974). The observation in BM biopsies of osteoclasts at resorptive bone surfaces adjacent to infiltrations of myeloma cells supported the conclusion that the increase of osteoclastic bone resorption in MM is due to the production of OAF by the myeloma cells (Durie et al., 1981). Recent advances in biotechnology made it possible to produce virtually pure cytokines in sufficient quantities so that their biologic activity could be investigated. Some of them showed bone resorbing activity *in vitro*, e.g. lymphotoxin (LT, TNFβ), tumor necrosis factor (TNFα), interleukin-1 (IL-1) and transforming growth factor β (TGFβ). OAF appeared to be identical to IL-1β (Dewhirst et al., 1985). TNFβ or LT and IL-1β could be detected in myeloma cell cultures (Garrett et al., 1987; Cozzolino et al., 1989; Kawano et al., 1989; Lichtenstein et al., 1989).

Normally, IL-1β and TNFβ are not detected in mature B cells. The presence of these proteins in the myeloma cells suggests that they play a role in myeloma growth and differentiation. Several cytokines that stimulate growth and differentiation of human B cells have now been described (Kishimoto, 1987). In general they are termed B-cell growth factors (BCGF) and B-cell stimulatory factors (BSF). BCGF are IL-2 (Mingari et al., 1984), BCGFI and BCGFII (IL-5) (Kishimoto et al., 1985; Muraguchi et al., 1985) and γ-interferon (IFN-γ) (Romagnani et al., 1986). BSF are IL-4 (BSF-1; Defrance et al., 1987) and IL-6 (BSF-2, IFN-β2, HGF) (Hirano et al., 1985;1986). IL-1 and complement factors are also involved in B cell activation and proliferation (Howard et al., 1983; Lipsky et al., 1983; Daha et al., 1984).

IL-1β has many different effects on various cell types. Next to osteoclast-activation, it can also activate T-cells (Le and Vicek, 1987), induce B-cell proliferation (Freedman et al., 1988), B-cell differentiation (Jelinek and Lipsky, 1987) and presumably the stimulation of myeloma cell growth through the induction of IL-6 (Kawano et al., 1989). On the other hand, Anderson et al. (1989) could neither detect proliferation nor Ig secretion in purified myeloma cells in response to IL-1α or IL-1β, which was also the case for IL-2 and IL-4.

IL-5 is produced by the myeloma cell line RPMI 8226 and acts as an autocrine growth factor in this cell line (Klein et al., 1987). Some freshly purified populations of myeloma cells can also proliferate to IL-5 (Anderson et al., 1989). Whether these myeloma cells can produce IL-5 themselves is as yet unclear.

IL-6 is a potential growth factor for B cell hybridomas, plasmacytomas, EBV-transformed B-lymphoblastoid cells and myeloma cells (Poupart et al., 1987; Van Damme et al., 1987; Muraguchi et al., 1988; Tosato et al., 1988; Anderson et al., 1989). Kawano et al. postulated an autocrine growth pattern for myeloma cells by IL-6 based on the observation that cultured purified myeloma cells express IL-6 mRNA, secrete IL-6, express receptors for IL-6, and proliferate in a specific manner to exogenous

recombinant IL-6 (Asaoku et al., 1988; Kawano et al., 1988). This observation has been questioned by Klein et al. (1989) who noted that the IL-6 activity resided entirely in the adherent cells of the BM. They therefore favored a paracrine growth mechanism (see also Chapter 6).

Most cytokines produced by myeloma cells induce the production and secretion of other cytokines by a variety of different cell types present in the BM. Because of this cascade of induction and/or suppression of a large variety of factors, it is not easy to investigate which events are primary or secondary results of a particular cytokine. Unraveling this cytokine network will be of great importance to clarify the mechanisms involved in the abnormal proliferation and maturation of myeloma cells and may eventually result in new therapies.

1.3. Oncogenes

It is widely accepted that neoplastic development in general results from multiple genetic changes. Much effort has been devoted to the identification of genetic sequences responsible for the oncogenic event. Our current knowledge about these oncogenes is derived from two important discoveries:

1)   acute transforming retroviruses contain specific genes responsible for their oncogenicity (Huebner and Todaro, 1969; Martin, 1970), called viral oncogenes (v-onc); these genes arose from normal cellular genes (proto-oncogenes), apparently picked up by an originally non-transforming virus during the course of infection;

2)   gene transfection experiments demonstrated that DNA fragments from different human tumor cell lines and fresh tumor tissues were able to transform non-neoplastic cells with high efficiency (Cooper et al., 1980; Cooper, 1982; Pulciani et al., 1982).

Some of these transforming genes in DNA transfection assays and v-oncogenes are homologous, e.g. transforming gene from human bladder carcinoma and the oncogene of Harvey sarcoma virus (H-ras) (Chang et al., 1982).

Since the initial discovery of oncogenes more then 40 oncogenes have been identified and the number is still growing. As a consequence, great progress has been made in understanding their normal function in cell growth and differentiation. A substantial number of the proteins encoded by proto-oncogenes can be arranged in three families:

1)   the protein-tyrosine kinases (Hunter and Cooper, 1985); these enzymes represent cell surface receptors like erb-B which codes for the transmembrane receptor EGF (Downward et al., 1984) or a signal transducers, for example src, which codes for a peripheral membrane protein;

2)   the ras proteins (Ellis et al., 1982), which also represent peripheral membrane proteins but their functions are analogous to G proteins (Toda et al., 1985);

3)   diverse nuclear proteins (Bishop, 1985), which may be involved in regulation of transcription or regulation of DNA replication; for example the myc product,

which is suggested to augment transcription from other genes (Kingston et al., 1985).

Alterations in a proto-oncogene can lead to aberrant expression of this gene which can disturb the normal growth control machinery of the cell, leading to malignant transformation. Activation of these genes can be caused by retroviral insertions, DNA deletions or amplifications, point-mutations and/or chromosomal translocations. In the process of cancer development, the activation of one proto-oncogene seems not to be sufficient. Co-operation among several oncogenes is required to achieve full transformation, explaining perhaps the multistep process of carcinogenesis. For example, *ras* and *myc* have been shown to co-operate in the transformation of certain primary cells (Land et al., 1983). Experiments with transgenic mice, which had received the c-*myc* on its own, induced an excessive proliferation of B-lymphoid cells but this activity was limited and benign. However, these mice almost all developed malignancies of the B-cell lineage after variable latency periods, suggesting that this abnormally expanded B-cell population is more susceptible to malignant conversion, generated by a secondary event, presumably a genetic accident (Harris et al., 1988; Cory et al., 1989).

1.3.1. Oncogenes in B-cell neoplasia

There are several different biological mechanisms whereby oncogenes are deregulated in B-cell neoplasia. In Burkitt's lymphoma, three characteristic translocations are found, involving the c-*myc* oncogene and the three Ig loci. The most common one is the 8;14 translocation (t(8;14)) in which the c-*myc* gene is translocated from chromosome 8 to the IgH locus at chromosome 14 in a head to head orientation (Taub et al., 1982; Adams et al., 1983). In the variant t(2;8) and t(8;22) translocations, part of the IgL chain genes (located on the human chromosomes 2 and 22, respectively) is moved to the 3' side of the c-*myc* gene on chromosome 8 in a head to tail orientation (De la Chapelle et al., 1983; Erikson et al., 1983). In mouse plasmacytomas, a similar recombination between the IgH or IgL genes and c-*myc* occurs (Klein, 1983). An obvious explanation for the involvement of the Ig genes in these chromosomal abnormalities is that these genes are undergoing chromosomal breakage and rejoining in the process of formation of the mature Ig gene. Chromosomal translocations therefore presumably occur as a result of errors in this natural process (Haluska et al., 1986).

Regardless of where the breakpoints occur, the c-*myc* gene associated with the translocation is deregulated and expressed, while the normal c-*myc* gene is silent in almost all cases (Nishikura et al., 1983). Thus, the oncogenic potential of the c-*myc* gene product results from the inappropriate expression of the gene and not from the generation of an altered c-*myc* gene product. Deregulated expression of c-*myc* may be due to cis-activation by different Ig-gene elements. The normal IgH chain gene enhancer can activate c-*myc* transcription (Fahrlander et al., 1985; Feo et al., 1986). On the other hand, the first exon of c-*myc* is frequently mutated in Burkitt's lymphoma, which may effect transcriptional activation (Cesarman et al., 1987). Activation of c-*myc*

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probably can also take place over a long distance, as is obviously the case in variant Burkitt's lymphoma in which the breakpoints cluster in a *pvt*-like region, located about 300 kb 3' of c-*myc* (Graham and Adams, 1986; Mengle-Gaw and Rabbitts, 1987). The *pvt*-1, the major locus of murine plasmacytoma variant translocations, was first cloned by Webb et al. (1984). In variant plasmacytomas with t(6;15), the Ck locus (on murine chromosome 6) is exchanged with the *pvt*-1 locus (on chromosome 15), which is located at least 85 kb 3' of c-*myc* (Banerjee et al., 1985; Cory et al., 1985; Graham et al., 1985). *Pvt*-1 is a common site of proviral integration in retrovirally induced murine T-lymphomas (Graham et al., 1985) and is equivalent to the *mis*-1 locus, a common proviral integration site in rat T-lymphomas (Villeneuve et al., 1986).

Alterations within this *pvt*-region appear to be associated with a deregulation of the c-*myc* gene, suggesting that a putative oncogene is located here which product has an effect on c-*myc* transcription over a long distance (Adams et al., 1986).

Co-amplification of the *pvt*-region and c-*myc* is observed in several human tumor cell lines, a colon carcinoma cell line, two small-cell lung carcinoma cell lines, a large-cell lung carcinoma cell line (Mengle-Gaw and Rabbits, 1987) and one case of acute non-lymphocytic leukemia (Asker et al., 1988), indicating that *pvt* is involved in tumor aetiology in several different cell types.

Evidence that the *pvt*-region indeed contains a gene that is actively transcribed, was obtained only recently by Shtivelman et al. (1989) for humans and mice and by Tsichlis et al. (1989) for rats. In human cell lines, transcripts range in size from 1-11 kb, in mouse liver cells the transcripts are 0.5 and 4.8 kb, in mouse osteosarcoma cell lines additional 0.6-0.7 kb mRNA were detected and in rat T cell lymphoma a ± 10 kb mRNA was found (Shtivelman and Bishop, 1989; Shtivelman et al., 1989; Tsichlis et al., 1989). The functional role of these transcripts in oncogenesis have yet to be elucidated.

Translocations involving the IgH gene other than the t(8;14) have been reported in different subtypes of lymphoma and chronic lymphocytic leukemia (CLL). In patients with CLL and diffuse small or large cell lymphoma a t(11;14) is often found in which the breakpoint on chromosome 11 clusters in a region defined as *bcl*-1 (Yunis,1983; Erikson et al., 1984; Tsujimoto et al., 1984[a], 1985[a]). Follicular cell lymphoma is almost invariably associated with a t(14;18) in which the breakpoint on chromosome 18 is clustered in a region defined as *bcl*-2 (Yunis, 1983; Tsujimoto et al., 1984[b]; Cleary and Sklar, 1985[a]; 1985[b]). Both translocations are probably the consequence of aberrant V-D-J joining events leading to the activation of a putative oncogene at the *bcl*-1 or *bcl*-2 breakpoint regions. Translocations of *bcl*-2 sequences from chromosome 18 to the J_H segment at chromosome 14 causes high steady state levels of *bcl*-2 mRNA (Tsujimoto et al., 1985[b]). The *bcl*-2 gene encodes two proteins, Bcl-2α and Bcl-2β (Tsujimoto and Croce, 1986). The Bcl-2α gene product is located at the inner surface membrane and has GTP-binding activity just like the H-*ras* gene product (Haldar et al., 1989). The translocation results in transcription of mRNA of aberrant sizes but does not affect the *bcl*-2 protein coding sequences (Tsujimoto and Croce, 1986). A *bcl*-1 product has not been identified until now.

1.3.2. Oncogenes in multiple myeloma

While advances in chromosomal banding and molecular techniques have clarified the role of cellular oncogenes in some B cell lymphomas and leukemias, the difficulty in obtaining adequate metaphase chromosomes and the absence of specific karyotypic aberrations have hindered similar progress in MM. As the c-*myc* gene is often deregulated in murine plasmacytomas (Fahrlander et al., 1985; Potter,1986), c-*myc* and other oncogenes implicated in B-cell lymphomas and leukemias, have been studied also in MM. Elevated *myc* mRNA expression has been detected in 9 out of 37 patients with MM but resulted only rarely from DNA rearrangements (two out of 37) and was not associated with DNA amplification (Selvanayagam et al., 1988). In one case the rearranged DNA was cloned and appeared to be derived entirely from chromosome 8, indicating a mechanism of c-*myc* activation different from that in Burkitt's lymphoma. In the other cases the mechanism of c-*myc* activation was not clear. Point-mutations in the first exon of c-*myc* leading to elevated *myc* mRNA expression as in variant Burkitt's lymphoma (Cesarman et al., 1987) could not be detected in these cases. Meltzer et al. (1987), on the other hand, reported mutations in the 3' region of the first exon of c-*myc* (*Pvu*II and *Alu*I endonuclease sites) in 10 out of 16 human myeloma samples and cell lines.

Rearrangement of the c-*myc* gene was also observed in the plasma cell myeloma cell line NCI-H929 as well as in the original tumor material (Gazdar et al., 1986). A complex translocation, in which only chromosome 8 seemed to be involved, had interrupted the third exon of the c-*myc* gene. As a result, a chimeric mRNA was expressed that contained the c-*myc* coding region but in which the c-*myc* 3' untranslated region had been replaced by sequences introduced by the translocation event. This chimeric c-*myc* mRNA was over seven times more stable than c-*myc* transcripts with intact 3' ends (Hollis et al., 1988).

A c-*myc* gene amplification with a concomitantly increased level of expression was found in 2 out of 3 cases of plasma cell leukemia but not in 21 cases of MM (Sümegi et al., 1985).

Most of the handful of available true human plasma cell lines have been initiated from malignant effusions (a characteristic feature of the aggressive phase) or from circulating leukemic cells (Garewal and Durie, 1982; Durie et al., 1985; Sümegi et al., 1985; Gazdar et al., 1986) in which c-*myc* gene alterations can be found. Since c-*myc* gene alteration is not a common feature of all human myelomas it is suggested that altered expression of the c-*myc* gene, by amplification or rearrangement, contributes to the highly malignant nature of plasma cell tumors in the leukemic and aggressive phases. Such tumors might be more capable of *in vitro* proliferation.

A second transforming gene, N-*ras*, was identified in the myeloma cell line NCI-H929 by transfection experiments (Ernst et al., 1988), suggesting that there is a co-operation between c-*myc* and N-*ras* which leads to a more aggressive stage as was demonstrated in transgenic mice (Sinn et al., 1987). It is possible that the disease course is associated with and dependent on the sequential activation of transforming genes such as *myc* and *ras*, which fits in the multi-step theory of carcinogenesis.

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Next to a higher c-*myc* mRNA expression in 25% of MM patients, the H-*ras* gene was also highly expressed in 17 out of 23 cases studied (Tsuchiya et al., 1988). High levels of H-*ras* p21 protein were present in aneuploid plasma cells suggesting the involvement of the H-*ras* oncogene in the pathophysiology of MM, which was further supported by a shorter survival among patients with high p21 levels. The mechanism of the H-*ras* gene activation in these MM cases is as yet unclear.

Rearrangements of *bcl-2* (commonly involved in follicular lymphoma) have not been found in MM, consistent with the rarity of t(14;18) translocations. *Bcl*-1 rearrangements have been detected in five out of 120 patients but without corresponding mRNA elevations (Selvanayagam et al., 1987).

1.4. A mouse model for multiple myeloma

The availability of tumor models and tumor derived cell lines has greatly increased our current knowledge about the pathological processes that occur in a variety of malignancies. Several models of plasma cell malignancies have been reported in the literature. Spontaneous plasma cell neoplasia have been observed in dogs, mice, rats and monkeys (Lingeman, 1969). But most of them cannot be used as experimental model for studies on MM because they occur very infrequently and transplantation of the malignant cells into allogeneic recipients of the same species leads to rejection. A few currently available inbred strains of mice are particularly prone to the development of a plasma cell tumor.

The BALB/c mouse strain is sensitive to induction of a plasmacytoma by intraperitoneal injection of mineral oils (Potter and Boyer, 1962). The primary BALB/c plasmacytomas occur in the peritoneal connective tissue and most of them secrete IgA immunoglobulins. Cells of these plasmacytomas have not been observed to metastasize into the BM. Furthermore, the bone lesions which are characteristic for MM, are absent (Kobayashi et al., 1961). Only after intravenous transplantation of the BALB/c plasmacytoma cells in a sufficient number, the malignant cells infiltrated the BM and led to the development of bone lesions in more than 70% of the recipients (Kobayashi et al., 1961). Cytogenetic analysis of these plasmacytomas showed nonrandom translocations involving chromosome 15, which bears the c-*myc* gene. Reciprocal translocations occurred between chromosome 15 and chromosome 12, and between chromosome 15 and chromosome 6 (Klein, 1983; reviewed by Potter, 1986). The breakpoints in chromosomes 15, 12 and 6 occurred in c-*myc*, IgH and IgL-κ, respectively. These translocations lead, just as in Burkitt's lymphoma (see Section 1.3.1.), to an activation of the c-*myc* oncogene. This murine plasmacytoma model has appeared to be useful to elucidate some oncogenic events that occur also in Burkitt's lymphoma, but is a model for a localized plasma cell malignancy rather than for MM.

Aging mice of the inbred C57BL/KaLwRij strain frequently develop proliferative B-cell disorders (Radl, 1981). In addition to the relatively frequently occurring idiopathic paraproteinaemia (benign monoclonal gammapathy) some old animals of this strain

revealed the presence of MM (Radl et al., 1985). Typical myeloma cells showing a monoclonal expansion were found in the BM and the spleen, in contrast with primary plasmacytomas which originate and grow only locally without involvement of the BM. The skeleton of these MM mice revealed osteoporosis with occasional osteolytic lesions. This MM could easily be propagated *in vivo* by intravenous transfer of BM cells into recipients of the same strain. Different MM lines (the 5T lines) maintained the properties of the original neoplasia in subsequent transplantation generations. The primary 5T MM are comparable to MM in man. They originate spontaneously, their localization is mainly in the BM, there is a positive correlation of their incidence with age, the most frequently observed isotype of the myeloma protein is IgG and bone lesions are present.

1.5. Introduction to the experimental work

In this thesis studies were performed at the DNA and RNA level of human and mouse MM in order to search for genetic defects, associated with MM. The aim of the study was to gain more insight into the molecular aspects that lead to the development of MM.

In order to investigate the presence of activated oncogenes which can lead to the transformation of normal cells into malignant myeloma cells, transfection studies were performed using DNA from several mouse MM lines (Chapter 2). DNA derived from three 5T MM lines was transfected to a mouse fibroblast cell line (NIH/3T3). Transfected cells were injected into nude mice, resulting in tumor development. The appearance of novel restriction fragments in the H-*ras* banding pattern in DNA isolated from tumors derived from two out of three 5T lines studied, may suggest a pathogenic role for H-*ras* in the MM development but no firm evidence for this suggestion was obtained.

In Chapter 3 the c-*myc* oncogene was studied in the same mouse model. The involvement of this oncogene in other B-cell malignancies in both man and mice made it a possible candidate to be also activated in MM. By Southern blot analysis of DNA derived from BM cells and ascitic cells of several 5T MM lines, data were obtained about possible c-*myc* rearrangements and/or amplifications. Such structural genetic changes may activate oncogenes leading to tumor development.

In Chapter 4 alterations in c-*myc* and *pvt*, two genes that are involved in Burkitt's lymphoma, were investigated in human MM BM samples. This was done at the DNA and RNA level to see whether any rearrangements, amplifications and/or overexpression of these genes could be detected.

In order to study the expression of particular genes in MM BM cells as an indication for activation, we set up an *in situ* hybridization technique to detect mRNA at the single cell level. We combined this technique with immunofluorescence labeling of cell surface markers to more precisely detect the cell type in which the mRNA was present (Chapter 5). With this technique the expression of two cytokines, IL-6 and IL-1β, was investigated in human MM cells. The rationale for this study was that IL-6

was suggested to be an autocrine growth factor for myeloma cells and IL-1β could be one of the major factors that is responsible for bone destruction, a characteristic feature of MM (Chapter 6).

In Chapter 7, the General discussion, the results of the experimental work are discussed in the context of the literature.

1.6. References

Adams, J.M., Gerondalus, S., Webb, E., Corcoran, L.M. and Cory, S. (1983) Cellular *myc* oncogene is altered by chromosome translocation to an immunoglobulin locus in murine plasmacytomas and is rearranged similarly in human Burkitt lymphomas. Proc Natl Acad Sci USA, 80: 1982.

Adams, J.M., Harris, A.W., Pinkert, C.A., Brinster, R.L., Palmitter, R.D., Corcoran, L., Alexander, W.S., Graham, M.W. and Cory, S. (1986) C-*myc* induced lymphomagenesis in transgenic mice and the role of the *pvt*-1 locus in lymphoid neoplasia. Curr. Top. Microbiol. Imm. 132: 1.

Anderson, K.C., Jones, R.M., Morimoto, C., Leavitt, P. and Barut, B.A. (1989) Response patterns of purified myeloma cells to hematopoietic growth factors. Blood, 73: 1915.

Asaoku, H., Kawano, M., Iwato, K., Tanabe, O., Tanaka, H., Hirano, T., Kishimoto, T. and Kuramoto, A. (1988) Decrease in BSF-2/IL-6 response in advanced cases of multiple myeloma. Blood, 72: 429.

Asker, C., Mareni, C., Coviello, D., Ingvarsson, S., Sessarego, M., Origone, P., Klein, G. and Sumeigi, J. (1988) Amplification of c-*myc* and *pvt*-1 homologous sequences in acute non-lymphatic leukemia. Leuk Res, 12: 523.

Bagg, A., Becker, P., Bezwada, W., Van Rensburg, L. and Mendelow, B. (1989) Circulating monotypic B-cells in multiple myeloma: association with lambda paraproteins. Brit J Haematol, 72: 167.

Banerjee, M., Wiener, F., Spira, J., Babonits, M., Nilsson, M.G., Sumegi, J. and Klein, G. (1985) Mapping of the c-*myc*, *pvt*-1 and immunoglobulin kappa genes in relation to the mouse plasmacytoma-associated variant (6;15) translocation breakpoint. EMBO J, 4: 3183.

Barlogie, B., Alexanian, R., Dixon, D., Smith, L., Smallwood, L. and Delasalle, K. (1985) Prognostic implications of tumor cell DNA and RNA content in multiple myeloma. Blood, 66: 338.

Bast, E.J.E.G., Van Camp, B., Reynaert, P., Wieringa, G. and Ballieux, R.E. (1982) Idiotypic peripheral blood lymphocytes in monoclonal gammopathy. Clin Exp Immunol, 47: 677.

Berenson, J., Wong, R., Kim, K., Brown, N. and Lichtenstein, A. (1987) Evidence for peripheral blood B lymphocyte but not T lymphocyte involvement in multiple myeloma. Blood, 70: 1550.

Bishop, J.M. (1985) Viral oncogenes. Cell, 42: 23.

Blattner, W.A. (1980) Epidemiology of multiple myeloma and related plasma cell disorders. In: Progress in Myeloma. Ed. M. Potter, Amsterdam, Elsevier North Holland Inc., pp. 1-67.

Cesarman, E., Dalla-Favera, R., Bentley, D. and Groudine, M. (1987) Mutations in the first exon are associated with altered transcription of c-*myc* in Burkitt lymphoma. Science, 238: 1272.

Chang, E.H., Furth, M.E., Scolnick, E.M. and Lowy, D.R. (1982) Tumorigenic transformation of mammalian cells induced by a normal human gene homologous to the oncogene of Harvey murine sarcoma virus. Nature, 297: 479.

Chiu, E.K.W., Ganeshaguru, K., Hoffbrand, A.V. and Mehta, A.B. (1989) Circulating monoclonal B lymphocytes in multiple myeloma. Br J Haematol, 72: 28.

Cleary, M.L. and Sklar, J. (1985[a]) DNA rearrangements in non-Hodgkin's lymphomas. Cancer Surveys, 4: 331.

Cleary, M.L. and Sklar, J. (1985[b]) Nucleotide sequence of a t(14;18) chromosomal breakpoint in follicular lymphoma and demonstration of a breakpoint cluster region near a transcriptionally active locus on chromosome 18. Proc Natl Acad Sci USA, 82: 7439.

Clofent, G., Klein, B., Commes, T., Ghanem, N., Lefranc, M.P. and Bataille, R. (1989) No detectable malignant B cells in the peripheral blood of patients with multiple myeloma. Br J Haematol, 71: 357.

Cooper, G.M., Olleirquist, S. and Silverman, L. (1980) Transforming activity of DNA of chemically transformed and normal cells. Nature, 284: 418.

Cooper, G.M. (1982) Cellular transforming genes. Science, 218: 801.

Cory, S., Graham, M., Webb, E., Corcoran, L. and Adams, J.M. (1985) Variant (6;15) translocations in murine plasmacytomas involve a chromosome 15 locus at least 72 kb from the c-*myc* oncogene. EMBO J, 4: 675.

Cory, S., Alexander, W.S., Rosenbaum, H., Vaux, D.L. Langdon, W.Y., Bath, M.L., McNeal, J., Webb, E., Adams, J.M. and Harris, A.W. (1989) Transgenic mice as models for the development of

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haemopoietic neoplasia. In: Progress in Immunology, Vol. 7. Ed.: F. Melchers, Springer-Verlag Berlin Heidelberg New York London Tokyo Hong Kong, pp. 494-501.

Cozzolino, F., Torcia, M., Aldinucci, D., Rubartelli, A., Miliani, A., Shaw, A.R., Lansdorp, P.M. and Duglielmo, R. (1989) Production of interleukin-1 by bone marrow myeloma cells. Blood, 74: 380.

Croce, C.M., Shander, M., Martinis, J., Cicurel, L., D'Ancona, G.G., Dolby, T.W. and Koprowski, H. (1979) Chromosomal location of the genes for human immunoglobulin heavy chains. Proc Natl Acad Sci USA, 76: 3416.

Daha, M.R., Bloem, A.C. and Ballieux, R.E. (1984) Immunoglobulin production by human peripheral lymphocytes induced by anti-CD3 receptor antibodies. J Immunol, 132: 1197.

De la Chapelle, A., Lenoir, G., Boué, J., Boué, A., Galano, P., Huerre, C., Szajnert, M.F., Jeanpierre, M., Lalonel, J.M. and Kaplan, J.C. (1983) Lambda Ig constant region genes are translocated to chromosome 8 in Burkitt's lymphoma with t(8;22). Nucl Acid Res, 11: 1133.

Defrance, T., Vanbervliet, B., Aubry, J.P., Takebe, Y., Arai, N., Miyajima, A., Yokota, T., Lee, F., Arai, K., de Vries, J.E. and Banchereau, J. (1987) B cell growth-promoting activity of recombinant human interleukin 4. J Immunol, 139: 1135.

Dewhirst, F.E., Stashenko, P.P., Mole, J.E. and Tsurumachi, T. (1985) Purification and partial sequence of human osteoclast-activating factor. Identity with interleukin 1β. J Immunol, 135: 2562.

Downward, J., Yarden, Y., Mayes, E., Serace, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J. and Waterfield, M.D. (1984) Close similarity of epidermal growth factor receptor and v-erb B oncogene protein sequences. Nature, 307: 521.

Durie, B.G.M., Salmon, S.E. and Mundy, G.R. (1981) Relation of osteoclast activating factor production to extent of bone disease in multiple myeloma. Br J Haematol, 47: 21.

Durie, B.G.M. and Crogan, T.M. (1985) CALLA-positive myeloma: An aggressive subtype with poor survival. Blood, 66: 229.

Durie, B.G.M., Vela, E., Baum, V., Leibovits, A., Payne, C., Richter, L.C., Crogan, T.M. and Trent, J.M. (1985) Establishment of two new myeloma cell lines from bilateral pleural effusions: Evidence for sequential in vivo clonal change. Blood, 66: 548.

Durie, B.G.M. and Salmon, S.E. (1982) The current status and future prospects of treatment for multiple myeloma. Clin Haematol, 11: 181.

Durie, B.G.M., Crogan, T.M., Spier, C., Vela, E., Baum, V., Rodriguez, M.A. and Frutiger, Y. (1989) Myelomonocytic myeloma cell line (LB 84-1). Blood, 73: 770.

Ellis, R.W., Lowy, D.R. and Scolnick, E.M. (1982) In: Advances in Viral Oncology. Ed. G. Klein, Raven Press, Vol. 1. pp. 107-126.

Epstein, J., Barlogie, B., Kodzmann, J. and Alexanian, R. (1988) Phenotypic heterogeneity in aneuploid multiple myeloma indicates pre-B cell involvement. Blood, 71: 861.

Erikson, J., Nishikura, K., Ar-Rushdi, A., Finan, J., Emanuel, B.S., Lenoir, G., Nowell, P.G. and Groce, C.M. (1983) Translocation of an immunoglobulin k locus to a region 3' of an unrearranged c-myc oncogen enhances c-myc transcription. Proc Natl Acad Sci USA, 80: 7581.

Erikson, J., Martinis, J. and Croce, C.M. (1981) Assignment of the genes for human l immunoglobulin chains to chromosome 22. Nature, 294: 173.

Erikson, J., Finan, J., Tsujimoto, Y., Nowell, P.C. and Croce, C.M. (1984) The chromosome 14 breakpoint in neoplastic B cells with the t(11;14) translocation involves the immunoglobulin heavy chain locus. Proc Natl Acad Sci USA, 81: 4144.

Ernst, T.J., Gazdar, A., Ritz, J. and Shipp, M. (1988) Identification of a second transforming gene, N-ras, in a human multiple myeloma line with a rearranged c-myc allele. Blood, 72: 1163.

Fahrlander, P.D., Sumegi, J., Yang, J.O. , Wiener, F., Marcu, K.B. and Klein, G. (1985) Activation of the c-myc oncogene by the immunoglobulin heavy-chain gene enhancer after multiple switch region-mediated chromosome rearrangements in a murine plasmacytoma. Proc Natl Acad Sci USA, 82: 3746.

Feo, S., Harvey, R., Showe, L. and Croce, C.M. (1986) Regulation of translocated c-myc genes transfected into plasmacytoma cells. Proc Natl Acad Sci USA, 83: 706.

Freedman, A.S., Freeman, G., Whitman, J., Segil, J., Daley, J. and Nadler, L.M. (1988) Pre-exposure of human B cells to recombinant IL-1 enhances subsequent proliferation. J Immunol, 141: 3398.

Fudenberg, H.H. and Virella, G. (1980) Multiple myeloma and Waldenström's macroglobulinaemia. Unusual presentations. Sem Hematol, 17: 63.

Garewal, H. and Durie, B. (1982) Aggressive phase of multiple myeloma with pulmonary plasma cell infiltrates. J Am Med Assoc, 248: 1875.

Garrett, R., Durie, B.G.M., Nedwin, G.E., Gillespie, A., Bringman, T., Sabatini, M., Bertolini, D.R. and Mundy, G.R. (1987) Production of lymphotoxin, a bone-resorbing cytokine, by cultured human myeloma cells. N Engl J Med, 317: 526.

Gazdar, A.F., Oie, H.K., Kirsch, I.R. and Hollis, G.F. (1986) Establishment and characterization of a

human plasma cell myeloma culture having a rearranged cellular *myc* proto-oncogene. Blood, 67: 1542.

Graham, M. and Adams, J.M. (1986) Chromosome 8 breakpoint far 3' of the c-*myc* oncogene in a Burkitt's lymphoma 2;8 variant translocation is equivalent to the murine *pvt*-1 locus. EMBO J, 5: 2845.

Graham, M., Adams, J.M. and Cory, S. (1985) Murine T lymphocytes with retroviral inserts in the chromosomal 15 locus for plasmacytoma variant translocations. Nature, 314: 740.

Greipp, P.R., Raymond, N.M., Kyle, R.A. and O'Fallon, W.M. (1985) Multiple myeloma: Significance of plasmablastic subtype in morphological classification. Blood, 65: 305.

Greipp, P.R. and Kyle, R.A. (1983) Clinical morphological and cell kinetic differences among multiple myeloma, monoclonal gammopathy of undetermined significance, and smoldering multiple myeloma. Blood, 62: 166.

Griesser, H., Feller, A., Lennert, K., Tweedale, M., Messner, H.A., Zalcberg, J., Minden, M.D. and Mak, T.W. (1986) The structure of the T cell gamma chain gene in lymphoproliferative disorders and lymphoma cell lines. Blood, 68: 592.

Griesser, H., Tkachuk, D., Reis, M.D. and Mak, T.W. (1989) Gene rearrangements and translocations in lymphoproliferative diseases. Blood, 73: 1402.

Grogan, T.M., Durie, B.G.M., Spier, C.M., Richter, L. and Vela, E. (1989) Myelomonocytic antigen positive multiple myeloma. Blood, 73: 763.

Haldar, S., Beatty, C., Tsujimoto, Y. and Croce, C.M. (1989) The *bcl*-2 gene encodes a novel G protein. Nature, 342: 195.

Haluska, F.G., Finger, S., Tsujimoto, Y. and Croce, C.M. (1986) The t(8;14) chromosomal translocation occuring in B-cell malignancies, results from mistakes in V-D-J joining. Nature, 324: 158.

Harris, A.W., Pinkert, C.A., Crawford, M., Langdon, W.Y., Brinster, R.L. and Adams, J.M. (1988) The Eμ-*myc* transgenic mouse. A model for high-incidence spontaneous lymphoma and leukemia of early B cells. J Exp Med, 167: 353.

Hirano, T., Yasukawa, K., Harada, H., Taga, T., Watanabe, Y., Matsuda, T., Kashiwamura, S., Nakajima, K., Koyama, K., Iwamatsu, A., Tsunasawa, S., Sakiyama, F., Matsui, H., Takahara, Y., Taniguchi, T. and Kishimoto, T. (1986) Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. Nature, 324: 73.

Hirano, T., Taga, T., Nakano, N., Yasukawa, K., Kashiwamura, S., Shimuzu, K., Nakajima, K., Puym, K.H. and Kishimoto, T. (1985) Purification to homogeneity and characterization of human B-cell differentiation factor (BCDF or BSF p-2). Proc Natl Acad Sci USA, 82: 5490.

Hollis, G.F., Gazdar, A.F., Bertness, V. and Kirsch, I.R. (1988) Complex translocation disrupts c-*myc* regulation in a human plasma cell myeloma. Mol Cell Biol, 8: 124.

Howard, M., Mizel, S.B., Lachman, L., Ansel, J., Johnson, B. and Paul, W.E. (1983) Role of interleukin-1 in anti-immunoglobulin-induced B cell proliferation. J Exp Med, 157: 1529.

Howell, G.M. and Alexanian, R. (1976) Multiple myeloma in young persons. Ann Intern Med, 84: 441.

Huebner, R.J. and Todaro, G.J. (1969) Oncogenes of RNA tumor viruses as determinants of cancer. Proc Natl Acad Sci USA, 64: 1087.

Humphries, J.E. and Williams, M.E. (1989) Immunoglobulin gene and c-*myc* oncogene rearrangement in early and advanced myeloma. Blood, 74: 375A.

Hunter, T. and Cooper, J.A. (1985) Protein-tyrosine kinase. Ann Rev Biochem, 54: 897.

Jelinek, D.F. and Lipsky, P.E. (1987) Enhancement of human B cell proliferation and differentiation by tumor necrosis factor-a and interleukin 1. J Immunol, 139: 2970.

Jernberg, H., Nilsson, K., Zeck, L., Lutz, D., Nowotny, H. and Scheirer, W. (1987) Establishment and phenotypic characterization of three new human myeloma cell lines (U-1957, U-1958 and U-1996). Blood, 69: 1605.

Kapadia, S.B. (1980) Multiple myeloma: a clinicopathologic study of 62 consecutive autopsied cases. Medicine, 59: 380.

Katagiri, S., Takeshi, Y., Kuyama, J., Kanayama, Y., Nishida, K., Tatuso, A., Tamaki, T., Ohnishi, M. and Tarni, S. (1985) Two distinct human myeloma cell lines originating from one patient with myeloma. Int J Cancer, 36: 241.

Kawano, M., Hirano, T., Matsuda, T., Taga, T., Horii, Y., Iwato, K., Asaoku, H., Tang, B., Tanabe, O., Tanaka, H., Kuramoto, A. and Kishimoto, T. (1988) Autocrine generation and requirement of BSF-2/II-6 for human multiple myeloma. Nature 332: 83.

Kawano, M., Yamamoto, I., Iwato, K., Tanaka, H., Asaoku, H., Tanabe, O., Ishikawa, H., Nobuyoshi, M., Ohmoto, Y., Hirai, Y. and Kuramoto, A. (1989) Interleukin-1 beta rather than lymphotoxin as the major bone resorbing activity in human multiple myeloma. Blood, 73: 1646.

Kingston, R.E., Baldwin, A.S., Jr. and Sharp, P.A. (1985) Transcription control by oncogenes. Cell, 41: 3.

Kishimoto, T., Yoshisalu, K., Okada, M., Kuritani, T., Kikutani, H., Sakaguchi, N., Miki, Y., Kishi, H.,

Nakagawa, T., Shimizu, K., Fukanaga, K. and Taga, T. (1985) Growth and differentiation factors and activation of human B cells. Lymphokines, 10: 15.

Kishimoto, T. (1987) B cell stimulatory factors (BSFs): Molecular structure, biologic function, and regulation of expression. J Clin Immunol, 5: 343.

Klein, B., Jourdan, M., Vazquez, A., Dugas, B. and Bataille, R. (1987) Production of growth factors by human myeloma cells. Cancer Res, 47: 4856.

Klein, B., Zhang, X.G., Jourdan, M., Content, J., Houssiau, F., Aarden, L., Piechaczyk, M. and Bataille, R. (1989) Paracrine rather than autocrine regulation of myeloma cell growth and differentiation by interleukin-6. Blood, 73: 517.

Klein, G. (1983) Specific chromosomal translocations and the genesis of B cell derived tumors in mice and men. Cell, 32: 311.

Kobayashi, H., Potter, M. and Dunn, T.B. (1961) Bone lesions produced by transplanted plasma-cell tumors in BALB/c mice. J Natl Canc Inst, 28: 648.

Kubagawa, H., Voghler, L.B., Capra, J.D., Conrad, M.E., Lawton, A.R. and Cooper, M.D. (1979) Studies on the clonal origin of multiple myeloma. Use of individually specific (idiotype) antibodies to trace the oncogenic event to its earliest point of expression in B cell differentiation. J Exp Med, 150: 792.

Kyle, R.A. (1975) Multiple Myeloma. Review of 869 cases. Mayo Clinic Proc, 50: 29.

Kyle, R.A. (1985) Malignant B-cell monoclonal gammopathies. In: Monoclonal gammapathies - Clinical significance and basic mechanisms, Vol. 5. Eds.: J. Radl, W. Hijmans, B. Van Camp. Eurage, Rijswijk, The Netherlands, pp. 15-23.

Land, H., Parada, L.F. and Weinberg, R.A. (1983) Cellular oncogenes and multistep carcinogenesis. Science, 222: 771.

Latreille, J., Barlogie, B., Johnston, D.A., Drewinko, B. and Alexanian, R. (1982) Ploidy and proliferative characteristics in monoclonal gammopathies. Blood, 59: 43.

Le, J. and Viček, J. (1987) Biology of disease. Tumor necrosis factor and interleukin 1: cytokines with multiple overlapping biological activities. Lab Invest, 56: 234.

Lichtenstein, A., Berenson, J., Norman, D., Chang, M.P. and Carlile, A. (1989) Production of cytokines by bone marrow cells obtained from patients with multiple myeloma. Blood, 74: 1266.

Lingeman, C.H. (1969) Plasma cell neoplasms of man and animals. Natl Canc Inst Monograph, 32: 303.

Lipsky, P.E., Thompson, P.A., Rosenwasser, L.J. and Dinarello, C.A. (1983) The role of interleukin-1 in human B cell activation: inhibition of B cell proliferation and the generation of immunoglobulin secreting cells by an antibody against human leucocyte pyrogen. J Immunol, 130: 2708.

Lokhorst, H.M., Boom, S.E., Bast, E.J.E.G. and Ballieux, R.E. (1985) Identification and functional significance of a novel type of proliferating B lymphoid cell in multiple myeloma (MM). In: Monoclonal gammapathies - Clinical significance and basic mechanisms. Vol. 5, Eds.: J. Radl, W. Hijmans, B. Van Camp. Eurage, Rijswijk, The Netherlands, pp. 123-126.

Mackenzie, M.R. and Lewis, J.P. (1985) Cytogenetic evidence that the malignant event in multiple myeloma occurs in a precursor lymphocyte. Cancer Genet Cytogenet, 17: 13.

Martin, G.S. (1970) Rous carcinoma virus: A function required for the maintenance of the transformed state. Nature, 227: 1021.

Mellstedt, H., Hammerstian, S. and Holm, G. (1974) Monoclonal lymphocyte population in human plasma cell myeloma. Clin Exp Immunol, 17: 371.

Meltzer, P., Shadle, K. and Durie, B. (1987) Somatic mutation alters a critical region of the c-*myc* gene in multiple myeloma. Blood, 70: 985A.

Mengle-Gaw, L. and Rabbitts, T.H. (1987) A human chromosome 8 region with abnormalities in B cell, HTLV-1^{+} T cell and c-*myc* amplified tumours. EMBO J, 6: 1959.

Mingari, M.C., Gerosa, F., Carra, G., Acolla, R.S., Moretta, A., Zubler, R.H., Waldmann, T.A. and Moretta, L. (1984) Human interleukin-2 promotes proliferation of activated B-cells via surface receptors similar to those of activated T-cells. Nature, 312: 641.

Mundy, G.R., Raisz, L.G., Cooper, R.A., Schechter, G.P. and Salmon, S.E. (1974) Evidence for the secretion of an osteoclast stimulating factor in myeloma. N Engl J Med, 291: 1041.

Muraguchi, A., Kehrl, J. and Fauci, A.S. (1985) Activation, proliferation and differentiation of human B lymphocytes. Lymphokines, 10: 33.

Muraguchi, A., Hirano, T., Tang, B., Matsuda, T., Horii, Y., Nakajiama, K. and Kishimoto, T. (1988) The essential role of B cell stimulatory factor-2 (BSF-2/IL-6) for the terminal differentiation of B cells. J Exp Med, 167: 332.

Nishikura, K., Ar-Rushdi, A., Erikson, J., Watt, R., Rovera, G. and Croce, G.M. (1983) Differential expression of the normal and of the translocated human c-*myc* oncogenes in B-cells. Proc Natl Acad Sci USA, 80: 4822.

Potter, M. and Boyer, C.R. (1962) Induction of plasma cell neoplasms in BALB/c mice with mineral oil adjuvants. Nature, 193: 1086.

Potter, M. (1986) Plasmacytomas in mice. Semin Oncol, 13: 275.

Poupart, P., Vandenabeele, P., Cayphas, S., Van Snick, J., Haegeman, G., Kruys, V., Fiers, W. and Content, J. (1987) B cell growth modulating and differentiating activity of recombinant human 26-kd protein (BSF-2, Hu IFN-beta 2, HPGF). EMBO J, 6: 1219.

Pulciani, S., Santon, E., Lanver, A.V., Long, L.K., Aeronson, S.A. and Barbacid, M. (1982) Oncogenes in solid human tumours. Nature, 300: 539.

Radl, J. (1981) Immunoglobulin levels and abnormalities in aging humans and mice. In: Immunological Techniques Applied to Aging Research. Eds.: W.H. Adler and A.A. Nordin. CRC Press, Boca Raton, FL, pp. 121-139.

Radl, J. (1982) Effects of aging on immunoglobulins. In: Protein abnormalities. Vol. 2. Pathology of immunoglobulins - diagnostic and clinical aspects. Ed.: S.E. Ritzmann. Alan R. Liss Inc., N.Y. pp. 55-69.

Radl, J. (1985) Monoclonal gammapathies. An attempt at a new classification. Neth J Med, 28: 134.

Radl, J., Croese, J.W., Zurcher, C., Brondijk, R.J. and Van den Enden-Vieveen, M.H.M. (1985) Spontaneous multiple myeloma with bone lesions in the aging C57BL/KaLwRij mouse as a natural model of human disease. In: Topics in Aging Research in Europe. Eds.: J. Radl, W. Hijmans and B.Van Camp. Eurage, Rijswijk, The Netherlands. Vol. 5, pp 191-194.

Rodriguez, L.H., Finkelstein, J.B., Shullenberger, C.C. and Alexanian, R. (1972) Bone healing in multiple myeloma with melphalan chemotherapy. Ann Int Med, 76: 551.

Romagnani, S., Grazia Guidizi, M., Biagotti, R., Almerigogna, F., Mingari, C., Maggi, E., Liang, C. and Morette, L. (1986) B cell growth factor activity of interferon gamma. Recombinant human gamma interferon promotes proliferation of anti-μ activated human B lymphocytes. J Immunol, 136: 3513.

Rosen, S.M., Buxbaum, J.N. and Frangione, H. (1986) The structure of immunoglobulins and their genes, DNA rearrangement and B cell differentiation, molecular anomalies of some monoclonal immunoglobulins. Semin Oncol, 13: 260.

Rubenstein, M.A. (1949) Multiple myeloma as a form of leukaemia. Blood, 4: 1049.

Schedel, I., Peest, D., Stunkel, K., Fricke, M., Eckert, G. and Deicher, H. (1980) Idiotype-bearing peripheral blood lymphocytes in human multiple myeloma and Waldenström's macro- globulinaemia. Scand J Immunol, 11: 437.

Segi, M. (1977) Graphic presentation of cancer incidence by site, area and population compiled from the data published in "Cancer incidence in five continents, volume III". Segi Institute of Cancer, Nagoya, Japan.

Selvanayagam, P., Goodacre, A., Strong, L., Saunders, G. and Barlogie, B. (1987) Alterations of bcl-1 oncogene in human multiple myeloma. AACR, Abstract #76.

Selvanayagam, P., Blick, M., Narni, F., van Tuinen, P., Ledbetter, D.H., Alexanian, R., Saunders, G.F. and Barlogie, B. (1988) Alteration and abnormal expression of the c-myc oncogene in human multiple myeloma. Blood, 71: 30.

Shander, M., Martinis, J. and Croce, C.M. (1980) Genetics of human immunoglobulins: assignment of the genes for m, a and g immunoglobulin chains to human chromosome 14. Transpl Proc, 12: 417.

Shimizu, K., Murate, T. and Kunii, A. (1980) Circulating immunoglobulin-secretin cells in patients with plasma cell dyscrasia. Blood, 55: 590.

Shtivelman, E. and Bishop, J.M. (1989) The PVT gene frequently amplifies with MYC in tumor cells. Mol Cell Biol, 9:1148.

Shtivelman, E., Henglein, B., Groitl, P and Bishop, M. (1989) Identification of a human transcription unit affected by the variant chromosomal translocations 2;8 and 8;22 of Burkitt lymphoma. Proc Natl Acad Sci USA, 86: 3257.

Siegelman, M.H., Cleary, M.L., Warnke, R. and Sklar, J. (1985) Frequent biclonality and Ig gene alterations among B cell lymphomas that show multiple histologic forms. J Exp Med, 161: 850.

Sinn, E., Muller, W., Pattengale, P., Tepler, I., Wallace, R. and Leder, P. (1987) Coexpression of MMTV/v-Ha-ras and MMTV/c-myc genes in transgenic mice: synergystic action of oncogenes in vivo. Cell, 49: 465.

Sümegi, J., Hedberg, T., Björkholm, M., Godal, T., Mellstedt, H., Nilsson, M.G., Pereman, C. and Klein, G. (1985) Amplification of the c-myc oncogene in human plasma cell leukemia. Int J Cancer, 36: 367.

Taub, R., Kirsch, I., Morton, C., Lenoir, G., Swan, D., Tronick, S., Aaronson, S. and Leder, P. (1982) Translocation of the c-myc gene into immunoglobulin heavy chain locus in human Burkitt lymphoma and murine plasmacytoma cells. Proc Natl Acad Sci USA, 79: 7837.

Toda, T., Uno, I., Ishikawa, T., Powers, S., Kataoka, T., Broek, D., Cameron, S., Broach, J., Matsumoto, K. and Wigler, M. (1985) In yeast, RAS proteins are controlling elements of adenylate cyclase. Cell, 46: 27.

Tonegawa, S. (1983) Somatic generation of antibody diversity. Nature, 362: 575.

Tosato, G., Seamon, K.B., Goldman, N.D., Sehgal, P.B., May, L.T., Washington, G.C., Jones, K.D. and

23

Pike, S.E. (1988) Monocyte-derived human B-cell growth factor identified as interferon beta 2 (BSF-2, IL-6). Science, 239: 502.

Tsichlis, P.N., Shepherd, B.M. and Bear, S.E. (1989) Activation of the *Mlvi*-1/*mis*1/*pvt*-1 locus in Moloney murine leukemia virus-induced T-cell lymphomas. Proc Natl Acad Sci USA, 86: 5487.

Tsuchiya, H., Epstein, J., Selvanayagam, P., Dodman-Gallich, G., Alexanian, R. and Barlogie, B. (1988) Correlated flow cytometric analysis of H-*ras* p21 and nuclear DNA in multiple myeloma. Blood, 72: 796.

Tsujimoto, Y., Yunis, J., Onorato-Showe, L., Erikson, J., Nowell, P.C. and Croce, C.M. (1984[a]) Molecular cloning of the chromosomal breakpoint of B-cell lymphomas and leukemias with the t(11;14) chromosome translocation. Science, 224: 1403.

Tsujimoto, Y., Finger, L.R., Yunis, J., Nowell, P.C. and Croce, C.M. (1984[b]) Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. Science, 226: 1097.

Tsujimoto, Y., Cossman, J., Jaffe, E. and Croce, C.M. (1985[a]) Involvement of the *bcl*-2 gene in human follicular lymphoma. Science, 228: 1440.

Tsujimoto, Y., Jaffe, E., Cossman, J., Gorham, J., Nowell, P.C. and Croce, C.M. (1985[b]) Clustering of breakpoints on chromosome 11 in human B-cell neoplasms with the t(11;14) chromosome translocation. Nature, 315: 340.

Tsujimoto, Y. and Croce, C.M. (1986) Analysis of the structure, transcripts and protein products of the *bcl*-2. The gene involved in human follicular lymphoma. Proc Natl Acad Sci USA, 83: 5214.

Van Acker, A., Conte, F., Hullin, N. and Urbain, J. (1979) Idiotypic studies on myeloma B cells. Eur J Cancer, 15: 627.

Van Damme, J., Opdenakker, G., Simpson, R.J., Rubira, M.R., Cayphas, S., Vink, A., Billiau, A. and van Snick, J. (1987) Identification of a human 26-KD protein, interferon b2 (IFN-b2), as a B cell hybridoma (plasmacytoma) growth factor induced by interleukin 1 and tumor necrosis factor. J Exp Med, 165: 914.

Van Riet, I., Heirman, C., Lacor, P., De Waele, M., Thielemans, T. and Van Camp, B. (1989) Detection of monoclonal B lymphocytes in bone marrow and peripheral blood of multiple myeloma patients by immunoglobulin gene rearrangement studies. Brit J Haematol, 73: 289.

Villeneuve, L., Rassart, E., Jolicoeur, P., Graham, M. and Adams, J.M. (1986) Proviral integration site *mis*-1 in rat thymomas corresponds to the *pvt*-1 translocation breakpoint in murine plasmacytomas. Mol Cell Biol, 6: 1834.

Webb, E., Adams, J.M. and Cory, S. (1984) Variant t(15;6) translocation in a murine plasmacytoma occurs near immunoglobulin Ck gene but far from the *myc* oncogene. Nature, 312: 777.

Yancopoulos, G.D. and F.W. Alt (1986) Regulation of the assembly and expression of variable region genes. Ann Rev Immunol, 4: 339.

Yunis, J.J. (1983) The chromosomal basis of human neoplasia. Science, 221: 227.

2. TRANSFORMING CAPACITY OF DNA FRAGMENTS FROM MURINE MULTIPLE MYELOMA CELLS

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SUMMARY

High molecular weight DNA from fresh bone marrow cells from three different multiple myeloma (5T2, 5T7 and 5T14) bearing mice were assayed for the presence of transmissible activating transforming genes. This was done by a DNA-mediated gene transfer technique using NIH/3T3 cells. After DNA transfer the cells were inoculated into nude mice. In all three cases tumors developed within 3 weeks while mice injected with control NIH/3T3 DNA transfected NIH/3T3 cells did not develop tumors within 4 weeks. Secondary transfection with DNA from these tumors ligated to a dominant selective marker in order to distinguish between tumor DNA and NIH/3T3 DNA, gave also rise to tumor development in nude mice. This dominant marker, however, may well be able to transform NIH/3T3 into tumorigenic cells by itself as the ligated NIH/3T3 DNA transfected NIH/3T3 cells also gave rise to tumor formation.

Tumor DNA did not show alterations in banding pattern when hybridized with the Ki-*ras*, N-*ras*, B-*lym* or *mos* probes. However, a clear alteration was observed in the H-*ras* banding pattern of the DNA from tumors derived from NIH/3T3 cells transfected with DNA from 5T7 and 5T14. After a second round of transfection, the same H-*ras* alteration was found in the case of 5T14. Whether this changed H-*ras* fragment is associated with any genetic abnormality in murine multiple myeloma is still unclear.

INTRODUCTION

The genetic alterations involved in the neoplastic transformation in multiple myeloma (MM) are still unknown. In many tumors the presence of oncogenes has been detected by their ability to induce transformation of NIH/3T3 cells (1). In the case of MM, however, so far transformation could not be established with DNA from fresh bone marrow cells, although it did occur with DNA from two established human MM cell lines (2). The gene(s) responsible for this transformation has not been identified.

In recent years spontaneously appearing MM in mice were observed (3). These MM are transplantable and resemble the human MM very closely in most respects (4). Three of these 5T MM lines were analyzed in the tumorigenicity assay described by Blair et al. (5). This assay relies on the ability of transformed NIH/3T3 cells to form tumors in nude mice.

MATERIALS AND METHODS

Cells and cell culture

5T2, 5T7 and 5T14 bone marrow cell suspensions were prepared as described (6). NIH/3T3 clone 224 cells were cultured in a mixture of Ham's F10/Dulbecco's modified Eagle's medium supplemented with 4% fetal calf serum (Boehringer, Mannheim, FRG) and 4% new born calf serum (Flow, Irvine, Scotland).

Transfection assay

High molecular weight (MW) genomic DNA of 5T2, 5T7, 5T14 and NIH/3T3 (200 μg), in coprecipitate with plasmid pSV_2 neo (10 μg) (7) containing the neomycin resistance (neo^r) marker, was transfected to 10 dishes with NIH/3T3 (2 x 10^5 cells/dish) using a modification of the calcium phosphate precipitation method developed by Graham and Van der Eb (8). The precipitate remained on the cells for 17 hours. Three days after transfection, the culture medium was replaced by similar medium containing 300 μg/ml G418 (Gibco, Paisly, Scotland). Four weeks later resistant cells were trypsinized, pooled and injected into nude mice as described below. In a secondary transfection high MW genomic DNA from tumors originating from the primary transformants was partially digested to approximately 50 kb fragments with BamHI and ligated to a modified pMCS-vector (9) containing a neo^r marker and a human 'Alu' repetitive sequence (pMCS-Alu) and transfected to NIH/3T3 as described above.

Test for tumorigenicity

Transfected NIH/3T3 cells were injected subcutaneously (s.c.) in athymic BALB/c nude mice (2 x 10^6 cells/mouse). Tumors appearing at the site of injection (5-21 days) were characterized by Southern blot hybridization and in vitro culture.

<u>DNA analysis</u>

Isolation of genomic, high MW DNA and plasmid DNA, restriction enzyme digestion, ligation, gel electrophoresis and Southern blot hybridization were done according to standard procedures as described by Maniatis et al. (10). DNA probes were ^{32}P-labeled by random priming (11). The probes used were the 2.6 kb HindIII fragment of chicken B-*lym* (12), the 580 bp Sal/EcoRI fragment of a human N-ras cDNA (13), the 1.0 kb HincII fragment of viral Ki-*ras* (14), a 0.8 kb and 2.9 kb SstI fragment of a human H-*ras* (14) oncogene and a 2.75 kb EcoRI fragment of a human *mos* gene (16).

RESULTS

High MW genomic DNA from 5T2, 5T7 and 5T14 MM bone marrow cells was transfected to NIH/3T3 cells in coprecipitation with plasmid pSV_2neo, carrying a neo[r] gene. Neo[r] NIH/3T3 transformants were counted, pooled and scored for tumorigenic potential by s.c. injection into nude mice 4 weeks after transfection. Some colonies had a characteristically transformed phenotype. The number of neo[r] colonies was about 2000/200 µg DNA in this transfection round. After 18 days, all mice developed tumors at the site of injection. In control experiments in which NIH/3T3 cells were transfected with NIH/3T3 DNA and injected into recipient mice, tumors did not appear (Table 1). Cell lines were established from the tumors. They all were resistent against neomycin, indicating that they originated from the transfected NIH/3T3 cells.

Table 1. Tumorigenicity assay of murine Multiple Myeloma DNA

DNA	FIRST FOUND[a]		SECOND ROUND[b]	
	Tumor appearance (days)	Number of tumors/number of mice injected	Tumor appearence (days)	Number of tumors/number of mice injected
5T2	13	2/2	13	4/4
5T7	18	2/2	10	4/4
5T14	14	2/2	21	3/4
NIH/3T3	-	0/2	14	4/4
T24[c]	7	2/2	N.T.[d]	N.T.

a.   In the first round NIH/3T3 were exposed to both pSV_2neo and DNA from the indicated cell lines.

b.   In the second round NIH/3T3 cells were exposed to DNA from the first round tumors ligated to pMCS-*Alu*. The negative control was NIH/3T3 DNA ligated to pMCS-*Alu*.

c.   T24 is a human bladder carcinoma with an activated H-*ras* oncogene.

d.   N.T. means not tested.

For the second round of transfection, DNA of approximately 50 kb was prepared from tumors from the first round of transfection and ligated to pMCS-*Alu*, carrying a neo[r] gene. In this cases about 7500 neo[r] colonies/200 μg DNA were obtained after transfection. Upon s.c. injection into nude mice, all mice developed tumors at the site of injection. In this case, however, in the control group in which NIH/3T3 cells were transfected with NIH/3T3 DNA ligated to MCS-*Alu*, all mice developed tumors within 14 days (Table 1). This also occurred in a second independent experiment (data not shown). In both cases, the cell lines established from these tumors were neo[r].

To determine whether the oncogenic transformation of NIH/3T3 was due to the transfer of activated oncogenes present in the genome of 5T2, 5T7 and 5T14 cells, chromosomal DNA of the nude mice tumors of the first and second round of transfection and of the original bone marrow cells of 5T2, 5T7 and 5T14 MM bearing mice, was digested with several restriction endonucleases, size fractionated by gel electrophoresis and after transfer to nitrocellulose hybridized with various ^{32}P-labeled probes. Transfection usually results in rearrangements around the qeue selected for and therefore will yield altered restriction fragments when enzymes are used that cleave at a sufficient distance from the transfected oncogene. In this way it is possible to discriminate between transferred (mouse) oncogenes and the endogenous copies. Hybridization with N-*ras*, Ki-*ras*, B-*lym* and *mos* revealed no other fragments than those of the endogeneous NIH/3T3 genes. When a probe of the human H-*ras* gene was applied was applied, however, an additional hybridizing fragment of 4.3 kb was detected in tumors derived from the 5T7 and 5T14 primary transformants and the 5T14
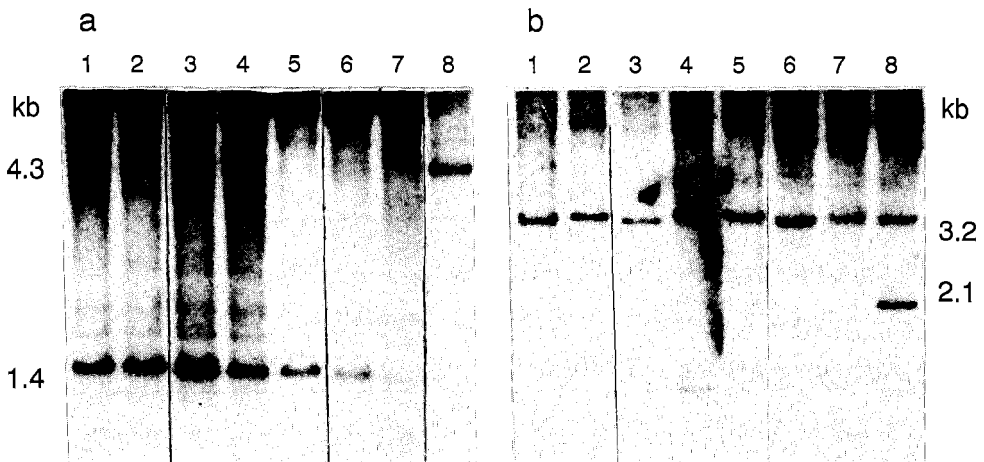
Figure 1. Presence of a novel fragment hybridizing to a human H-ras probe in tumor DNA derived from murine MM transfected NIH/3T3. DNA was digested with EcoRI (a) and BamHI (b). <u>Lane</u> 1, NIH/3T3; 2, NIH/3T3 ligated to pMCS-Alu primary transfectant; 3, 5T7; 4, 5T7 in coprecipitate with pSV₂neo primary transfectant; 5, 5T7 ligated to pMCS-Alu secondary transfectant; 6, 5T14; 7, 5T14 in coprecipitate with pSV₂neo primary transfectant; 8, 5T14 ligated to pMCS-Alu secondary transfectant. 5T2 DNA was not analyzed.

secondary transformants after digestion with EcoRI. A BamHI digest revealed only an additional fragment of 2.1 kb in tumors derived from the 5T14 secondary trans formants. The tumor DNA derived from the control NIH/3T3 transformants showed only the endogenous H-*ras* gene (Fig. 1). All tumors contained plasmid sequences (data not shown) indicating incorporation of the cotransfected or ligated DNA.

DISCUSSION

In the present study, a unique mouse model of MM was investigated for the presence of oncogenes capable of transforming NIH/3T3 cells. Using cotransfer and tumorigenicity in nude mice, tumors developed in the three 5T MM cases and none in the NIH/3T3 control. This strongly suggests that 5T MM DNA contains transforming genes. In order to detect this murine transforming fragment in the murine genome of NIH/3T3, we ligated the DNA from the tumors derived from primary transformants to pMCS-*Alu* in order to select for neomycine resistance in culture and for the presence of pMCS-*Alu* in the genome. We considered that, when the secondary transformants would be tumorigenic, the DNA fragment next to pMCS-*Alu* had to be of 5T origin and should contain the transforming sequences. Partial digestion of the DNA in 50 kb pieces, in order to ligate it to pMCS-*Alu*, was not expected to completely destroy the transforming gene as the largest known *ras* gene is about 40 kb. The tumor development, however, in the control experiment in which NIH/3T3 DNA itself was ligated to pMCS-*Alu* made it impossible to conclude that the transforming sequences next to pMCS-*Alu* were of 5T origin. They might also be of NIH/3T3 DNA origin. An explanation for the tumorigenicity of the ligated NIH/3T3 may be found in the structure of pMCS-*Alu*. This plasmid contains the SV40 promotor sequence (9). Therefore, during BamHI ligation, this promotor is put in the very near vicinity of the adjacent DNA fragments. This could lead to the activation of a proto-oncogene by SV40 promotor insertion. However, other explanations, such as spontaneous mutation of NIH/3T3 in culture or activation of a proto-oncogene due to the DNA manipulation during the transfection by other mechanisms than SV40 promoter insertion, are also possible.

Southern blot hybridization revealed changes in the H-*ras* banding pattern in the tumor DNA from 5T7 and 5T14 primary transfectants and from the 5T14 secondary transfectants. The additional fragments are presumably derived from the 5T DNA as the NIH/3T3 derived tumor did not reveal these bands. The new band might have been generated in the DNA extraction and manipulation or by excision during the transforming process. If restriction sites closely linked to the transferred gene were destroyed during the transfection process, DNA prepared from tumors derived from transformants would contain novel restriction fragments hybridizing to H-*ras*. Tumors derived from 5T14 secondary transformants showed the same novel H-*ras* hybridizing fragment as the tumors derived from primary transformants, suggesting that the H-*ras* gene is responsible for tumor development. This is in agreement with the

observation that most of the NIH/3T3 transforming genes are members of the *ras* gene family (17). The possible pathogenic role of H-*ras* in murine MM, however, is unclear and needs further investigations.

In conclusion, the results from the first round transfection assay show that the 5T DNA has transforming capacity and that presumably the gene responsible for the neoplastic transformation of NIH/3T3 cells is H-*ras*. Secondary transfection data supported these conclusions in the case of 5T14 but not in the case of 5T7. However, the 5T7 secondary transfection data could not be interpreted because NIH/3T3 DNA ligated to pMCS-*Alu* was tumorigenic by itself so that the assay did not select for an activated H-*ras* gene.

REFERENCES

1. Wigler, M., Sweet, R., Sim, G.K., Wold, B., Pellicer, A., Lacy, A., Maniatis, T., Silverstein, S. and Axel, R. (1979) Cell, 16, 777-785.
2. Lane, M.-A., Sainten, A. and Cooper, G.M. (1982) Cell, 28, 873-880.
3. Radl, J., Croese, J.W., Zurcher, C., Brondijk, R.J. and Van den Enden-Vieveen, M.H.M. (1985) In: Radl, J., Hijmans, W.van Van Camp, B. (Eds.) Monoclonal gammapathies. Clinical significance and basic mechanisms, EURAGE, Rijswijk, pp. 191-194.
4. Radl, J., Croese, J.W., Zurcher, C., Van den Enden-Vieveen, M.H.M. and de Leeuw, A.M. (1988) Amer. J. Pathol., 132, 593-598.
5. Blair, D.G., Cooper, C.S., Oskarsson, M.K., Fader, L.A. and Van de Woude, G.F. (1982) Science, 281, 1122-1125.
6. Croese, J.M., Vas Nunes, C.M., Radl, J., Van den Enden-Vieveen, M.H.M., Brondijk, R.J. and Boersma, W.J.A. (1987) Br. J. Cancer, 56, 555-560.
7. Song, K.-Y., Chekuri, L., Rauth, S., Ehrlich, S. and Kucherlapati, R. (1985) Mol. Cell. Biol. 5, 3332-3336.
8. Graham, F.L. and Van der Eb, A.J. (1973) Virology, 52, 456-467.
9. Grosveld, F.G., Lund, T., Murray, E.J., Mellor, A.L., Dahl, H.H.M., Flavell, R.A. (1982) Nucleic Acid Res., 10, 6715-6732.
10. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular cloning. A Laboratory Manual, Cold Spring Harbor Laboratories, New York.
11. Friedberg, A.P. and Vogelstein, B. (1983) Anal. Biochem., 132, 6.
12. Goubin, G., Goldman, D.S., Luce, J., Nieman, P.E. and Cooper, G.M. (1983) Nature, 302, 114-119.
13. Taparowsky, E., Shimizu, K., Goldfarb, M. and Wigler, M. (1983) Cell, 34, 581-586.
14. Tsuchida, N. and Uesuchi, S. (1981) J. Virol., 38, 720-727.
15. Fasano, O., Taparowski, E., Fiddes, J., Wigler, M. and Goldfarb, M. (1983) J. Molec. Appl. Genet., 2, 173-180.
16. Watson, R., Oskarsson, M. and Van de Woude, G.F. (1982) Proc. Natl. Acad. Sci. USA, 79, 4078-4082.
17. Weinberg, R.A. (1984) Blood, 64, 1143-1145.

3.   THE 5T MOUSE MULTIPLE MYELOMA MODEL: ABSENCE OF

C-*MYC* ONCOGENE REARRANGEMENT IN EARLY TRANSPLANT GENERATIONS

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SUMMARY

Consistent chromosomal translocations involving the c-*myc* cellular oncogene and one of the three immunoglobulin loci are typical for human Burkitt's lymphoma, induced mouse plasmacytoma (MPC) and spontaneously arising rat immunocytoma (RIC). Another plasma cell malignancy, multiple myeloma (MM), arising spontaneously in the aging C57BL/KaLwRij mice, was investigated in order to see whether the MM cells contain c-*myc* abnormalities of the MPC or RIC type. Rearrangement of the c-*myc* oncogene was found in the bone marrow cells only in 5T2 MM transplantation line in a mouse of the 24th generation and in none of the seven other MM of the 5T series which were of earlier generations. Since the mouse 5T MM resembles the human MM very closely, including the absence of consistent structural c-*myc* oncogene abnormalities, it can serve as a useful experimental model for studies on the etiopathogenesis of this disease.

INTRODUCTION

Consistent chromosomal translocations involving the c-*myc* cellular oncogene and one of the three immunoglobulin loci have been reported in human Burkitt's lymphoma, in induced mouse plasmacytoma (MPC) and in spontaneously arising rat immunocytoma (RIC) (rev. by Croce & Nowell, 1985; Potter, 1986; Pear et al., 1986; Enrietto, 1987). Translocation is believed to play an important role in the development of these tumors (Klein, 1986). Multiple myeloma (MM) in man is a neoplasm of B cells at a differentiation stage comparable to that of MPC and RIC. However, a rearrangement of the c-*myc* oncogene was reported only in three cases of MM (Gazdar et al., 1986; Selvanayagam et al., 1988) and in one case of plasma cell leukemia (Yamada et al., 1983).

In the recent years, several transplantation lines derived from spontaneously arising MM in aging C57BL mice became available in our institute. This mouse MM resembles the human disease very closely in several aspects (Radl et al., 1988). It is of interest to investigate whether the cells of the experimental 5T MM series contain c-*myc* abnormalities of the MPC type or whether they resemble the human MM also in this respect. The results of this study show that rearrangement of the c-*myc* oncogene was found in the bone marrow of only one of the 5T MM transplantation lines and was possibly due to a late event in the progression of this malignancy.

MATERIALS AND METHODS

Mice

Male and female C57BL/KaLwRij mice from the colony of the Institute for Experimental Gerontology in Rijswijk, The Netherlands, were used in this study. Detailed information on this inbred strain of mice has been published elsewhere (Zurcher et al., 1982; Van Zwieten et al., 1984).

5T mouse multiple myeloma

The different 5T MM originated spontaneously in aging C57BL/Ka- LwRij mice (Radl et al., 1988). The individual 5T MM were further propagated by intravenous transfer of bone marrow or spleen cells into young recipients of the same strain. An attempt was also made to grow the individual 5T MM in an ascitic form by transplanting bone marrow cells into the peritoneal cavity of young recipient mice. In four instances this was successful. The main characteristics of the individual 5T MM lines pertinent to this study are given in Table I.

Cell preparations

Cell suspensions from bone marrow and spleen were prepared as described (Croese et al., 1987). The percentage of 5T MM cells in these samples was estimated by morphology, cytoplasmic immunoperoxidase staining and by analysis of the cellular

Table I. C57BL/KaLwRij mouse 5T multiple myeloma lines of spontaneous origin

5T MM nr	Isotype	Transplantation generation	Growth pattern	Remark
5T2	IgG2a-κ	24 (17)	moderately progressive	several sublines (also ascitic form)
5T7	IgG2b-κ	7	'smoldering MM'	
5T13	IgG2b-κ	4	moderate	
5T14	IgG1-κ	10 (59)	aggressive	different sublines (also ascitic form)
5T21	IgD-κ	11	atypical	
5T30	IgG2a-κ	3 (3)	aggressive	(also ascitic form)
5T33	IgG2b-κ	5 (34)	moderately progressive	(also ascitic form)
5T41	IgG3-κ	1	moderate	

Note: generations of the MM in ascitic form are given between parentheses.
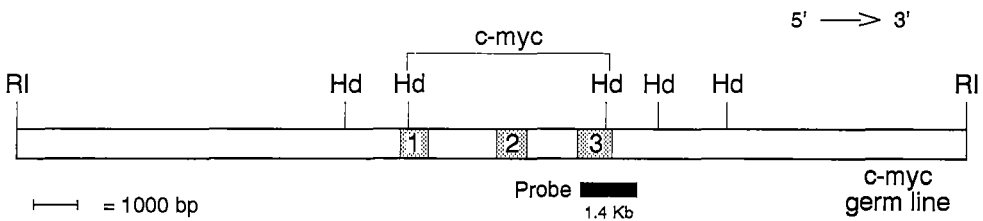
Figure 1. Molecular map of the murine c-myc gene. Restriction sites: RI, EcoRI; Hd, HindIII. The third exon probe used is denoted by a black box.

DNA content. Cytoplasmic examination was performed on cytocentrifuge preparations (Hijmans et al., 1965) of the suspensions, using PO-labeled antibodies (Nordic Immunological Laboratories, Tilburg, The Netherlands) specific for the isotype of the given 5T MM immunoglobulin (Table I). The DNA from bone marrow or spleen cells was stained with propidium iodine (PI) according to Taylor (1980). The cellular DNA content was analysed by measuring the PI fluorescence intensity by a fluorescence-activated cell sorter (FACS-II, Becton Dickinson, Mountain View, Ca, U.S.A.).

Southern blot analysis

High molecular weight DNA was extracted from 0.5 - 1.0 x 10^8 bone marrow, spleen or ascitic cells by the method of Kunkel et al. (1977). The chromosomal DNA was digested with the restriction enzymes *Hind*III or *Eco*RI under conditions

recommended by the manufacturer (Gibco-BRL, Breda, The Netherlands). Digested DNA was electrophoresed on 0.6 - 0.8 % agarose gels in buffer consisting of 89 mM TRIS, 89 mM boric acid and 0,2mM EDTA, pH=8.0 and transferred to Gene Screen Plus filters in 0.4N NaOH and 0.6M NaCl (16). Filters were prehybridized for two hours at 65°C in a solution of 50 mM Tris/HCl,pH 7.5, 10mM EDTA, 1M NaCl, 1% SDS, 0.1% sodium pyrophosphate, 0.2% Ficoll, 0.2% polyvinylpyrrolidone 100 μg/ml salmon sperm DNA and hybridized in the same solution overnight with 25 ng of c-*myc* probe with specific activity of approximately 10^9 cpm/μg DNA, a 1.4 kb *Cla*I-*Eco*RI fragment containing the third exon of the human c-*myc* gene, which was random-primed ^{32}P labeled (Boehringer, Mannheim, FRG). After hybridization, filters were washed to a stringency of 0.5 x S.S.C. (1 S.S.C. = 75 mM NaCl,7.5mM sodium citrate, 1% SDS) 0.1% sodium pyrophosphate at 65°C, and exposed overnight at -70°C to Kodak XAR-5 X-ray films. A schema of the murine c-*myc* gene and the relevant restriction sites are shown in figure 1.

Sensitivity of the technique and controls

Bone marrow cells from normal mice and from RPC-20 mouse plasmacytoma with a known c-*myc* rearrangement were used as controls. The detection sensitivity limit of the c-*myc* rearrangement in this technique was determined by admixing isolated SP2/0 hybridoma cells to normal bone marrow cells in different proportions and was found to be about 3 to 4%. In the different 5T MM preparations, the percentages of MM cells varied from 15 to 70% and from 7 to 44% for bone marrow and spleen cells, respectively.
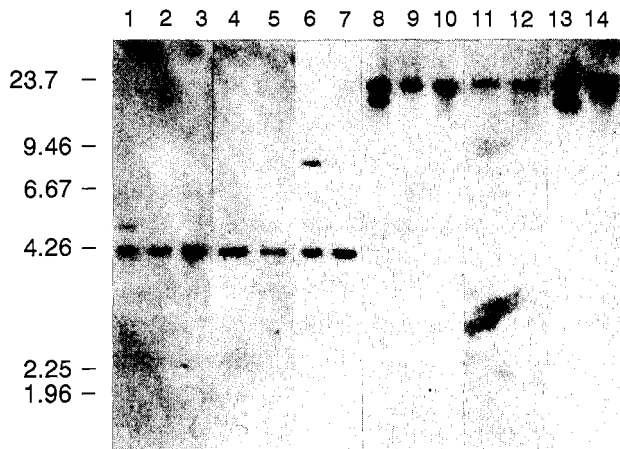
Figure 2. Autoradiogram of DNA from bone marrow cells digested with HindIII (lanes 1 - 7) and EcoRI (lanes 8 - 14). Lanes 1 and 8: 5T2; lanes 2 and 9: 5T7; lanes 3 and 10: 5T14; lanes 4 and 11: 5T33; lanes 5 and 12: 5T41; lanes 6 and 13: RPC-20; lanes 7 and 14: normal bone marrow cells. Analyses of MM 5T13, 5T21 and 5T30 are not shown. For details see the text.

RESULTS

No rearrangement of the c-*myc* oncogene was found in any of the individual 5T MM (Table I) when spleen cells were investigated. Similar results were obtained when bone marrow cells were analysed, however, with one exception. The mouse 5T7, 5T13, 5T14, 5T21, 5T30, 5T33 and 5T41 MM showed only the germ line fragments of 4.6 kb (*Hind*III) and 22 kb (*Eco*RI) as in normal mouse bone marrow (Fig. 2). However, the 5T2 MM showed an additional hybridizing fragment of 5.4 kb (*Hind*III) and 15 kb (*Eco*RI). The same pattern was observed when DNA from 5T2 MM cells originating from an animal with an ascitic form of 5T2 MM was investigated (Fig. 3). This indicates that the c-*myc* rearrangement took place in the common donor of both sublines in an earlier generation.

Three other 5T MM were shown to be able to grow in the peritoneal cavity of unprimed recipient mice: 5T14, 5T30 and 5T33 MM. The DNA isolated from the 5T30 and 5T33 MM showed only the germ line configuration, while that of the 5T14 MM produced an additional band of 1.3 kb (*Hind*III) and 5.8 kb (*Eco*RI) (Fig. 3).
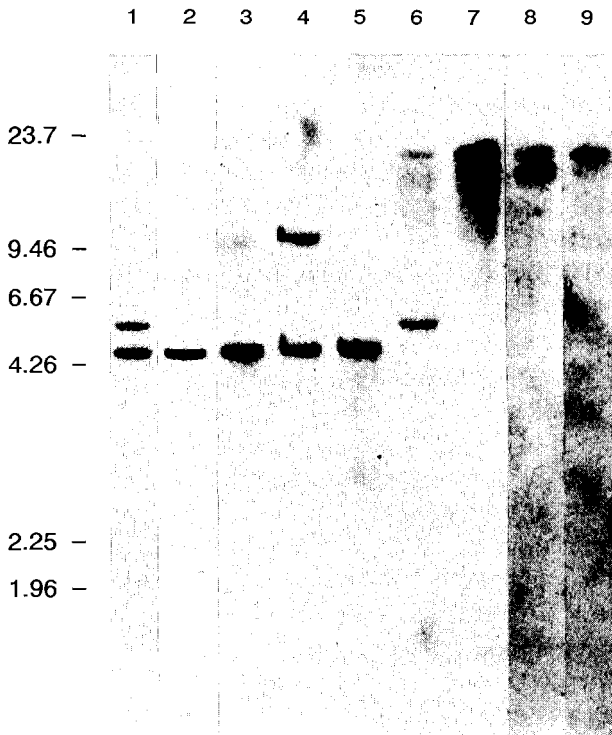
Figure 3. Autoradiogram of DNA from ascitic cells digested with HindIII (lanes 1 - 5) and EcoRI (lanes 6 - 9). Lane 1: 5T2; lanes 2 and 6: 5T14; lanes 3 and 9: 5T33; lanes 4 and 8: RPC-20; lane 5: normal bone marrow cells. Analysis of 5T30 MM is not shown.

DISCUSSION

The most common structural alteration in human Burkitt's lymphoma, mouse plasmacytoma and rat immunocytoma is an interruption of the c-*myc* gene upstream of its second exon (Mushinski et al., 1987). In many MPC, the breakpoint is within the first intron between E1 and E2, in some MPC, the breakpoint in c-*myc* occurs 300 to 500 base pairs 5' of E1 (Potter, 1986). This kind of abnormality, indicated by a rearrangement of the c-*myc* oncogene within the bone marrow tumor cells of the 5T MM series was found only in the 5T2 MM bearing mouse in the 24th transplantation generation and in none of the seven other 5T MM, which were of earlier generations. The bone marrow is the major site of this malignancy in both man and the C57BL mouse (Radl et al., 1988). Therefore, any structural abnormalities within different onco-genes, if they were of basic importance for the development of this malignancy, should primarily be present in the MM cells of the bone marrow compartment. In humans, rearrangement of the c-*myc* oncogene was found only in three cases (one of them being a very progressive IgA MM involving pleural tissue) and in one case of plasma cell leukemia (Gazdar et al., 1986; Selvanayagam et al., 1988; Yamada et al., 1983). In this context, it is interesting that in the 5T14 MM, being able to grow in the peritoneal tissue, a rearrangement of the c-*myc* was found in ascitic cells but not in the bone marrow cells. Moreover, the 5T2 MM in an advanced stage can develop features of a plasma cell leukemia (Ebbeling et al., 1985). These findings indicate that structural abnormalities of the c-*myc* oncogene of the most common MPC types are not a prerequisite for the development of MM. Our data, together with those of others on human MM, can be interpreted as indicating that such rearrangement can take place, possibly as a late event in the progression of this malignancy or due to its location in peritoneal or pleural tissue, where it can obtain selective growth advantage. Our investigation does not exclude some other structural abnormalities which would occur outside the analyzed region.

Cytogenetic investigations performed in this multiple myeloma of the 5T series (Van den Akker et al., in preparation) showed near triploid chromosome numbers in 4 lines (5T2, 5T7, 5T14 and 5T41) and hypotetraploid numbers in one (5T33). All karyotypes showed 1 or 2 copies of normal chromosome 15 and markers involving chromosome 15. 5T2 and 5T14 (transplant generation 11) showed markers with partial deletion of chromosome 15. No consistent abnormalities involving chromosomes 6, 12 or 16 with the three immunoglobulin gene loci were found. To detect more subtle changes, if present, within the first exon of the *myc* gene, studies on the *myc* RNA message will be performed after establishing cell lines of the 5T multiple myelomas *in vitro* (work in progress).

The human MM and the mouse 5T MM show a close resemblance in several aspects (Radl et al., 1985; Radl et al., 1988), including possibly also the c-*myc* pattern. Therefore, these 5T MM series offer an excellent experimental model for studies on the etiology and pathogenesis of multiple myeloma. In addition, this mouse B-cell malig-nancy, expressed mainly at the differentiation stage of a plasma cell, shows clear-cut

differences when compared with MPC and RIC, both also involving a B cell at its last differentiation stage (Radl et al., 1988). Investigation of these differences may shed new light on possible microheterogeneity of the plasma cell and its malignant counterparts evolving either into local plasmacytoma or diffuse multiple myeloma.

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REFERENCES

CROCE, C.M., & NOWELL, P.C. (1985). Molecular basis of human B cell neoplasia. In RNA Tumor Viruses, Oncogenes, Human Cancer and AIDS, (eds) Furmanski, P., Hager, J.C. and Rich, M.A., p. 116. Mart. Nijhoff: Boston, Dordrecht and Lancaster.

CROESE, J.W., VAS NUNES, C.M., RADL, J., V.D. ENDEN-VIEVEEN, M.H.M., BRONDIJK, R.J. & BOERSMA, W.J.A. (1987). The 5T2 mouse multiple myeloma model: Characterization of the 5T2 cells within bone marrow. Br. J. Cancer, **56**, 555.

EBBELING, S.B., LOKHORST, H.M., RADL, J., CROESE, J.W., BAST, E.J.E.G., BALLIEUX, R.E. (1985). Phenotypic and kinetic aspects of idiotype cells in the murine C57BL/KaLwRij/5T2 multiple myeloma. In Monoclonal Gammapathies- Clinical Significance and Basic Mechanisms; Topics in Aging Research in Europe, (eds) Radl, J., Hijmans, W. and V. Camp, B. Vol. 5, p. 205. EURAGE: Rijswijk.

ENRIETTO, P.J. (1987). The myc oncogene in avian and mammalian carcinogenesis. Cancer Surweys, **6**, 85.

GAZDAR, A.F., OIE, H.K., KIRSCH, I.R. & HOLLIS, G.F. (1986). Establishment and characterization of a human plasma cell myeloma culture having a rearranged cellular myc proto-oncogene. Blood, **67**, 1542.

HIJMANS, W., SCHUIT, H.R.E. & KLEIN, F. (1965). An immunofluorescence procedure for the detection of intracellular immunoglobulins. Clin. exp. Immunol., **4**, 457.

KLEIN, G. (1986). Oncogene activation by chromosomal translocations in B cell-derived tumors. Progress in Immunology VI, Acad. Press, p. 630.

KUNKEL, L.M., SMITH, K.D., BOYER, S.H. & 6 others (1977). Analysis of human Y-chromosome-specific reiterated DNA in chromosome variants. Proc. Natl. Acad. Sci. USA, **74**, 1245.

MUSHINSKI, J.F., DAVIDSON, W.F. & MORSE, H.C.III. (1987). Activation of cellular oncogenes in human and mouse leukemia-lymphomas: spontaneous and induced oncogene expression in murine B lymphocytic neoplasms. Cancer Invest. **5**, 345.

PEAR, W.S., INGVARSSON, S., STEFFEN, D. & 5 others (1986). Multiple chromosomal rearrangements in a spontaneously arising t(6;7) rat immunocytoma juxtapose c-myc and immunoglobulin heavy chain sequences. Proc. Natl. Acad. Sci. USA, **83**, 7376.

POTTER, M. (1986). Plasmacytomas in mice. Seminars in Oncology, **13**, 275.

RADL, J., CROESE, J.W., ZURCHER, C., V.D. ENDEN-VIEVEEN, M.H.M., DE LEEUW, A.M. (1988). Animal model of human disease. Multiple myeloma. Amer. J. Pathol., **132**, 593.

RADL, J., CROESE, J.W., ZURCHER, C., BRONDIJK, R.J. & V.D. ENDEN-VIEVEEN, M.H.M. (1985). Spontaneous multiple myeloma with bone lesions in the aging C57BL/KaLwRij mouse as a natural model of human disease. In Monoclonal Gammapathies- Clinical Significance and Basic Mechanisms; Topics in Aging Research in Europe, (eds) Radl, J., Hijmans, W., V. Camp, B. Vol. 5, p. 191. EURAGE: Rijswijk.

REED, K.C. & MANN, D.A. (1985). Rapid transfer of DNA from agarose gels to nylon membranes. Nucleic Acids Res., **13**, 7207.

SELVANAYAGAM, P., BLICK, M., NARNI, F. & 5 others (1988). Alteration and abnormal expression of the c-myc oncogene in human multiple myeloma. Blood, **71**, 30.

TAYLOR, I.W. (1980). Rapid single step staining technique for DNA analysis by microfluorometry. J. Histochem. Cytochem., **28**, 1021.

VAN ZWIETEN, M.J., ZURCHER, C., SOLLEVELD, H.A. & HOLLANDER, C.F. (1981). Pathology. In Immunological Techniques Applied to Aging Research, (eds) Adler, W.H. and Nordin, A.A., p. 1. CRC Press, Boca Raton, FL.

YAMADA, K., SHIONOYA, S., AMANO, M. & IMAMURA, Y. (1983). A Burkitt type 8;14 translocation in a case of plasma cell leukemia. Cancer Genet. Cytogenet., **9**, 67.

ZURCHER, C., VAN ZWIETEN, M.J., SOLLEVELD, H.A. & HOLLANDER, C.F. (1982). Aging Research. In The Mouse in Biomedical Research, (eds) Foster, H.L., Small, J.D. and Fox, J.G. Biomedical Research, Vol IV, p. 11. Academic Press: New York.

4. AMPLIFICATION OF THE C-MYC AND THE PVT-LIKE REGION

IN HUMAN MULTIPLE MYELOMA

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ABSTRACT

Genetic alterations that lead to the clonal expansion of differentiated cells in multiple myeloma have still to be elucidated. Many chromosomal aberrations have been found, but until now, none of them is typically associated with multiple myeloma. In search for genetic defects in multiple myeloma we studied the structure and expression of the c-myc oncogene and the pvt-like region because of their frequent association with other B-cell malignancies. Here we report co-amplification of the c-myc oncogene and the 5' part of the pvt-like region in two out of 26 cases of multiple myeloma. In both cases only κ-light chains were produced. The amplification manifested itself also at the RNA level. Total RNA was analyzed in one of these two cases showing abundant c-myc mRNA. In the same RNA sample we also detected a strong hybridizing band of about 7 kb, when the pSS.4 probe, representing the 5' part of the pvt-like region, was used. This band was not present in total RNA from normal bone marrow cells or bone marrow from multiple myeloma patients without the amplification of c-myc and the pvt-like region. Until now, transcripts of the pvt-like region were only found in a few human cell lines ranging in size from 1 to 11 kb. This is the first case in which a high expression of an about 7 kb transcript of the pvt-like region is found in freshly obtained tumor material, probably due to a pvt-amplification. The occurrence of abnormalities in the c-myc and the pvt-like regions in advanced cases of multiple myeloma suggests their involvement in the progression of this type of tumor.

INTRODUCTION

Multiple myeloma (MM) is a B-cell neoplasia characterized by the clonal expansion, mainly in the bone marrow (BM), of malignant plasma cells producing a monoclonal immunoglobulin (Ig) (Mellstedt et al., 1984; Barlogie & Alexanian, 1986). The genetic alterations involved in the neoplastic transformation in MM are still unknown. Evidence for the presence of activated cellular oncogenes have been shown in several human malignancies but for MM the involvement of oncogenes remains unclear. This is probably due to the low proliferative activity of myeloma plasma cells which makes it difficult to obtain adequate metaphase chromosomes and to establish representative cell lines (Latreille et al., 1982; Durie et al., 1985; Katagiri et al., 1985; Gazdar et al., 1986; Jernberg et al., 1987). Many different chromosomal aberrations have been found, but until now none of them is typically associated with MM (Lewis & MacKenzie et al., 1984; Ferti et al., 1984; DeWald et al., 1985; Ranni et al., 1987). As cytogenetic studies didn't lead to any clue which genes might be involved in MM, we searched for alterations in genes that are known to be associated with other B-cell malignancies, c-*myc* and *pvt*. In this context, the best described gene is the c-*myc* oncogene. The c-*myc* oncogene is often structurally altered in human Burkitt's lymphoma and in mouse plasmacytoma (Potter & Mushinski, 1984; Croce & Nowell, 1985). In rare occasions, c-*myc* was also found to be altered in MM and/or human MM derived cell lines and in one 5T mouse MM line (Sümegi et al., 1985; Gazdar et al., 1986; Meltzer et al., 1987; Selvanayagam et al., 1988; Pegoraro et al., 1989; Radl et al., 1990).

The human *pvt*-like region is situated about 300 kb distal from the c-*myc* gene and is frequently involved in t(2;8) translocations, occurring in variant Burkitt's lymphomas (Mengle-Gaw et al., 1987). The human *pvt*-like region is homologous to the mouse *pvt*-1 and the rat *mis*-1 gene, which are frequent proviral integration sites in retrovirally induced lymphomas (Graham et al., 1985; Graham & Adams, 1986). In mouse plasmacytomas with t(6;15) translocations, most of the breakpoints cluster in the *pvt*-region, resulting in relocation of the Cκ-locus from chromosome 6 to the *pvt*-region on chromosome 15 (Banerjee et al., 1985; Cory et al., 1985; Graham et al., 1985). Possibly in mice there is a functional relationship between the c-*myc* proto-oncogene and the *pvt*-1 locus, in which *pvt* regulates the transcription of c-*myc* over a long distance (Adams et al., 1986). Transcripts of the *pvt*-locus have only been found recently in a variety of human cell lines (Shtivelman & Bishop, 1989; Shtivelman et al., 1989), indicating that this locus indeed contains a functional gene. In human MM, involvement of the *pvt*-locus has not been explored. Therefore we searched for alterations in this gene and the c-*myc* gene in 26 MM BM samples.

Table 1. Clinical and molecular features of 26 patients with multiple myeloma.

Patient	%PC	Paraprotein	J_H re-arrangement	c-*myc* amplification	*pvt*-1 amplification pSS.4	pJBL.BR1	pJBLSS15
1 (normal BM)	5	-	-	-	-	-	-
2	87	IgG-λ	R	-	-	-	-
3	22	IgG-κ	R	-	-	-	-
4	25	IgA-λ	R	-	-	-	-
5	50	IgG-λ	R	-	-	-	-
6	15	IgG-κ	R	-	-	-	-
7	39	κ	-	-	-	-	-
8	45	IgG-κ	R	-	X^3	-	-
9	30	IgG-κ	R	-	-	-	-
10	30	IgA-κ	R	-	-	-	-
11	15	IgG-κ	-	-	-	-	-
12	34	κ	R	-	-	-	-
13	90	λ	-	-	-	-	-
14	35	IgG-κ	-	-	-	-	-
15	<1	IgG-λ	-	-	-	-	-
16	20	κ	R^1	A	A	-	-
17	75	IgA-κ	R	-	-	-	-
18	12	IgA-κ	R	-	-	-	-
19	58	λ	-	-	-	-	-
20	24	IgG-κ	-	-	-	-	-
21[2]	>90	IgG-κ	R	-	-	-	-
22	76	IgA-κ	R	-	-	-	-
23	50	κ	R	-	-	-	-
24	30	κ	R	-	-	-	-
25	60	κ	R	A	A	-	-
26	95	κ	R	-	-	-	-

Abbreviations: R, rearranged; A, amplified; X, extra band; PC, plasma cells; -, not detected.
1) rearranged band is very weak.
2) blood sample from a patient with plasma cell leukemia.
3) extra band of undetermined significance.

RESULTS

DNA analysis

Immunoglobulin genes.   Analysis of Ig gene rearrangement has helped to determine the clonality and differentiation stage in lymphoproliferative disorders (Siegelman et al., 1985; Griesser et al., 1986). When applied to BM samples from MM patients, discrete rearranged bands of the Ig heavy-chain genes were observed in most cases when the DNA was digested with *Bgl*II or *Eco*RI (Table 1 and Fig. 1). The intensity of the autoradiographic signal of rearranged Ig gene bands corresponded with the degree of BM plasmacytosis, as estimated by immunofluorescent analysis.
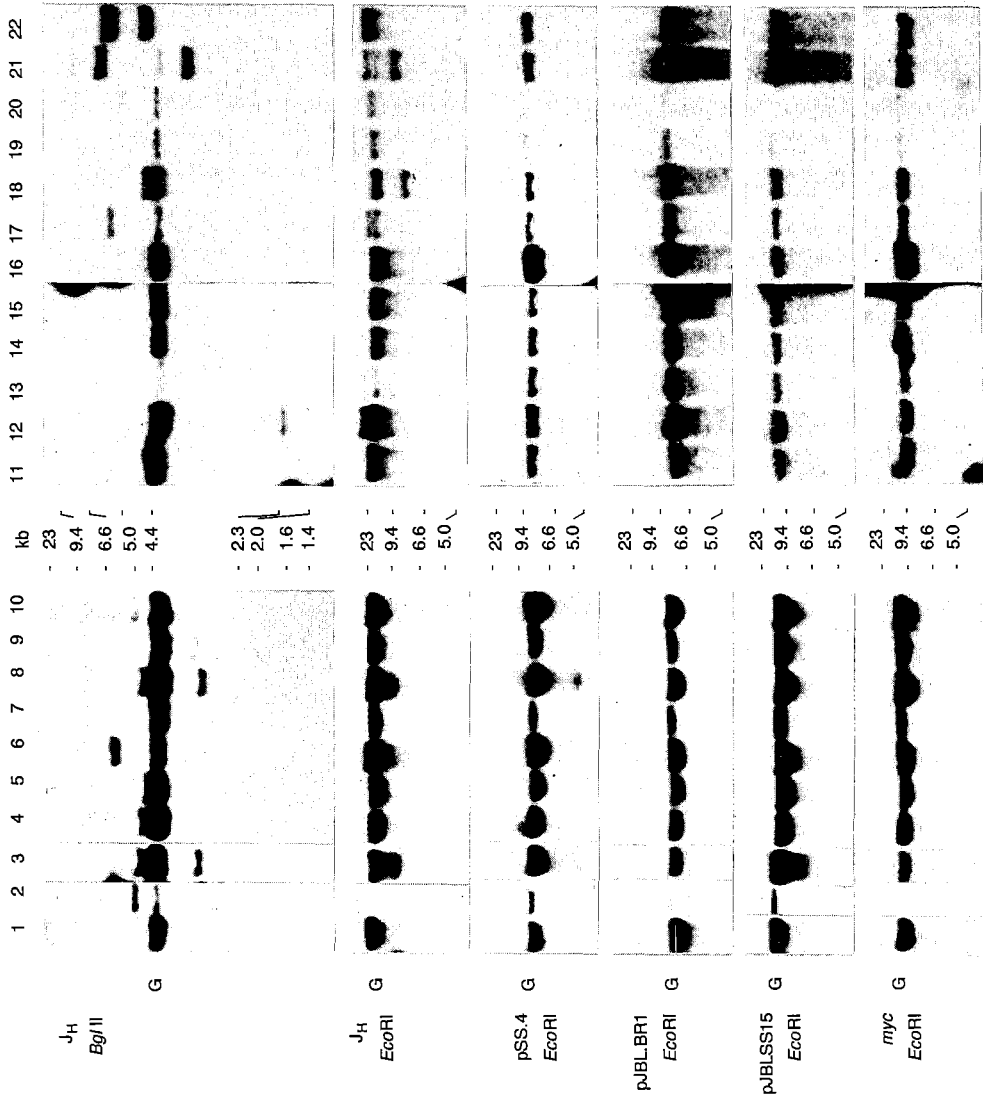
Figure 1. Southern blot analysis of MM DNA digested with BglII and EcoRI. 10 μg DNA from the BM of 20 MM patients, one plasmacytoma patient (nr 21) and one normal control (nr 1) were digested with the indicated enzymes and hybridized to the indicated probes. The numbers correspond to the patient numbers in Table 1. The same filter was used for all hybridizations.

The appearance of rearranged Ig bands in the total DNA extracted from the mononuclear cell (MNC) fraction of the BM samples, ensured us that the malignant clone could be detected which allowed us to detect abnormalities in the DNA from this clone. It should be noted that in some samples (patients nrs 7, 13, 14, 19 and 20) no rearranged J_H fragment could be detected although the number of plasma cells was apparently high enough (see table 1) to expect detection of the rearranged Ig gene. For patients nrs 13, 19 and 20 this could be due to the low amount of DNA present on the Southern blot but for patients nrs 7 and 14 some other explanation must be considered. The inability to detect clonal Ig rearrangements in some cases of MM is a phenomenon also observed by others (Humphries and Williams, 1989; Thielemans, personal communication).

Myc gene. For c-*myc* gene analysis, the filters used for Ig-analysis were rehybridized to the *myc* exon-3 probe. Rearranged bands were not detected in the
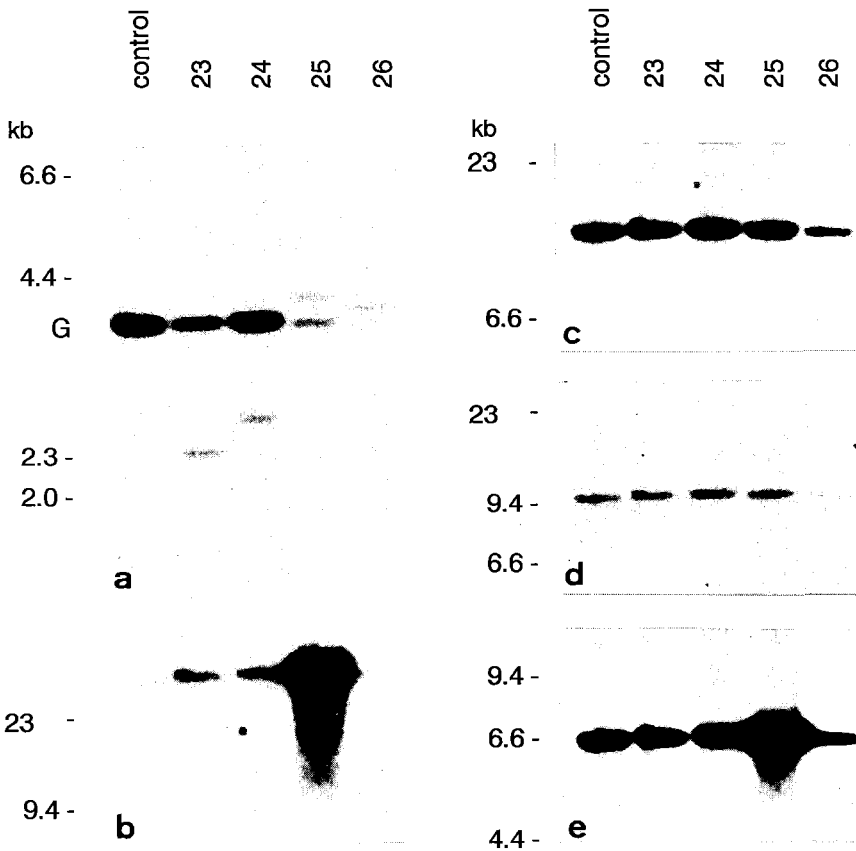
Figure 2. Southern blot analysis of MM DNA digested with BamHI. 10 µg DNA from four κ-LCD and one control was hybridized to the J_H (A), the c-myc (B), the pJBLSS15 (C), the pJBL.BRI (D) and the pSS.4 probe (E). The numbers correspond with the patient numbers in Table 1. The same filter was used for all hybridizations.
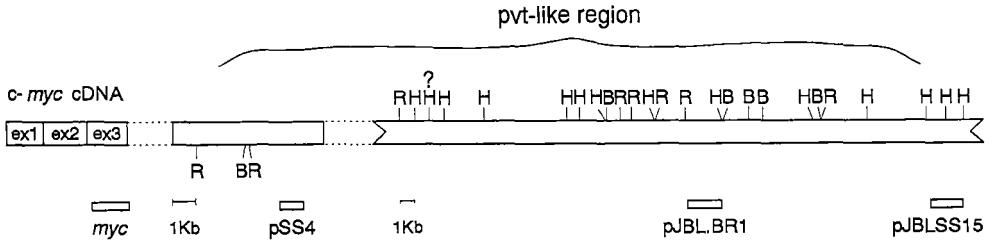
Figure 3. Schematic diagram of the pvt-like region. Probes representing different regions of pvt are depicted in a linear order on the chromosome. Restriction sites, R:EcoRI, H:HindIII, B: BamHI.
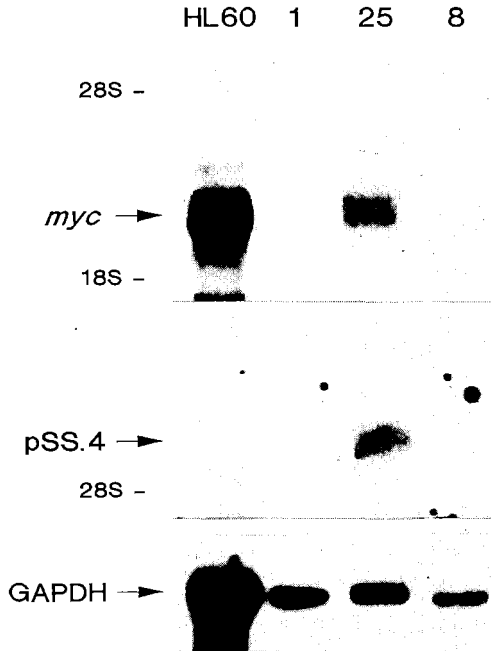

Figure 4. Northern blot analysis of myc and pvt mRNA from three MM patients. Approximately 15 μg of total RNA were loaded per lane and hybridized with the c-myc, the pSS.4 and the GAPDH probe. HL60 is a control cell line with known myc amplification. The numbers correspond with the patient numbers in Table 1. The same filter was used for all hybridizations.

DNA samples tested. In two cases an amplification was found. The first was found in a case of κ-light chain disease (LCD) (patient nr. 16, Fig. 1). The amplification was about 5 times as estimated from dilution experiments (data not shown). In a subsequent series of κ-LCD cases, another case showing an amplification was found (patient nr. 25, Fig. 2). In this case the c-myc fragment was about 10 times amplified, as estimated from dilution experiments (data not shown).

Pvt-like region. To examine whether the DNA of MM showed rearrangements within the pvt-1 like region, the filters were rehybridized with the pSS.4, pJBL.BR1 and pJBLSS15 probes. The positions of these probes in the pvt-like region are shown in Fig. 3 in relation to the c-myc locus. In one DNA sample, digested with EcoR1 (patient nr. 8), an extra band was observed when hybridized with probe pSS.4. The intensity of this band corresponded with the intensity of the rearranged band detected with the J_H probe, suggesting that it was derived from the malignant plasma cell clone. When the same DNA sample was digested with BglII, HindIII, SalI, XbaI or BamHI, no extra band was observed (data not shown). Amplification of the pvt-locus was observed in the same two patients (nrs 16 and 25) that contained the c-myc amplification (Figs. 1 and 2). The degree of amplification was in the same order of magnitude as for myc. In both cases, the amplification was only detected with probe pSS.4 and not with probe JBLSS15 or pJBL.BR1.

RNA analysis

The possibility that amplification of the c-myc gene was also reflected in enhanced levels of myc mRNA could only be analyzed in patient nr. 25. A Northern blot with total RNA from this patient, from normal BM and from a patient without an amplification was hybridized with the c-myc probe and the pvt-probe. The expression of c-myc mRNA was higher in patient nr. 25 as compared to patient nr. 8 and normal BM (Fig. 4). A pvt transcript of about 7 kb was detected with probe pSS.4 in patient nr. 25. No transcripts were found in normal BM and in patient nr. 8 (Fig. 4). Hybridization with probes pJBL.BR1 and pJBLSS15 also failed to reveal positive hybridization signals (data not shown).

DISCUSSION

In search for molecular mechanisms underlying the oncogenic event occurring in MM, we studied the structure and expression of the c-myc proto-oncogene. This gene is often deregulated in B-cell tumors of mouse and man. Indeed, we were able to show that the c-myc gene is amplified in two out of 26 cases of MM. It is of interest, that in both cases, only κ-light chains were produced and progression of the disease was aggressive. Rearrangement or amplification of c-myc is not a common phenome-non in MM. It has only been found in those cases in which the disease was already in an advanced stage with plasma cells in the pleura or in the blood (Gazdar et al., 1986). This suggests activation of the c-myc proto-oncogene as a late event step, promoting the clonal evolution of a more malignant cell variant. The amplification of the c-myc gene resulted in a high expression of myc mRNA in at least the one case that could be analyzed. Because of the association of c-myc expression with pvt-1 alterations in murine plasmacytomas with t(6;15) and in murine T lymphomas with proviral integrations in pvt-1 (Cory et al., 1985; Tsichlis et al., 1989), we studied the structure of the pvt-1 locus in the same 26 patients. Amplification of this region was detected

in the same cases as in which a c-*myc* amplification was found. Amplification concerned only the most 5' part of the *pvt*-like locus, as it could only be detected with probe pSS.4 and not with two probes specific for sequences more distal from the c-*myc* gene.

Amplifications of the *pvt*-like region together with c-*myc* amplifications have already been found in various cell lines from small cell lung carcinomas, a large cell lung carcinoma cell line and a colon carcinoma cell line (see Mengle-Gaw & Rabbits, 1987), and also in a case of acute non-lymphocytic leukemia (Asker et al., 1988). In three cases, the c-*myc* amplification terminated within the *pvt*-like region. Whether the c-*myc* and *pvt* amplification represent the same amplicon in these two cases of MM was not examined, but is likely because the magnitude of amplification was the same for *myc* and *pvt*.

Rearrangements involving the *pvt*-like region, as in variant Burkitt's lymphoma (Mengle-Gaw & Rabbitts, 1987), were not detected in this study. The origin of the extra pSS.4 band, observed in patient nr. 8, is unclarified. As the intensity of the band was similar to the rearranged J_H band, it was probably derived from the malignant clone. However, it did not occur in hybridization patterns using other restriction enzymes like *Bam*HI, *Xba*I, *Sal*I, *Hind*III or *Bgl*II. This extra band might be due to a small mutation in the *pvt*-like region, resulting in an *Eco*RI polymorphism. The c-*myc* expression in this case did not differ from that observed in the other patients (data not shown).

Whether the co-amplification of c-*myc* and the *pvt*-locus has any implications for tumor development in MM, remains to be analyzed. Amplifications of N-*myc* in neuroblastomas and c-*myc* in small cell lung cancer are correlated with a poor prognosis (Brodeur et al., 1984; Little et al., 1983; Seeger et al., 1985). The c-*myc* gene encodes a nuclear protein that appears to be involved in DNA synthesis (Classon et al., 1987). Interference with normal level of c-*myc* expression can block the programmed transition of the cell to the resting state and may thereby contribute to tumor progression (see Cole, 1986). Involvement of the *pvt*-like region in various tumors and cell lines suggests an important role in tumorigenesis. The relationship to the c-*myc* gene function is speculative. As in man the normal distance of the *pvt* to the c-*myc* locus is about 300 kb, regulation must be considered to take place over a long distance. The observation of an abundant *pvt*-transcript of about 7 kb in one patient confirms the existence of a functional *pvt*-gene, of which the product may be involved in regulation of c-*myc* transcription. Recently, *pvt*-transcripts ranging in length from 1.0-11 kb were found in a variety of human cell lines (Shtivelman & Bishop, 1989; Shtivelman et al., 1989). In our case, we could not detect other transcripts than the about 7 kb transcript, probably because normal expression of the *pvt*-locus is very low and only in case of an amplification, the expression reaches levels above the detection level of Northern blot analysis. In the two cases of MM presented here, c-*myc* and *pvt* appear to be deregulated in parallel, suggesting that they may be functionally related and that they probably both play a role in oncogenesis. In the other 24 cases studied, c-*myc* and *pvt* do not seem to be involved in the oncogenesis of MM, which means that other, still unknown genes have to be responsible for tumor

formation.

The mechanism of transformation that leads to the formation of MM has still to be elucidated. The fact that amplifications of c-*myc* and *pvt* are only found in advanced stages of MM, suggests that alterations in the c-*myc* and *pvt*-region may promote the clonal evolution of a more malignant plasma cell variant.

MATERIALS AND METHODS

Bone marrow samples

BM aspirates were obtained from 24 MM patients. Mononuclear cells (MNC) were obtained from these samples by Ficoll-Paque (1.077 kg/l, Pharmacia, Uppsala, Sweden) density gradient centrifugation. The degree of marrow plasmacytosis was defined by immunological staining for cytoplasmic Ig light chains as described (Van Dongen et al., 1987). A BM sample from a healthy donor served as control. The MNC samples were frozen and stored in liquid nitrogen.

Nucleic acid analysis

High-molecular weight DNA and total RNA were co-extracted from BM cells by a guanidine isothiocyanate method with cesium chloride modification (Chirgwin et al., 1979). DNA was digested with restriction enzymes and electrophoresed on 0.7% agarose gels. The gels were transferred to Ny13 N Nytran filters (Schleicher and Schuell, Dassel, F.R.G.) in 10 x SSC (1 x SSC is 0.15 M NaCl, 15 mM sodium citrate, pH 7.0). After blotting, the filters were rinsed in 6 x SSC before drying and baked for two hours at 80°C. Hybridization was done in 0.5 M NaHP04 (pH 7.2), containing 1% bovine serum albumin (BSA, Boehringer, Mannheim, F.R.G.), 1 mM EDTA, 3% sodium dodecyl sulphate (SDS), and 200 μg/ml salmon sperm DNA at 65°C. Final washings were performed in 40 mM NaHP04, 1% SDS, 1 mM EDTA at 65°C. Filters were rinsed in 100 mM NaHP04 and sealed in plastic bags. To remove hybridized probe, filters were rinsed in 0.4 M NaOH at 43°C for 20 min, followed by a rinse in 0.1 x SSC; 0.1 x SDS at 42°C for 30 min.

For Northern blot analysis, 15 μg of total RNA was electrophoresed on a 1.0% agarose gel in the presence of 6% formaldehyde, blotted onto Zeta-Probe filters (Bio-Rad Laboratories Inc., Richmond, CA) and hybridized in 10% dextran, 50% formamide (Maniatis et al., 1982) with 0.5% SDS at 42%C. Final washing was performed in 0.2 x SSC; 0.5% SDS at 65°C. To remove hybridized probes, filters were rinsed in boiling 0.01 x SSC; 0.01% SDS 4 times for 2 to 3 min.

DNA probes were labeled according to Feinberg & Vogelstein (1983). Autoradio-graphy on X-ray films was performed at -70°C for various lengths of time.

Gene probes

To detect structural abnormalities in the c-*myc* gene, we used a 1.6 kb *Cla*I-*Eco*RI fragment consisting of the third exon and the 3' flanking sequences of the

human c-*myc* gene (Dalla-Favera et al., 1983), and 3 probes for the *pvt*-1 like region, pSS.4, pJBL.BR1 and pJBLSS15 (kindly provided by Dr. T.H. Rabbitts, see Mengle-Gaw and Rabbitts, 1987).

Immunoglobulin gene rearrangements were examined with a 2.4 kb *Sau*3A fragment containing the human J_H 2-6 region (Siegelman et al, 1985).

The GAPDH (a 0.8 kb *Eco*RI-*Pst*I fragment; Benham et al., 1984) probe served as a control for the amount of RNA per lane in the Northern blot analysis.

REFERENCES

Adams, J.M., Harris, A.W., Pinkert, C.A., Brinster, R.L., Palmitter, R.D., Corcoran, L., Alexander, W.S., Graham, M.W. and Cory, S. (1986) Curr. Top. Microbiol. Imm. 132, 1-8.
Asker, C., Mareni, C., Coviello, D., Ingvarsson, S., Sesrarego, M., Origone, P., Klein, G. & Sümegi, J. (1988). Leuk. Res., 12, 523-527.
Banerjee, M., Wiener, F., Spira, J., Babonits, M., Nilsson, M.G., Sümegi, J. & Klein, G. (1985). EMBO J., 4, 3183-3188.
Barlogie, B. & Alexanian, R. (1986). Multiple myeloma and other paraproteinemias. Delamore, I.W. (ed.). Churchill Livingstone : Edinburgh. pp. 154-168.
Benham, F.J., Hodgkinson, S. & Davies, K.E. (1984). EMBO J., 3, 2635-2640.
Brodeur, G.M., Seeger, R.C., Schwab, M., Varmus, H.E. & Bishop, J.M. (1984). Science, 224, 1121-1124.
Chirgwin, J.M., Przybyla, A.E., MacDonald, J. & Rutter, W.J. (1979). Biochemistry, 18, 5294-5299.
Classon, M., Henriksson, M., Sumegi, J., Klein, G. & Hammarskjöld, M.L. (1987). Nature, 330, 272-274.
Cole, M.D. (1986). Ann. Rev. Genet., 20, 361-384.
Cory, S., Graham, M., Webb, E., Corcoran, L. & Adams, J.M. (1985). EMBO J., 4, 675-681.
Croce, C.M. & Nowell, P.C. (1985). Blood, 65, 1-7.
Dalla-Favera, R., Martinotti, S. & Gallo, R.C. (1983). Science, 219, 963-967.
DeWald, G.W., Kyle, R.A., Hicks, G.A. & Greipp, R.R. (1985). Blood, 66, 380-390.
Durie, B.G.M., Vela, E., Baum, V., Leibovits, A., Payne, C.M., Richter, L.C., Crogan, T.M. & Trent, J.M. (1985). Blood, 66, 548-555.
Feinberg, A.P. & Vogelstein, B. (1983). Ann. Biochem., 132, 6-13.
Ferti, A., Panani, A. & Sotirius, R. (1984). Cancer Genet. Cytogenet., 12, 247-253.
Gazdar, A.F., Oie, H.K., Kirsch, I.R. & Hollis, G.F. (1986). Blood, 67, 1542-1549.
Graham, M., Adams, J.M. & Cory, S. (1985). Nature, 314, 740-743.
Graham, M. & Adams, J.M. (1986). EMBO J., 5, 2845-2851.
Griesser, H., Feller, A., Lennert, K., Tweedale, M., Messner, H.A., Zalcberg, J., Minden, M.D. & Mak, T.W. (1986). Blood, 68, 592-594.
Humphries, J.E. & Williams, M.E. (1989). Blood, 74, 375A.
Jernberg, H., Nilsson, K., Zeck, L., Lutz, D., Nowotny, H. & Scheirer, W. (1987). Blood, 69, 1605-1612.
Katagiri, S., Takeshi, Y., Kuyama, J., Kanayama, Y., Nishida, K., Tatuso, A., Tamaki, T., Ohnishi, M. & Tarui, S. (1985). Int. J. Cancer, 36, 241-246.
Latreille, J., Barlogie, B., Dosik, G., Johnston, D.A., Drewinko, B. & Alexanian, R. (1982). Blood, 59, 43-51.
Lewis, J.P. & MacKenzie, M.R. (1984). Hematol. Oncol., 2, 307-317.
Little, C.D., Nau, M.M., Carney, D.N., Gazdar, A.F. & Minna, J.D. (1983). Nature, 306, 194-196.
Maniatis, T., Fritsch, E.F. & Sambrook, J. (eds.). (1982). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press : New York.
Mellstedt, H., Holm, G. & Björkholm, M. (1984). Adv. Cancer Res., 41, 257-284.
Mengle-Gaw, L. & Rabbitts, T.H. (1987). EMBO J., 6, 1959-1965.
Meltzer, P., Shadle, K. & Durie, B. (1987). Blood, 70, 985A.

Radl, J., Punt, Y.A., van den Enden-Vieveen, M.H.M., Bentvelzen, P.A.J., Bakkus, M.H.C., van den Akker, Th.W. & Benner, R. (1990). Br.J.Cancer, **61**, 276-278.

Pegoraro, L., Malawasi, F., Bellone, G., Massaia, M., Boccadoro, M., Saglio, G., Guerrasio, A., Benetton, G., Lombardi, L., Coda, R. & Avanzi, G.C. (1989). Blood, **73**, 1020-1027.

Potter, M. & Mushinski, J.F. (1984). Cancer Invest., **2**, 285-300.

Ranni, N.S., Slavutski, I., Wechsler, A. & Brieux, S. (1987). Cancer Genet. Cytogenet., **25**, 309-316.

Seeger, R.C., Brodeur, G.M., Sather, H., Dalton, A., Siegel, S.E., Wong, U.Y. & Hammond, D. (1985). N. Engl. J. Med., **313**, 1111-1116.

Selvanayagam, P., Blick, M., Narni, F., Van Tuinen, P., Ledbetter, D.H., Alexanian, R., Saunders, G.F. & Barlogie, B. (1988). Blood, **71**, 30-35.

Siegelman, M.H., Cleary, M.L., Warnke, R. & Shlar, J. (1985). J. Exp. Med., **161**, 850-863.

Shtivelman, E. & Bishop, J.M. (1989). Mol. Cell. Biol., **9**, 1148-1154.

Shtivelman, E., Henglein, B., Grotl, P., Lipp, M. & Bishop, J.M. (1989). Proc. Natl. Acad. Sci. USA, **86**, 3257-3260.

Sümegi, J., Hedberg, T., Björkholm, M., Godal, T., Mellstedt, H., Nilsson, M.G., Pereman, C. & Klein, G. (1985). Int. J. Cancer, **36**, 367-371.

Tsichlis, P.N., Shepherd, B.M. & Bear, S.E. (1989). Proc. Natl. Acad. Sci. USA, **86**, 5487-5491.

Van Dongen, J.J.M., Adriaansen, H.J. & Hooijkaas, H. (1987). Applications of monoclonal antibodies in tumor pathology. Ruiter, D.J., Fleuren, G.J. & Warnaar, S.O. (eds.). Martinus Nijhoff : Dordrecht. pp. 87-116.

5. DETECTION OF ONCOGENE EXPRESSION BY FLUORESCENT *IN SITU* HYBRIDIZATION IN COMBINATION WITH IMMUNOFLUORESCENT STAINING OF CELL SURFACE MARKERS

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ABSTRACT

To study oncogene expression in heterogeneous cell populations we developed and optimized a non-radioactive *in situ* hybridization technique using biotinylated single-stranded RNA probes and combined this technique with immunofluorescent staining of cell surface markers. As a model for our studies we used HL60 cells. In these cells we detected c-*myc* mRNA molecules by *in situ* hybridization following staining of the pan myeloid cell surface marker CD33, by a monoclonal antibody. Hybrids were detected by streptavidin-FITC and CD33 by a TRITC-conjugated anti- body. Controls involved pretreatment with RNAse, hybridization with sense RNA probes and blocking with an excess of unlabeled antisense probes. The integrity of the RNA in the cell was shown by hybridization with the GAPDH antisense probe. Essential for successful double-labeling was the choice of a fixation procedure that was suitable for the *in situ* hybridization and mild enough not to destroy the cell surface marker staining. This fluorescent *in situ* hybridization in combination with cell surface marker staining will be useful for studying gene expression in phenotypically well-defined cell populations.

INTRODUCTION

The detection of RNA sequences within individual cells by *in situ* hybridization (ISH) allows the analysis of gene transcription at the single cell level. Although Northern and dot blot analyses of heterogeneous cell samples can provide important qualitative data on gene expression, these methods are not sensitive enough to detect a mRNA that is present in less than about 5% of the cell population. Moreover, these techniques do not allow one to differentiate between samples in which only a few cells have a high gene expression and samples in which the majority of the cells have a relatively low expression of the gene under study. The ISH technique, on the other hand, is more informative and more suitable in those cases where only a few cells are available for analysis and/or only a few cells express the gene of interest. A combination of ISH to detect oncogene expression and cell surface marker staining to phenotype the cells may be an extremely powerful diagnostic tool in cancer, especially in hemato-oncology. By using non-radioactive probes, the ISH can be easily performed in a routine fashion as these probes are easy to handle and to detect, and can be stored for an extended period of time. The use of biotin-labeled probes, labeled by nick translation or random primer methods (Coghlan et al., 1985) or in RNA polymerase systems (Hoefler et al., 1985) is currently the most explored method and several detection systems are now available. These non-radioactive DNA or RNA probes have mainly been used to detect DNA targets (Bhatt et al., 1988; Garson et al., 1987; Hopman et al., 1986; Lawrence et al., 1988). Studies on detection of mRNA sequences are still scarce and most reports deal with the detection of abundant mRNA species (see e.g. Dirks et al., 1988; Bauman et al. (1988); Singer et al., 1986). Only a few investigators reported on the detection of low copy number mRNA molecules by using RNA probes and a fluorescent detection system (Bresser et al., 1987). These systems are claimed to detect oncogene expression in haematopoietic cells for as few as 5 copies per cell. We explored this method and determined the conditions that can permit simultaneous detection of cell surface markers and specific mRNA sequences in the individual cell. In this report we describe the detection of c-*myc* expression in HL60 cells, a promyeloid leukemia cell line, in combination with the cell surface marker CD33. This was done by first labeling the cells with a monoclonal antibody (MoAb) followed by ISH with a biotinylated RNA probe.

RESULTS

Biotin-labeling of RNA probes

Comparison of the labeling of RNA probes with bio-11-UTP, allyl-UTP or photobiotin revealed that the amount of RNA probe synthesized was about 4-7 μg RNA per 1 μg template when unlabeled UTP was used. In the presence of bio-11-UTP instead of unlabeled UTP, the yield of RNA was about four times lower, probably due to inefficient incorporation of bio-UTP by the RNA-polymerase. The allyl-UTP could be
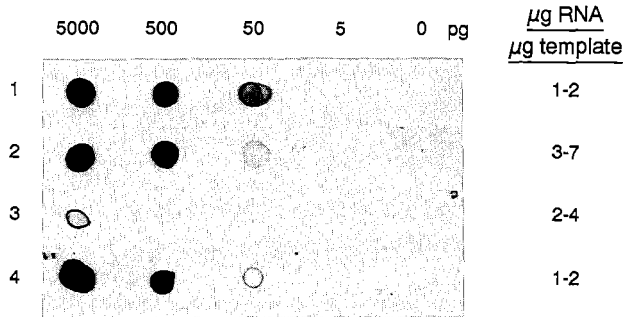
Figure 1. Direct-spot assay of biotin-labeled RNA. Comparison between biotin-labeling with (1) bio-UTP, (2) allyl-UTP, (3) photobiotin once and (4) photobiotin twice. The right column shows the yield of RNA transcripts.

incorporated more efficiently into RNA, resulting in a yield of about 3-8 μg RNA per 1 μg template. After photobiotinylation of the unlabeled probe and the attachment of a biotin-group to the allylamine labeled probe with the CAB-NHS ester the probes were compared for detectability by spotting them onto nitrocellulose. The bio-11-UTP labeled probe and the allyl-UTP labeled probe were both readily detectable at the 5 pg level (fig. 1). The photobiotinylated RNA probes were detectable at levels ranging from 10 pg to 1 ng. Labeling with photobiotin for a second or third time increased the level of biotinylation but caused considerable loss of RNA probably due to the precipitation steps involved as well as to degradation of the RNA during the photobiotinylation step. In order to get large amounts of well-biotinylated probes we therefore choose to use allyl-UTP for subsequent experiments.

Probe specificity

Northern blots with total RNA from HL60 and other cell lines were hybridized with ^{32}P-labeled *myc* antisense or sense RNA probes. The antisense probe detected the specific *myc* mRNA band of 2.3 kb. No cross-hybridization was observed with the ribosomal RNA bands. No signal was detected when the sense probe was used (fig. 2). Spotblots containing antisense and sense *myc* RNA and *myc* DNA were hybridized with biotin-labeled *myc* sense and antisense RNA probes. Hybridization signals were observed only with complementary RNA spots and DNA spots (data not shown). The antisense GAPDH RNA probe detected the 1.2 kb mRNA band on a Northern blot while the sense GAPDH probe did not (data not shown). The probes used were all degraded by limited alkaline hydrolysis. The time needed to degrade the probe to about 100-200 nucleotides varied between 30 and 60 min for the *myc* RNA probe dependent on different synthesis batches of the same probe. Therefore the length was checked at different hydrolysis time points. An example is shown in fig. 3.
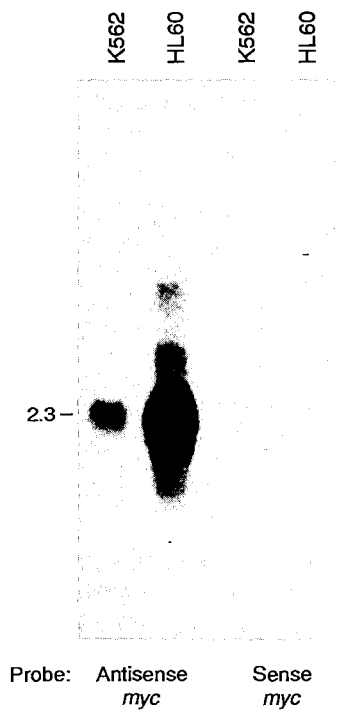
K562　HL60　K562　HL60

2.3 –

Probe:　Antisense　　Sense
　　　　　　myc　　　　　myc

Figure 2.　*Filter hybridization of sense and antisense myc RNA probes to total RNA from HL60 and K562 cells. Only the antisense probe can detect the 2.3 kb myc mRNA.*
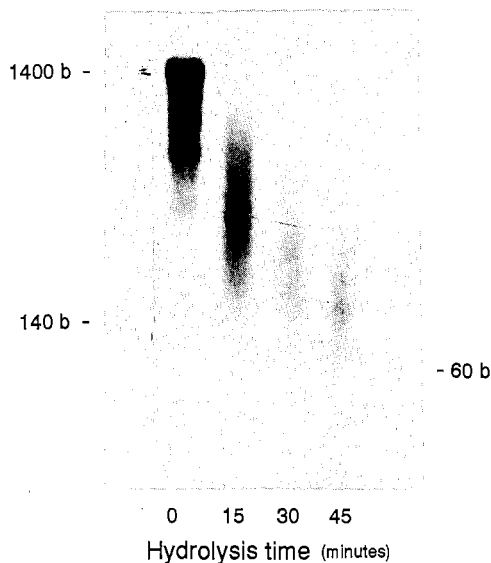
1400 b –

140 b –

　　　　　　　　　　　　　　　　　　– 60 b

0　　15　　30　　45
Hydrolysis time (minutes)

Figure 3.　*Estimation of the length of biotin-labeled antisense myc RNA probe by Northern blot analysis at different time points during alkaline hydrolysis. The RNA is detected with streptavidine-alkaline phosphatase (BluGENE, BRL). Size markers are depicted on both sides.*

Simultaneous detection of mRNA transcripts and cell surface markers

To develope an oncogene *in situ* hybridization in combination with cell surface marker staining we used HL60 cells as a model since these cells are known to express the *myc* oncogene at a high level (Westin et al., 1982). The *myc* and the GAPDH RNA probes were used, in combination with the CD33 MoAb My9, to determine which fixative should be used and which conjugates for detecting the MoAb and biotin-labeled hybrids were the best. Several storage procedures of cells and slides were tested to determine which procedure was the best in terms of RNA and surface marker retention.

Fixation. The best results were obtained with 4% PF fixation. Cell morphology and RNA retention were good, which is in agreement with the findings of Lawrence et al. (1985). Also the labeling with the MoAb against CD33 was well preserved (see next section). The ISH signals on ethanol/glacial acid and methanol/acetone fixed cells were found to be variable and as glutaraldehyde fixation was not useful because of severe autofluorescence we choose PF fixation for further experiments. It should be

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remarked that only cytocentrifuge preparations with a good cytomorphology as determined by phase-contrast microscopy resulted in optimal ISH signals. Therefore, cytocentrifuge preparations were checked for their quality before they were subjected to the ISH procedure.

Detection. The detection of the CD33 MoAb My9 with a tetramethylrhodamine isothiocyanate (TRITC)-conjugated antibody was in favor above a fluorescein isothiocyanate (FITC)-conjugated antibody because there was a considerable loss of FITC-label during the ISH procedure, especially during the hybridization step in 50% formamide at 42°C. Although there was also some loss of TRITC-label, it was less considerable than when using the FITC-label. Reducing the hybridization time from 16 hours to 4 hours, resulted in hardly any loss of TRITC-label but in this case the ISH signal was lower. This could be resolved by increasing the probe concentration from 1 ng/μl to 10 ng/μl. However, in order to save probe we choose the overnight hybridization method. The CD33 staining could be increased by using a second unlabeled rabbit anti-mouse antibody and a third TRITC-conjugated goat anti-rabbit antibody. However, a slight reduction of the ISH signal was found due to the larger time period needed for the labeling procedure in which endogenous RNAse could decrease the ISH.

The best conjugate for the detection of intracellular biotinylated hybrids appeared to be the streptavidine (SA)-FITC from Zymed. The use of avidine-FITC resulted in high nonspecific nuclear staining. The use of anti-biotin antibodies and extravidine-FITC resulted in weaker signals. In order to enhance the ISH signal SA-FITC was added before the washing procedure which would allow the formation of a complex of SA-FITC with intracellular bound and unbound biotin-labeled probe as stated by Bresser et al. (1987). In our hands this procedure increased the background to such a level that discrimination between specific and non-specific binding was no longer possible. The removing of excess probe before adding SA-FITC resulted in a weaker signal but the signal : noise ratio was much better. The use of acetic anhydride increased the signal : noise ratio even further.

Storage procedures. Cytocentrifuge preparations were stored at different steps during the fixation: before fixation (dry at room temperature), after fixation in 70% ethanol at 4°C, and after the graded alcohol series (dry at room temperature). It appeared that after fixation in 4% PF the slides could be stored for at least 2 month in 70% ethanol at 4°C without significant loss of ISH signal or surface marker staining. For optimal results cytocentrifuge preparations of the cells should be made within 2 hours after harvesting. In the meantime the cells should be kept on melting ice at high density. After centrifugation, the slides could be kept dry at room temperature for several hours without any decrease of signal. But after 2 days the signal became weaker. The slides could also be stored dry at room temperature after passing through the whole fixation procedure ending with dehydration in graded alcohols. Also in this case the signal changed and became more diffuse after 2 days. The best and most convenient way was to fix the slides immediately and store them in 70% ethanol at 4°C until needed. Good ISH signals were obtained also with cells that had been stored in
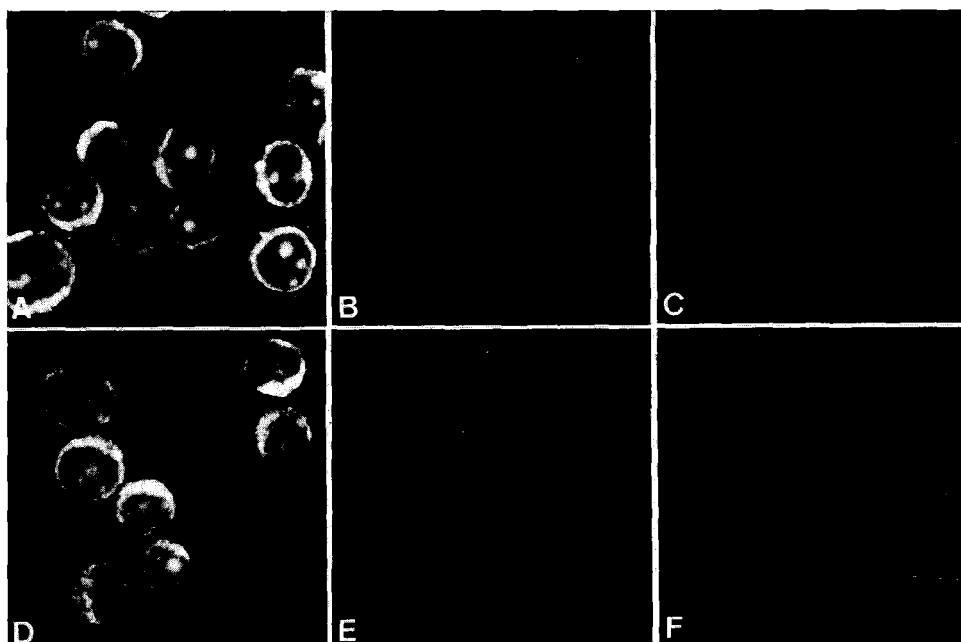
Figure 4. In situ hybridization of HL60 cells with biotinylated RNA probes. A: antisense GAPDH probe (1 ng/µl); B: sense GAPDH probe (1 ng/µl); C: RNAse treated cells, antisense GAPDH probe (1 ng/µl); D antisense myc probe (1 ng/µl); E: sense myc probe (1 ng/µl); F: RNAse treated cells, antisense myc probe.
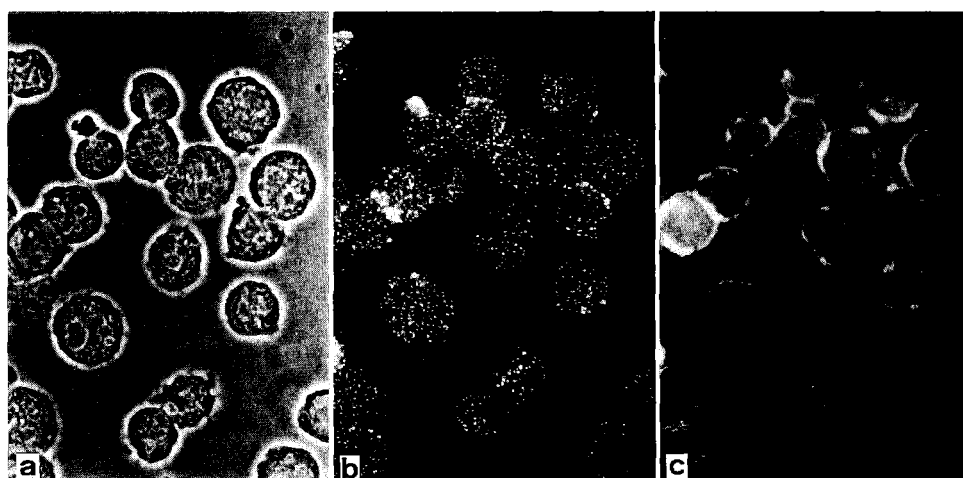

Figure 5. In situ hybridization of CD33 labeled HL60 cells. A: phase-contrast morphology; B: CD33 staining (TRITC); C: antisense myc probe (1 ng/µl, FITC). The three micrographs represent the same field.

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liquid nitrogen provided that extra care was taken in the thawing procedure to minimize cell death.

Staining patterns

GAPDH. The antisense GAPDH probe (1 ng/μl) was used as a positive control probe for the ISH studies. In every cell this GAPDH mRNA has to be present. Virtually all HL60 cells hybridized with this probe showed a cytoplasmic fluorescent staining indicating a good retention of mRNA (fig. 4A). The signal had a patch-like pattern with often a more intense staining around the nucleus. Also some nuclear staining was observed which, in contrast to the cytoplasmic staining, turned out to be sensitive to RNAse treatment after hybridization. No hybridization signal was observed when the sense GAPDH probe (1 ng/μl) was used (fig. 4B) or when the slides were first treated with RNAse (fig. 4C).

Myc. When using the antisense *myc* probe (1 ng/μl) a similar pattern of fluorescent staining was seen in most cells (fig. 4D). The specificity of the ISH signal was confirmed in three ways. First, hybridization with the sense *myc* probe (1 ng/μl) did not yield fluorescent staining above background (fig. 4E). Second, the signal was reduced by adding a high amount of unlabeled antisense *myc* RNA to the hybridization mixture (data not shown). Third, RNAse pretreatment prevented staining of the cells (fig. 4F).

CD33 in combination with ISH. HL60 cells are known to express the CD33 marker at a high level on the cell surface membrane (Paietta et al., 1987). The marker was still detectable after the cells were taken through the ISH procedure. But only after enhancement of the signal by a second unlabeled antibody the speckled TRITC-staining became apparent (fig. 5A). The *myc* mRNA was also detectable in these labeled cells (fig. 5B). However, there was some loss of specific hybridization signal, probably due to RNAse contamination of the antibody preparations used and/or loss of cellular integrity during the labeling procedure in suspension. The same results were obtained when the antisense GAPDH probe was used together with the CD33 MoAb (data not shown). The CD33 staining did not cause any aspecific binding of the probes as no hybridization with the sense GAPDH probe occurred (data not shown). The same results were obtained when the antisense GAPDH probe was used together with the CD33 MoAb (data not shown).

Detection of myc mRNA in PB samples

To test whether this ISH procedure was also suitable for peripheral blood (PB) cells we hybridized mononuclear cells (MNC) of a chronic myelocytic leukemia (CML) patient with a lymphoblastic blast crisis, with the antisense and sense *myc* probes. With the antisense probe strong FITC fluorescent staining was seen in virtually all leukemic blast cells (fig. 6B) implicating an overexpression of the gene. Hardly any signal was observed in the smaller surrounding cells, presumably T cells. No hybridization signal was observed with the sense *myc* probe, indicating, together with the appearance of negative cells in the antisense *myc* ISH, the specificity of the hybridization.
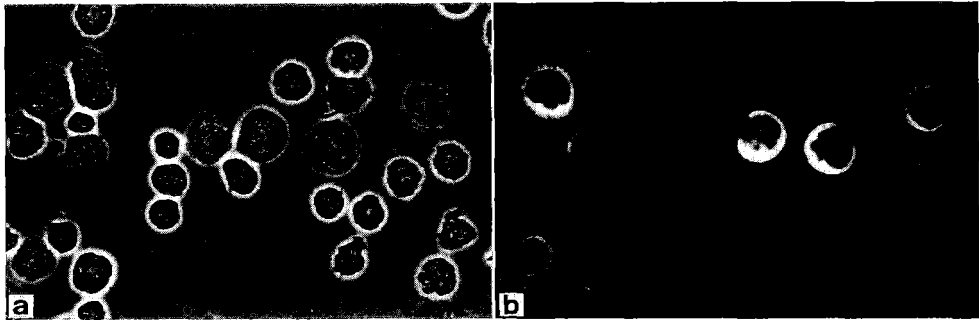
Figure 6. In situ hybridization of PB cells from a patient with CML-BC. A: Phase-contrast morphology; B: antisense myc probe (1 ng/μl). Only the blast cells are expressing myc mRNA.
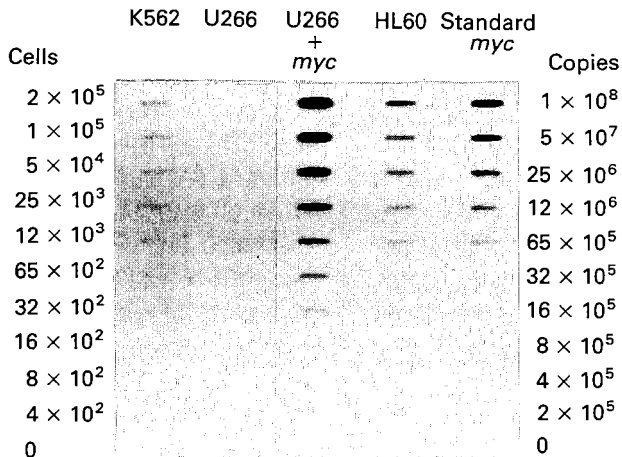
Cells	K562	U266	U266 + myc	HL60	Standard myc	Copies
2×10^5			●	─	─	1×10^8
1×10^5			●	─	─	5×10^7
5×10^4			●	─	─	25×10^6
25×10^3			●	─	─	12×10^6
12×10^3			●			65×10^5
65×10^2			●			32×10^5
32×10^2						16×10^5
16×10^2						8×10^5
8×10^2						4×10^5
4×10^2						2×10^5
0						0

Figure 7. Quantitation of the mean number of myc mRNA copies per cell in different cell lines by dot blot analysis. The hybridization signals were analyzed by a densitometer and compared to the myc copy-standard (lane 5). Loss of RNA during the isolation step was determined by adding 3000 myc RNA copies/cell to U266 (lane 3), having no detectable myc mRNA on his own (lane 2). The level of myc mRNA expression was about 200 copies/cell in HL60 and 30 copies/cell in K562.

Calculation of the number of myc copies in HL60, K562 and U266

Myc-specific hybridization signals on dot blots with total RNA from a fixed number of cells were compared to hybridization signals of a standard series of myc sense RNA, synthesized on the pGEM myc template (fig. 7). After analysing the signals by means of a video densitometer the myc copy-number could be calculated. HL60 cells contained about 200 copies per cell, comparable to the findings of Holt et al. (1988). In our subline of K562 cells we calculated about 30 copies per cell which was

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higher than the 5-10 copies present in the subline used by Evinger-Hodges et al. (1988). In U266 cells the level of *myc* mRNA was below our level of detection. To calculate the loss of RNA due to the isolation procedure we added 3000 copies of *myc* sense RNA per cell to the U266 cell lysate. Dot blot analysis of U266 cells artificially made *myc* positive showed that there were about 3000 copies per cell, indicating that there was no substantial loss of RNA during the isolation procedure (fig. 7).

DISCUSSION

ISH is becoming a widely used technique to detect gene expression in individual cells. To determine more precisely the cell type in which the mRNA of interest is localized, a combination of ISH with immunofluorescent staining of surface or cytoplasmic antigens would be very useful. In this study we show that it is possible to detect *myc* mRNA in CD33 labeled HL60 cells. To obtain an optimal hybridization signal, the starting material was fixed and prepared in a way that leads to optimal RNA retention, tissue preservation, and accessibility of the probe. Several fixatives have been described in the literature that fullfil this purpose (see e.g. Singer et al., 1986; Dirks et al., 1989; Bauman et al., 1988). In our hands PF fixation gave the most reproducible results in terms of ISH signal and cell surface marker staining.

The advantage of single-stranded (ss) RNA probes over double-stranded (ds) DNA probes includes a higher affinity and thermal stability of RNA-RNA hybrids (Angerer & Angerer, 1981; Cox et al., 1984, Meinkoth et al., 1984) which leads to increased sensitivity. Furthermore, competitive hybridization to the complementary strand, which can occur with dsDNA probes, is excluded with ssRNA probes. The use of plasmids containing two RNA polymerase promoters at both sides of the subcloned fragment makes it possible to produce large amounts of ssRNA transcripts in both orientations.

Our ISH studies clearly revealed localization of both *myc* and GAPDH mRNA in the cytoplasm of HL60 cells. The specificity of the ISH was shown by several controls. First, hybridization with the sense *myc* and the sense GAPDH probe in the same amounts as the antisense probes did not reveal fluorescent staining. Second, RNAse pretreatment of the slides prevented hybridization. Third, adding of an excess of unlabeled antisense *myc* probe reduced the hybridization signal to background levels. Furthermore, the *myc* signal occurred only in the morphologically determined blast cells in a PB sample of a patient with a CML-BC and not in the non-malignant cells in the same sample. High expression of c-*myc* is known to occur in CML (Ferrari et al., 1985), but not in normal PB cells and is probably indicative for the unregulated growth of the leukemic cells. These differences in levels of c-*myc* expression within the same sample provide a good internal control. With this ISH technique we can clearly detect *myc* mRNA in HL60 cells which indicates about 200 copies per cell as is shown by dot blot analysis. In K562 cells we observed also *myc* hybridization signals, which were clearly distinguishable from the *myc* negative U266 cells (data not shown). We estimate

therefore that we can detect with this technique about 30 copies per cell.

The combination of ISH with immunocytochemistry has been described earlier (Brahic et al., 1984; Shivers et al., 1986; Dirks et al., 1988). Most of these papers concerned ISH on sections, using radioactively labeled probes and avidin-alkaline phosphatase cytochemistry or DAB cytochemistry. In this study we show that it is possible to use fluorescent labels for both the ISH detection and the surface marker staining of cell suspensions. Hybridization signals were slightly reduced as a consequence of primary and secondary antibody treatment, which takes time enabling RNA destruction by endogenous RNAse. Decrease of the ISH signal can also be due to RNAse contamination of the antibody preparations. To avoid this as much as possible, antibodies should be diluted in RNAse free buffers. For surface marker staining the TRITC label was in favor over the FITC label in terms of resistance to 50% formamide at 42°C overnight.

Since we would like to use the ISH technique for routine analysis of oncogene expression in bone marrow and blood samples, we also investigated the best conditions for storing the cells and slides with minimal loss of ISH signal. It appeared that the best way to store the slides was first to fix the slides in 4% PF and subsequently to store them in 70% EtOH at 4°C. Up to 2 months no reduction in ISH signal was observed. Especially for mRNA's with a short half-life such as *myc*, it is necessary to fix the cells as soon as possible. It has been reported that cytocentrifuge preparations, after fixation, can be stored air dry at room temperature for at least five months (Bresser et al., 1987). In our hands, however, by storing the slides air dry, the ISH signal decreased already after one week, which was not the case when the slides were stored in 70% EtOH at 4°C. Also cells stored in liquid nitrogen could be used for ISH studies, provided they were frozen rapidly after sampling and provided they were thawed very carefully.

In conclusion, ISH can be performed conveniently using biotin-labeled RNA probes and fluorescence based detection methods. The combination of ISH with cell surface marker staining may become a useful diagnostic tool for early detection of malignancies in which overexpression of oncogenes occur which cannot be addressed by Northern blotting. Furthermore, the method enables one to determine whether a particular protein, present in a cell, is also produced by this cell itself and to study the relationship between the concentration of a particular species of mRNA and the amount of protein encoded by this mRNA. This method can also serve as a monitor of therapy which aims to down-regulate particular genes or to extinct cells with high oncogene expression.

MATERIALS AND METHODS

Cells

Cell lines. The HL60 promyelocytic leukemia cell line (Collins et al., 1977; Gallagher et al., 1979), the K562 chronic myelocytic leukemia cell line (Lozzio et al.,

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1975) and the U266 myeloma cell line (Nilsson, et al., 1970) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal calf serum (FCS; Boeringer, Mannheim, F.R.G.), penicillin (100 µg/ml), streptomycine (100 µg/ml), L-glutamine (4mM) and HEPES (25 mM). Cells were maintained in log phase.

Leukemic cell samples. Peripheral blood (PB) samples were obtained from a patient with a chronic myelocytic leukemia in blast crisis (CML-BC). The leukemic cells had a lymphoblastic morphology and expressed CD10 (VIL-A1). Mononuclear cells (MNC) from PB were isolated by Ficoll-Paque (density, 1.077 g/ml; Pharmacia, Uppsala, Sweden) centrifugation.

Molecular probes

For ISH studies, single-stranded biotin-labeled antisense and sense RNA probes were prepared. DNA probes were subcloned in vectors containing RNA polymerase promoters. Vectors were linearized by digestion with suitable restriction enzymes that would cut just behind or in front of the subcloned fragment revealing templates for RNA production. For c-*myc* RNA production a 1.4 kb ClaI-EcoRI fragment, containing the third exon of the human c-*myc* gene (Dalla-Favera et al., 1983), was subcloned into the pGEM-1 vector (Promega Biotec, Madison, WI). This vector contains the promoters for Sp6 and T7 polymerases. The template linearized by HindIII digestion was transcribed with the T7 polymerase, yielding antisense transcripts. The template linearized by EcoRI digestion was transcribed with the Sp6 polymerase encoding sense transcripts. For GAPDH RNA production an 0.8 kb EcoRI-PstI fragment (Benham et al., 1984) was subcloned into the bluescribe vector (Vector Cloning System, San Diego, CA). This vector contains the promoters for T7 and T3 polymerases. The template linearized by digestion with HindIII was transcribed with the T7 polymerase yielding antisense transcripts. The template linearized by digestion with EcoRI was transcribed with the T3 polymerase encoding sense transcripts. The transcriptions were performed as suggested by the manufacturer except for the concentration of the ribonucleotides which was increased 2-fold to 1 mM, the amount of enzyme was doubled and after each 15 min 5 additional units of polymerase were added. The reaction was allowed to continue for 2 hours at 40°C (Melton et al., 1984), resulting in full length transcripts. The DNA template was then digested with DNAseI (Promega Biotec) and the probes were purified by centrifugation chromatography through a Sephadex-G50 column, eluted with 0.1 x SSC (1 x SSC is 0.15 M NaCl, 15 mM tri-sodium citrate, pH 7.0) containing 0.1% sodium dodecyl sulphate (SDS). The biotinylation of the probes was achieved either by replacing the unlabeled UTP by biotin-11-UTP (BRL, Gaithersburg, MD) in the synthesis reaction or by replacing the unlableled UTP by allylamine-UTP (BRL) followed by biotinylation of the allyl-group with a CAB-NHS ester (BRL) or with Photobiotin (Vestor Laboratories, Inc., Burlingame, CA) as suggested by the manufacturer. The RNA yield was measured under UV light by ethidium-bromide staining in comparison with a standard series of known RNA concentrations. The RNA yield ranged from 1 to 10 µg RNA per µg template. The biotinylation of the probes was checked by direct filterspot tests following directions as described for biotinylated DNA

probes (BluGENE detection system, BRL), except for the RNA dilution which was diluted in 1 x SSC, with 0.1 μg/μl tRNA. The probes were degraded before use by limited alkaline hydrolysis (Cox et al., 1984) to a length of 100-200 nucleotides. To check the size of the probes they were electrophorized through a 2% agarose/formaldehyde gel, together with biotin-labeled ssRNA molecular weight markers. These markers were prepared on templates containing a 60 bp fragment and a 140 bp fragment. The gel was transferred to a nitrocellulose membrane filter and visualized according to the BluGENE system (BRL) with streptavidin-alkaline phosphatase (SA-AP). The specificity of the RNA probes was checked on Northern blots containing total cellular RNA extracts from HL60 cells. The RNA probes used for this purpose were labeled with [^{32}P-]α-CTP (800 Ci/mmol; Amersham, UK) according to the manufacturer. The incorporation of the [^{32}P]α-CTP was about 75% and the specific activity of the probe was about 5 x 10^8 dpm/μg. Probes were stored in 0.1 x SSC, 0.1% SDS at -70°C.

In situ hybridization (ISH)

Labeling of cell surface membrane markers. Cells were washed twice in phosphate-buffered saline (PBS, pH 7.8) containing 0.5% bovine serum albumin (BSA; Boehringer), counted, and adjusted to 10 x 10^6 cells per ml. HL60 cells were incubated with the CD33 murine monoclonal antibody (MoAb) My9 (Coulter Clone, Hialeah, FL) and subsequently with a TRITC-conjugated goat anti-mouse Ig antiserum (Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands) as a second-step reagent as described elsewhere (Van Dongen et al., 1987). The signal could be enhanced by using a rabbit anti-mouse Ig antiserum (Nordic Immunological Laboratories, Tilburg, The Netherlands) as a second-step reagent and a TRITC-conjugated goat anti-rabbit antiserum (Supertechs, Bethesda, MD) as a third-step reagent. Labeled cells were cytocentrifuged (Cytofuge, Nordic Immunological Laboratories) on ethanol pre-cleaned slides, air-dried at room temperature for about 15 min and fixed immediately.

Fixation. Several fixatives for fixation of the cytocentrifuge preparations were tested: 1) ethanol/glacial acetic acid (80:20) for 10 min, followed by 5 min in 96% ethanol and air-dried, 2) methanol/acetone (50:50) for 15 min and air-dried, 3) 4% paraformaldehyde in PBS (pH 7.8) containing 5mM MgCl$_2$, followed by at least 15 min in 70% ethanol at 4°C.

Acetylation. Fixed slides were washed in PBS 5mM MgCl$_2$ (pH 7.8) for 15 min; acetylated in freshly prepared 0.25% acetic anhydride in 0.1 M tri-ethanolamine (pH 8.0) for 10 min (Hayashi et al., 1978; Hoefler et al., 1986), washed in 2 x SSC for 1 min, washed in PBS-5mM MgCl$_2$ for 1 min, treated with 0.1 M glycine in 0.1 M Tris-HC1 (pH 7.0) for 30 min, washed in 2 x SSC for 10 min, dehydrated for 5 min each through 70%, 80%, 95%, 100% ethanol. Finally the slides were air-dried and hybridized.

Hybridization. Slides were preincubated with 15 μl of 50% deionized formamide (Merck, Darmstadt, F.R.G.) in 2 x SSC for 10 min and then hybridized with 10 μl hybridization mix containing the biotinylated probe (0.5-10 ng/μl) diluted in a

hybridization buffer containing 50% deionized formamide, 5 x SSC, 0.1 M sodium phosphate (pH 7.4), 1 mg/ml E.coli tRNA (Boehringer), 10 mM dithiotreitol and 10 mM vanadyl ribonucleoside complexes (New England Biolabs, Inc., Westburg, The Netherlands). Hybridization was performed at 42°C in a chamber moistened with 50% formamide in 2 x SSC for 16 hr. After hybridization, slides were washed twice with 1 x SSC containing 0.01% Triton for 15 min at 55°C and once with 0.5 x SSC, for 15 min at 55°C.

Detection.   Several conjugates for biotin-detection were tested: streptavidine-FITC (SA-FITC, BRL), SA-FITC (Pharmacia), SA-FITC (Zymed, San Francisco, CA), extravidine-FITC (Sigma, St. Louis, MO), avidine-FITC (E.Y. Laboratories, San Mated, CA) and anti-biotin-FITC (Sigma). Fifteen μl of optimally titrated (often an 1:50 dilution) conjugate (in PBS-0.5% BSA) were applied to the cells and the slides were incubated for 30 min in a moist chamber. Excess of conjugate was removed by a wash in PBS-5mM $MgCl_2$ (pH 7.8) for 15 min. The slides were embedded in an antifade solution consisting of 90% glycerol in PBS (pH 8,6) and either 100 mg/ml 1,4-diazobicyclo-(2,2,2)-octane (DABCO) or 1 mg/ml p-phenylenediamine (BDH Chemicals, Poole, UK).

Controls.   The specificity of the ISH was tested by several controls. First: by RNAse pretreatment. Cytocentrifuge preparations were fixed as described above but before they were dehydrated in graded alcohols incubated with RNAse A (Boehringer), 100 μg/ml in 2 x SSC for 30 min at 37°C. Subsequently the slides were washed extensively in 2 x SSC and processed for hybridization as described above. Second: by adding a high amount of unlabeled antisense RNA probe to the hybridization mixture. The unlabeled probe concentration was 200 x more than the labeled probe cencentration. Third: by hybridization with the sense RNA probe. The preservation of RNA in the cells was tested by GAPDH hybridization.

Microscopes.   Zeiss (Carl Zeiss, Oberkochen, FRG) and Leitz (Ernst Leitz Wetzlar, FRG) microscopes were used for the evaluation of the fluorescence staining. The microscopes were equipped with HBO mercury lamps (Osram, Berlin, FRG), phase-contrast facilities and filter combinations for the selective visualization of FITC and TRITC.

Storage.   Several storage procedures for the cells and the cytocentrifuge preparations were tested. Cells were stored in liquid nitrogen in DMEM containing 40% FCS and 10% DMSO for time periods of several weeks up to several years, or cells were stored at 4°C or room temperature for a couple of hours to one night. Cytocentrifuge preparations were stored at different steps during the fixation: before fixation (dry at room temperature), after fixation in 70% ethanol at 4°C, and after the graded alcohol series (dry at room temperature).

Estimation of myc copy number

Total RNA was isolated from 2 x 10^6 HL60 cells, K562 cells and U266 cells respectively according to Chirgwin et al. (1979). The RNA was spotted on nitrocellulose in a two-fold dilution, starting with one tenth of the isolated RNA, comparable to 2 x 10^5 cells. Sense *myc* RNA, synthesized on the pGEM-*myc* template, was used as a

standard and spotted on nitrocellulose in a two-fold dilution starting with 73.33 pg, comparable to 1×10^8 copies *myc* RNA (our *myc* transcript is 1.4 kb). The filter was hybridized to a $[^{32}P]$-labeled cDNA *myc* probe in 10% dextran, 50% formamide (Maniatis et al., 1982). Final washing was performed in 0.3 x SSC, 0.1% SDS at 65°C. Hybridization signals of the different cell types were compared to the hybridization signal of the standard. The signals were analyzed by a densitometer (Bio-Rad Laboratories Inc., Richmond, CA). The loss of RNA during the isolation procedure was estimated by adding a fixed amount of sense *myc* RNA to the lysate of 2×10^6 U266 cells and scoring the amount of this sense *myc* RNA after isolation and spotting onto nitrocellulose by comparing the *myc* hybridization signal in U266 with the standard hybridization signal.

REFERENCES

Angerer, L.M. & Angerer, R.C. (1981). Nucleic Acids Res., **9**, 2819-2840.
Bauman, J.G.J., Bentvelzen, P. (1988). Cytometry, **9**, 517-524.
Benham, F.J., Hodgkinson, S. & Davies, K.E. (1984). EMBO J, **3**, 2635-2640.
Bhalt, B., Burns, J., Flannery, D. & McGee, J.O.D. (1988). Nucleic Acids Res., **16**, 3951-3961.
Brahic, M., Haase, A.T. & Cash, E. (1984). Proc. Natl. Acad. Sci. USA, **81**, 5445-5448.
Bresser, J. & Evinger-Hodges, M.J. (1987). Gene Anal. Techn., **4**, 89-104.
Chirgwin, J.M., Prsybyla, A.E., MacDonald, R.J. & Rutter, W.J. (1979). Biochemistry, **18**, 5294-5299.
Coghlan, J.P., Aldred, P., Haralambidis, J., Niall, H.D., Penschow, J.D. & Tregear, G.W. (1985). Anal. Biochem., **149**, 1-28.
Collins, S., Gallo, R. & Gallagher, R. (1977). Nature, **270**, 347-350.
Cox, K.H., Deleon, D., Angerer, L.M. & angerer, R.C. (1984). Devel. Biol., **101**, 485-502.
Dalla-Favera, R., Martinotti, S. & Gallo, R.C. (1983). Science, **219**, 963-967.
Dirks, R.W., Raap, A.K., Van Minnen, J., Vreugdenhil, E., Smit, A.B. & Van Der Ploeg, M. (1989). J. Histochem. Cytochem., **37**, 7-14.
Evinger-Hodges, M.J., Bresser, J., Brouwer, R., Cox, J., Spitzer, G. & Dicke, K. (1988). Leukemia, **2**, 45-49.
Ferrari, S., Torelli, U., Selleri, L., Donelli, A., Venturelli, A., Narni, F., Moretti, L. & Torelli, G. (1985). Cancer Res., **7**, 833-842.
Gallagher, R., Collins, S., Trujilo, J., McCredie, M., Ahearn, M., Tsai, S., Metzgar, R., Aulakh, G., Ting, R., Ruscetti, F. & Gallo, R. (1979). Blood, **54**, 713-733.
Garson, J.A., Van Den Berghe, A. & Kemshead, J.T. (1987). Nucleic Acids, Res., **15**, 4761-4770.
Hayashi, S., Gillam, I.C., Delaney, A.D. & Tener, G.M. (1978). J. Histochem. Cytochem., **26**, 677-679.
Hoefler, H., Childers, H., Montminy, M.R., Lechan, R.M., Goodman, R.H. & Wolfe, H.J. (1986). Histochem. J, **18**, 597-604.
Holt, J.T., Redner, R.L. & Nienhuis, A.W. (1988). Molec. Cell Biol., **8**, 963-973.
Hopman, A.H.N., Wiegant, J., Tesser, G.I. & Van Duijn, P. (1986). Histochem., **85**, 1-4.
Lawrence, J.B. & Singer, R.H. (1985). Nucleic Acids Res., **13**, 1777-1799.
Lawrence, J.B., Vilnave, C.A. & Singer, R.H. (1988). Cell, **52**, 51-61.
Lozio, C.B. & Lozio, B.B. (1975). Blood, **45**, 321-334.
Maniatis, T., Fritsch, E.F. & Sambrook, J. (eds). Molecular Cloning: A Laboratory Manual. (1982). Cold Spring Harbor Laboratory Press: New York.
Meinkoth, J. & Wahl, G. (1984). Anal. Biochem., **138**, 267-284.
Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K. & Green, M.R. (1984). Nucleic Acids

Res., **12**, 7035-7056.

Nilsson, K., Bennich, H., Johansson, S.G.O. & Pontén, J. (1970). Clin. exp. Imm., **7**, 477-489.

Paietta, E., Stockert, R.J., Calvelli, T., Papenhausen, P., Seremetis, S.V., Fotine, M., Wiermik, P.H., Chang, L. & Bollum, F.J. (1987). Blood, **70**, 1151-1160.

Shivers, B.D., Harlan, R.E., Pfaff, D.W. & Schachter, B.S. (1986). J. Histochem. Cytochem., **34**, 39-43.

Singer, R.H., Lawrence, J.B. & Villnave, C. (1986). Biotechn., **4**, 230-250.

Van Dongen, J.J.M., Adriaansen, H.J. & Hooijkaas, H. (1987). Application of monoclonal antibodies in tumor pathology. Ruiter, D.J., Fleuren, G.J., Warnaar, S.O. (eds). Martinus Nijhoff: Dordrecht. pp. 87-116.

Westin, E., Wong-Staal, F., Gelmann, E., Dalla-Favera, R., Papas, T., Lautenberger, J., Allessandra, E., Reddy, E., Tronick, S., Aeronson, S. & Gallo, R. (1982). Proc. Natl. Acad. Sci. USA, **79**, 2490-2494.

6. DETECTION OF INTERLEUKIN-1β AND INTERLEUKIN-6 EXPRESSION IN HUMAN MULTIPLE MYELOMA BY FLUORESCENT IN SITU HYBRIDIZATION

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ABSTRACT

Using fluorescent in situ hybridization together with cell surface marker staining we studied the expression of mRNA of IL-6 and mRNA of IL-1β in bone marrow samples from human multiple myeloma patients. It is known that IL-6 can stimulate B cell growth and differentiation. Recently it has been suggested that IL-6 is responsible for autocrine growth stimulation of myeloma cells. IL-1 may play a role in bone resorption. These interleukins have previously been detected in the supernatants of cultured myeloma cells. Here we report the expression of IL-1β mRNA by plasma cells, T cells and macrophages according to morphology and immunologic marker analysis, suggesting that not only myeloma cells but also other cell types can contribute to the production of IL-1β and thus to bone-resorption. IL-6 mRNA could not be detected in plasma cells from bone marow aspirates but did occur in monocytes and T cells, suggesting that in vivo IL-6 stimulates the growth of myeloma cells in a paracrine instead of an autocrine way.

INTRODUCTION

Multiple myeloma (MM) is a malignancy characterized by the clonal expansion of malignant plasma cells in the bone marrow (BM). These tumor cells produce a monoclonal immunoglobulin (Ig) defined by its idiotypic determinants (1). Bone lytic lesions often occur in advanced stages of the disease. Myeloma cells in culture produce several cytokines which may be involved in the regulation of tumor growth and differentiation and/or the activation of osteoclasts. Especially interleukin-6 (IL-6), a potential growth factor for B-cell hybridomas (3), EBV-transformed B-lymphoblastoid cells, plasmacytomas and myeloma cells (2-6), is suggested to play a role in autocrine stimulation of myeloma cells (7), although direct evidence for this suggestion is lacking.

The mechanism of bone destruction in MM is also a major point of investigation. Several cytokines are thought to be responsible for bone resorption in vitro, such as interleukin-1 (IL-1), tumor necrosis factor (TNFα), lymphotoxin (LT/TNFβ), and transforming growth factor β (TGFβ) (10-13). These cytokines are found in culture supernatants of normal activated peripheral blood mononuclear cells. Garrett et al. reported that cultured human myeloma cells produce LT, not IL-1, and that the secreted LT may be responsible for bone destruction in MM (14). In contrast with Garrett's findings, Kawano et al. and Cozzolino et al. reported that cultured human myeloma cells do produce IL-1 which has bone resorbing activity (15,16).

To investigate the capacity of myeloma cells to produce cytokines in the in vivo situation we performed in situ hybridization (ISH) on BM samples of MM patients to detect mRNA of IL-1β and IL-6 at the single cell level. We used biotinylated single stranded (ss) RNA probes for these two cytokines in combination with a fluorescent streptavidine conjugate. This was combined with cell surface marker staining to phenotype the cells.

MATERIALS AND METHODS

Bone marrow samples

BM aspirates were obtained from 17 MM patients. Mononuclear cells (MNC) were isolated from these samples by Ficoll-Paque (1.077 kg/l; Pharmacia, Uppsala, Sweden) density centrifugation. The degree of marrow plasmacytosis was defined by immunological staining for cytoplasmic Ig light chains as described (17). A BM sample from a healthy donor served as control. These MNC samples were frozen and stored in liquid nitrogen.

Northern blot analysis

Total RNA was isolated from MNC by a guanidine isothiocyanate method with cesium chloride modification (18). RNA (10 μg) was electrophoresed on a 1% agarose gel in the presence of 6% formaldehyde, blotted onto Biotrans Nylon membranes (FCN, Irvine, CA) and hybridized in 10% dextran, 50% formamide (19) at 42°C. Final

washing was performed in 0.2 x SSC; 0.5% SDS at 65°C. To remove hybridized probe, filters were rinsed in boiling 0.01 x SSC; 0.01% SDS 4 times for 2 to 3 min. DNA probes (IL-1β, 1.3 kb cDNA, Genetics Institute, Cambridge, MA, and IL-6, 0.3 kb cDNA, see ref. 20) were labeled according to Feinberg & Vogelstein (21). Autoradiography on X-ray films was performed at 70°C for various durations.

The GAPDH (a 0.8 kb EcoRI-PstI fragment; 21) served as a control for the amount of RNA per lane in the Northern blot analysis.

RNA probes

IL-1β and IL-6 DNA probes were subcloned into the bluescribe vector (Vector Cloning System, San Diego, CA). RNA transcription, biotinylation and control of the probes were performed as described previously (23). Briefly, the vectors were linearized and transcribed with either T7 or T3 RNA polymerase (Boehringer, Mannheim, FRG) in the presence of allylamine-UTP (BRL, Gaitherburg, MO), CTP, GTP, and ATP. The allyl-group was biotinylated with a CAB-NHS ester (BRL). Probes were degraded by limited alkaline hydrolysis to a length of 100-200 nucleotides. The concentration of the probes used in the ISH ranged from 1 to 5 ng/μl.

In situ hybridization

ISH was performed on cells which were stained by monoclonal antibodies as described previously (23). In brief, MNC were labeled with monoclonal antibodies against CD3(Leu-4; Becton Dickinson, San Jose, CA) to detect T cells, CD14 (My4; Coulter Clone, Hialeah, FL) to detect monocytes, RFD9 (Dr. L.W. Poulter, London, U.K.) to detect macrophages and CD38 (OKT10; Ortho Diagnostic Systems, Raritan, NJ) which is expressed on the surface membrane of plasma cells. A TRITC-labeled goat anti-mouse Ig antiserum (Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands) was used as a second step to detect the immunologic markers. Data from the immunologic marker analysis of the BM samples from some MM patients studied in detail are shown in Table 2. For ISH, samples of labeled cells were cytocentrifuged and fixed in 4% paraformaldehyde for 1 min, and stored for at least 15 min in 70% ethanol at 4°C. Slides were acetylated, washed and treated with 0.1M glycine, again washed and dehydrated in a graded alcohol series. Air-dried slides were hybridized with biotin-labeled ssRNA probes in 50% formamide, 5 x SSC, 0.1M sodium phosphate containing 1 mg/ml E.coli tRNA (Boehringer), 10 mM dithiotreitol and 10 mM vanadyl ribonucleoside complexes (New England Biolabs, Inc., Berkeley, CA) at 42°C overnight. After hybridization, slides were washed twice in 1 x SSC, 0.01% Triton for 15 min at 55°C. The biotin-labeled hybrids were detected by streptavidine-FITC (Zymed, San Francisco, CA) incubation for 30 min at room temperature. The slides were washed for 15 min in phosphate buffered saline (PBS) and embedded in an antifade solution consisting of 90% glycerol in PBS (pH 8.6) and 1 mg/ml p-phenylenediamine (BDH Chemicals, Poole, UK).

The specificity of the ISH was checked as described (20-23). Only the antisense RNA probes detected a 1.8 kb IL-1β mRNA or a 1.3 kb IL-6 mRNA on Northern blots

as the sense probes did not. Integrity of RNA in the cell was checked by hybridization with the antisense GAPDH probe. Hybridization with the sense GAPDH probe served as a negative control. Only cells in which the hybridization signal was clearly higher than the background were scored as positive.

Microscopes

Zeiss (Carl Zeiss, Oberkochen, FRG) and Leitz (Ernst Leitz, Wetzlar, FRG) microscopes were used for the evaluation of the fluorescent staining. The microscopes were equipped with HBO mercury lamps (Osram, Berlin, FRG), phase-contrast facilities and filter combinations for the selective visualization of FITC and TRITC.

RESULTS

Expression of IL-1β and IL-6 in MM detected by Northern blot analysis

IL-1β mRNA could be detected in 5 out of 17 BM samples from MM patients and in one normal BM sample (Table I). In patients nrs. 1, 4 and 5 the expression was abundant (see also Fig. 1). In 12 BM samples we were not able to detect IL-1β mRNA probably due to the detection limit of Northern blot analysis. GAPDH hybridization

Table 1   Clinical features and interleukin expression in 17 patients with multiple myeloma

Patient	Stage	Paraprotein	Bone-destruction	%PC	IL-1β*	IL-6*
normal BM				5	+	-
1	IIIB	IgA/κ	+	75	+ +	+
2	IA	IgG/κ	-	15	+	-
3	IA	IgG/λ	-	23	+	-
4	IIIA	IgG/λ	+	55	+ + +	+
5	IIA	IgG/κ	?	55	+ +	-
6	IIIB	IgA/κ	+	30	-	-
7	IA	IgG/κ	-	30	-	-
8	?	κ-LCD	+	39	-	-
9	IIIB	IgG/κ	+	15	-	-
10	IIA	IgG/λ	+	50	-	-
11	?	IgA/λ	?	25	-	-
12	IIIB	IgG/λ	+	22	-	-
13	IIIA	IgG/κ	+	57	-	-
14	IIIA	κ-LCD	+	34	-	-
15	IIIA	IgA/κ	-	76	-	-
16	IIA	IgG/κ	-	68	-	-
17	IIB	IgG/κ	+	10	-	-

Abbreviations: LCD, light chain disease; %PC, percent plasma cells per MNC; ?, not known; -, not detected.
* Detected by Northern blot analysis; an example is shown in Fig. 1.

clearly revealed the presence of GAPDH mRNA in these 12 BM samples (data not shown). IL-6 mRNA could be detected in 2 out of 17 BM samples from MM patients (Table I). These two BM samples revealed a high expression of IL-1β mRNA. The IL-6 expression was much lower than the IL-1β expression. To see which cells were responsible for the production of these two interleukins we performed ISH on BM samples from three patients.
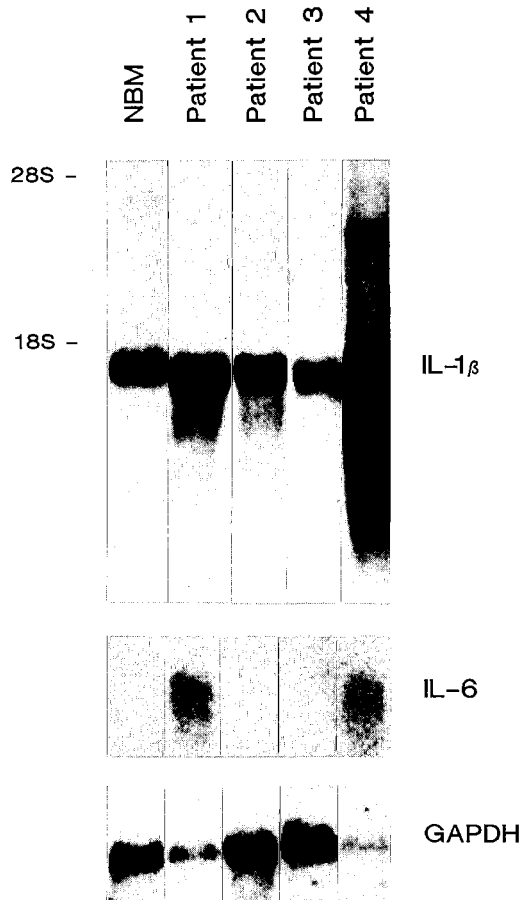

Figure 1. Northern blot analysis of four MM samples and one normal control probed with IL-1β, IL-6 and GAPDH probes. Abbreviation: NBM, normal bone marrow.

Expression of IL-1β and IL-6 in MM detected by ISH

We performed ISH on BM aspirates from patients nr 1, 2 and 3 (see Table 2) and on one normal BM sample. IL-1β mRNA was clearly present in morphologically defined plasma cells which were CD38 positive as shown in Fig. 2. There were also IL-1β^+ cells present which were CD38$^-$.
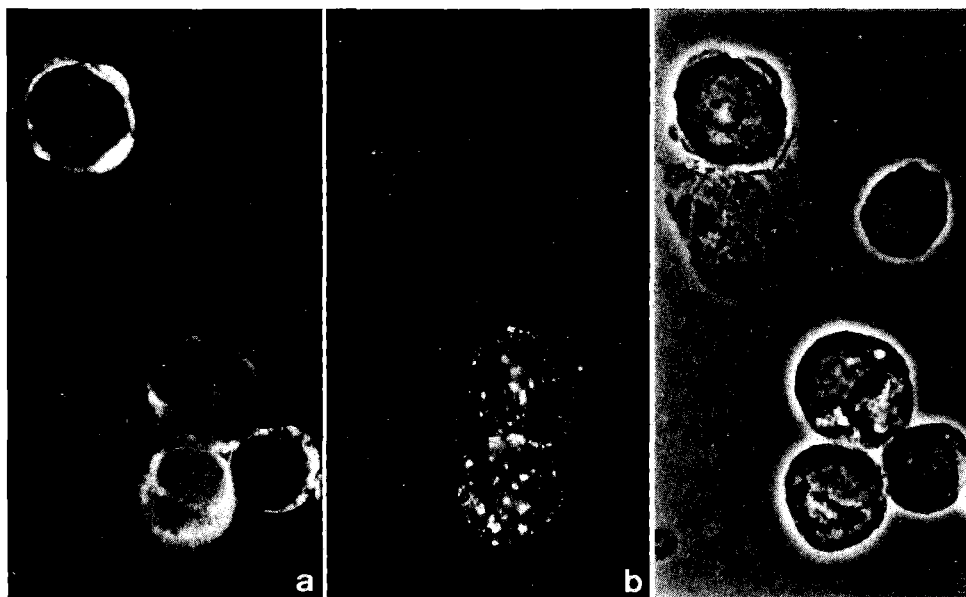
71

Figure 2. IL-1 <u>in situ</u> hybridization on CD38 labeled BM cells from a patient with MM. (a) antisense IL-1β probe (1 ng/μl, FITC); (b) CD38 staining (TRITC); (c) phase-contrast morphology. The three micrographs represent the same field.
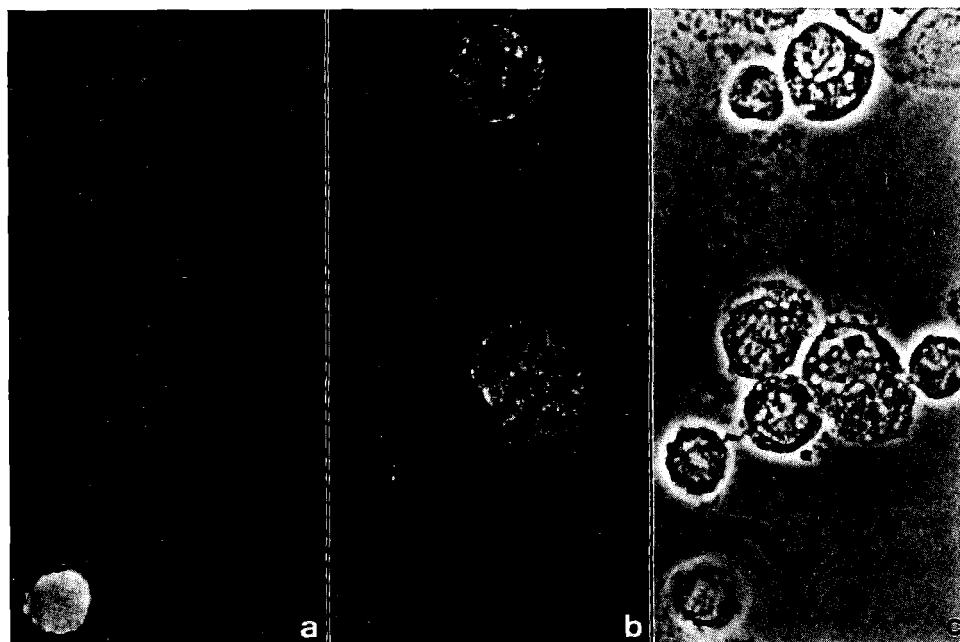

Figure 4. IL-6 in situ hybridization on CD38 labeled BM cells from a patient with MM. (a) antisense IL-6 probe (1 ng/μl, FITC); (b) CD38 staining (TRITC); (c) phase-contrast morphology. The three micrographs represent the same field.

In double labeling studies with other markers it appeared that these IL-1β^+ cells were macrophages (RFD9$^+$) and CD3$^+$ T cells but not CD14$^+$ monocytes (Fig. 3A). The proportion of CD38$^+$ cells that expressed IL-1β mRNA was at least twice as high in the three MM patients as in normal BM (Fig. 3A). Expression of IL-1β mRNA by CD3$^+$ T cells in MM BM was also more frequently found than in normal BM (Fig. 3A). Most of the RFD9$^+$ macrophages expressed the IL-1β mRNA in contrast with the CD14$^+$ monocyte population, in which less than 10% of the cells expressed IL-1β mRNA. The distribution of CD38, CD3, CD14 and RFD9 positive cells in the separate BM samples is shown in Table 2.

Table 2   Immunologic marker analysis of three MM cell samples and one normal BM sample

	Immunologic markers			
Cell samples	CD38 (OKT10)	CD3 (Leu-4)	CD14 (My4)	RFD9
Normal BM	18	6	17	NT
Patient 1	99	9	5	11
Patient 2	35	9	20	20
Patient 3	53	21	NT	NT

The figures represent percent positivity per MNC. The cell sample numbers correspond with the numbers in Table 1. NT, not tested.
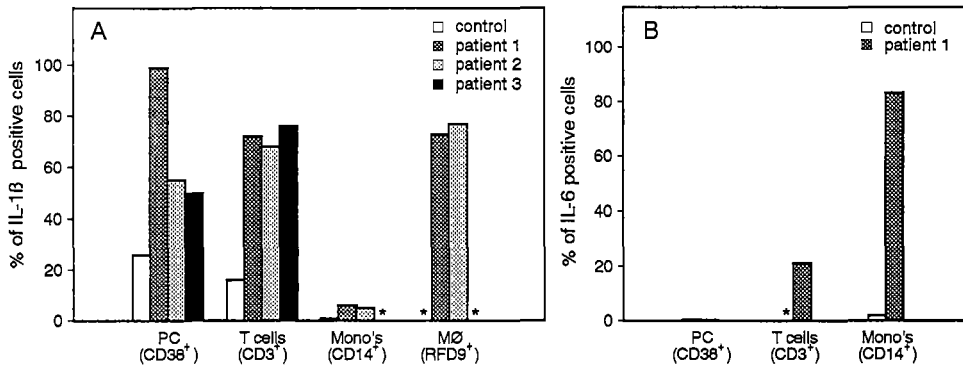

Figure 3. Expression of IL-1β (A) and IL-6 (B) by the different cell types present in the BM sample. The percentage of IL-1β en IL-6 cells were estimated by counting the IL-1β^+ or IL-6$^+$ cells within the population of cell surface marker positive cells (CD38, CD3, CD14 and RFD9 respectively). The distribution of CD38, CD3, CD14 and RFD9 in the different bone marrow samples is shown in Table 2. Abbreviations: PC, plasma cells; Mono's, monocytes; Mϕ, macrophages; *, not tested.

IL-6 expression was analyzed in one patient (nr. 1). IL-6 mRNA was only detected in small cells which where CD38$^-$. The CD38$^+$ plasma cells were clearly IL-6$^-$ (Fig. 4). The IL-6$^+$ cells appeared to be monocytes (CD14$^+$) and T cells (CD3$^+$) as analyzed by double labeling studies (Fig. 3B).

DISCUSSION

In this study we have examined the cellular localization of IL-1β and IL-6 mRNA in BM samples of MM patients. By Northern blot analysis we could detect IL-1β mRNA in five out of seventeen MM samples and in one normal BM sample. Two of these five IL-1β mRNA$^+$ MM samples expressed also IL-6 mRNA, although weak. The cell type(s) responsible for expression of IL-1β mRNA and IL-6 mRNA were identified by double fluorescent ISH. Plasma cells, macrophages and T-cells were found to express IL-1β mRNA while monocytes and T-cells expressed IL-6 mRNA. IL-1β is suggested to be one of the principal mediators of bone resorption (12). The patients in which on Northern blots an abundant expression of IL-1β was demonstrated indeed had bone lytic lesions but there were also patients with bone lytic lesions in which we could not detect IL-1β mRNA (Table 1). This may be due to the limited sensitivity of Northern blot analysis or to other bone resorbing factors like TNFα, LT/TNFβ and TGF.

In addition to the plasma cells, also the majority of the T cells may contribute to the elevated IL-1β expression in these MM patients. A striking difference was found between the T cells in the BM of the healthy control and the T cells in the myeloma BM with regard to IL-1β expression. About 4 times more T cells contributed to the IL-1β expression in MM BM than in the normal BM. It may be that these T cells are activated due to the disease and consequently release a variety of interleukins. In adult T cell leukemia (ATL) primary leukemic ATL cells also express IL-1β mRNA and this is frequently associated with hypercalcemia and thus increased osteolysis (21-22). Our findings together with the ATL data suggest a major role for T cells in the production of IL-1β which could be involved in bone resorption in MM.

With the ISH technique we could not detect IL-6 mRNA in plasma cells. This is in contrast with the findings of Kawano et al. (7) who reported that cultured myeloma cells produce IL-6 and express IL-6 receptors on their surface suggesting an autocrine growth pattern for myeloma cells. Our findings are in agreement with data from Klein et al. who reported that IL-6 was mainly produced by cells of the BM environment and not by the myeloma cells (26). We support therefore their suggestion of a paracrine rather than an autocrine growth regulation of myeloma cells, in which monocytes and T-cells produce IL-6, triggering proliferation of myeloma cells which in turn produce IL-1β causing bone destruction. Because IL-1β is also a major promotor of IL-6 expression in several cell types (2), the production of IL-1β by plasma cells, T cells and macrophages may be responsible for the induction of IL-6 production in monocytes and T cells. This data suggests a "loop" in which malignant plasma cells produce IL-1β which stimulates other cell types to produce IL-6 which in turn stimulates the plasma

cells again.

There are some contradicting reports about factors produced by myeloma cells. Most of them concern data obtained from purified cultured myeloma cells (6,7,15). Because it is difficult to obtain highly purified myeloma cells and because culturing of cells may induce artefacts the only way to determine the true facts in cytokine production is by *in vivo* studies. By means of the ISH technique we were able to detect gene expression directly in the in vivo situation, circumventing purification and culturing of BM cells and thus excluding a minor contaminating cell population obfuscating the results. ISH for gene expression studies is therefore a powerful tool in studying the capacity of the different cell types in the myeloma BM to produce factors involved in the pathogenesis of the disease.

REFERENCES

1. Mellstedt H, Holm G, Björklom M: Multiple myeloma, Waldenström's macroglobulinemia, and benign monoclonal gammapathy: Characteristics of the B-cell clone, immunoregulatory cell populations and clinical implications. Adv Cancer Res 41: 257, 1984.
2. Van Damme J, Opdenakker G, Simpson RJ, Rubira MR, Cayphas S, Vink A, Billiau A, Van Snick J: Identification of the human 26-Kd protein, interferon beta 2 (IFN beta 2), as a B cell hybridoma/plasmacytoma growth factor induced by interleukin 1 and tumor necrosis factor. J Exp Med 165: 914, 1987.
3. Muraguchi A, Hirano T, Tang B, Matsuda T, Horii Y, Nakajiama K, Kishimoto T: The essential role of B cell stimulatory factor 2 (BSF-2/IL-6) for the terminal differentiation of B cells. J Exp Med 167; 332, 1988.
4. Poupart P, Vandenbeele P, Cayphas S, Van Snick J, Haegeman G, Kruys V, Fiers W, Content J: B cell growth modulating and differentiating activity of recombinant human 26-Kd protein (BSF-2, HuIFN-beta 2, HPGF). EMBO J 6: 1219, 1987.
5. Tosato G, Seamon KB, Goldman ND, Seghel FB, May LT, Washington GC, Jones KD, Pike SE: Monocyte-derived human B-cell growth factor identified as interferon beta 2 (BSF-2, IL-6). Science 239: 502, 1988.
6. Anderson KC, Jones RM, Morimoto C, Leavitt P, Barut BA: Response patterns of purified myeloma cells to hematopoietic growth factors. Blood 73: 1915, 1989.
7. Kawano M, Hirano T, Matsuda T, Taga T, Horii Y, Iwato K, Asaoku H, Tang B, Tanabe O, Tanaka H, Kuramoto A, Kishimoto T: Autocrine generation and requirement of BSF-2/IL-6 for human multiple myelomas. Nature 332: 83, 1988.
8. Kishimoto T: The Biology of Interleukin-6. Blood 74: 1, 1989.
9. Aarden LA, De Groot ED, Schaap OL, Lansdorp PM: Production of hybridoma growth factor by monocytes. Eur J Immunol 17: 1411, 1987.
10. Bertolini DR, Nedwin GE, Bringman TS, Smith DD, Mundy GR: Stimulation of bone resorption and inhibition of bone formation in vitro by human tumor necrosis factors. Nature 319: 516, 1986.
11. Dewhirst FE, Stashenko PP, Jole JE, Tsurumachi T: Purification and partial sequence of human osteoclast-activiting factor. Identity with interleukin 1β. J Immunol 135: 2562, 1985.
12. Gowen M, Wood DD, Ihrie CJ, McGuire MKB, Rusell RGG: An interleukin 1 like factor stimulates bone resorption in vitro. Nature 306: 378, 1983.
13. Tashijan AH, Voelkel EF, Lazzaro M, Singer FR, Roberts AB, Derynck R, Winkler ME, Levine L: Alpha and beta human transforming growth factors stimulate prostaglandin production and bone resorption

in cultured mouse calvaria. Proc Natl Acad Sci USA 82: 4535, 1985.

14. Garrett R, Durie BGM, Neckvin GE, Gillespie A, Bringman T, Sabatini M, Bertolini DR, Mundy GR: Production of lymphotoxin, a bone-resorbing cytokine, by cultured human myeloma cells. N Engl J Med 317: 526, 1987.

15. Kawano M, Yamamoto I, Iwato K, Tanaka H, Asaoku H, Tanabe O, Ishikawa H, Nobuyoshi M, Ohmato Y, Hirai Y, Kuramoto A: Interleukin-1 beta rather than lymphotoxin as the major bone resorbing activity in human multiple myeloma. Blood 73: 1646, 1989.

16. Cozzolino F, Torcia M, Aldinucci D, Rubartelli A, Miliami A, Shaw AR, Lansdorp PM, Duglielmo R: Production of interleukin-1 by bone marrow myeloma cells. Blood 74: 380, 1989.

17. Van Dongen JJM, Adriaansen HJ, Hooijkaas H: Immunological marker analysis of cells in the various hematopoietic differentiation stages and their malignant counterparts. In: Ruiter DJ, Fleuren GJ, Warnaar SO (eds): Application of monoclonal antibodies in tumor pathology. Dordrecht, Martinus Nijhoff, 1987, p. 87.

18. Chirgwin JM, Przybyla AE, MacDonald J, Rutter WJ: Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18: 5294, 1978.

19. Maniatis T, Fritsch EF, Sanbrook J: Molecular Cloning: A Laboratory Manual. New York, Cold Spring Harbor Laboratory Press, 1982.

20. Brakenhoff JPJ, de Groot ER, Evers RF, Pannekoek H, Aarden LA: Molecular cloning and expression of hybridoma growth factor in Escherichia coli. J Immunol 139: 4116, 1987.

21. Benham FJ, Hodgkinson S, Davies KE: A glyceraldehyde-3-phosphate dehydrogenase pseudogene on the short arm of the human X chromosomes defines a multigene family. EMBO 3: 2635, 1984.

22. Feinberg AP, Vogelstein B: A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132: 6, 1983.

23. Bakkus MHC, Brakel-van Peer KMJ, Adriaansen HJ, Wierenga-Wolf AF, van den Akker TW, Dicke-Evinger MJ, Benner R: Detection of oncogene expression by fluorescent in situ hybridization in combination with immunofluorescent staining of cell surface markers. Oncogene 4: 1255, 1989.

24. Grossman B, Schechter GP, Horton JE, Pierce L, Jaffe E, Wahl L: Hypercalcemia associated with T-cell lymphoma-leukemia. Am J Clin Pathol 75: 149, 1981.

25. Yamaguchi K, Nishimura H, Kawano F, Kohrogi H, Jono M, Migamoto Y, Takatsuki K: A proposal for smoldering adult T-cell leukemia diversity in clinical pictures of adult T-cell leukemia. Jpn J Clin Oncol 13: 189, 1983.

26. Klein B, Zhang XG, Jourdan M, Content J, Houssian F, Aarden L, Piechaczijk M, Bataille R: Paracrine rather than autocrine regulation of myeloma-cell growth and differentiation by interleukin-6. Blood 73: 517, 1989.

7.   GENERAL DISCUSSION

This study aims to contribute to the understanding of the underlying mechanism that leads to tumor development in MM. Genetic disorders like chromosomal translocations, gene amplifications and/or deletions play a fundamental role in malignancies, like the t(9;22) in CML and the t(8;14) in Burkitt's lymphoma (see also Chapter 1). These genetic disorders can lead to the activation of proto-oncogenes which are involved in cell growth control and differentiation. Deregulation of such genes can disturb the normal growth pattern of the cell resulting in tumor development. Several of such oncogenes have already been identified. Their putative involvement in MM tumor cell formation has still to be elucidated. By studying the organization and expression of oncogenes in the myeloma cells we tried to determine the difference between the malignant cell and its normal counterpart. This should lead to a better understanding of tumor aetiology and perhaps to the finding of a tumor specific marker for MM.

7.1.   Cytogenetic analysis

It has been recognized for many years that chromosomal translocations are common in tumor cells and that certain aberrations of chromosomes occur repeatedly in certain types of tumors (Rowley, 1980; 1982; 1983; Yunis, 1983). Much attention has been directed to the issue whether proto-oncogenes known to be residents of translocated chromosomes might be positioned close to the breakpoints and whether their structure or expression is altered as a consequence of such translocation (see also Chapter 1). Also, the appearance of homogeneously staining regions (HSR) or double minute chromosomes (DM), which are karyotypical markers of gene amplifications (Cowell, 1982), may be indicative for oncogene activation (Alitalo et al.,1983; Schwab et al.,1983).

The data so far obtained from the cytogenetic analysis of MM BM cells did not reveal any aberration that was typically associated with MM (Van den Berghe et al., 1984; Ferti et al., 1984; DeWald et al., 1985; Philip and Drivsholm, 1976; Liang et al., 1979; Philip, 1980; Philip et al., 1980; Lewis and MacKenzie, 1984; Chu et al., 1986; Ranni et al., 1987). Instead, many abnormalities have been detected, involving several chromosomes, but not a single one in every patient. As a consequence no information about the primary genetic defect in MM is available from cytogenetic analysis. Therefore, other ways of detecting oncogenes in MM had to be explored.

7.2. Transfection experiments

By means of the NIH/3T3 transfection assay we have tried to detect activated oncogenes in a mouse model for MM, the 5T model. This assay is based on the assumption that neoplastic transformation of the recipient cell is dependent upon the acquisition of a mutant allele. This mutant allele is believed to act in a dominant manner in both donor and recipients cells. The oncogenic activity of DNA from a wide variety of tumor cells has been identified by this assay, frequently employing NIH/3T3 cells as acceptor cells (reviewed by Varmus, 1984). Characterization of the transformed NIH/3T3 DNA proved that most of the times a mutant member of the *ras* gene family was responsible for the neoplastic transformation (Weinberg, 1984).

Our data described in Chapter 2 revealed that DNA from various mouse MM lines also had the potential to transform NIH/3T3 cells. Further investigations by transplanting the transfected cells to athymic nude mice did not lead to the identification of a specifically activated oncogene because tumor formation was also observed in the negative control, NIH/3T3 cells transfected with NIH/3T3 DNA. It is known from the literature that sheared DNA from normal cells can transform NIH/3T3 cells at a low frequency (Cooper et al., 1980). So great care must be taken in interpreting the data derived from transfection experiments. New approaches to detect mutated *ras* genes in tumor tissue have become available. By the use of specific oligonucleotides it is possible to detect a single base pair substitution in the different *ras* genes of the primary tumor (Bos et al., 1987). Single base pair substitution is the mechanism by which *ras* genes are activated (Capon et al., 1983). As a consequence, the use of transfection experiments to detect activated *ras* genes in tumor material has become somewhat outdated. However, new oncogenes may still be discovered with this technique, especially when other recipient cells are used, more related to the cell type of the tumor.

The suggestion that one or more *ras* oncogenes may be involved in the pathogenesis of MM is supported by three observations. The first one came from Tsuchiya et al. (1988) who detected an overexpression of the H-*ras* gene at the protein level in 47% of human MM cases studied. He observed a shorter survival among patients with high p21 (*ras* protein) levels. The second came from Ernst et al. (1988) who detected a second transforming gene, N-*ras*, in a human MM line with a rearranged c-*myc* allele. The third came from Seremetis et al. (1989) who showed that introducing H-*ras* or N-*ras* oncogenes into immortalized B lymphoblasts could lead to malignant transformation and terminal differentiation into plasma cells. These data all point to a possible role for activated *ras* genes in secondary transforming events leading to a more malignant stage in the disease.

7.3. Gene rearrangements and amplifications

As no typically MM associated chromosomal aberration is known and our transfection experiments were not sufficiently successful and informative, we went on

searching for oncogenes that were already known to be involved in other B cell malignancies.

A large number of B cell lines of both human and murine origin harbor translocations that join the c-*myc* gene to one of the three Ig loci in the manner originally predicted by Klein (1981). We have searched for such c-*myc* rearrangements in the 5T MM mouse model and in fresh human MM BM samples (see Chapters 3 and 4). The Southern blot data revealed that c-*myc* rearrangement is not a common feature in MM. In the mouse model, only one (5T2) of the 8 lines studied showed a c-*myc* rearrangement in BM cells which was also present in ascitic cells (see Chapter 3). Another line, 5T14, showed only a c-*myc* gene rearrangement in the ascitic cells and not in the BM. No amplifications were detected. In the human situation, no c-*myc* gene rearrangements were detected in 26 cases studied. Amplification of the c-*myc* gene together with an increased level of expression was found in only 2 out of 24 cases (Chapter 4). These data are in agreement with the findings of others. Selvanayagam et al. (1988) reported c-*myc* rearrangements in 2 out of 37 cases of MM. Gazdar et al. (1986) reported a c-*myc* rearrangement in a myeloma cell line. Sumegi et al. (1985) detected c-*myc* rearrangements in plasma cell leukemia, but not in 21 cases of MM.

It is suggested that altered expression of the c-*myc* gene, by amplification or rearrangement, contributes to the highly malignant nature of plasma cell tumors in the leukemic and aggressive phase (Sumegi et al., 1985). Our data are in agreement with this hypothesis because: (a) 5T2, the mouse MM line with c-*myc* rearrangement in the BM and ascites, can develop features of a plasma cell leukemia (Ebbeling et al., 1985); (b) 5T14 MM showed only a c-*myc* rearrangement in the ascites cells, and the ascitic form is considered to be a more aggressive phase of MM; and (c) the two human cases with c-*myc* amplification both produced κ-light chains, a characteristic feature of a progressive MM (Durie, 1986).

We studied also the *pvt*-like region in MM because of its involvement in variant Burkitt's lymphoma and plasmacytoma (see Section 1.3.1. and Chapter 4). Rearrangements of the *pvt*-region were not detected in the 26 MM cases studied, although there is some doubt about one case (nr 8) in which an additional fragment was observed in *Eco*RI digested DNA when the Southern blot was hybridized with a probe situated on the 5' site of the *pvt*-locus. This result could not be confirmed using other restriction enzymes, which argues against a major rearrangement event. A possible explanation for this result could be the occurrence of a minor mutation in this area, which is only reflected in the *Eco*RI digest.

Amplification of the *pvt*-like region was observed in two MM cases (nr 16 and nr 25). These cases were the same ones as in which c-*myc* amplification was found. This *pvt* amplification concerned only the 5' part of the *pvt*-like region as it was only detected with the most 5' *pvt* probe and not with *pvt* probes situated in the middle and at the 3' part of the locus. The c-*myc* and *pvt* amplifications were in the same order of magnitude. This suggests that c-*myc* and *pvt* are situated on the same amplicon. The distance between c-*myc* and *pvt* is about 300 kb (Mengle-Gaw and Rabbitts, 1987). Amplicons of about this size containing c-*myc* sequences have been detected in a

colon carcinoma cell line (COLO 320) and in small cell lung carcinoma cell lines (N417 and U1285; Kinzler et al., 1986). These amplicons also terminate in the *pvt*-like region (Mengle-Gaw and Rabbits, 1987). The mechanism of these amplifications still has to be resolved.

The amplification of c-*myc* and *pvt* was also reflected at the RNA level. A transcript from the *pvt*-like region was detected in the patient with *pvt* amplification (nr 25). Only recently it was discovered that the *pvt*-like region harbored a functional gene. *Pvt* transcripts ranging in size from 1 to 11 kb were detected in the polyadenylated RNA fraction of a variety of human cell lines (Shtivelman and Bishop, 1989; Shtivelman et al., 1989). The fact that we could detect a transcript of about 7 kb in the total RNA fraction from fresh tumor material is likely to be due to the *pvt* gene amplification. Without this amplification expression is probably very low and therefore undetectable in the total RNA fraction.

The biological function of *pvt* transcripts is still unclear. Likely the *pvt*-like region plays a role in tumorigenesis because of its association with tumor specific abnormalities and involvement in different cell lines. It has been hypothesized that alterations in the *pvt*-locus had an influence on c-*myc* expression because c-*myc* was expressed in most tumors with structural changes in the *pvt*-region (Adams et al., 1986). It might be that the *pvt*-region harbors an oncogene of which the normal function is c-*myc* regulation. Another possibility is that the effects of *pvt* alterations can be transmitted to c-*myc* along the chromosome over a remarkable distance (Adams et al., 1986). The discovery of *pvt* transcripts in tumor cell lines (Shtivelman and Bishop, 1989; Shtivelman et al., 1989) and MM tumor material (Chapter 4) makes the first model more favorable.

The c-*myc/pvt* amplification is a rare phenomenon in MM, just as c-*myc* rearrangement. Therefore it must probably be considered as a late event in carcinogenesis, perhaps leading to the formation of a more malignant subclone.

7.4. Gene over-expression *in situ*

In addition to the gene rearrangement and amplification studies to identify candidate oncogenes in MM we also wanted to directly measure gene expression in tumor cells. Detection of abnormal expression of certain genes is an alternative way to get information about candidate oncogenes. Northern blot analysis of total RNA isolated from BM cells of MM patients had proven to be a valuable technique in those cases in which gene amplification led to abundant expression (see Chapter 4). As the number of tumor cells in these BM samples was relatively high, it was possible to detect this over-expression. But in most cases the number of tumor cells is relatively low in MM BM samples. As a consequence, the transcript of a putative oncogene in the tumor cells is considerably diluted by mRNA from normal cells. In that case masking the over-expression by Northern blot analysis. Therefore we set up the RNA *in situ* hybridization technique (ISH) to be able to detect mRNA at the single cell level (see Chapter 5). By combining this technique with immunofluorescent staining of cell

surface markers we were able to detect more precisely the cell type in which the mRNA was present. We used HL60 cells with known c-*myc* amplification and an abundant c-*myc* expression as a model system to set up the technique. We prepared biotinylated ssRNA probes to detect the mRNA. ssRNA probes are more sensitive than DNA probes and the labeling with biotin makes it possible to use different detection methods, e.g. streptavidine or avidine conjugated to different reporter molecules or anti-biotin antibodies detectable with fluorochrome- or enzyme-linked second antibodies (Bresser and Evinger-Hodges, 1987). By avoiding radioactively labeled probes, the ISH technique becomes suitable for routine diagnostics.

An important step in the ISH assay is the fixation of the cells. The cell surface must remain intact and the antigenic determinants have to be preserved. On the other hand, the cell surface has to become permeable to let the probe enter the cell. These conditions are often contradictory but with the use of the right fixative an appropriate result can be obtained. For this purpose we tested several fixatives. In our hands paraformaldehyde gave the best results with regard to both cell surface marker staining and preservation of the ISH signal. Bresser et al. (1987) favored the ethanol/acetic acid fixative, but in our hands this fixative did not give sufficiently consistent results. With our modified procedure it was possible to detect c-*myc* expression together with the cell surface marker CD33 in HL60 cells. The detection limit was about 30 copies per cell as estimated by dilution experiments (Chapter 5).

Using this technique we studied IL-1β and IL-6 expression in human MM BM cells (see Chapter 6). The IL-1β gene was depicted because of its involvement in bone destruction, a major problem in MM (see Section 1.2.3.). IL-1β had been detected in myeloma cell cultures (Cozzolino et al., 1989; Kawano et al., 1989) as well as in uncultured myeloma BM cells from patients with extensive bone disease (Lichtenstein et al., 1989). We wanted to know which cell type was responsible for the production of IL-1β.

The IL-6 gene was depicted because of the recent observation that myeloma cells could produce IL-6 constitutively and expressed the IL-6 receptor (Kawano et al., 1988). Exogeneous IL-6 stimulated DNA synthesis in some cases suggesting that IL-6 may function as an autocrine growth factor in MM. If the above statement is correct, IL-6 could be a possible oncogene involved in the transformation process in MM just as TGF acts as an autocrine growth factor in sarcoma virus-transfected cells (Todaro and De Larco, 1978). Also the observation that IL-6 was involved in the generation of plasmacytomas in mice made this gene a good candidate for being involved in MM. We therefore wanted to know whether the myeloma cells themselves could produce IL-6 in the *in vivo* situation.

We have studied a few MM BM samples for IL-1β and IL-6 expression by ISH. The samples were chosen because of their positivity on Northern blot for IL-1β and/or IL-6 and the availability of sufficient cells. The results described in Chapter 6 indicate that in three MM BM samples, positive for IL-1β on Northern blot, IL-1β mRNA was mainly expressed by plasma cells, T cells and macrophages. In one MM BM sample, positive for IL-6 on Northern blot, IL-6 mRNA was mainly expressed by T cells and

monocytes. We could not detect IL-6 mRNA in the plasma cells of this patient. Two other MM patients, of which we didn't have Northern blot data, also showed no ISH positivity for IL-6 mRNA in the plasma cells (data not shown).

These results indicate that myeloma plasma cells can indeed produce IL-1β but not IL-6. The number of plasma cells expressing the IL-1 mRNA was clearly higher than in normal BM. The high number of plasma cells in these BM samples made this cell type the major source of IL-1β. These patients also had lytic bone lesions which is in agreement with the assumption that IL-1β is the cytokine that causes the bone destruction in MM. The fact that we were unable to detect IL-1β mRNA by Northern blot analysis in every MM patient having lytic bone lesions, argues for the presence of another osteoclast activating factor active in these patients. Possible candidates are lymphotoxin and tumor necrosis factor which have been shown to be present in some MM BM cultures and in the RNA fraction of uncultured MM BM cells (Bertolini et al., 1986; Garret et al., 1987; Lichtenstein et al., 1989).

Our data on IL-6 expression are in contrast with the finding of Kawano et al. (1988) who reported the expression of IL-6 mRNA by purified cultured myeloma cells and by the MM cell line U266. But Klein et al. (1989) reported that they could not detect IL-6 mRNA in myeloma cells and the U266 cell line. We also tested this cell line and also were unable to detect IL-6 mRNA in U266. Klein et al. detected only IL-6 mRNA in the adherent cell fraction of the BM cells. These adherent cells are likely to represent the monocytes which have been shown positive for IL-6 by our ISH technique. An explanation for the presence of IL-6 in the myeloma cell cultures of Kawano et al. (1988) could be a contamination with monocytes in the purified myeloma cell fraction. They claim that their myeloma cell fractions are more than 95% pure. However, only a few monocytes are needed to explain the observed IL-6 positivity (Helle et al., 1988).

Although the number of MM cases examined in Chapter 6 is too limited to allow firm conclusions, these results together with the results of Klein et al. (1989) are more indicative for a paracrine growth control of IL-6 in MM than an autocrine one. This conclusion is supported by the observation that transgenic mice which had received the IL-6 gene fused with a human IgH-enhancer, did not generate plasma cell neoplasia (Suematsu et al., 1989). These mice showed a polyclonal increase of IgG1 in the blood and a massive plasmacytosis. The plasma cells were not transpantable to syngeneic mice and did not have chromosomal aberrations like c-*myc* rearrangements. Thus, deregulated IL-6 gene expression alone is not sufficient to induce plasmacytoma.

It is suggested that additional genetic changes are required for the generation of plasma cell neoplasia. IL-6 can be a major factor involved in this oncogenic transformation by the fact that it can trigger plasma cell proliferation. These proliferating plasma cells may be more susceptible for malignant conversion by additional genetic changes. This situation resembles the situation in transgenic mice which had received the c-*myc* oncogene. These mice also show excessive benign proliferation, not of plasma cells but of B-cells. After variable latency periods most of these mice developed

B-cell malignancies suggesting the occurrence of a second transforming event in this expanded B-cell population (Harris et al., 1988).

In the case of MM IL-1 and IL-6 can act synergystically in myeloma cell proliferation. IL-1β, produced by the MM plasma cells, could induce IL-6 production in monocytes and the stromal cells of the BM (Van Damme et al., 1987; Nemunaitis et al., 1989). This IL-6 could bind to the IL-6 receptor on the MM plasma cells, triggering their proliferation, giving rise to more plasma cells, which in turn produce more IL-1β causing bone destruction and inducing IL-6 production by monocytes etc. (see Fig. 1). These proliferating plasma cells could then undergo secondary transforming events leading to a more malignant stage of the disease. The fact that in some cases of MM the response to IL-6 decreases (Asaoku et al., 1988), is in agreement with this phenomenon and with the general hypothesis that during progression of the disease dependence on growth factor(s) decreases (Weinberg, 1985).
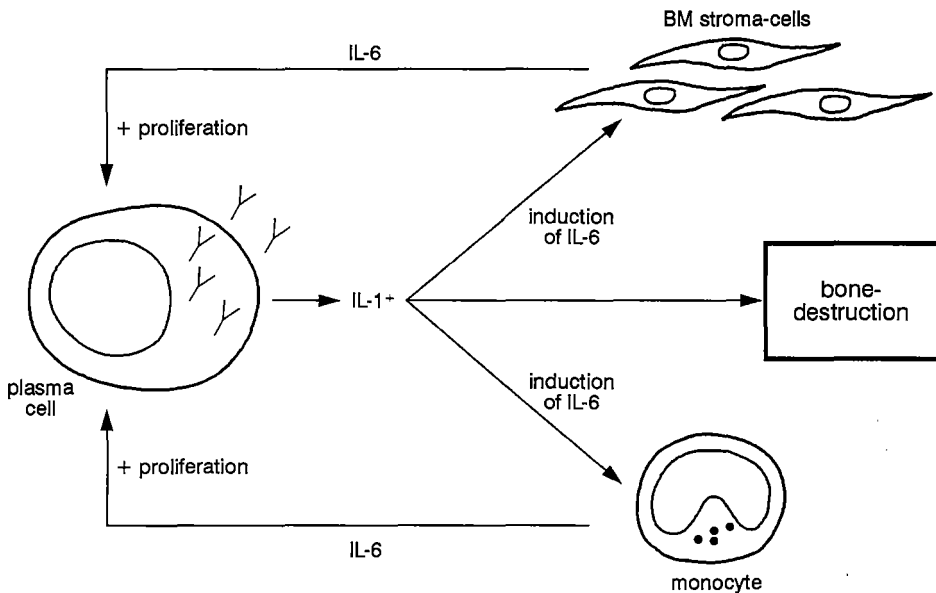

Figure 1. *A possible mechanism for stimulation of plasma cell proliferation and bone destruction in MM.*

Other cell types and cytokines may also be involved in this proliferation model for MM, like T cells and macrophages, IL-5 and IL-3. This model may be adequate for some cases of MM but an alternative model should be proposed for the cases in which we did not detect IL-1β or IL-6 gene expression. However, lack of IL-1β and IL-6 expression might also be due to the detection limit of the Northern blot assay.

Therefore the ISH studies should be expanded to other cytokines, more phenotypic markers and more MM BM samples to unravel the true growth factor network in MM. Understanding the requirements for growth factors in MM should lead to a more successful intervention therapy. In the laboratory it will simultaneously facilitate the production of MM cell lines, which will stimulate studies of the biology of MM. This may lead to more adequate karyotypic analysis of MM and thereby to the discovery of a tumor specific marker for MM.

7.5. References

Adams, J.M., Harris, A.W., Pinkert, C.A., Brinster, R.L., Palmitter, R.D., Corcoran, L., Alexander, W.S., Graham, M.W. and Cory, S. (1986) C-*myc* induced lymphomagenesis in transgenic mice and the role of the *pvt*-1 locus in lymphoid neoplasia. Curr Top Microbiol Imm, 132: 1.

Alitalo, K., Schwab, M., Lin, C.C., Varmus, H.E. and Bishop, J.M. (1983) Homogeneously staining chromosomal regions contain amplified copies of an abundantly expressed cellular oncogene (c-*myc*) in malignant neuro endocrine cells from a human colon carcinoma. Proc Natl Acad Sci USA, 80: 1707.

Asaoku, H., Kawano, M., Iwato, K., Tanabe, O., Tanaka, H., Hirano, T., Kishimoto, T. and Kuramoto, A. (1988) Decrease in BSF-2/IL-6 response in advanced cases of multiple myeloma. Blood, 72: 429.

Bertolini, D.R., Nedwin, G.E., Bringman, I.S., Smith, D.D. and Mundy, G.R. (1986) Stimulation of bone resorption and inhibition of bone formation *in vitro* by human tumor necrosis factors. Nature, 319: 516.

Bresser, J. and Evinger-Hodges, M.J. (1987) Comparison and optimalization of *in situ* hybridization procedures yielding rapid, sensitive mRNA detections. Gene Anal Tech, 4: 89.

Bos, J.L., Verlaan-de Vries, M., van der Eb, A.J., Janssen, J.W.G., Delwel, R., Löwenberg, R. and Colly, L.P. (1987) Mutations in N-*ras* predominate in acute myeloid leukemia. Blood, 69: 1237.

Capon, D.J., Chen, E.Y., Levinson, A.D., Seeburg, P.H. and Goeddel, D.B. (1983) Complete nucleotide sequences of the T24 human bladder carcinoma oncogene and its normal homologue. Nature, 302: 33.

Chu, K.-C., Bevan, P.C. and Mattews, J.G. (1986) Analysis of G banded karyotypes in myeloma cells. J Clin Pathol, 39: 260.

Cooper, G.M., Okenquist, S. and Silverman, L. (1980) Transforming activity of DNA of chemically transformed and normal cells. Nature, 284: 418.

Cowell, J.K. (1982) Double minutes and homogeneously staining regions: Gene amplification in mammalian cells. Ann Rev Genet, 16: 21.

Cozzolino, F., Torcia, M., Aldinucci, D., Rubartelli, A., Miliani, A., Shaw, A.R., Lansdorp, P.M. and Duglielmo, R. (1989) Production of interleukin-1 by bone marrow myeloma cells. Blood, 74: 380.

DeWald, G.W., Kyle, R.A., Hicks, G.A. and Greipp, P.R. (1985) The clinical significance of cytogenetic studies in 100 patients with multiple myeloma, plasma cell leukemia or amyloidosis. Blood, 66: 380.

Durie, B.G.M. (1986) Staging and kinetics of multiple myeloma. Sem Oncol, 13: 300.

Ebbeling, S.B., Lokhorst, H.M., Radl, J., Croese, J.W., Bast, E.J.E.G. and Ballieux, R.E. (1985) Phenotypic and kinetic aspects of idiotype cells in the murine C57BL/KaLwRij/5T2 multiple myeloma. In: Monoclonal gammapathies - Clinical significance and basic mechanisms; Vol. 5. Eds.: J. Radl, W. Hijmans, B. Van Camp. Eurage, Rijswijk, The Netherlands, pp. 205.

Ernst, T.J., Gazdar, A., Ritz, J. and Shipp, M. (1988) Identification of a second transforming gene, N-*ras*, in a human multiple myeloma line with a rearranged c-*myc* allele. Blood, 72: 1163.

Ferti, A., Panani, A., Arapakis, G. and Sotirius, R. (1984) Cytogenetic study in multiple myeloma. Cancer Genet Cytogenet, 12: 247.

Garrett, R., Durie, B.G.M., Nedwin, G.E., Gillespie, A., Bringman, T., Sabatini, M., Bertolini, D.R. and Mundy, G.R. (1987) Production of lymphotoxin, a bone-resorbing cytokine, by cultured human myeloma cells. N Engl J Med, 317: 526.

Gazdar, A.F., Oie, H.K., Kirsch, I.R. and Hollis, G.F. (1986) Establishment and characterization of a human plasma cell myeloma culture having a rearranged cellular *myc* proto-oncogene. Blood, 67: 1542.

Harris, A.W., Pinkert, C.A., Crawford, M., Langdon, W.Y., Brinster, R.L. and Adams, J.M. (1988) The Eµ-*myc* transgenic mouse. A model for high-incidence spontaneous lymphoma and leukemia of early B cells. J Exp Med, 167: 353.

Helle, M., Boeije, L. and Aarden, L. (1988) Functionel discrimination between interleukin 6 and interleukin

1. Eur J Immunol, 18: 1535.

Helle, M., Boeije, L. and Aarden, L.A. (1989) IL-6 is an intermediate in IL-1 induced thymocyte proliferation. J Immunol, 142: 4335.

Houssiau, F.A., Coulie, P.G., Olive, D. and Van Snick, J. (1988) Synergystic activation of human T cells by interleukin 1 and interleukin 6. Eur J Immunol, 18: 653.

Kawano, M., Hirano, T., Taga, T., Hrii, Y., Iwato, K., Asaoku, H., Tang, B., Tanabe, O., Tanaka, H., Kuramoto, A. and Kishimoto, T. (1988) Autocrine generation and requirement of BSF-2/IL-6 for human multiple myelomas. Nature, 332: 83.

Kawano, M., Yamamoto, I., Iwato, K., Tanaka, H., Asaoku, H., Tanabe, O., Ishikawa, H., Nobuyoshi, M., Ohmoto, Y., Hirai, Y. and Kuramoto, A. (1989) Interleukin-1 beta rather than lymphotoxin as the major bone resorbing activity in human multiple myeloma. Blood, 73: 1646.

Kinzler, K.W., Zehnbauer, B.A., Brodeur, G.M., Seegers, R.C., Trent, J.M., Meltzer, P.S. and Vogelstein, B. (1986) Amplification units containing human N-*myc* and c-*myc* genes. Proc Natl Acad Sci USA, 83: 1031.

Klein, G. (1981) The role of gene dosage and genetic transpositions in carcinogenesis. Nature, 294: 313.

Klein, B., Zhang, X.G., Jourdan, M., Content, J., Houssiau, F., Aarden, L., Piechaczyk, M. and Bataille, R. (1989) Paracrine rather than autocrine regulation of myeloma cell growth and differentiation by interleukin-6. Blood, 73: 517.

Lewis, J.P. and MacKenzie, M.R. (1984) Non-random chromosomal aberrations associated with multiple myeloma. Hematol Oncol, 2: 307.

Liang, W., Hopper, J. and Rowley, J.D. (1979) Karyotypic abnormalities and clinical aspects of patients with multiple myeloma and related paraproteinemic disorders. Cancer, 44: 630.

Lichtenstein, A., Berenson, J., Norman, D., Chang, M.P. and Carlile, A. (1989) Production of cytokines by bone marrow cells obtained from patients with multiple myeloma. Blood, 74: 1266.

Mengle-Gaw, L. and Rabbitts, T.H. (1987) A human chromosome 8 region with abnormalities in B cell, HTLV-1^{+} T cell and c-*myc* amplified tumours. EMBO J, 6: 1959.

Nemunaitis, J., Andrews, D.F., Mochozuki, D.Y., Lilly, M.B. and Singer, J.W. (1989) Human marrow stromal cells: response to interleukin-6 (IL-6) and control of IL-6 expression. Blood, 74: 1929.

Philip, P. and Drivsholm, A. (1976) G-banding analysis of complex aneuploidy in multiple myeloma bone marrow cells. Blood, 47: 69.

Philip, P. (1980) Chromosomes of monoclonal gammapathies. Cancer Genet Cytogenet, 2: 79.

Philip, P., Drivsholm, A., Nansen, N.E., Jensen, M.K. and Killman, S. (1980) Chromosomes and survival in multiple myeloma. Cancer Genet Cytogenet, 2: 243.

Potter, M. and Boyer, C.R. (1962) Induction of plasma cell neoplasms in BALBc mice with mineral oil adjuvants. Nature, 193: 1086.

Ranni, N.S., Slavutski, I., Wechsler, A. and Brieux, S. (1987) Chromosome findings in multiple myeloma. Cancer Genet Cytogenet, 25: 309.

Rowley, J.D. (1980) Chromosome abnormalities in human leukemia. Ann Rev Genet, 14: 17.

Rowley, J.D. (1982) Identification of the constant chromosome regions involved in human hematologic malignant disease. Science, 216: 749.

Rowley, J.D. (1983) Human oncogene locations and chromosome aberrations. Nature, 301: 290.

Schwab, M., Alitalo, K., Varmus, H.E., Bishop, J.M. and George, D. (1983) A cellular oncogene (c-Ki-*ras*) is amplified, over-expressed, and located within karyotypic abnormalities in mouse adrenocortical tumor cells. Nature, 303: 497.

Selvanayagam, P., Blick, M., Narni, F., van Tuine, P., Ledbetter, D.H., Alexanian, R., Saunders, G.F. and Barlogie, B. (1988) Alteration and abnormal expression of the c-*myc* oncogene in human multiple myeloma. Blood, 71: 30.

Seremetis, S., Inghirami, G., Ferrero, D., Newcomb, E.W., Knowles, D.M., Dotto, G.P. and Dalla-Favera, R. (1989) Transformation and plasmacytoid differentiation of EBV-infected human B lymphoblasts by *ras* oncogenes. Science, 243: 660.

Shtivelman, E. and Bishop, J.M. (1989) The *pvt* gene frequently amplifies with *myc* in tumor cells. Mol Cell Biol, 9: 1148.

Shtivelman, E., Henglein, B., Groitl, P. and Bishop, M. (1989) Identification of a human transcription unit affected by the variant chromosomal translocations 2;8 and 8;22 of Burkitt lymphoma. Proc Natl Acad Sci USA, 86: 3257.

Suematsu, S., Matsuda, T., Aozasa, K., Akira, S., Nakarro, N., Ohne, S., Miyazaki, J., Yamamura, K., Hirano, T. and Kishimoto, T. (1989) IgG1 plasmacytosis in interleukin-6 transgenic mice. Proc Natl Acad Sci USA, 86: 7547.

Sümegi, J., Hedberg, T., Bjorkholm, M., Godal, T., Mellstedt, H., Nilsson, M.G., Pereman, C. and Klein, G. (1985) Amplification of the c-*myc* oncogene in human plasma-cell leukemia. Int J Cancer, 36:

367.

Tsuchiya, H., Epstein, J., Selvanayagam, P., Dedman, J.R., Gallick, G., Alexanian, R. and Barlogie,B. (1988) Correlated flow cytometric analysis of H-*ras* p21 and nuclear DNA in multiple myeloma. Blood, 72: 796.

Todaro, G.J. and De Larco, J.E. (1978) Growth factors produced by Sarcoma virus-transformed cells. Cancer Res, 38: 4147.

Van Damme, J., Cayphas, S., Opdenakker, G., Billiau, A. and Van Snick, J. (1987) Interleukin 1 and poly(rI).poly(rC) induce production of a hybridoma growth factor by human fibroblasts. Eur J Immunol, 17: 1.

Van den Berghe, H., Vermaelen, K., Louwagie, A., Criel, A., Mecucci, C. and Vaerman, J.P. (1984) Hight incidence of chromosome abnormalities in IgG3 myeloma. Cancer Genet Cytogenet, 11: 381.

Varmus, H.E. (1984) The molecular genetics of cellular oncogenes. Ann Rev Genet, 18: 553.

Weinberg, R.A. (1984) *Ras* oncogenes and the molecular mechanisms of carcinogenesis. Blood, 64: 1143.

Weinberg, R.A. (1985) The action of oncogenes in the cytoplasm and nucleus. Science, 230: 770.

Yunis, J.J. (1983) The chromosomal basis of human neoplasia. Science, 221: 227.

SUMMARY

Multiple myeloma (MM) is a malignant proliferating disorder of the B lymphocyte lineage, characterized by an increasing proportion of plasma cells in the bone marrow, a high and progressively increasing concentration of a homogeneous immunoglobulin in the blood and the occurrence of osteolytic bone lesions. It is predominantly a disease of the elderly. The incidence of MM in the Netherlands is approximately 3 in 100,000 inhabitants. The prognosis for survival is about 3 years.

In this thesis a study was made of genetic defects responsible for the transforming event in MM. The purpose of this study was to increase our insight into the underlying mechanism of tumor formation and to find tumor specific markers that would improve the diagnosis MM in an early stage of the disease.

Since the discovery of oncogenes in the early eighties, much progress has been made in understanding tumor development. Oncogenes can play an important role in the process of malignant transformation. Oncogenes are activated proto-oncogenes, which in normal cells regulate growth and differentiation. Alterations in such genes by translocations, amplifications, point-mutations or deletions can disturb the normal growth pattern of the cell leading to malignant conversion.

An approach to detect oncogenes in tumor material is the use of the NIH/3T3 transfection assay. This assay is based on the neoplastic transformation of murine NIH/3T3 recipient cells upon acquisition of an oncogene. DNA derived from three *in vivo* propagated murine MM lines (5T2, 5T7 and 5T14) was transfected into NIH/3T3 cells (Chapter 2). After isolation and injection of the transfected cells into nude mice, tumor development was observed in all three cases studied. Secondary transfection rounds were performed with DNA derived from these tumors, ligated to a dominant selective marker in order to distinguish between tumor DNA and NIH/3T3 DNA. Tumor development was again seen in all cases but also in the negative control, which hampered the isolation of the transforming gene from the murine MM lines.

Further investigations of oncogenes that might be involved in MM were focussed on oncogenes known to be involved in other B-cell neoplasia. C-*myc*, for example, is involved in Burkitt's lymphoma and mouse plasmacytoma. It is activated by a translocation event in which one of the three immunoglobulin loci are involved. The presence of such rearrangements of c-*myc* was investigated in eight murine MM lines and 21 human MM patients (Chapters 3 and 4). Southern blot data from DNA derived from MM bone marrow cells revealed that c-*myc* rearrangement is not a common phenomenon in MM. Rearrangements were only observed in the bone marrow and ascites cells of the 5T2 MM and in the ascites cells of the 5T14 MM. Amplification of c-*myc*, as is often observed in plasma cell leukemias, was only detected in two out of

21 human MM cases. This amplification was also reflected at the RNA level. Northern blot data revealed an abundant expression of c-*myc* mRNA in one MM patient with c-*myc* amplification. The *pvt*-region, often involved in translocations in variant Burkitt's lymphoma and plasmacytoma, was also studied in human MM. No clear rearrangements were detected but amplification of the *pvt* locus was found in the same two patients that also showed c-*myc* amplification. This amplification concerned only the 5' part of the locus. The amplification was in the same order of magnitude for both c-*myc* and *pvt*, which argues for the presence of c-*myc* and *pvt* on the same amplicon. The distance between c-*myc* and *pvt* on chromosome 8 is about 300 kb. Amplicons of that size containing *myc* sequences and ending within the *pvt*-region have been shown to be present in other tumors as well. The *pvt* gene amplification in MM also led to an abundant expression of a transcript of about 7 kb. So far, *pvt*-transcripts (ranging from 1-11 kb) had only been described in some tumor cell lines. Thus, detection of a *pvt* transcript in de total RNA fraction from fresh tumor material is exceptional.

The biological function of the *pvt* transcript is yet unclear. It is suggested that *pvt* may be involved in c-*myc* regulation. Because c-*myc* and *pvt* amplifications were detected in two MM patients that produced only κ-light chains, a characteristic feature of a progressive stage of the disease, it was presumed that c-*myc* and *pvt* were only activated in a late stage of the disease. This presumption was confirmed by the fact that in the mouse model for MM c-*myc* rearrangements were only detected in the 5T2 MM line that can develop features of a plasma cell leukemia and in the ascites of 5T14 MM, a more aggressive phase of MM. This activation of c-*myc* and *pvt* is probably the course of the development of a more malignant cell variant.

To get more information about aberrant expression of certain genes in MM, we set up the RNA *in situ* hybridization (ISH) technique combined with immunofluorescent staining of cell surface markers. With this technique it is possible to detect a specific mRNA in the cytoplasm of a single cell. Cell morphology was sufficiently preserved, as were antigenic determinants. By double staining of antigens on the cell surface and mRNA in the cytoplasm of the cells it was possible to determine more precisely the cell type in which the mRNA is expressed (Chapter 5). This information about gene expression in a well defined cell type cannot be achieved by conventional methods like the Northern blot assay. ISH is a useful technique, especially for the study on MM, because the proportion of malignant plasma cells in the MM bone marrow is in an early phase of the disease relatively low, which makes it difficult to answer questions on gene expression in these cells.

Using the ISH technique we have studied the expression of IL-1ß and IL-6 in 17 MM bone marrow samples (Chapter 6). IL-1ß was choosen because of its osteoclast activating activity which leads to bone destruction, an important feature of MM. IL-6 was choosen because of its growth factor activity on plasma cells. From *in vitro* studies it was postulated that IL-6 could act as an autocrine growth factor in MM, making it a candidate oncogene involved in the transformation process. We tested this hypothesis in a few human MM by the ISH technique. Biotinylated ssRNA probes specific for IL-6 and IL-1ß were generated and used in the ISH assay together with cell surface marker

staining to distinguish plasma cells from other cell types. These experiments showed that malignant plasma cells were the major cell type in the MM bone marrow that expressed IL-1ß. This is indicative for the important role of the malignant plasma cell clone itself in the characteristic bone destruction. IL-6 expression, on the other hand, could not be detected in the plasma cells. Monocytes were the major population expressing this gene. This finding argues against an autocrine growth factor activity of IL-6 in MM. Instead, IL-6 is more likely acting as a paracrine growth factor for MM plasma cells.

IL-1β and IL-6 may act synergystically on plasma cell proliferation. A model has been postulated in which plasma cells can produce IL-1, which induces IL-6 production in monocytes and stromal cells in the bone marrow. This IL-6 stimulates the plasma cell to proliferate, giving rise to more cells producing more IL-1β. This IL-1β again can cause plasma cell proliferation through IL-6 induction and bone-destruction. Because MM is a heterogeneous disease, this model is probably not true for every case. Extension of the ISH studies with other cytokine specific probes on more MM samples is needed to unravel the cytokine network in MM.

The studies described in this thesis did not lead to the discovery of the primary genetic defect responsible for the onset of MM, but have contributed to the identification of genes possibly involved in a more advanced stage of the disease and to the understanding of the role of IL-1β and IL-6 in MM.

SAMENVATTING

Het multipel myeloom (MM) is een kwaadaardige aandoening uitgaande van cellen van de B lymfocyten reeks. Deze ziekte wordt gekenmerkt door de aanwezigheid van een toenemend percentage plasmacellen in het beenmerg, een hoog en progressief toenemend gehalte aan een homogeen immunoglobuline in het bloed, en botlesies. MM komt voornamelijk voor bij oudere mensen en de incidentie in Nederland is ongeveer 3 per 100.000 inwoners. De prognose is slechts 3 jaar.

Dit proefschrift beschrijft onderzoek naar de genetische defecten die verantwoordelijk zijn voor de transformerende gebeurtenis in MM. Het doel van het onderzoek was om een beter inzicht te krijgen in het mechanisme dat ten grondslag ligt aan de tumorvorming en het opsporen van tumor specifieke markers waarmee de diagnose van MM in een vroeg stadium verbeterd zou kunnen worden.

De ontdekking van oncogenen in het begin van de jaren tachtig heeft geleid tot een grote vooruitgang in ons begrip van tumorontwikkeling. Oncogenen spelen een belangrijke rol in de transformatie van een normale cel tot een tumorcel. Oncogenen zijn geaktiveerde proto-oncogenen die normaal een funktie vervullen in de regulatie van celgroei en -differentiatie. Veranderingen in zulke regulatie-genen, bijvoorbeeld door translocaties, punt-mutaties, amplificaties en/of deleties, kunnen aanleiding geven tot een verstoring van het normale groeipatroon van de cel, waardoor kanker kan ontstaan.

Een manier om oncogenen op te sporen is de NIH/3T3 transformatietest. Deze test is gebaseerd op de neoplastische transformatie van muize NIH/3T3 ontvangercellen door de opname van een oncogen. Door ons werd DNA van drie getransplanteerde muize MM lijnen (5T2, 5T7 en 5T14) getransfekteerd naar NIH/3T3 cellen (Hoofdstuk 2). Na isolatie en injectie van deze getransformeerde cellen in naakte muizen worden er tumoren gevonden in alle drie gevallen. Met het DNA afkomstig van deze tumoren werd een tweede transfektieronde uitgevoerd. Om onderscheid te kunnen maken tussen het tumor DNA en het NIH/3T3 DNA werd het tumor DNA geligeerd aan een dominante selektieve marker. Ook deze laatste getransfekteerde cellen veroorzaakten tumoren in naakte muizen. Echter, ook de negatieve controle, NIH/3T3 DNA geligeerd aan de selektieve marker, gaf aanleiding tot tumorontwikkeling. Dit verhinderde de isolatie van eventueel transformerende genen uit de muize MM lijnen.

Verder onderzoek naar de rol van oncogenen in MM werd gericht op oncogenen waarvan bekend was dat zij betrokken waren bij andere B cel neoplasieën, zoals c-*myc*, betrokken bij het Burkitt's lymfoom en het plasmacytoom. Dit gen wordt geaktiveerd door een translokatie waarbij één van de drie immunoglobuline loci

betrokken is. De aanwezigheid van eventuele herschikkingen van c-*myc* werd bestudeerd in acht muize MM lijnen en in 21 MM patiënten (Hoofdstuk 3 en 4). De resultaten van Southern blot analyse van DNA van beenmergcellen toonden aan dat c-*myc* herschikking niet een algemeen verschijnsel is in MM. Herschikking werd alleen gevonden in beenmerg en ascites cellen van 5T2 en in ascites cellen van 5T14. Amplificatie van c-*myc*, dat vaak gevonden wordt in plasmacel leukemieën, kon slechts in twee gevallen van MM worden gedetecteerd.

De *pvt*-regio, een sequentie die vaak betrokken is bij het variant Burkitt's lymfoom, werd ook onderzocht in MM (Hoofdstuk 4). Er werden geen duidelijke herschikkingen gevonden maar wel een amplificatie van het 5' gedeelte van deze regio in dezelfde twee patiënten waarin ook een c-*myc* amplificatie was gevonden. De mate van amplificatie was gelijk voor zowel c-*myc* als *pvt*. Dit is een aanwijzing voor de aanwezigheid van c-*myc* en *pvt* op hetzelfde amplicon. De afstand tussen c-*myc* en *pvt* op chromosoom 8 bedraagt ongeveer 300 kb. Amplicons van die grootte die c-*myc* sequenties bevatten en in de *pvt*-regio eindigen werden reeds gevonden in andere tumoren. Deze *pvt* amplificatie gaf ook aanleiding tot een verhoogde expressie van een *pvt* transcript van ongeveer 7 kb. *Pvt* transcripten, variërend in grootte van 1-11 kb, werden reeds eerder in enkele tumor cellijnen gevonden. De detectie van een *pvt* transcript in de totale RNA fraktie van vers tumor materiaal is dus uitzonderlijk. De biologische betekenis van dit *pvt* transcript is nog niet duidelijk. Er is wel gesuggereerd dat *pvt* betrokken zou zijn bij de regulatie van c-*myc*.

Het feit dat c-*myc* en *pvt* amplificaties slechts werden gevonden in twee MM patiënten die beiden alleen κ-lichte ketens produceerden, een karakteristiek kenmerk voor een progressief stadium van de ziekte, doet vermoeden dat c-*myc* en *pvt* slechts geaktiveerd worden in een late fase van de ziekte. Dit vermoeden werd bevestigd in het muizemodel voor MM, waar c-*myc* veranderingen alleen werden gevonden in de 5T2 lijn die zich kan ontwikkelen tot een plasmacel leukemie en in de ascites vorm van 5T14, een agressieve vorm van MM. De aktivering van c-*myc* en *pvt* zou aanleiding kunnen geven tot de ontwikkeling van een meer maligne celvariant van de tumor.

Om meer informatie te verkrijgen over eventueel veranderde genexpressie in het MM hebben we de *in situ* hybridisatie (ISH) techniek opgezet, gecombineerd met een immunofluorescentie kleuring van celoppervlakte antigenen. Met deze techniek is het mogelijk om specifiek mRNA te detekteren op "single cell" niveau. De celmorfologie en antigene determinanten bleken daarbij in voldoende mate behouden te kunnen worden. Door dubbelkleuring van antigenen op het celoppervlak en mRNA in het cytoplasma van de cel kon het celtype worden bepaald waarin het mRNA aanwezig was (Hoofdstuk 5). Zulke informatie kan niet verkregen worden m.b.v. conventionele methoden zoals de Northern blot assay. De ISH techniek is een waardevolle techniek, speciaal voor het bestuderen van RNA expressie in MM, omdat in een vroeg stadium van de ziekte het aantal maligne plasmacellen in de beenmerg aspiraten relatief laag is, waardoor het moeilijk is om een uitspraak te doen over de genexpressie specifiek in deze cellen.

Met behulp van de ISH techniek hebben we de expressie bestudeerd van IL-1β

en IL-6 in zeventien MM beenmerg monsters (Hoofdstuk 6). IL-1β was uitgekozen vanwege zijn osteoclast activerend vermogen, welke aanleiding kan geven tot botafbraak, een belangrijk kenmerk van MM. IL-6 was uitgekozen vanwege zijn groeifaktor activiteit voor plasmacellen. Op basis van *in vitro* onderzoek is door anderen gepostuleerd dat IL-6 een autocrine groeifaktor is voor MM, dus een kandidaat-oncogen in de transformatie bij MM. We hebben deze hypothese getoetst in de *in vivo* situatie door gebruik te maken van de ISH techniek. Specifieke, gebiotinyleerde ssRNA probes voor IL-6 en IL-1β werden gemaakt. Deze werden gebruikt voor ISH in combinatie met verschillende oppervlakte markers om de plasmacellen te kunnen onderscheiden van andere celtypen. Uit de ISH experimenten bleek dat in beenmerg van een MM patiënt, de maligne plasmacel het belangrijkste celtype was waarin IL-1β expressie aangetoond kon worden. Dit is een indikatie voor de belangrijke rol die de maligne plasmacel kan vervullen in de botafbraak. IL-6 daarentegen, kon niet worden aangetoond in de plasmacellen. Het bleek dat de monocyten de belangrijkste celpopulatie vormden die IL-6 tot expressie bracht. Deze bevindingen zijn in tegenspraak met de vermeende autocriene groeifaktor aktiviteit van IL-6 in MM. IL-6 lijkt meer een paracriene groeifaktor te zijn in MM.

IL-1β en IL-6 zouden synergistisch kunnen werken in de stimulering van de plasmacel proliferatie. Plasmacellen produceren IL-1β dat de IL-6 productie in monocyten en stromale cellen van het beenmerg kan induceren. Dit IL-6 kan op zijn beurt de plasmacel proliferatie stimuleren, waardoor er meer plasmacellen ontstaan die IL-1β produceren. Dit IL-1β kan weer plasmacel proliferatie induceren via IL-6 en botdestruktie veroorzaken. Omdat MM een zeer heterogeen ziektebeeld is, gaat dit model waarschijnlijk niet op voor elk geval van MM. Een uitbreiding van de ISH studies met andere cytokine probes bij een groter aantal gevallen van MM zal nodig zijn om het cytokine netwerk in MM te ontrafelen.

De studies die in dit proefschrift beschreven zijn, hebben niet geleid tot de opheldering van het primaire genetische defekt dat verantwoordelijk is voor het ontstaan van MM, maar hebben wel bijgedragen tot de identifikatie van genen die mogelijk betrokken zijn in een meer gevorderd stadium van de ziekte, en tot een beter begrip van de rol van IL-1β en IL-6 in MM.

ABBREVIATIONS

AP	alkaline phosphatase
ATL	adult T-cell leukemia
BCGF	B-cell growth factor
B-CLL	B-cell chronic lymphocytic leukemia
BM	bone marrow
BMG	benign monoclonal gammapathy
BrdU	5-bromo-2-deoxyuridine
BSA	bovine serum albumin
BSF	B cell stimulatory factor
C	constant immunoglobulin gene segment
CAB-NHS	ϵ-caproylamidobiotin-N-hydroxysuccinimide ester
cDNA	complementary deoxyribonucleic acid
CML-BC	chronic myeloid leukemia with lymphoblastic blast crisis
c-onc	cellular oncogene
cpm	counts per minute
D	immunoglobulin diversity gene segment
DM	double minute
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EBV	Epstein Barr virus
EGF	epidermal growth factor
FACS	fluorescent activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GTP	guanosine 5'-triphosphate
HGF	hepatocyte growth factor
HSR	homogeneously staining region
IFN	interferon
IgH	immunoglobulin heavy chain
IgL	immunoglobulin light chain
IL	interleukin
ISCN	international system for human cytogenetic nomenclature
ISH	in situ hybridization
J_H	joining region of the heavy chain locus
kb	kilobase

LT	lymphotoxin
MG	monoclonal gammapathy
MM	multiple myeloma
MNC	mononuclear cells
MoAb	monoclonal antibody
MPC	mouse plasmacytoma
mRNA	messenger ribonucleic acid
MW	molecular weight
neo^r	neomycine resistant
OAF	osteoclast activating factor
(^{32}P)a-dCTP	^{32}P labeled deoxycytidine triphosphate
PB	peripheral blood
PBS	phosphate buffered saline
PCL	plasma cell leukemia
PF	paraformaldehyde
pvt	plasmacytoma variant translocation
RIC	rat immunocytoma
RNA	ribonucleic acid
SA	streptavidin
SDS	sodium dodecyl sulphate
ss	single stranded
SSC	standard saline citrate
t	translocation
T-ALL	T cell acute leukemia
TGF	transforming growth factor
TNF	tumor necrosis factor
tRNA	transfer ribonucleic acid
TRITC	tetramethyl rhodamine isothiocyanate
V	variable immunoglobulin gene segment
v-*onc*	viral oncogene

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LIST OF PUBLICATIONS

Burbach, J.P.H., Van Tol, H.H.M., Bakkus, M.H.c., Schmale, H. and Ivell, R. (1986) Quantitation of vasopressin mRNA and oxytocin mRNA in hypothalamic nuclei by solution hybridization assays. J. Neurochem. 47, 1814-1821.

Bakkus, M.H.C., Punt, Y.A., Croese, J.W., Radl, J. and Van den Akker, Th.W. (1987) Cytogenetic analysis and transforming capacity of DNA in murine multiple myeloma. J. of Cellular Biochemistry, 11A, p. 190.

Bakkus, M.H.C., Sontag, Y., Keijzer, W., Punt, Y.A., Radl, J. and Van den Akker, Th.W. (1989) Transforming capacity of DNA fragments from murine multiple myeloma cells. In: Monoclonal gammapathies. II. Clinical significance and basic mechanisms. Eds: J. Radl, B. van Camp, p. 117-123. Eurage.

Bakkus, M.H.C., Versnel, M.A., Adriaansen, H.J. and Van den Akker, Th.W. (1989) Non-isotopic RNA *in situ* hybridization: the detection of low copy number mRNA molecules in bone marrow cells of multiple myeloma patients. In: Monoclonal gammapathies. II. Clinical significance and basic mechanisms. Eds: J. Radl, B. van Camp, p. 113-116, Eurage.

Van den Enden-Vieveen, Punt, Y.A., Bakkus, M.H.C., Benner, R. and Radl, J. (1989) Rearrangement of the c-*myc* oncogene in mouse multiple myeloma, morbus waldenström and plasmacytoma. In: Monoclonal gammapathies. II. Clinical significance and basic mechanisms. Eds: J. Radl, B. van Camp, p. 245-248.

Bakkus, M.H.C., Versnel, M.A., Adriaansen, H.J., Evinger-Hodges, M.J. and Van den Akker, Th.W. (1989) Simultaneous detection of oncogene expression and cell surface markers. J. of Cellular Biochemistry, 13B, p. 25.

Bakkus, M.H.C., Brakel-van Peer, K.M.J., Adriaansen, H.J., Wierenga-Wolf, A.F., Van den Akker, Th.W., Dicke-Evinger, M.J. and Benner, R. (1989) Detection of oncogene expression by fluorescent *in situ* hybridization in combination with immunofluorescent staining of cell surface markers. Oncogene 4, p. 1255-1262.

Radl, J., Punt, Y.A., Van den Enden-Vieveen, M.H.M., Bentvelzen, P.A.J., Bakkus, M.H.C., Van den Akker, Th.W. and Benner, R. (1990) The 5T mouse multiple myeloma model: absence of c-*myc* oncogene rearrangement in early transplant generations. Br. J. Cancer 61, 276-278.

Bakkus, M.H.C., Brakel-van Peer, K.M.J., Adriaansen, H.J. and Benner, R. Detection of IL-1β and IL-6 in human multiple myeloma by fluorescent *in situ* hybridization. Submitted.

Bakkus, M.H.C., Brakel-van Peer, K.M.J., Michiels, J.J., Van 't Veer, M.B. and Benner, R. Amplification of the c-*myc* and the *pvt*-like region in human multiple myeloma. Submitted.

Prens, E.P., Benne, K, Bakkus, M.H.C., Brakel-van Peer, K.M.J., Van Damme, J. and Van Joost, Th. IL-1 and IL-6 in psoriasis. Submitted.

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