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CD34 selections from myeloma peripheral blood cell autografts contain residual tumour cells due to impurity, not to $CD34^+$ myeloma cells

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Summary. Malignant cells in haemopoietic autografts can contribute to post-transplant relapse. Engraftment of myeloma patients with $CD34^+$ peripheral blood progenitors selected from total autografts reduces the number of tumour cells infused by 2·7–4·5 logs. Residual tumour cells detected in $CD34^+$ selected cells may be due to selection impurity or the existence of malignant $CD34^+$ progenitors. In three patients we evaluated the CD34 purity and tumour load of total autografts, $CD34^+$ progenitors selected with immunomagnetic beads and highly purified $CD34^+$ progenitors obtained in two rounds of selection (combining magnetic with flow cytometry activated cell sorting) to determine the cause of residual tumour cells in CD34 selections.

Using allele-specific oligonucleotides (ASO) complementary to the unique Ig heavy chain sequence (CDRIII region) of the malignant clone, semi-quantitative ASO-PCR was capable of detecting one malignant cell in 10^4–10^5 normal white blood cells. Selection of $CD34^+$ cells from bone marrow (BM) with approximately 20% malignant plasma cells resulted in a 1·4 log reduction of tumour burden. Using two-colour flow-cytometry we observed $CD34^-$, $BB4^+$ malignant plasma cells contaminating this CD34 selection. Prior to sorting, peripheral blood cell autografts (PBCA) contained approximately 0·1% malignant cells. Selection of >99% pure $CD34^+$ cells using immunomagnetic beads (Dynal) resulted in an approximate 2 log reduction of malignant cells, but residual tumour cells were still detectable. ASO-PCR detected no malignant cells in >99·9% pure $CD34^+$ peripheral blood progenitors obtained with two rounds of selection (combining magnetic with flow cytometry activated cell sorting). We conclude that $CD34^+$ malignant cells are not detectable in myeloma PBCA and that residual tumour cells in CD34 selections are due to contaminating CD34-negative cells.

Keywords: PBCA, myeloma, CD34, immunoglobulin, ASO-PCR.

Autologous bone marrow transplantation as a treatment for multiple myeloma patients frequently results in early relapse (Barlogie *et al*, 1987; Gore *et al*, 1989; Jagannath *et al*, 1990). These relapses can result from residual tumour cells in the autologous graft as was shown for AML (Brenner *et al*, 1993), follicular lymphomas (Gribben *et al*, 1991) and CML (Deisseroth *et al*, 1994). Using selected $CD34^+$ peripheral blood progenitors rather than total peripheral blood cell autografts (PBCA) reduces the number of tumour cells infused by 2·7–4·5 logs (Schiller *et al*, 1995). In myeloma some residual tumour cells have been detected in selections of $CD34^+$ cells (Takishita *et al*, 1994; Belch *et al*, 1994; Bersagel *et al*, 1994; Schiller *et al*, 1995), suggesting either the existence of malignant $CD34^+$ progenitors or impurity of the CD34 selection. If malignant $CD34^+$ progenitors contributing to post-transplant relapse exist in myeloma, CD34 selection would be inadequate as a purging strategy for myeloma autografts. On the other hand, if residual tumour cells are due to impurity of the CD34 selection, better CD34 selection methods are needed. To determine the cause of residual tumour cells in selections of $CD34^+$ cells we quantified the tumour load and evaluated CD34 purity of total autografts, $CD34^+$ progenitors selected with immunomagnetic beads and $CD34^+$ progenitors purified with two rounds of selection (combining magnetic and flow cytometry activated cell sorting).

Using oligonucleotide primers complementary to the unique immunoglobulin heavy chain CDRIII sequence of the malignant clone, we developed tumour-specific PCRs capable of detecting one tumour cell in a background of 10^4

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normal cells. Using these sensitive PCRs we show that multiple myeloma PBCA contain about 0·1% tumour cells and that immunomagnetic bead selection of $CD34^+$ cells results in an approximate 2 log depletion of tumour cells. Residual tumour cells in CD34 selections were due to contaminating CD34-negative cells.

MATERIAL AND METHODS

Patients. All three patients (K, S and T) included in this study had stage III multiple myeloma according to Durie & Salmon (1975) and had been previously treated with alkylating agents and prednisone. Patients S and K subsequently received repeated courses of steroids with vincristine and doxorubicin administered by continous infusion (VAD). Patients T and S achieved partial remission but patient K was refractory to prior treatment. For all three patients peripheral blood stem cells were harvested after high-dose cyclophosphamide (7 g/m^2) and G- or GM-CSF (patients T and K). Stem cells were re-infused after high-dose melphalan (140 mg/m^2) and TBI (9 Gy).

Cell isolations. Bone marrow cells were obtained by aspiration from the sternum of the patients after informed consent. PBCA were collected in four to six aphereses procedures with a continuous flow cell separator (Fenwal CS 3000, Baxter Healthcare, Deerfield, U.S.A.; Areman *et al*, 1990). Cytospin preparations of BM and PBCA were stained with May Grünwald Giemsa (MGG) and differential morphology of 200 nucleated cells was scored by two independent investigators. Cells were layered over Ficoll Hypaque and the mononuclear layer was collected after density centrifugation and washed in phosphate-buffered saline (PBS). These cells were cryopreserved at −196°C in small aliquots.

Cell sortings. The cryopreserved cells were thawed, washed and resuspended in RPMI 1640 supplemented with 10% FCS. More than 40×10^6 thawed cells were washed and resuspended in PBS with 1% BSA (PBSB) to a concentration of 10^7 cells/ml. $CD34^+$ cells were selected according to the manufacturer's protocol with M450 beads directly coated with '561' antibody (Dynal). Bead to cell ratio was 1:2. After 30 min incubation at 4°C with CD34 beads, a magnetic separation was performed. Selected cells were concentrated in 150 μl PBSB and detachment of beads was performed by adding 50 μl CD34 Detach-a-Bead (Dynal). After incubation for 1 h at room temperature, beads were magnetically separated from detached cells. Purity of CD34 selection was flow-cytometrically monitored. In brief, detached cells were washed twice and concentrated in 100 μl PBSB and incubated with a mixture of phycoerythrin (PE)-conjugated CD34 (HPCA-2, Becton Dickinson) and fluorescein isothyocyanate (FITC)-conjugated BB4 (Immuno Quality Products). As a control cells were labelled with IgG1-FITC/IgG1-PE. Flow cytometric analysis and FACS was performed with an

Fig 1(a). Outline of the method used to develop ASO-PCR. Schematic representation of the rearranged heavy chain locus amplified by CDR3 primers.

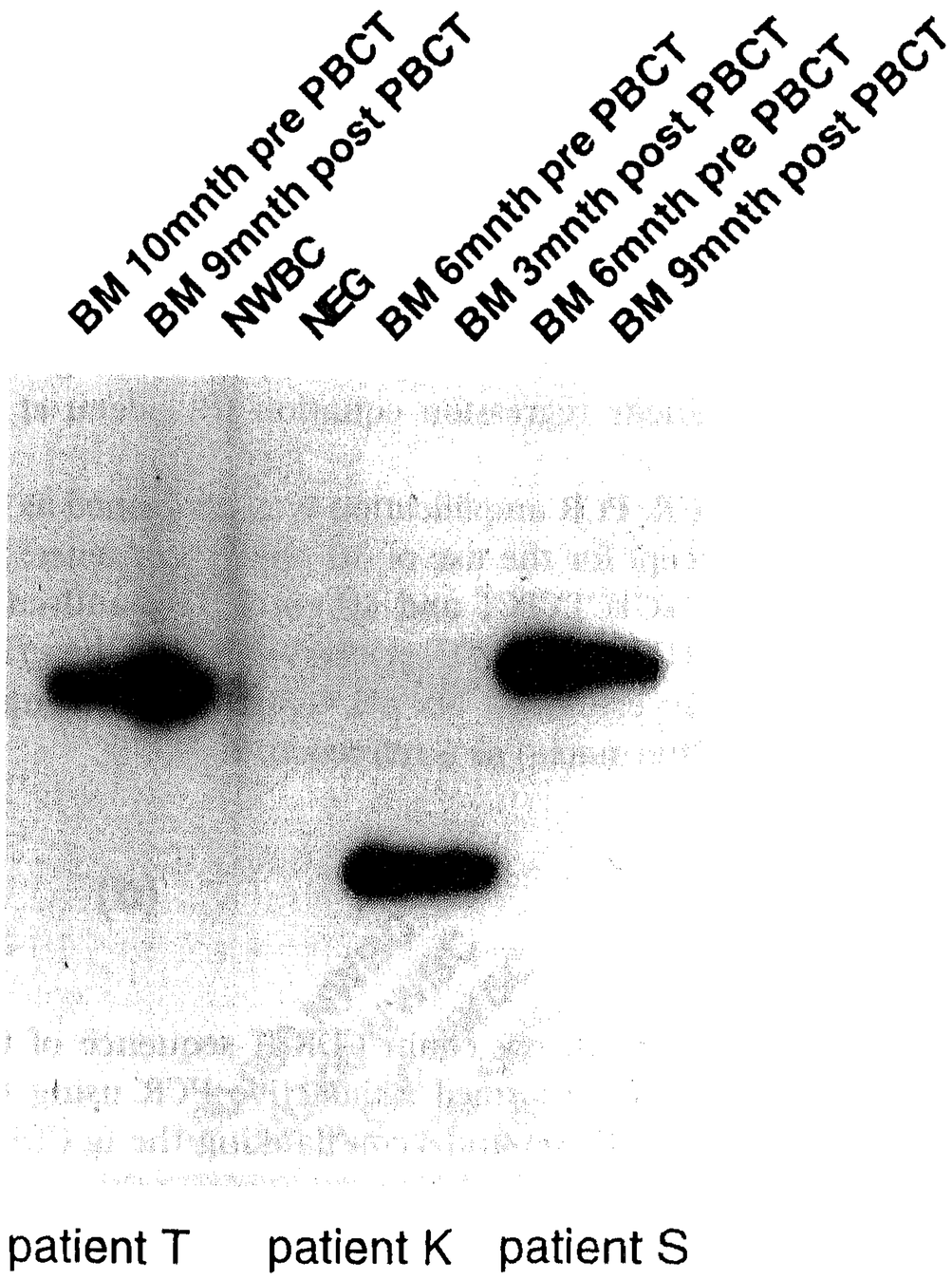


Fig 1(b). IgH CDR3 PCR products generated using consensus primers. The radioactive products are separated on a 6% non-denaturing polyacrylamide gel.

Epics Elite flow-cytometer (Coulter Corporation, Hialeah, U.S.A.) equipped with a 15 mW argon laser (488 nm). Cells were flow-cytometrically sorted with a data rate of between 1000 and 2000 cells per second into a PBSB foam layer on top of 100 μl PBSB in 1 ml Eppendorf vials. An aliquot of each sorted fraction was re-analysed on the flow-cytometer to assess the purity. 50 μl of each sorted fraction was cytocentrifuged and stained with MGG to assess the morphology of sorted cells.

DNA extraction. DNA was extracted by making whole-cell lysates. If present, erythrocytes were lysed with NH_4Cl. The NH_4Cl was then washed away with PBS. Cells in PBS were transferred to Eppendorf tubes and pelleted in an Eppendorf centrifuge (5 min, 400 *g* at 4°C). The supernatant was removed and the pellet loosened by vigorous rasping. The pellet of $\geqslant 10^5$ cells was resuspended in PCR buffer supplemented with 0·5% NP40 and 0·5% Tween 20 to a concentration of 10^4 cells/μl. 1 μl of proteinase K (10 mg/ml) was added for every 100 μl cell lysate. The cell lysate was incubated at 55°C for 1 h, after which the proteinase K was inactivated by a 10 min incubation at 95°C. When $\leqslant 10^5$ cells were available, pelleted cells were resuspended to a concentration of 10 cells/μl. In those cases 1 μl of proteinase K (0·1 mg/ml) was added for every 100 μl. Whole-cell lysates were frozen at −70°C. 10 μl of cell lysate was used in each PCR.

Amplification of CDRIII using consensus primers. Whole-cell lysates were subjected to PCR in a 100 μl PCR solution containing: 31 μM dNTP, 0·2 μl [α-^{32}P]dCTP (Amersham International, Amersham, U.K., 3000 Ci/mmol, 10 mCi/ml), 50 mM KCl, 10 mM Tris-HCl pH 8·4, 0·0001% gelatine, 2·5 Units *Taq* DNA polymerase (Life Technologies) and 30 pmol of each consensus primer (Fig 2). Primers were synthesized on a 391A DNA synthesizer (Applied Biosystems, Warrington, U.K.). PCR was performed for 35 cycles of 1 min 95°C, 1 min 55°C and 1 min 72°C, supplemented with a final 10 min extension at 72°C in a Perkin-Elmer Cetus™ thermocycler. Radioactive products were separated on a 6% non-denaturing polyacrylamide gel.

Analysis of CDRIII sequence. For direct sequencing double-stranded DNA was sequenced by the dideoxy chain

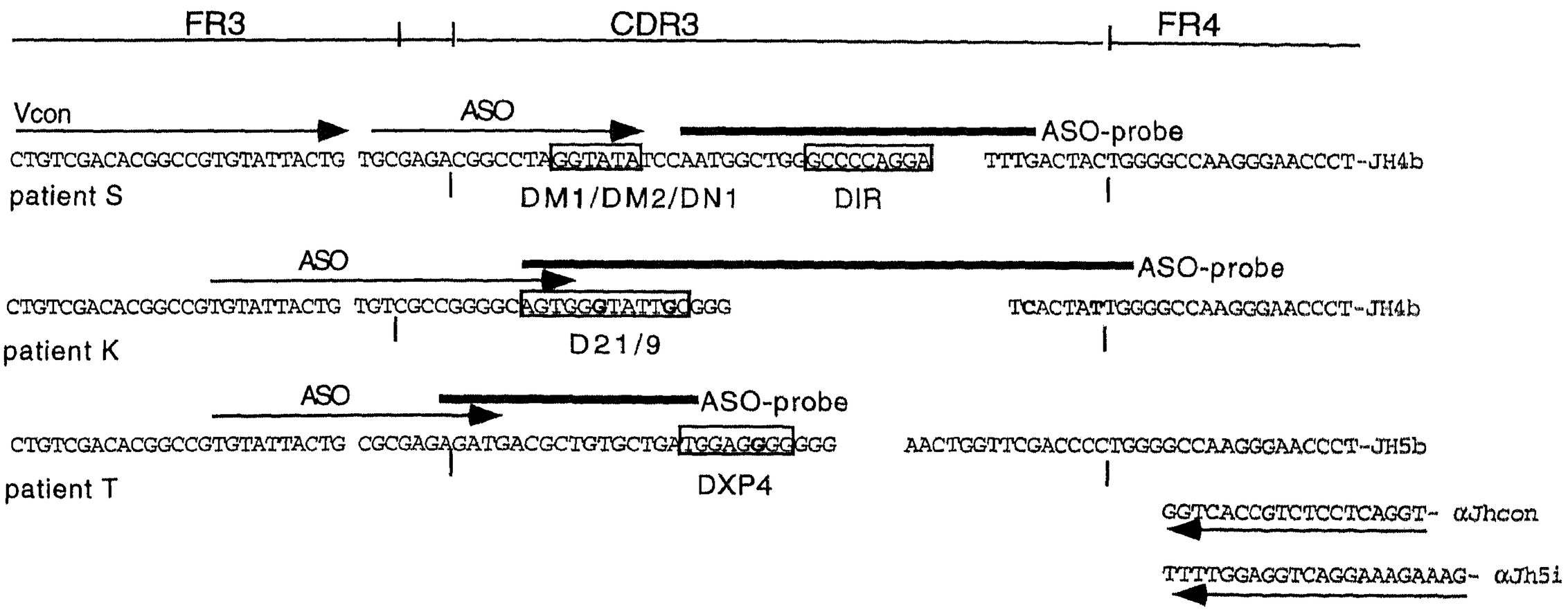


Fig 2. DNA sequences of IgH VDJ junctions from predominant clones in myeloma BM and location of primers used in ASO-PCR strategy. The 3′ end of the third framework region (FR3) and 5′ end of the fourth framework region (FR4) are indicated according to Kabat *et al* (1991). The boxed regions designate putative D and DIR sequences (Ichihara *et al*, 1988). The Jh gene used, the amount of trimming, and possible somatic mutations (bold capitals) are indicated based upon published sequences (Ravetch *et al*, 1981).

termination method (Innis *et al*, 1988) in a 10-cycle PCR using 5 pmol of one of the end-labelled CDRIII consensus primers (Fig 2) and 1 μl (10 ng) template. CDRIII sequences were searched for homology (Yamada *et al*, 1991) with published DIR (Dh gene containing irregular spacer signals), D gene segments (Ichihara *et al*, 1988; Matsuda *et al*, 1990; Buluwela *et al*, 1988) and Jh gene segments (Ravetch *et al*, 1981).

Tumour-specific PCR of patient samples. To achieve highest PCR specificity allele specific oligonucleotides (ASOs) were designed complementary to the CDRIII region with the highest variability amongst different B-cell clones (Fig 2). A non-radioactive amplification was performed essentially as described for CDRIII consensus PCR except for the use of 30 pmol 5′ASO primer instead of Vconsensus primer (Fig 2) and a dNTP concentration of 250 μM. PCR products were separated on 2% agarose gel, transferred to nylon membranes (Hybond N+) and probed with end-labelled ASO probes under stringent conditions. Radioactive signals were visualized on X-ray film (Kodak) and quantified by densitometrical scanning on a LKB laser densitometer. Allele-specific calibration curves were generated after serially diluting patient marrow DNA into PCR lysate buffer in 10-fold decrements, supplemented with normal white bood cell (NWBC) lysate to yield a concentration of 10^4 cells per μl PCR lysate buffer and 10^5 cells per PCR.

Statistical estimation of tumour load. Quantified PCR product is given in OD (optical density, arbitrary units). Least squares was used to fit a linear regression equation for ln (OD) as a function of ln (tumour fraction) for each patient. The number of tumour cells in patient samples and its 95% Scheffe's confidence interval was computed using this patient-specific linear regression equation (Billadeau *et al*, 1991).

β2m control PCR. PCR amplification was performed as for the ASO-PCR except for the use of 30 pmol β2m sense: 5′-CTCGCGCTACTCTCTCTTTCT and 30 pmol β2m antisense: 5′-CTAAACTTGTCCCGACCCTC primers. Size of the β2m PCR product was estimated on 2% agarose gels using a 100 bp ladder (Pharmacia) as a reference.

RESULTS

CDRIII sequencing

To determine the Ig heavy chain CDRIII sequence of the malignant clone we performed radioactive PCR using the consensus primers αJhcon and Vcon flanking the Ig CDRIII

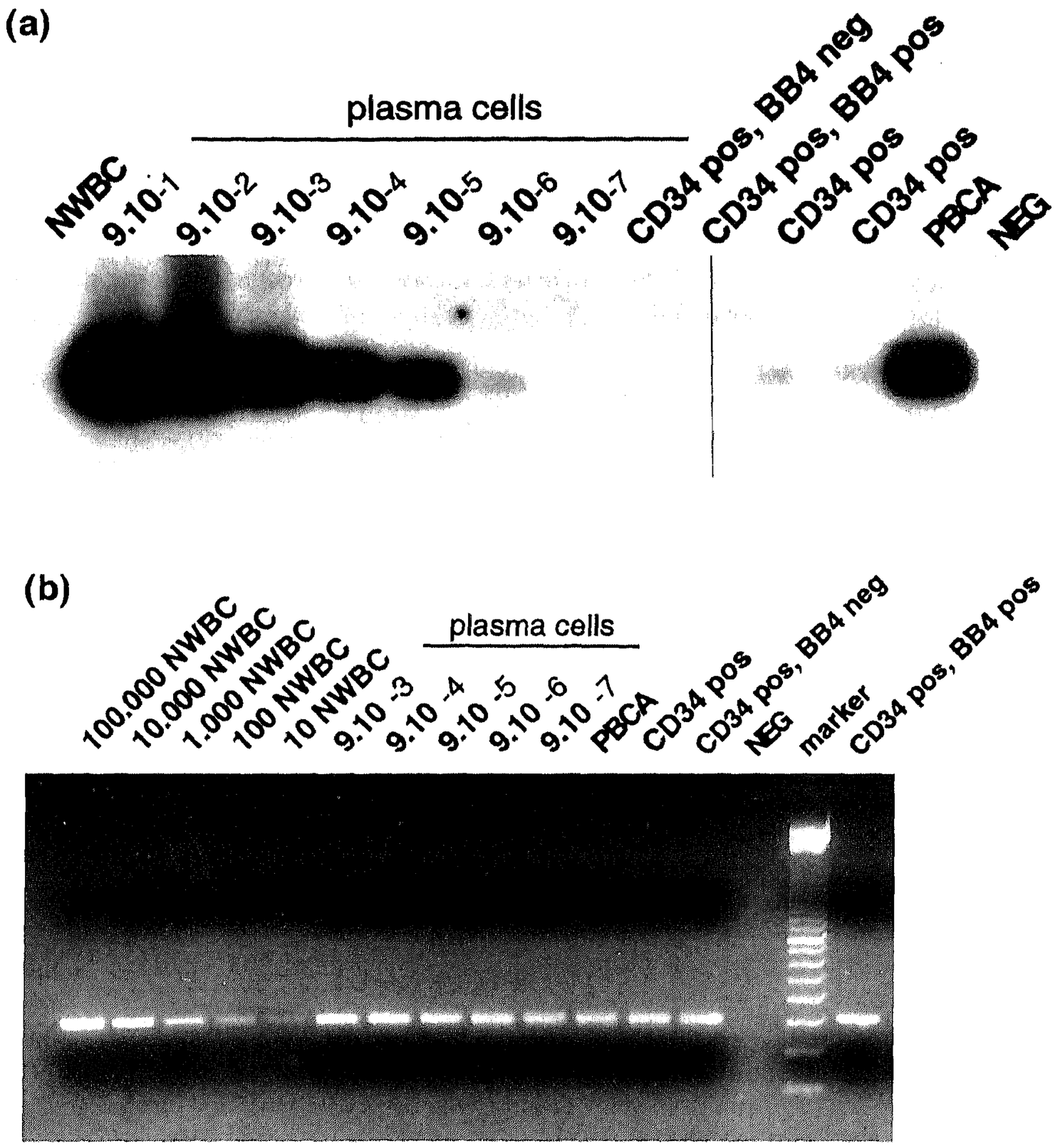


Fig 3. (a) ASO-PCR analysis of tumour cells in PBCA fractions of patient S. The dilutions of BM DNA samples used to obtain the calibration curve are shown on the left. CD34 pos columns represent duplicate PCRs of the same selection of $CD34^+$ cells with immunomagnetic beads. An aliquot of this $CD34^+$ selection was then subdivided by flow sorting. The columns CD34 pos, BB4 neg and CD34 pos, BB4 pos represent ASO-PCR results performed with DNA isolated from flow sorted $CD34^+$, $BB4^-$ and $CD34^+$, $BB4^+$ cells.
(b) Ethidium bromide stained 2% agarose gel showing β2m control products of patient S DNA samples used in ASO-PCR.

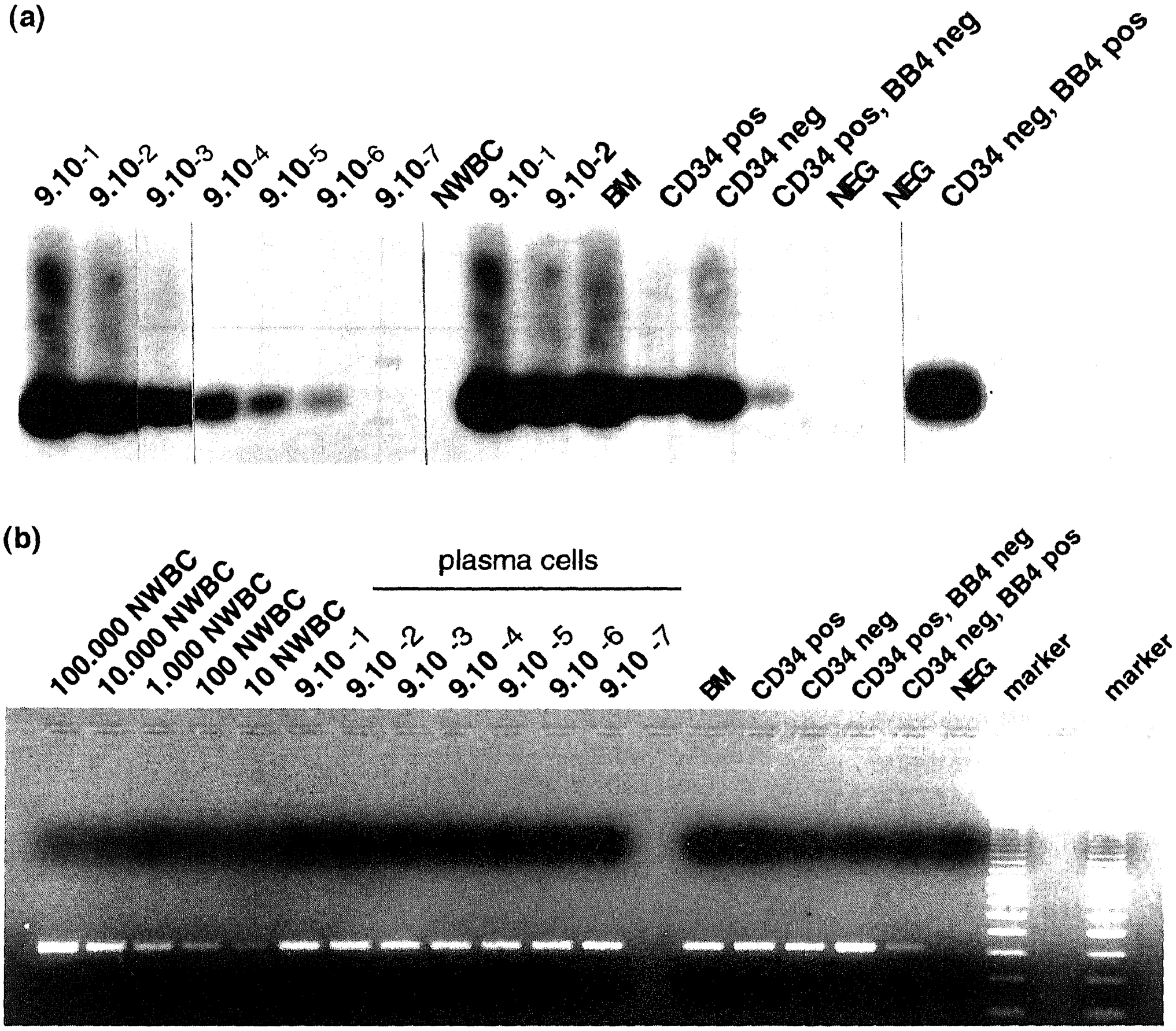


Fig 4. (a) ASO-PCR analysis of myeloma tumour in BM fractions of patient S. The dilutions of BM DNA samples used to obtain the calibration curve are shown on the left. Sample description is as in Fig 3. The CD34 neg column represent ASO-PCR results performed with DNA isolated from bone marrow samples that were depleted of CD34-positive cells. (b) Ethidium bromide stained 2% agarose gel showing β2m control products of patient S DNA samples used in ASO-PCR.

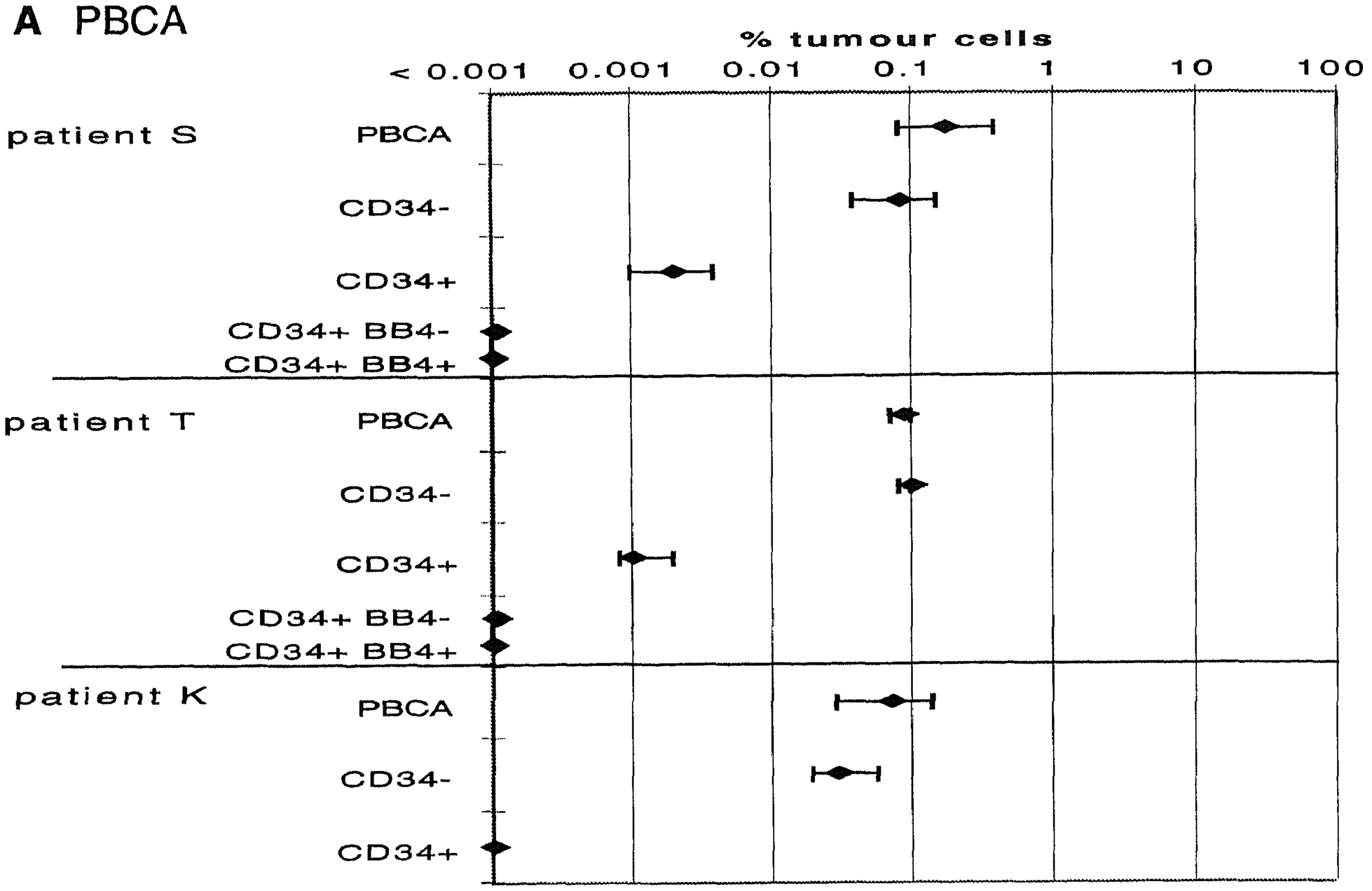
region (Fig 1a). Performing this PCR with a DNA template isolated from polyclonal B-cell compartments (NWBC) resulted in multiple PCR products differing in length and sequence. The use of DNA template isolated from multiple myeloma bone marrow samples (containing over 10% plasma cells) resulted in just one PCR product (Fig 1b). Direct sequencing of these PCR products consistently revealed only one CDRIII sequence at different time points (before and after high-dose chemotherapy) in a patient's disease.

Sensitivity of ASO-PCR to specifically detect malignant cells

For each patient two allele-specific oligonucleotides (ASO) were synthesized (Fig 2). Using a 5′ ASO and the αJhcon or αJh5i primer a PCR was developed that was specific for the malignant clone. ASO-PCR products were blotted and hybridized with a radiolabelled internal ASO-probe (Fig 1a). Quantification by densitometrical scanning shows that each tumour-specific PCR detects only malignant cells in one of the multiple myeloma patients but not in several NWBC preparations. Also ASO probes specifically designed for each patient never hybridized with ASO-PCR products of other multiple myeloma patients. By serially diluting the DNA derived from myeloma bone marrow samples (containing $>30\%$ plasma cells) in DNA derived from NWBC we were able to show that sensitivity of the ASO-PCRs ranged from detection of one tumour cell in 10^4–10^5 normal white blood cells (Figs 3 and 4).

Quantification of malignant cells in myeloma autografts

As a control we performed PCR with β2m primers on all samples tested in the tumour-specific PCRs. Only minor differences in signal intensity of the β2m PCR products were observed (Figs 3 and 4). We conclude that the DNA



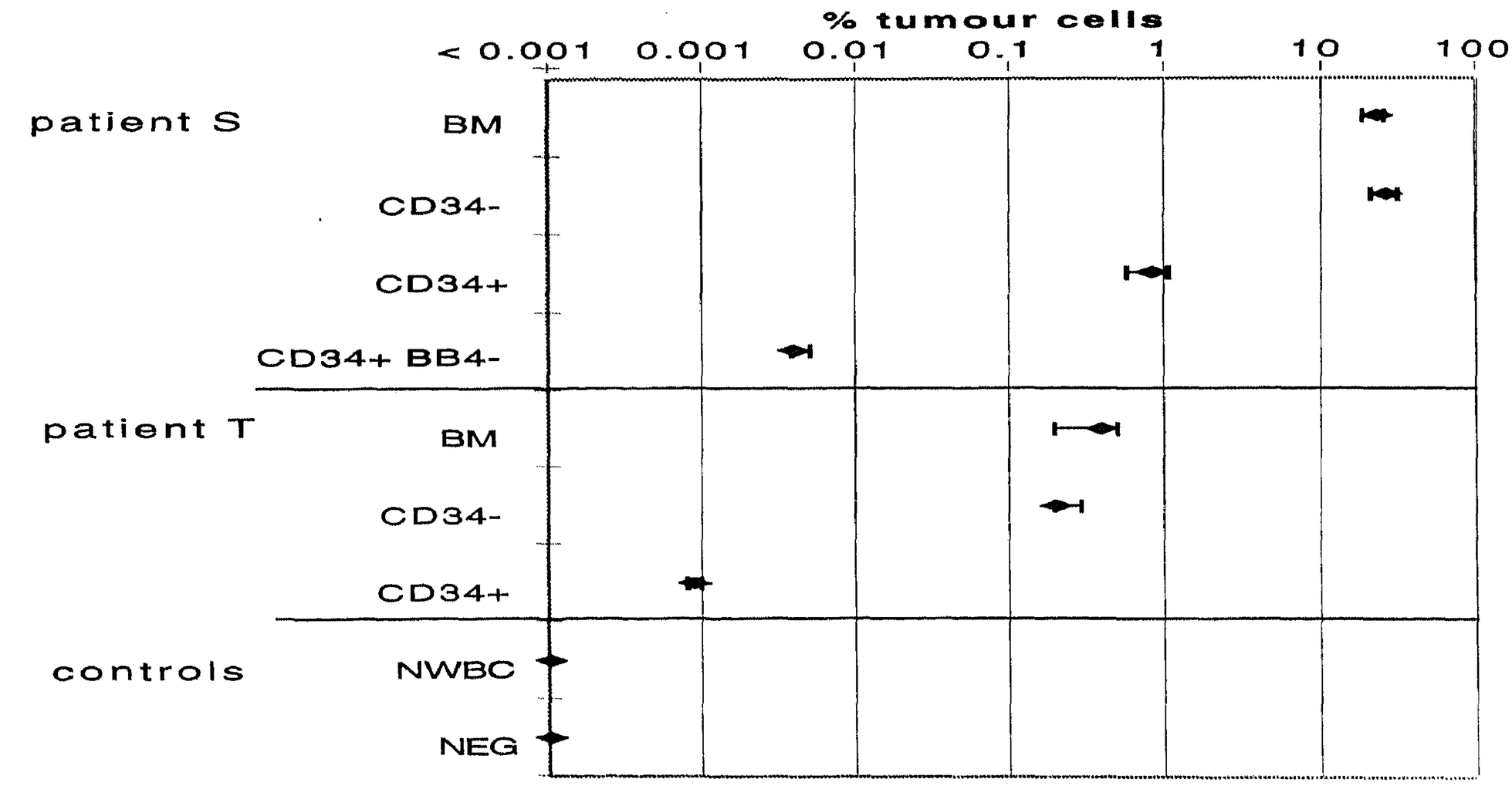
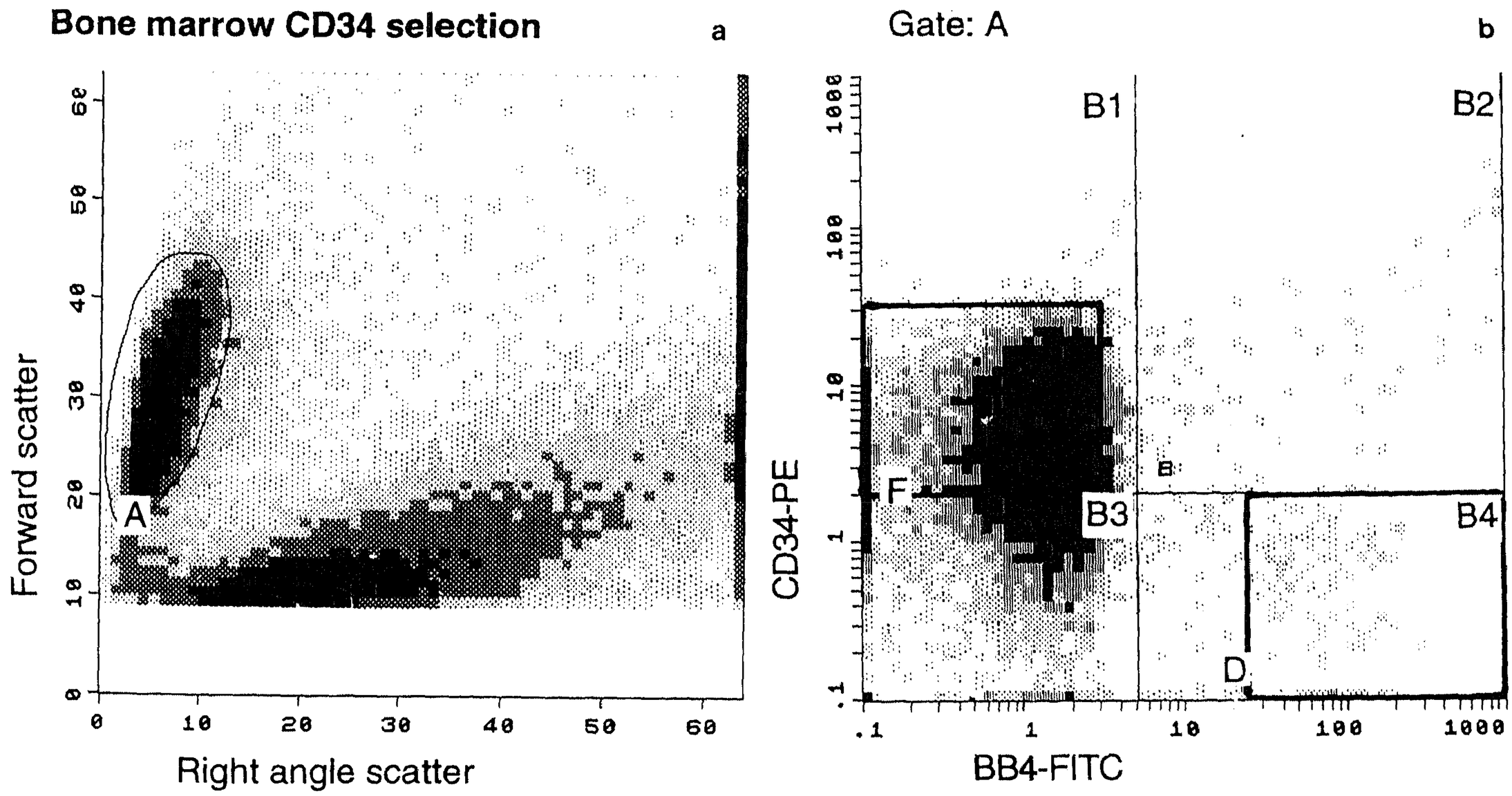


Fig 5. Percentage tumour burden in peripheral blood cell autografts (A) and BM samples (B) of myeloma patients as detected by ASO-PCR. Values are given with statistically generated confidence intervals (see Material and Methods) and < 0·001% tumour cells indicates that the number of tumour cells in the sample is below the detection limit of ASO-PCR. $CD34^+$ samples were selected with CD34 immunomagnetic beads and the CD34-depleted fraction was called $CD34^-$; $CD34^+$, $BB4^-$ samples are cells sorted with CD34 immunomagnetic beads and subsequent flow cytometry. $CD34^-$ are samples depleted of $CD34^+$ cells. Aliquots of these $CD34^+$ selection were then subdivided by flow sorting into $CD34^+$ $BB4^+$ cells and $CD34^+$ $BB4^-$ cells and their tumour burden was also assessed.

Fig 6. Flow cytometric analysis of cells obtained with CD34 immunomagnetic bead selection from bone marrow (a, b) and PBCA (c, d) of patient S. Analysis of forward and side scatter (a) and CD34 (PE) and BB4 (FITC) expression (b). For bone marrow as well as PBCA material $CD34^+$, $BB4^-$ cells in gate F and $CD34^+$, $BB4^+$ cells in gate D were further purified by FACS. Purity of the $CD34^+$, $BB4^-$ cellsort was monitored by flow cytometric analysis of CD34 (PE) and BB4 (FITC) expression (d). The quadrant B indicates green and red fluorescence of IgG-PE/IgG-FITC control antibodies.


Bone marrow CD34 selection
a
Forward scatter
Right angle scatter
A
gate A: 33.5%
Gate: A
b
B1
B2
B3
B4
F
D
B
CD34-PE
BB4-FITC
gate D: 0.9%
gate F: 70.8%
quadrant B1: 73.7%
quadrant B2: 0.6%
quadrant B3: 24.5%
quadrant B4: 1.2%
PBCA CD34 selection
c
gate D: 0.2%
gate F: 92.7%
quadrant B1: 99.0%
quadrant B2: 0.7%
quadrant B3: 0.3%
quadrant B4: 0.0%
d
gate D: 0.0%
gate F: 100%
quadrant B1: 100%
quadrant B2: 0.0%
quadrant B3: 0.0%
quadrant B4: 0.0%

preparation was of sufficient quality to allow PCR amplification of each sample with comparable efficiency. ASO-PCR detected between 0·07% and 0·17% malignant cells in PBCA in three patients in duplicate PCR experiments (Fig 5). Plasma cells were never detected in cytospin preparations of PBCA whereas in cytospins of bone marrow aspirates 2–5% plasma cells were found (results not shown).

Isolation of CD34$^+$ progenitors using immunomagnetic beads

CD34$^+$ cells were selected from PBCA and BM using CD34$^+$-immunomagnetic beads (Dynal). Flow-cytometric analysis of detached cells, stained with CD34 phycoerythrin showed that purity of CD34$^+$ peripheral blood progenitor cells was always higher than 99% (Fig 6c). Using this procedure, PBCA or BM were depleted of 99% of the CD34$^+$ cells and we consequently called these fractions CD34$^-$. Tumour-specific ASO-PCR detected < 0·002% malignant cells in CD34$^+$ immunomagnetic bead preparations and 0·03–0·1% malignant cells in CD34$^-$ PBCA fractions (Figs 3 and 5a). Thus the majority of malignant cells were not selected with CD34 immunomagnetic beads and CD34 selection from PBCA using immunomagnetic beads (Dynal) reduced malignant cells with approximately 2 logs. Selection of CD34$^+$ cells from bone marrow of patient T resulted in a 2·6 log reduction in tumour cells (Fig 5b). We used BM of patient S (who had progressed to plasma cell leukaemia) to test if, in bone marrow heavily infiltrated with malignant cells, the same reduction of tumour cells could be accomplished with CD34 selection. Flow-cytometric analysis of this BM sample stained with the plasma cell (PC) marker BB4-FITC (Pellat-Deceunynck *et al*, 1994) revealed 20·7% PC. In MGG-stained cytospins 22% PC were detected (results not shown). When the selected CD34$^+$ cells were stained with BB4 and CD34, purity was higher than 75%. BB4$^+$ PC (1%) were, however, contaminating this selection (Fig 6b). Tumour-specific ASO-PCR detected 22·2% malignant cells in this BM aspirate of patient S and 25·7% in the CD34$^-$ fraction. In the CD34$^+$ fraction 0·8% malignant cells were detected (Figs 4 and 5b). We conclude that selection of CD34$^+$ cells from highly contaminated BM results in a 1·4 log reduction of tumour cells.

Isolation of CD34$^+$ progenitors using immunomagnetic beads followed by flow cytometrically activated cell sorting (FACS)

The selection of CD34$^+$ progenitors from BM of patient S was contaminated with BB4$^+$ PC (Fig 6b). Using FACS we sorted 10^3 BB4$^+$ PC (Fig 6b, gate D) from CD34 immunomagnetic bead fractions. Cytospin preparations of sorted BB4$^+$ cells showed only cells with typical plasma cell morphology (results not shown). ASO-PCR showed that BB4$^+$ PC belong to the malignant clone (Fig 4). Over 90% of the BB4$^+$ cells were CD34 negative, suggesting that most plasma cells contaminate the CD34 selections in a non-specific manner. If this were true it would mean that increasing the purity of CD34$^+$ cells could further reduce the contamination of malignant cells. Highly purified CD34$^+$ BM progenitors were obtained in two rounds of selection combining magnetic with flow cytometry activated cell sorting (Fig 6b, gate F). Purity of these flow-sorted CD34$^+$ cells was > 99·9%. ASO-PCR showed that a second round of purification with FACS results in a 2·3 log reduction of malignant cells (Figs 4 and 5b). Flow-cytometric analysis of CD34$^+$ immunomagnetic bead selections from PBCA of patients S and T showed 0·1% CD34$^+$, BB4$^+$ cells but no CD34$^-$, BB4$^+$ PC. Using FACS, we sorted 10^5 CD34$^+$, BB4$^+$ and 10^5 CD34$^+$, BB4$^-$ cells. Purity of flow sorted CD34$^+$ peripheral blood progenitors was > 99·9% (Fig 6d). ASO-PCR could not detect tumour cells in either of these fractions (Fig 3 and5a). Consequently malignant cells with a CD34$^+$ progenitor phenotype were not detectable in PBCA of three patients and residual tumour cells detected in CD34 selections were due to contaminating CD34 negative myeloma cells.

DISCUSSION

In this study we showed that residual tumour cells in CD34 selections of myeloma autografts are due to impurity. We used tumour-specific PCR, capable of detecting one malignant cell in a background of 10^5 normal white blood cells. In accordance with other studies (Mariette *et al*, 1994; Bird *et al*, 1994) we found between 0·07% and 0·17% malignant cells in PBCA. CD34 selection using immunomagnetic beads resulted in an approximately 2 log depletion of malignant cells in myeloma PBCA of three patients. Even when highly contaminated BM (22% PC) was used for CD34 selection, a 1·4 log reduction in tumour cells was achieved. Assuming that most tumour cells do not express the CD34 marker, a 2 log reduction in malignant cells is in agreement with the 2 log increase of CD34$^+$ cells achieved by CD34 selection. An additional advantage of autologous CD34$^+$ transplants over total peripheral blood cell transplants is that fewer cells are needed, resulting in a 2·7–4·5 log reduction of malignant cells in clinical trials (Schiller *et al*, 1995).

Since the CD34 selection was not 100% pure we reasoned that positive ASO-PCR signals might result from contamination with CD34-negative cells. Starting with BM heavily infiltrated with plasma cells we showed that contaminating malignant plasma cells were the major cause of impurity in CD34 immunomagnetic beads selections. In those cases the purging of BB4$^+$ PC in combination with CD34 selection using immunomagnetic beads should provide further tumour cell reduction. A contamination phenomenon was also reported by Vescio *et al* (1994). They found positive ASO-PCR signals when CD34 cells were collected from BM with one round of selection using a immunoadsorption column, whereas a second round of CD34 selection with FACS resulted in a negative ASO-PCR. Others have suggested that residual tumour cells in selections of CD34$^+$ cells are malignant CD34$^+$ progenitors (Takishita *et al*, 1994; Belch *et al*, 1994; Bersagel *et al*, 1994). Since no PCR data of preselected material are shown it cannot be excluded that the positive ASO-PCR signals Takashita *et al* (1994) report after having selected CD34 or CD20 cells from PB of myeloma patients with FACS arise from contamination. To prove that positive ASO-PCR signals in CD34 selections arise from contamination with CD34-negative tumour cells not from malignant CD34$^+$ progenitors we

performed a second round of CD34 selection with FACS resulting in > 99·9% pure $CD34^+$ cells. We found a clearcut reduction of malignant cells with ASO-PCR when we compared 10^5 CD34 immunomagnetic bead selected cells with 10^5 highly purified $CD34^+$ cells (isolated with two rounds of CD34 selection) in patients S and T. In 10^5 highly purified $CD34^+$ cells from PBCA we were not able to detect malignant cells with ASO-PCR. These experiments show that ASO-PCR signals in CD34 selections arise from contamination with CD34-negative tumour cells.

Our results argue against the presence of malignant $CD34^+$ precursors. One could reason, however, that malignant $CD34^+$ stem cells that have yet to undergo Ig gene rearrangement or malignant pre B cells that could still undergo Vh replacements and somatic hypermutation could exist and escape detection by ASO-PCR. If such precursor cells exist, the plasma cell progeny should have an extremely diverse Ig gene sequence. However, after sequencing multiple clones in each patient, Bakkus *et al* (1992) and Vescio *et al* (1993) found no evidence of intraclonal diversity. They concluded there was no ongoing somatic hypermutation. In addition, others found no evidence for ongoing Vh replacements (Takishita *et al*, 1994). Therefore the scenario of a malignant pre-B-lymphocyte or stem cell as a precursor for malignant plasma cells is unlikely. It has been suggested that a change in the predominant tumour clone may occasionally occur in multiple myeloma (Bird *et al*, 1994), resulting in tumour cells that are not detected by ASO-PCR. We detected the same monoclonal tumour population before and after peripheral blood cell transplantation without major additional clones appearing. We conclude that there was no clonal evolution in our patients and tumour cells are thus adequately quantified.

In summary, residual tumour cells detected in CD34 selections are due to impurity. Removal of all tumour cells from the graft is not possible with CD34 selection protocols that are currently applied in clinical trials (Schiller *et al*, 1995). We and others (Vescio *et al*, 1994), however, consistently achieved negative ASO-PCR signals with two rounds of CD34 selection, or a combination of counterflow elutriation, treatment with phenylalanine methylester and flow sorting of $CD34^+$, Lin^-, Thy^+ stem cells (Gazitt *et al*, 1995). Since purity of CD34 selection seems the most essential parameter in obtaining tumour-free autografts, we are currently evaluating the purity of different $CD34^+$ isolation methods and their applicability in the clinical setting of autologous transplantation.

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