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Isolation and characterization of human multiple myeloma cell enriched populations

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Abstract

We developed a simple and rapid method to enrich tumor cells within bone marrow (BM) aspirates from patients with multiple myeloma (MM). Thirty patients with a median of 50% (8–85%) MM cells by morphology and 55% (6–85%) MM cells identified by CD38 + CD45 − cell surface phenotype were studied. BM mononuclear cells (BMMCs) were isolated by Ficoll Hypaque sedimentation and incubated with a cocktail of mouse monoclonal antibodies (mAbs) directed against CD3 (T cells); CD11b and CD14 (monocytes); CD33 (myeloid cells), CD45 and CD45RA (leucocyte common antigen); CD32 as well as glycophorin A. After the addition of anti-mouse Fc Ig-coated immunomagnetic beads, mAb-bound cells were removed in a magnetic field. The residual cell populations were enriched for MM cells, evidenced by > 95% plasma cell morphology and > 95% CD38 + CD45RA − cell surface phenotype. Since this method requires only two short incubations, cell losses were minimal and the yield of MM cells was therefore high (> 95%). Viability of the MM-cell enriched fractions was 99%, and these cells were functional in assays of proliferation, cell cycle analysis and immunoglobulin secretion. This immunomagnetic bead depletion method therefore permits the ready isolation of homogeneous populations of patient MM cells for use in both cellular and molecular studies.

Keywords: Multiple myeloma; Enrichment methods; Immunomagnetic depletion; Dual fluorescence analysis

1. Introduction

Multiple myeloma (MM) is characterized by the presence of excess plasma cells (PCs) in the bone marrow (BM), in the context of monoclonal protein either in the blood and/or urine in most cases. However, the percentage infiltration of MM cells in the BM can vary and be heterogeneous. Multiple cellular and molecular studies have shown that the MM precursor cell is a pre-immunoglobulin class switch B cell (Bakkus et al., 1994). Such studies

Abbreviations: BM, bone marrow; MM, multiple myeloma; mAbs, mouse monoclonal antibodies; PC, plasma cell; CM, complete medium; MGG, May–Grünwald–Giemsa; FITC, fluorescein isothiocyanate; PE, phycoerythrin; HBSS, Hank's buffered saline solution

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have also defined the mechanisms whereby MM cells localize in the BM microenvironment (Teoh and Anderson, 1997), delineated mechanisms regulating MM cell growth and survival (Anderson and Lust, 1999), and led to strategies for enhancing autologous and allogeneic immune responses to MM cells (Anderson et al., 1999). Moreover, exciting studies have recently shown the potential role of accessory factors within the BM microenvironment, such as increased angiogenesis (Ribatti et al., 1999) and Kaposi's sarcoma herpesvirus infection (Rettig et al., 1997; Chauhan et al., 1999), in the growth and survival of MM cells. These studies in some cases have relied primarily on studies of human MM-derived cell lines, but whether they truly represent MM in vivo is unknown, and analogous studies of freshly isolated patient MM cells must confirm their relevance. However, studies of freshly isolated patient BM samples are often limited due to the difficulties inherent in isolating MM cells, which are often a minor subset, from BMMCs. Methods which reproducibly enrich for highly homogeneous populations of MM cells, with minimal cell losses, are therefore urgently needed in order to further characterize cellular and molecular events in the tumor cell versus the BM microenvironment.

A major limitation of positive selection techniques for the isolation of MM cells has been the lack of available reagents which specifically identify tumor cells (Anderson et al., 1983b, 1984; Tong et al., 1987; Stevenson et al., 1991; Huang et al., 1993; Goldmacher et al., 1994; Goto et al., 1994; Ellis et al., 1995). In addition, although MM is a B-cell neoplasm characterized by excess PCs, the malignant clone in MM extends from the pre-B cell to PC stage of differentiation (Szczepek et al., 1998; Van Riet et al., 1998), and techniques which isolate MM cells based upon positive selection of cells expressing PC antigens may lose earlier clonotypic pre-B and B cells. CD38 is the most commonly used PC marker, but it is also expressed on pre-B cells, immature T and activated T cells, and monocytes (Funaro et al., 1990). Although both normal PCs and MM cells highly express CD38, MM cells express CD56, but lack CD45 and are therefore frequently isolated by positive selection methods based upon their CD38 + CD45 − or CD38 + CD56 + cell surface phenotype (Harada et al., 1996). Portier et al. (1991) have also isolated MM cells with positive selection methods using magnetic beads coated with mAb B-B4, and achieved a purity of 99% MM cells. Borset et al. (1993) have used a similar method for the purification of MM cells, and in some cases, established primary cultures of homogeneous MM cells. However, cell viability was, on average, 80% (52–90%), and Wijdenes et al. (1996) have demonstrated that mAb B-B4 stained only those cells expressing CD38 strongly and also recognized both normal and malignant PCs. In addition, only 75% of PCs were labeled by the B-B4 mAb, and conversely, cells of other lineages, that is, myeloid cells, were also reactive with this mAb (Fillola et al., 1996).

Prior methods to purify human MM cells from patient samples have also been based upon depletion of non-MM cells. These techniques were multistep processes, with poor yields and large attendant cell losses (Kawano et al., 1989; Yamamoto et al., 1989). In the past, we have purified MM cells from patient samples by first staining with mouse monoclonal antibodies (mAbs) targeting those cells (monocytes, myeloid cells and T cells) to be depleted, and then performing immune rosetting using rabbit anti-mouse Ig-coated sheep red blood cells, as in our separation of B cells from normal donors (Anderson et al., 1983a). This multistep process can achieve homogeneous populations of MM cells, but is time consuming with significant losses. A recent report using immunomagnetic bead depletion to purify BM PCs (Fillola et al., 1996) suggested the potential utility of this approach for the separation of MM cells. In the present report, we have therefore developed a method for immunomagnetic bead depletion of T cells, monocytes, myeloid cells and leucocyte common antigen-positive cells from MM patient BM aspirates, which readily and reproducibly achieves high yields of viable, homogeneous populations of MM cells.

2. Materials and methods

2.1. BM specimens

Heparinized BM samples were freshly obtained from MM patients and from normal donors after appropriate informed consent. To isolate BM

mononuclear cells (BMMCs), the samples were first diluted with 1–2 vols. of RPMI 1640 medium or Hank's buffered saline solution (HBSS) (GIBCO BRL, Gaithersburg, MD) and then mixed with a pipette to disaggregate cell clumps. Excess tissue and clumps were removed by filtering the cell suspension through a 70-μm nylon tissue strainer (Falcon). The cell filtrate was then subjected to Ficoll-Hypaque density separation (1.077 g/l; Amersham Pharmacia Biotech). In brief, the cell filtrate was carefully layered over 15 ml of Ficoll in a 50-ml conical centrifuge tube and density gradient sedimentation was achieved by centrifugation at $400 \times g$ for 30 min at 16°C, in a swinging-bucket rotor without braking. Mononuclear cells were carefully collected from the medium-Ficoll interface, transferred into a 50-ml conical tube, and pelleted. Cell pellets were next washed twice in 2–3 vols. of RPMI 1640 medium or HBSS.

2.2. Cell preparation

Erythrocytes were next lysed using red blood cell lysing solution containing 0.83% NH_4Cl in 0.01 M Tris buffer. Two milliliters of red blood cell lysing solution was added to each cell pellet of $10–20 \times 10^6$ cells, and the cells were suspended by gentle pipetting. After incubation on ice for 5 min, the cell suspension was diluted with complete medium (CM): RMPI 1640 medium with 10% heat-inactivated fetal bovine serum, 25 IU/ml penicillin, 25 μg/ml streptomycin, and 2 mM L-glutamine. Cells were pelleted by centrifugation at $250–500 \times g$ for 7 min at 4°C, and washed once with CM. Lysing and washing is repeated once if necessary. After centrifugation, cells were then suspended in PBS/20% human AB serum (N.A.B.I., Boca Raton, FL) to block nonspecific Fc binding. In some cases, cells at this step were frozen for long-term storage in RPMI 1640 medium containing 5% DMSO and 80% autologous serum. At the time of thawing, cryopreserved cells were washed twice in CM and incubated at 37°C overnight; nonviable cells were removed by density gradient centrifugation using Ficoll-Hypaque. Viable cells were then resuspended in PBS (without Ca^{2+} and Mg^{2+})/20% human AB serum. The following procedure was performed in a cold room at 4°C, if not otherwise specified.

2.3. Antibody incubation and immunomagnetic cell depletion

Cells were suspended in PBS (without Ca^{2+} or Mg^{2+})/20% human AB serum for 15 min to block non-specific Fc receptor binding and then washed twice with washing solution (PBS without Ca^{2+} or Mg^{2+})/2% human AB serum). After a final wash, cells were resuspended in washing solution and the number of viable cells was determined by trypan blue staining. A single cell suspension was made in washing solution ($1–2 \times 10^7$ cells/ml) and a defined mAb cocktail was added with thorough mixing. This cocktail included mAbs directed at CD3 (T cells), CD11b and CD14 (monocytes), and CD33 (myeloid cells), CD45 and CD45RA (leucocyte common antigen), as well as CD32 and glycophorin A (immature erythrocytes); all from Immunotech-Coulter. Anti-CD32 blocks the non-specific binding of immunomagnetic labeling reagents to Fc RIIA. mAbs were used at 0.2–0.4 mg/ml, equivalent to 1–2 μg of each mAb per 10^6 total cells. Cells were incubated with mAbs for 20 min on ice on an apparatus that provides both gentle tilting and rotation, pelleted, and washed twice with washing solution to remove the residual unbound mAbs. Immunomagnetic beads coated with mouse anti-Fc IgG1 (Dynabeads M450 Goat anti-mouse IgG; Dynal, A.S, Oslo, Norway) were then added for a 15 min-incubation, with gentle tilting and rotation. These superparamagnetic beads would bind to the mAb-labeled cells, and the bead-target cell complexes were then attracted to the wall of the test tube in a magnetic field (Neodymium–Iron–Boron Magnets Particle Separator; Dynal MPC). Cells remaining in the supernatant, depleted of bead-coated cells, were readily and specifically removed by pipetting and transferred to a new tube. Viable cells were counted by trypan blue exclusion, and either used directly or incubated at 37°C in a humidified atmosphere containing 5% $C0_2$.

2.4. Cell culture

The RPMI 8226 human MM-derived cell lines were obtained from American Type Culture Collection (Rockville, MD) and maintained in CM. The SV MM cell line was developed in our laboratory from the BM aspirates of an MM patient with the CDR3

and CD38 + CD45RA − phenotype. They were also maintained in CM. These cell lines were used to define optimal conditions for use of the immunomagnetic depletion method. Mixtures of these MM cells (10% and 50%) in normal BMMCs were incubated with various mAb cocktails, followed by depletion of mAb-labeled cells as described above. In order to optimize mAb concentration and the bead-to-cell ratio, cell mixtures containing MM cells: BMMCs at 1:1 ratio were used.

2.5. Viability, morphologic and phenotypic analysis

The MM enriched cell populations were examined for viability, morphology and cell surface phenotype. Cell viability was assessed using trypan blue dye exclusion. Cytospins from initial samples and MM enriched samples were examined using direct/indirect cell surface staining. Percentage MM cells were first determined morphologically by counting at least 200 cells on a May–Grünwald–Giemsa (MGG) stained cytospin. Purity of MM enriched cell fractions was further examined with fluorescein isothiocyanate (FITC)-labeled anti-human CD38 mAb (Pharmingen) and phycoerythrin (PE)-labeled anti-human CD45RA mAb (Immunotech-Coulter) using dual fluorescence flow cytometric analysis (Coulter Epics XL, Coulter); MsIgG1-FITC (FITC-conjugated goat anti-mouse IgG1) and MsIgG1-PE (PE-conjugated goat anti-mouse IgG1) (Immunotech-Coulter) were used as isotype controls. For each sample, the monoclonal immunoglobulin (Ig) expression was examined by staining of cytospins with kappa-FITC and lambda-FITC (Immunotech-Coulter). Flow cytometric data were analyzed using WinMID 2.8 software (generously provided by Joe Trotter of the Scripps Research Institute).

2.6. Immunoblotting

As a further measure of cell enrichment achieved with immunomagnetic bead depletion, cell lysates were prepared from initial and MM-cell enriched populations as described previously (Tai et al., 1999). Immunoblotting was performed using standardized conditions. In brief, 20 μg of lysate from each sample was separated on SDS-PAGE (6% or 10%), transferred onto PVDF membranes (Millipore), and immunoblotted with anti-CD38 mAb (AT1: sc-7325, Santa Cruz Biotechnology, Santa Cruz, CA); and with anti-CD45RA mAb (Ab-1, Labvision, Fremont, CA). The membranes were then incubated with anti-mouse IgG-HRP Ab (Santa Cruz Biotechnology) for an hour and the reaction was visualized using the ECL detection system (Amersham). Blots were stripped in 62.5 mM Tris (pH 6.7)/2% SDS/100 mM 2-mercaptoethanol, blocked and reprobed with anti-α-tubulin DM1A mAb (Sigma; St. Louis, MO) to confirm equal protein loading. The ratio of CD38 to α-tubulin expression was determined using imag-

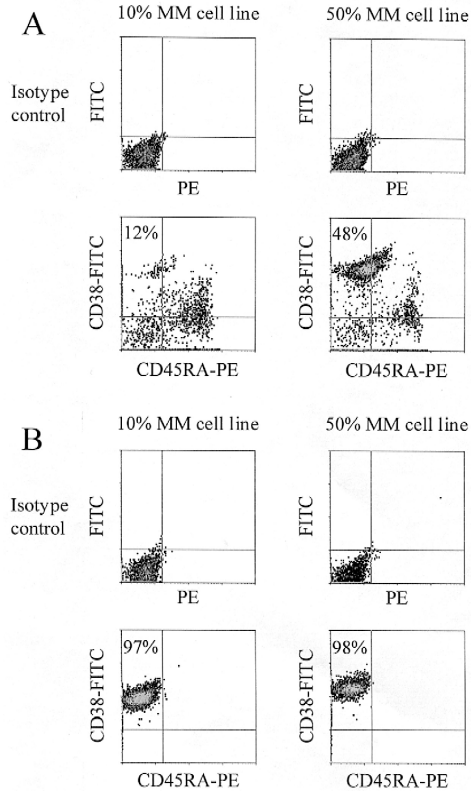

Fig. 1. Immunophenotypic assessment of immunomagnetic bead enrichment for MM cell lines. RPMI 8226 MM cells (10% and 50%) were mixed in normal bone marrow mononuclear cells. Cell mixtures were incubated with mAb cocktail targeting non-MM cells, followed by incubation with immunomagnetic beads coated with mouse anti-Fc IgG. Cells bound to immunobeads were removed in a magnetic field, and residual cell populations were enriched for MM cells. Initial (A) and MM enriched (B) cell populations were examined using dual fluorescence analysis with FITC-anti-CD38 and PE-anti-CD45RA mAbs.

ing densitometry (AlphaImager 2000, Alpha Innotech, San Leandro, CA) in order to determine relative CD38 expression in pre- and post-enrichment samples.

3. Results

3.1. Optimal conditions for the detection of MM

We first performed a series of experiments with mixtures of RPMI 8226 or SV MM cells and BMMCs from healthy donors to determine both the sensitivity and reproducibility of this immunomagnetic bead depletion methodology. RPMI 8226 cells (10% and 50%) were mixed with BMMCs from healthy donors. Mixtures were incubated with defined mAb cocktails (1 μg of each mAb per 10^6 total cells), followed by Dynabead M-450 separation. The mAb cocktail which we found to be most effective at depleting unwanted cells, with minimal MM cell losses, targeted CD3 (T cells); CD11b and CD14 (monocytes); CD33 (myeloid cells), CD45 and CD45RA (leucocyte common antigen); CD32 as well as glycophorin A. After immunomagnetic bead depletion of mAb-stained cells, MM enriched populations were collected and stained with FITC-anti-CD38 mAb and PE-anti-CD45RA mAb. As shown in Fig. 1, dual fluorescence flow cytometric analysis revealed 12% and 48% CD38 + CD45RA − cells initially (A) and > 95% CD38 + CD45RA − cells in the MM enriched populations (B). Yields of MM cells were > 95%, and viability, assessed by trypan blue exclusion, was > 99%. Similar results were obtained when SV MM cells were mixed with normal BMMCs (data not shown).

To optimize culture conditions, we mixed MM cell lines with BMMCs (1:1 ratio) and enriched for MM cells using different amounts of mAbs (1–2 μg of each Ab per 10^6 total cells) and various ratios (5:1 to 25:1) of beads: cells. The most homogeneous populations of MM cells were obtained using 1 μg of each Ab per 10^6 total cells, and bead: cell ratios of 10–13:1. These culture conditions were therefore utilized for enrichment of MM cells from patient BM samples.

Table 2
Yield of immunomagnetic bead enrichment for patient myeloma cells

Patient	No. of MM cells ($\times 10^6$), pre	No. of MM cells ($\times 10^6$), post	Yield (%)	Viability (%)
1	9.5	9.2	97	99
2	10.6	10.3	97	98
3	15.4	15.2	99	99
4	5.9	5.8	99	99
5	1.4	1.3	96	99
6	26.3	25.4	97	99
7	0.8	0.7	89	99
8	1.9	1.8	95	99
9	7.9	7.7	97	99
10	8.4	8.2	98	99

Table 1
Tumor cell enrichment using immunomagnetic bead method

Patient	Percentage of MM cells, pre (by morphology)	Percentage of MM cells, pre (by phenotype)	Percentage of MM cells, post (by morphology)	Percentage of MM cells, post (by phenotype)
1	30	27	> 94	95
2	85	82	> 96	98
3	75	55	> 94	99
4	70	85	> 95	99
5	50	45	> 95	96
6	80	75	> 98	96
7	8	6	> 75	85
8	15	15	> 95	95
9	30	26	> 95	96
10	65	65	> 95	95

3.2. Separation of MM cells in patient BM aspirates

BM aspirations were obtained from 10 patients with a median of 50% (8–85%) MM cells by morphology and 45% (6–82%) MM cells identified by CD38 + CD45 − cell surface phenotype. BMMCs were incubated with the mAb cocktail described above using each mAb at 1 μg per 10^6 cells. Immunomagnetic mouse anti-Fc IgG1-coated beads were added at bead: cell ratios of 10:1. As seen in Table 1, the purity of the enriched MM cell fractions, assessed both by morphology of MGG stained cytosmears and dual fluorescence flow cytometric analysis for CD38 + CD45RA − cells was > 95%, except in a single case (patient 7) which was enriched from 6–8% MM cells initially to 75–85% MM cells after immunomagnetic bead depletion. The yield of MM cells was > 95% in all cases except for patient 7 (yield 89%), and the viability in all cases was 99% (Table 2). An additional 20 MM BM samples were tested, with a median of 50% (8–85%) MM cells by morphology and 60% (6–85%) MM cells determined by CD38 + CD45RA − cell surface staining. The MM cell enriched population was ≥ 97% CD38 + CD45RA − tumor cells in 13 cases which initially contained ≥ 10% MM cells, whereas

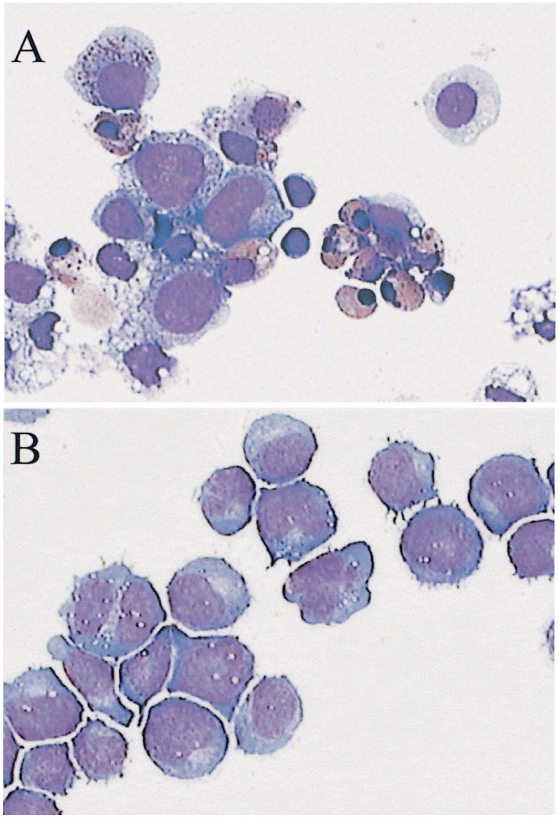

Fig. 2. Histologic assessment of immunomagnetic bead enrichment for patient MM cells. Bone marrow mononuclear cells obtained by Ficoll Hypaque centrifugation of a freshly obtained MM patient BM aspirate were incubated with mAb cocktail targeting non-MM cells, followed by incubation with immunomagnetic beads coated with mouse anti-Fc IgG. Cells bound to immunobeads were removed in a magnetic field, and residual cell populations were enriched for MM cells. Initial (A) and MM enriched (B) cell populations were examined using MGG staining (400× magnification).

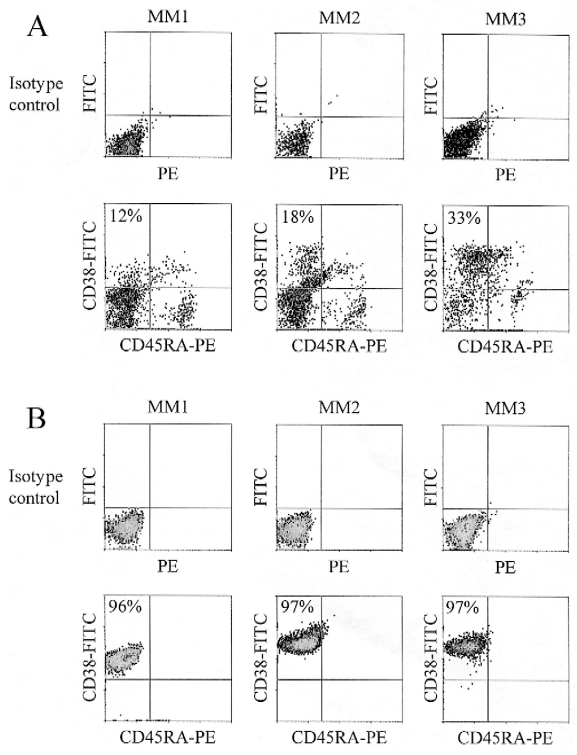

Fig. 3. Immunophenotypic assessment of immunomagnetic bead enrichment for patient MM cells. Bone marrow mononuclear cells obtained by Ficoll Hypaque centrifugation of three freshly obtained MM patient BM aspirates were incubated with mAb cocktail targeting non-MM cells, followed by incubation with immunomagnetic beads coated with mouse anti-Fc IgG. Cells bound to immunobeads were removed in a magnetic field, and residual cell populations were enriched for MM cells. Initial (A) and MM enriched (B) cell populations were examined using dual fluorescence analysis with FITC-anti-CD38 and PE-anti-CD45RA mAbs.

≥ 70% cells in the MM enriched fraction were CD38 + CD45RA − in the two cases starting with ≤ 10% MM cells (data not shown). Shown in Fig. 2 is a representative MGG stained cytosmear of initial and MM enriched populations from a single patient. Dual fluorescence flow cytometric analysis demonstrates 12–33% CD38 + CD45 − cells initially and 96–97% CD38 + CD45 − cells after purification of three MM patient BM aspirates (Fig. 3).

3.3. Immunoblotting of cell lysates from MM enriched cell populations

As a further assessment of enrichment for MM cells achievable with the immunomagnetic bead depletion methodology, cell lysates were prepared from initial (A) and MM enriched (B) cell populations from eight patient samples and immunoblotted with anti-CD38 and anti-CD45RA Abs. Blots were then stripped and reprobed with anti-α-tubulin to assure equal protein loading. As can be seen in Fig. 4, initial samples expressed both CD38 (45 kDa) and CD45RA (220 kDa), whereas MM enriched samples consistently expressed CD38, but either lacked, or only weakly expressed, CD45RA. Immunoblotting with anti-α-tubulin Ab confirmed integrity and equal loading of protein lysates. The expression of CD38 was significantly increased in post- versus pre-enrichment samples, as evidenced by relative densitometry: CD38 to α-tubulin expression was a median of 0.4 (0.05–0.5) in pre- versus 1.4 (1.2–1.5) in post-enrichment samples ($p < 0.01$).

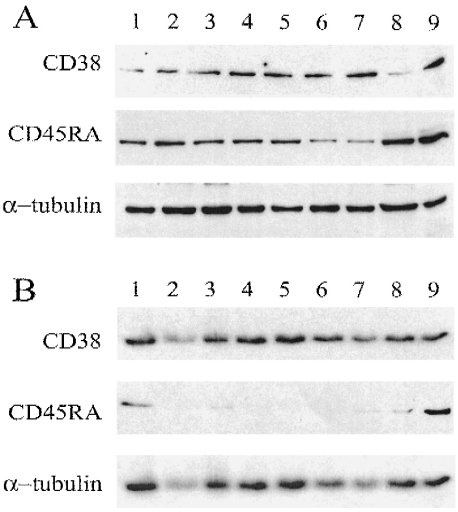

Fig. 4. Immunoblotting assessment of immunomagnetic bead enrichment for patient MM cells. Bone marrow mononuclear cells obtained by Ficoll Hypaque centrifugation of eight freshly obtained MM patient BM aspirates were incubated with mAb cocktail targeting non-MM cells, followed by incubation with immunomagnetic beads coated with mouse anti-Fc IgG. Cells bound to immunobeads were removed in a magnetic field, and residual cell populations were enriched for MM cells. Cell lysates were prepared from initial (A) (Lanes 1–8) and MM enriched (B) cell populations (Lanes 1–8) and immunoblotted with anti-CD38 and anti-CD45RA Abs. Cell lysates from RPMI and Jurkat cells were used as positive controls for CD38 (45 kDa) and CD45RA (220 kDa), respectively (Lane 9). Blots were stripped and reprobed with anti-α-tubulin Ab to assure equal protein loading. The relative intensity of expression was assessed using imaging densitometry.

4. Discussion

In this study, we describe a simple, reliable, and reproducible system for efficiently isolating tumor cells within BM from patients with MM. We designed our purification system to deplete unwanted cells of other lineages and to isolate cells with the CD38 + CD45RA − cell surface phenotype, known to be characteristic of MM cells. In our initial attempts to purify MM cells, we used immunomagnetic beads to sequentially deplete CD3 and then CD45 cells, followed by positive selection for cells expressing CD38; however, this method had large attendant cell losses and low yields of MM cells. We therefore utilized a single incubation with multiple mAbs targeting those cells to be removed, followed by a single immunomagnetic bead depletion step. Our defined mAb cocktail against CD3 (T-cells), CD11b and CD14 (monocytes), CD33 (myeloid cells), CD45 and CD45RA (leucocyte common antigen), CD32 as well as glycophorin A targeted non-MM cells, and optimal Ab concentration was 1–2 μg per 10^6 cells. In all cases where initial patient samples contained ≥ 5–10% MM cells, the MM enriched fractions were > 95% MM cells, evidenced both by MGG morphology and CD38 + CD45 − phenotype. In accordance with the observations by Fillola et al. (1996) and Borset et al. (1993), MM patient samples containing ≤ 5% MM cells initially

could be enriched to 75–85% MM cells. These MM cell enriched populations are $>99\%$ viable, and we routinely use then in assays of proliferation, cell cycle analysis, and Ig secretion.

Portier et al. (1991) and Borset et al. (1993) have reported that anti-CD138 (B-B4 mAb) was specifically reactive with MM cells. In this study, we found that $\geq 85\%$ of MM cell enriched samples (CD38 + CD45RA −) were also CD138 + (data not shown). In their study, high ($\geq 98\%$) purity of tumor was achieved based on positive selection of cells expressing CD138 using immunomagnetic beads. However, the MM cell viability varied, and in one case was only 52%. In this study and other reports using positive selection of MM cells, it is also possible that MM cells phagocytose the immunomagnetic beads, compromising either their viability or function. In contrast, using our two-step immunomagnetic enrichment method, we consistently obtain highly viable (99%) MM enriched populations. Moreover, by using this negative depletion technique, we have not depleted earlier B cells within the MM clone. Of interest, Partington et al. (1999) have recently developed a method of cell separation based upon immunomagnetic cell selection of cells expressing two surface antigens. Specifically, using two different surface markers and two different sizes of commercially available immunomagnetic beads and particles, it was possible to isolate very rare target cell populations that retained functional capacity. This technique could be employed to target pre B, B and PC in MM patient samples and thereby facilitate positive selection of the entire MM cell clone.

In summary, we have developed a simple and rapid method for the enrichment of MM tumor cells in BM aspirates from patients with MM. This is an alternative to cell sorting and positive magnetic bead selection for the isolation of tumor cells. The faster turnaround time, high purity, and low attendant cell losses using this method will permit both functional and molecular experiments of homogeneous MM cells versus non-MM cells in the BM microenvironment.

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