Circulating Monoclonal B Cells Expressing P Glycoprotein May Be a Reservoir of Multidrug-Resistant Disease in Multiple Myeloma

By Linda M. Pilarski and Andrew R. Belch

Multiple myeloma is basically an incurable cancer. Most patients respond initially to chemotherapy with reduction in bone marrow (BM) plasma cells and monoclonal Ig levels, but the disease nearly always recurs and becomes refractory to therapy. The objective of this study was to characterize the expression of the multidrug transport pump, P-glycoprotein 170 (P-gp), in myeloma. The great majority of B cells from peripheral blood mononuclear cells (PBMCs) in myeloma express P-gp, detected by the monoclonal antibody MRK-16. P-gp⁺ blood B cells exhibit extensive DNA hyperdiploidy, suggesting replicative abnormality characteristic of malignant growth. We speculate these represent a stem cell population in myeloma. The proportion of B cells expressing P-gp was comparable among untreated myeloma patients and those treated with chemotherapy, biologic response modifiers, or off treatment. Among BM cells, P-gp was absent or low in untreated myeloma patients but was expressed at high levels on BM cells from patients previously treated with chemotherapy. For untreated patients the majority of B/plasma cells expressing P-gp are located in PBMCs, not the BM cells. Flow cytometric analysis of rhodamine 123 dye efflux indicated a functional P-gp that was efficiently blocked by verapamil.

MULTIPLE MYELOMA is a hematologic cancer characterized by accumulations of monoclonal plasma cells in the bone marrow (BM), a high serum or urine concentration of monoclonal immunoglobulin, and lytic lesions of the bone. Although most patients respond to initial chemotherapeutic treatment, nearly all relapse and become refractory to further treatment. The mean lifespan postdiagnosis is approximately 3 years. Neither the decrement in M protein, nor degree of plasma cell kill correlate with survival of myeloma patients. Although most attention has focussed on the plasma cells resident in the BM, recent reports implicate a circulating B-lymphocyte population in the pathogenesis of myeloma. The blood of all myeloma patients often includes a very large component of monoclonal B-lineage cells, predominantly late-stage B cells. These B cells are monoclonal as defined by their heavy-chain Ig rearrangements, restricted expression of either κ or λ light chain mRNA, and the presence of monoclonal cytoplasmic light chain. These monoclonal B cells have extensive DNA aneuploidy, presumptive evidence of malignancy. They appear programmed for traffic and invasion based on their expression of CD11b, a J2 integrin required for extravasation, and β1 integrins required for binding to extracellular matrix. Upon chemotherapeutic treatment, plasma cells in the BM disappear, but circulating B cells persist and in some cases even increase in absolute number in blood. Multidrug resistance in many malignancies involves an adenosine triphosphate (ATP)-dependent transport pump, p-glycoprotein (P-gp), encoded by the MDRI gene, which mediates drug efflux. P-gp appears to play a role in the development of refractory disease in myeloma, but the expression of a functional P-gp transport pump on the BM plasma cells is controversial. Cytoplasmic P-gp is sometimes detectable, but others have not found P-gp on the surface or in the cytoplasm, or that the myeloma plasma cells contain mRNA. Treatment of myeloma patients with chemotherapy in the presence of agents known to block P-gp has met with some success. These include calcium channel blockers such as verapamil, and cyclosporin A (CsA). The latter inhibits drug transport by blocking P-gp has met with some success. These include calcium channel blockers such as verapamil, and cyclosporin A (CsA). The latter inhibits drug transport by blocking P-gp and may underlie the almost uniform fatal relapse in myeloma patients.
MULTIDRUG-RESISTANT B CELLS IN MYELOMA

We find consistently high expression and function of P-gp on B cells from nearly all patients tested. The function of the P-gp pump was blocked by verapamil and CsA. In vitro treatment of blood B cells from myeloma patients with Adriamycin plus CsA, but not adriamycin alone, resulted in extensive apoptosis within the B but not the T-cell compartment.

MATERIALS AND METHODS

Patients. A cohort of 161 patients with multiple myeloma were studied for a period of 14 months after informed consent and with ethical approval from the University of Alberta Human Ethics Committee. Clinical and experimental information was maintained in a set of linked databases using DbaseII+ (Borland, Scotts Valley, CA). The patients included 16 untreated (at diagnosis), 37 on melphalan/prednisone, 20 on vincristine/adriamycin/dexamethasone (VAD) 12 on biologic response modifiers interferon (IFN) or IFN plus interleukin-2 (IL-2), and 60 off chemotherapy. For some patients analyzed for prolonged periods of time, a single patient may appear in several of these groups. Patients on intermittent chemotherapy were studied at least 4 weeks after their last treatment. The definition of clinical stages was as described by Durie and Salmon.1 Among these patients, treatment groups included melphalan/prednisone, vincristine/adriamycin/high- or low-dose dexamethasone, or biologic agents such as IFN-α or IFN/IL-2. Patients were designated as off treatment 2 months after their last cycle of chemotherapy. However, most patients in this category had been off treatment for longer periods of time.

Purification of peripheral blood mononuclear cells (PBMCs). Venous blood samples were drawn into heparinized vacutainer tubes, and the PBMCs were isolated on a Ficoll-Paque (Pharmacia, Dorval, Quebec, Canada) density gradient. Cells harvested from the interface were washed twice in RPMI (GIBCO, Grand Island, NY) and resuspended in phosphate-buffered saline (PBS), containing 2% fetal calf serum (FCS) (HyClone Labs Logan, UT). Methods for depletion of adherent cells were carefully avoided, because the presumptively malignant B cells in the blood of myeloma patients have adherent properties and are depleted by such procedures.2

Antibodies. Leu-3PE (CD4), Leu-2PE (CD8), Leu-3PE (CD14), Leu-15PE (CD11b), and Leu-17PE (CD38) were purchased from Becton Dickinson (Ontario, Canada). From Coulter (Hialeah, FL), we purchased B1RDI (CD20). Monoclonal antibody (MoAb) FMC63 (CD19) was IgG2a from H. Zola (Findlers Medical Centre, South Australia).36 IgG2a and IgG1 isoforms of FITC- and phycoerythrin (PE)-conjugated isotype controls were purchased from Southern Biotech (Birmingham, AL). For detection of circulating CD19+ B cells in blood of myeloma patients, either FMC63 or B4 (Coulter) gave comparable results, but these cells were not reliably detected with Leu-12 (Becton Dickinson). The IgG2a MoAb MRK-16 detecting a surface epitope of P-gpP11 was a gift from Dr. T. Tsuruo (Tokyo University, Japan).3,12

Two-color immunofluorescence (IF). A two-color fluorescence staining procedure was used for the study of surface marker expression as previously described.7,12 PBMCs were incubated with MRK-16 MoAb in indirect IF, washed, blocked with mouse Ig, and stained with a direct FITC conjugate of FMC63 (CD19). A double-direct IF procedure was used for staining of cells with B1RDI (CD20), Leu-15PE (CD11b) or Leu-17PE (CD38) and FMC63 FITC. Stained cells were washed twice and fixed in 1% formalin for flow cytometric analysis.

Analysis of IF. Samples were analyzed using a FACScan (Becton Dickinson, San Jose, CA). Dead cells were excluded by electronic gating on forward angle light scatter and files of 10 to 20,000 cells collected. Files were electronically gated for CD19+ B cells and the expression of the second MoAb plotted as a histogram. In all cases, staining with a specific MoAb was compared with its appropriate isotype-specific control, with identical electronic gates for B cell subsets. To maintain a consistent evaluation of the intensity of staining among different patients, in all cases the intensity of staining was categorized moderate if it was placed between channels 332 and 516, or between 200 and 400 channel shift beyond the isotype-matched control; and high if the staining was greater than channel 516, or a 400 or more channel shift relative to the isotype control.

CD19+ cells were also evaluated for their physical properties as measured by forward (FALS) and side-angle scatter (SSc). Cells designated as small were those with SSc and FALS less than channel 400, whereas those designated as large had SSc and FALS greater than channel 400 on a linear scale.79

Dye-efflux analysis. This procedure measures the ability of cells to minimize retention of the mitochondrial-binding dye, rhodamine 123 (RH123), and determines whether or not the dye efflux observed, if any, is blocked by agents known to block the P-gp transport pump. A published procedure was used.34 Briefly, PBMCs from myeloma patients were loaded with RH123 (150 ng/mL) (Sigma, St Louis, MO), followed by washing and resuspension in medium with or without the blocking agent for 3 hours at 37°C to allow dye efflux. Blocking agents were either verapamil (50 mmol/L, or CsA)29,36 (Sandoz Pharmaceuticals, Dorval Quebec) at 1 μg/mL unless otherwise indicated. Cells were then stained with a direct PE conjugate of the indicated MoAbs, with the blocking agent included in all buffers for the samples treated with it, washed, resuspended in PBS with or without the blocking agent, and run immediately on the FACScan. The RH123 emission was collected in the FL1 channel and emissions from MoAbs defining surface phenotype in the FL2 channel. FACScan compensation was set to reduce as much as possible the RH123 emissions detected in FL2. For staining of B cells using dyes emitting in FL2, we used a cocktail of CD19-PE + CD20-RDI to optimize detection of B cells based on their coexpression of these two B-cell markers. This was necessary because detection of myeloma PBMC B cells using PE conjugates of CD19 was suboptimal and variable between patients, unlike the highly reliable staining with FITC conjugates. This true for both FMC63-PE and B4-RDI (Coulter). Controls for each sample established that the total number of B cells detected at the end of the functional dye-efflux assay was comparable with that before the pumping procedure, as measured with either CD19-FITC or the CD19-PE + CD20-RDI cocktail.

There were several important aspects to this procedure. It was essential that the cells be used on the same day the blood was collected. The dye efflux was compromised even if stored blood or buffy coats were loaded with RH123 and stored for 24 hours at 4°C. To control for these effects, aliquots of the blood were labeled with RH123 and run immediately on the FACScan. The RH123 emission was collected in the FL1 channel and emissions from MoAbs defining surface phenotype in the FL2 channel. FACScan compensation was set to reduce as much as possible the RH123 emissions detected in FL2. For staining of B cells using dyes emitting in FL2, we used a cocktail of CD19-PE + CD20-RDI to optimize detection of B cells based on their coexpression of these two B-cell markers. This was necessary because detection of myeloma PBMC B cells using PE conjugates of CD19 was suboptimal and variable between patients, unlike the highly reliable staining with FITC conjugates. This true for both FMC63-PE and B4-RDI (Coulter). Controls for each sample established that the total number of B cells detected at the cocktail of the functional dye-efflux assay was comparable with that before the pumping procedure, as measured with either CD19- FITC or the CD19-PE + CD20-RDI cocktail.

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samples were also stained in normal IF procedures to confirm phenotype and size. However, the size reductions prevented gating for B lineage subsets based on scatter properties. Previous work has shown that nearly all CD11b+ cells in myeloma PBMCs are CD19+ large cells and this was confirmed for each sample where dye efflux on CD11b+ cells was evaluated.

Files of 30 to 50,000 cells were gated for surface phenotype in the FL1 channel, and the amount of dye retained plotted as a histogram of FL1 staining. Analysis was performed using Lysis II (Becton Dickinson).

**In vitro culture of myeloma PBMCs with cytotoxic drug.** To test the ability of Adriamycin to kill B cells in myeloma PBMC, cells were cultured with various doses of adriamycin in the presence or absence of CsA as an inhibitor of extracellular drug transport. Culture was in RPMI plus 10% FCS/PBS, followed by permeabilization with 3 mL of 70% ethanol at 4°C. RNase treatment, and staining with DAPI (1.5 μg/mL final concentration). Sample were run immediately after staining on the ELITE (Coulter) flow cytometer with FITC and PE excited by the argon laser (488 nm), and DAPI excited at 353 nm by the water-cooled laser. As an internal standard, and to define the placement of the diploid peak, all samples included the addition of chicken red blood cells (CRBCs). Diploid human cells bind approximately three times as much DAPI as do CRBCs, but in our hands this varied somewhat for CRBCs from individual chickens from 3.05 to 3.54, although with any given preparation of CRBCs, this value remained constant. The value used for the definition of diploid was determined by staining unfractionated uncultured human thymocytes, which have a very distinct large diploid peak. Diploid human cells bind approximately three times as much DAPI as do CRBCs, and the DAPI profile is used for all the groups listed in Table 2, although there was considerable variation within each group.

Untreated patients had low or no expression of P-gp on BM B/plasma cells, in contrast to the abundant expression on PBMC B cells from these same patients. Fifty-four percent of untreated patients had little or no detectable P-gp expression in BM cells (Table 2, line 1). Analysis of treated patients categorized according to phase of disease, as compared with untreated patients, showed a significant increase in P-gp expression on CD19+ small BM cells in progression and relapse (Table 2, lines 3 and 4). For all treated groups, the number of P-gp+ large CD19+ BM cells was higher than in untreated patients. Nearly all patients, had greater than 20% P-gp+ CD19+ BM cells (Table 2, column 4).

In seven patients, five of whom were untreated, approximately 20% of BM cells were CD19+ large cells with expression of PCA-1 and a high density of CD38 characteristic of end stage plasma cells. For seven of seven patients, all of these end stage plasma cells from BM cells were negative for P-gp expression, confirming previous reports that well-differentiated myeloma plasma cells may lack P-gp.

**Expression of P-gp on B lineage PBMCs varies between...**

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**RESULTS**

P-gp is expressed on blood B cells from untreated and treated myeloma patients. The expression of P-gp, as defined by binding of MoAb MRK-16, which recognizes a surface epitope of the P-gp transport pump, was evaluated on PBMC B lineage cells from patients with multiple myeloma (Table 1). Patients were grouped according to their treatment status and type. Previous work with this same cohort of patients has shown 26% to 36% CD19+ B cells among PBMCs or 0.2 to 0.6 x 10^6 CD19+ cells/L blood, and confirmed monoclonality. The presence of monoclonal B cells in blood varies over the course of the disease with maximal numbers during progressive phases of the disease and in relapse.

Based on physical properties of forward and side scatter, PBMC B cells were divided into a small and a large subset. The small subset shares some properties with normal resting B cells but small B cells in myeloma PBMCs are phenotypically distinct in their expression of CD10, PCA-1 and CD45R0. The large subset consists of late stage B cells expressing CD11b, CD10, PCA-1 and CD45R0. Based on two-color IF analysis as illustrated in Fig 1, Table 1 shows that in all patients, irrespective of treatment, 42% to 75% of patients had expression of P-gp on 6% to 57% of the small B cells (columns 1 and 2). A major proportion of patients, 79% to 91%, had 75% to 84% of large B cells expressing P-gp. In all treatment categories, a small proportion of patients had no or very few B cells expressing detectable P-gp.

In myeloma, the multidrug resistance transport pump, P-gp, is expressed at moderate to high density on blood B cells before any chemotherapy, and the overall proportion of such B cells does not increase either in number or in density during chemotherapy.

**Expression of P-gp on myeloma BM B lineage cells is increased posttreatment for patients with progressive or relapsed disease.** The expression of P-gp on plasma cells in bone marrow (BM) of myeloma patients is controversial. Table 2 reports the expression of surface P-gp on CD19+ B lineage cells in BM cells taken during various phases of disease. In myeloma patients, phenotypic analysis detects abnormal numbers of CD19+ cells not always detectable by morphologic analysis as plasma cells. Myeloma BM cells were subdivided into small and large subsets. The mean number of CD19+ cells in either subset was comparable for all the groups listed in Table 2, although there was considerable variation within each group.

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MULTIDRUG-RESISTANT B CELLS IN MYELOMA

Table 1. Expression of P-Glycoprotein on B-Lineage Cells in Blood of Myeloma Patients

<table>
<thead>
<tr>
<th>Treatment Status</th>
<th>CD19 Small B Cells</th>
<th>CD19' Large B Cells</th>
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<tbody>
<tr>
<td></td>
<td>% B-Lineage Cells That Are MRK-16*</td>
<td>% Pts With &gt;20% P-gp' B Cells</td>
</tr>
<tr>
<td>Unt (16)</td>
<td>51 ± 7</td>
<td>75 ± 6</td>
</tr>
<tr>
<td>M/P (72)</td>
<td>54 ± 4</td>
<td>80 ± 3</td>
</tr>
<tr>
<td>VAD (47)</td>
<td>49 ± 6</td>
<td>80 ± 4</td>
</tr>
<tr>
<td>IFN/IL-2 (27)</td>
<td>51 ± 5</td>
<td>80 ± 5</td>
</tr>
<tr>
<td>Off Tr (102)</td>
<td>57 ± 4</td>
<td>84 ± 2</td>
</tr>
</tbody>
</table>

Values are the mean ± SE of the indicated number of samples. None of the groups had a value significantly different from the untreated group. PBMC samples were stained with CD19-FITC and MRK-16/goat-antimouse-lg PE. Files of 10 to 20,000 cells were gated for small or large CD19' B cells as indicated in Materials and Methods and the expression of P-gp plotted as a histogram. Positive staining with MRK-16 (P-gp) was compared with that of identically gated samples stained with an lgG2a isotype control. Intensity of staining ranged from 80 to 600 channels brighter than the isotype-control staining with the majority of samples giving an intensity in the range of 200 to 400 channels above the background on a logarithmic scale of 1024 channels. For most patients, 70% to 95% of the total staining with MRK-16 was accounted for by the CD19' population. All patient groups had mean values of B cells from 26% to 36% of PBMCs, approximately 4% of which were small B cells. Because in all groups, a proportion of patients showed low or no staining for P-gp among their B cells, mean values were calculated only for those patients with greater than 20% of the indicated subset of B cells expressing P-gp. The percent of patients with >20% of B cells expressing P-gp is indicated above. For those patients with less than 20% B cells expressing P-gp, the mean values were comparable for all treatment groups, ranging from 3 ± 3 to 5 ± 7 (mean ± SE) of B cells stained with MRK-16 MoAb.

Abbreviations: Pts, patients; Unt, untreated at diagnosis; Off Tr, off treatment for at least 2 months; M/P, melphalan/prednisone; VAD, Vincristine/Adriamycin/Dexamethasone; IFN/IL-2, treated with either interferon-α alone or a combination of IL-2 and interferon-α.

Table 2. Expression of P-gp on B-Lineage Cells From BM of Myeloma Patients

<table>
<thead>
<tr>
<th>Treatment Status</th>
<th>% of B-Lineage Subset That Is MRK-16*</th>
<th>% Pts With &gt;20% P-gp'</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>CD19 Small</td>
<td>CD19 Large</td>
</tr>
<tr>
<td>Unt (13)</td>
<td>8 ± 4</td>
<td>36 ± 11</td>
</tr>
<tr>
<td>Asympt (10)</td>
<td>38 ± 10*</td>
<td>84 ± 21</td>
</tr>
<tr>
<td>Prog (10)</td>
<td>57 ± 15*</td>
<td>68 ± 17</td>
</tr>
<tr>
<td>Relapse (9)</td>
<td>62 ± 10*</td>
<td>82 ± 12*</td>
</tr>
<tr>
<td>'Remis' (5)</td>
<td>40 ± 171</td>
<td>78 ± 16</td>
</tr>
</tbody>
</table>

Values are the mean ± SE. Staining was as described for Table 1, and the staining intensity for all treated groups was comparable with that of PBMC B cells. The staining with MRK-16 for BM cells from untreated patients was often quite low. Comparison of clinical evaluation and flow cytometric studies indicated that the majority of the B-lineage cells, including mature plasma cells, expressed a low but detectable density of CD19. B-lineage cells were subdivided into small and large subsets, but the relationship of small CD19' BM cells to those in PBMCs is unclear.

For untreated patients and those in the progressive phase of the disease, approximately 40% of small CD19' BM cells are P-gp+, and only a few samples are P-gp- (Fig 2, top). Patients in temporary remission have an increased proportion of P-gp' small B cells. Patients responding to that therapy, had a significant reduction in the proportion of P-gp' small B cells and a large number of patients lacked detect-
able P-gp on their small PBMC B cells. Patients unresponsive to treatment were not significantly different from untreated patients.

P-gp expression on large PBMC B cells was considerably increased in all treatment groups as compared with untreated patients, with the highest proportions, 80% to 90% of CD19+ B cells expressing P-gp, among patients with progressing disease and those in temporary "remission" (Fig 2, bottom).

Patients were evaluated for P-gp expression on PBMCs and BM B-lineage cells at multiple time points over a 14 month period. Figure 3 shows the dynamic nature of P-gp expression on B lineage subsets. For patient 1 (Fig 3, A) expression of P-gp on BM cells was on only a small subset and at low surface density, whereas on PBMCs, a substantial proportion of small and large B cells expressed P-gp at high surface density. The proportion of P-gp+ PBMC B cells increased with the initiation of VAD therapy and at the end of the VAD cycle. P-gp was expressed on nearly all blood and BM B-lineage cells, although this patient was in temporary remission. In late 1992, the patient received an autologous transplant. The 10/92 time point shows the high P-gp expression on PBMC B cells at the time of transplant, and on the BM cells transplanted. The expression of P-gp has decreased slightly posttransplant. Patient 2 (Fig 3, B) had P-gp+ cells in both blood and BM at the time of diagnosis. During treatment with melphalan/prednisone, expression of P-gp varied in an apparently cyclical manner among small PBMC B cells and in a less dramatic way for the large PBMC B cells. In a sample taken during temporary remission, BM cells had strong expression of P-gp.

These individual profiles illustrate the dynamic patterns of P-gp expression seen among the 161 patients analyzed, in whom the blood appears to be a persistent but variable reservoir of multidrug resistant B-lineage malignant cells.

P-gp+ blood B cells include a subset of DNA hyperdiploid cells. To confirm their presumptive malignant character, myeloma B cells expressing P-gp were analyzed for their DNA content. BM plasma cells exhibit extensive DNA aneuploidy.47-52 and recent work indicates that nearly all patients have circulating hyperdiploid B cells with restricted light-chain expression.11 Myeloma PBMCs from a patient in relapse, on treatment or untreated at diagnosis were stained for surface phenotype and DNA content (Fig 4, A through C), and the DNA content of P-gp+ B cells evaluated. Files were gated for small non-B cells and the position of the DNA peak compared with that of T cells in a replicate aliquot of PBMCs to establish the position of the diploid peak. B cells were considered to be hyperdiploid only if their DNA content exceeded that of T cells. Figure 3 shows that both small and large CD19+ P-gp+ B cells had a substantial DNA hyperdiploid subset with 5% to 10% excess DNA (Fig 4, top and middle panels). Hyperdiploid cells were less frequent among the P-gp+ B cells (Fig 4, bottom panels).

PBMC B cells in myeloma express functionally active P-gp. Although myeloma blood B cells express phenotypically defined P-gp, it was important to show that this was a functionally active transport pump. The ability of PBMC B cells from myeloma patients to retain or efflux dye was measured using flow cytometry that allows quantitation of

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**Figure 2.** Expression of P-gp on PBMC B cells from patients grouped according to clinical condition. Each dot represents a single PBMC sample. Staining was as described in Table 1, and the same set of patients are included here as in Table 1. Abbreviations: Unt, untreated; Prog, progressive disease at the time the sample was taken; Remis, temporary remission; Tr Resp, those patients responding to treatment at the time the blood sample was taken; Tr No Resp, those patients not responding to treatment at the time the blood sample was taken. The proportion of B lineage cells was significantly greater in the progressing and relapse groups (39% to 42% of PBMCs) than in untreated patients (26% of PBMCs). The values below the x axis are the mean ± SE of the percentage of CD19+ B-cell subset expressing P-gp for the sample group. Top: Files gated for small B cells in PBMCs and the percentage of MRK-16+ B cells enumerated from the gated histogram. Bottom: Files gated for large B cells and the percentage MRK-16+ B cells enumerated. *P ≤ .1 compared with the untreated group. **P ≤ .01 compared with the untreated group.
dye in individual cells. The involvement of P-gp in the dye efflux was confirmed by the demonstration that dye efflux was blocked by verapamil or CsA. To define subsets of PBMCs, cells were loaded with RH123, allowed to efflux dye in the presence or absence of CsA and then stained for surface CD19 or CD11b (Fig 5). The representative histograms for two patients are shown. For both patients, the majority of CD19+ cells were able to efflux dye, and this efflux was blocked by CsA. Gating for the CD11b+ PBMCs, which defines the CD19+ large B cells,5 showed efficient CsA-sensitive efflux in this subset.

The functional activity of P-gp was also assessed among the presumptively nonmalignant components of the PBMCs in myeloma patients. A representative experiment is shown in Fig 6. For this patient, CD4+ and CD8+ T cells included 39% to 42% of cells expressing functional P-gp. However, monocytes, defined by their expression of CD14, retained dye even in the absence of CsA, indicating a lack of P-gp or other transport mechanism on monocytes and confirming observations on monocytes from normal donors.6 The inability of monocytes within myeloma PBMCs to efflux dye contrasts strongly with the active dye efflux of the B cells represented by the CD11b+ PBMCs. This provides further evidence that the monoclonal CD19+ CD11b+ PBMCs described here and elsewhere6,9 are distinct from monocytes.

Table 3 summarizes the dye-efflux patterns in PBMC subsets for 32 patients, and the inhibitory effects of CsA or verapamil on this efflux. In all patients, the CD19+ B-cell subset and the CD11b+ cells exhibited CsA- or verapamil-sensitive dye efflux for 58% to 63% of the gated cells (Table 3, lines 1 and 2). For some of patients, a subset of the gated population had CsA- or verapamil-insensitive dye efflux. Among T cells, all dye efflux was CsA-sensitive. CD4+ cells had approximately half as many cells capable of effluxing dye as did the CD8+ cells, consistent with the properties of T cells from normal donors.54 Monocytes from myeloma patients had little or no detectable dye efflux either without or with CsA (Table 3, line 5). This work confirms that the CD19+ CD11b+ B cells in myeloma PBMCs are distinct from monocytes in the same PBMC sample.

**BM B-lineage cells in myeloma have a reduced ability to efflux dye.** Figure 7 shows the dye-efflux patterns of B-lineage cells from blood and BM samples taken from a myeloma patient immediately before autologous BM transplantation. CD19+ and CD11b+ B cells in PBMCs showed active dye efflux that was blocked by CsA. Among myeloma BM cells, the CD19+ cells had reduced dye efflux with only 40% effluxing dye in the absence of CsA. Gating to include only the CD38+ BM plasma cells yielded a population that lacked a functional transport pump and retained dye even in the absence of CsA. This figure confirms with a functional assay the conclusion that the predominant reservoir of drug-resistant disease resides among the blood B-cell population and on the less differentiated B cells of BMCs. A similar pattern was seen for three different myeloma BM samples.

**P-gp protects B cells in myeloma PBMCs from the cytotoxic effects of adriamycin.** Although it is a reasonable conclusion that dye efflux reflects resistance to drug-induced cell death, this assumption was tested by culture of myeloma PBMCs with various doses of adriamycin in the presence or absence of CsA (Fig 8). The steady-state concentration of adriamycin in the blood of treated patients is in the range of 0.01 μg/mL,28 and is known to be effluxed by P-gp.35-36 The effects of Adriamycin on the B and T cells present in myeloma PBMCs were determined by a multiparameter IF study of surface phenotype and DNA content as a measure of apoptotic cell death (Fig 8). The same aliquot of cells was stained for both CD19 and for CD4, files were gated for both
Fig 4. Extensive DNA hyperdiploidy among P-g+ blood B cells. Myeloma PBMCs were stained with CD19-FITC, MRK-16/goat-antimouse PE or isotype-matched control, and DAPI. Files of 50,000 cells were collected and analysed by gating for MRK-16+ or -CD19+ cells and the DAPI distribution plotted. All samples included CRBCs as an internal standard and in all cases a replicate sample was stained for CD19 and Leu-2/3 to compare DNA content of T and B cells. Solid lines represent the DNA content of the B-cell subset indicated. Dotted lines represent the DNA content of 'control' small non-B cells in the same aliquot of cells (diploid). Cells falling to the far left are apoptotic. CRBCs have been gated out of these histograms. The top panel derives from PBMCs of a patient in relapse, the middle is from a patient on intermittent chemotherapy, and the bottom from an untreated patient at diagnosis.
Fig 5. CD19+ CD11b+ B cells from myeloma PBMCs actively efflux dye. Samples from two patients (different from those shown in Fig 3) were stained and treated as for Fig 3. A 1 μg/mL concentration of CsA was used to block P-gp in this experiment. PBMCs were loaded with RH123, allowed to efflux dye either with or without CsA, and then stained with either a CD19-PE + CD20-ROD1 cocktail, or CD11b-PE. Files were gated for CD19/20+ or CD11b+ cells and the dye efflux plotted as the RH123 histogram. Thick lines represent dye retention in the absence of CsA and thin lines represent the dye retention in the presence of CsA for the gated population indicated. The numerical values are the proportion of cells effluxing dye in the absence of CsA.

DISCUSSION

This study shows the expression of the multidrug transport pump on PBMC B cells from myeloma patients renders them resistant to all but extremely high doses of cytotoxic drug. Inhibition of the pump by CsA allows a relatively selective B-cell kill by a lower dose of Adriamycin that spares most T cells.
Table 3. Dye Efflux by Subpopulations of White Blood Cells in Myeloma Patients

<table>
<thead>
<tr>
<th>Gated Subset (no. pts.)</th>
<th>% Dye Efflux</th>
<th>Pts With CsA/V</th>
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<tbody>
<tr>
<td>CD19+ (32)</td>
<td>58 ± 4</td>
<td>13 ± 2</td>
</tr>
<tr>
<td>CD11b+ (20)</td>
<td>63 ± 4</td>
<td>9 ± 2</td>
</tr>
<tr>
<td>CD4+ (16)</td>
<td>33 ± 3*</td>
<td>2 ± 0.2</td>
</tr>
<tr>
<td>CD8+ (16)</td>
<td>58 ± 4*</td>
<td>2 ± 0.5</td>
</tr>
<tr>
<td>CD14+ (10)</td>
<td>18 ± 2*</td>
<td>15 ± 3</td>
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Values are the mean ± SE. The subsets noted above derive from the same set of patients. The inhibitor used was CsA (1 μg/mL) for 20 patients and verapamil (50 μmol/L) for 12 patients. All patients assayed for dye efflux by CD4, CD8, or CD14 subsets were also assayed for CD19 and CD11b sets. Patients with CsA-insensitive dye efflux included those patients for whom greater than 20% of the indicated subset effluxed dye in the presence of CsA. For the most part, the patients indicated to have CsA-independent efflux, for eg, CD14, also had CsA-insensitive efflux for CD11b and CD19+ subsets. However, one patient had completely CsA-insensitive CD14+ dye efflux but completely CsA-sensitive CD11b+ efflux.

Abbreviations: V, verapamil.
* P ≤ .0001 compared with CD19+ cells.
† P ≤ .0001 compared with CD8+ cells.

Fig 6. T cells in myeloma PBMCs, but not monocytes, efflux dye. The same samples stained in the experiment of Fig 4 were also loaded with RH123, allowed to efflux dye, and stained with CD4, CD8, PE, CD38-PE, or CD14-PE. Files were gated for the indicated subset and the dye retention with or without CsA plotted as a RH123 histogram. Thick lines represent samples without CsA, and thin lines, those with CsA.

Sitive dye efflux. Among BM B-lineage cells, increased expression of P-gp was apparent after chemotherapy, but the proportion of P-gp+ CD19+ cells was equivalent for relapsed patients and those in temporary remission. For a substantial number of myeloma patients, a large proportion of monoclonal P-gp+ B cells were resident in cell populations used for autologous BM transplants. The malignant character of these circulating B cells was confirmed by their extensive abnormality of DNA content characteristic of malignant cells but absent from normal B cells. P-gp+ B cells included both diploid and hyperdiploid B-cell subsets, but P-gp+ B cells were predominantly diploid. This suggests that the majority of abnormal DNA replication occurs among the multidrug resistant B cells.

P-gp has homology to the MHC Class I peptide transport pump, transports peptides, has an associated volume-regulated chloride channel, and appears as part of the normal differentiation of human blood or splenic B cells. P-gp is expressed as part of a developmental pattern during the mature B-cell stage, and appears to be lost as B cells differentiate to end stage plasma cells. It is plausible that circulating monoclonal B cells in myeloma, which represent the least differentiated members of the malignant lineage, constitute a reservoir of drug resistant disease that is continuously replenished as differentiation proceeds.

The work reported here supports this view. End-stage plasma cells lacking CD19 and CD45 have presumably lost the capacity to respond to activating signals from the outside environment and are unlikely to require a peptide transport pump. In myeloma patients, seven of seven completely lacked expression of P-gp on CD19+ plasma cells. CD38+ plasma cells were unable to efflux dye, confirming the absence of a functional drug pump. Other work shows that BM samples from untreated myeloma patients have weak expression of MDRI mRNA or lack surface P-gp. In
contrast, circulating B cells expressed a high density of P-gp, and effectively mediated CsA-sensitive dye efflux. CD19^+ B cells in the BM have a relatively infrequent expression of P-gp and a limited ability to efflux dye as compared with PBMC B cells. In over half of the untreated patients, P-gp is absent or only weakly expressed among BM cells, but is strongly expressed on PBMC B cells. Because these patients have not yet been exposed to chemotherapy that might select for drug-resistant populations, the distribution of P-gp is likely to reflect developmental patterns within the malignant clone. The findings here of a high proportion of P-gp surface staining on a high proportion of CD19^+ BM cells from patients subjected to chemotherapy appears to contrast with an extensive study analyzing cytoplasmic P-gp expression on plasma cells.\textsuperscript{62} This conflict is likely to be more apparent than real, because the cells staining with CD19 as detected by flow cytometry may include a high proportion of cells not scored as plasma cells by Grogan et al.\textsuperscript{62} Our finding that CD19^− plasma cells from untreated patients lacked detectable P-gp expression confirms their work.

With the initiation of chemotherapy, the proportion of P-gp expressing cells within the malignant lineage increases in all compartments suggesting continuous kill of sensitive components and consequent selection of drug-resistant subsets. Even though these subsets are likely to differentiate, lose P-gp, and become sensitive themselves, they are probably continuously replenished by the generative compartment of myeloma. We predict that as the malignant clone differentiates in myeloma, P-gp^+ subsets are continuously generated and upon introduction of chemotherapy, are enriched through selective kill of P-gp^− differentiation stages. The introduction of altered developmental patterns by chemotherapy-induced kill may influence the course of disease more profoundly than would selective pressure on P-gp^+ B-cell stages. The perturbation of clonal growth by chemotherapy may be responsible for effusive production of monoclonal B cells in the blood, perhaps accounting for the increased numbers of blood B cells postchemotherapy in many patients.\textsuperscript{9,10}

The role of P-gp expression in myeloma remains to be established. Other mechanisms of drug resistance may play a role.\textsuperscript{26,27} Melphalan, the most commonly used therapeutic agent, is a substrate for the P-gp transporter in some
Fig 8. CsA potentiates the cytotoxicity of Adriamycin for circulating B cells in PBMCs of myeloma patients. PBMCs from myeloma patients were cultured for 3 days with the indicated drugs, or with no drugs added (not shown). At day 3, cells were harvested and stained with MoAb to CD19, CD4 and for DNA content using DAPI. CRBCs were used as an internal standard to define the diploid peak but have been gated out of the plots shown here. Thick lines represent the DAPI staining of B cells, and thin lines, the DAPI stain for T cells. The region marked as hypodiploid (H) includes 75% to 95% of the diploid DNA content. Regions marked as apoptotic (A) include 0% to 75% of the diploid DNA content. D, diploid region. Left column of histograms shows cells cultured with 0.1 μg/mL Adriamycin + 1 μg CsA. Right column of histograms shows cells cultured with 1 μg/mL Adriamycin + 1 μg/mL CsA.
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eflux mechanisms. Our work indicates that a putative generative compartment of the myeloma malignant clone present in the blood, the monoclonal CD19+ B cells, is also spared.

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Circulating monoclonal B cells expressing P glycoprotein may be a reservoir of multidrug-resistant disease in multiple myeloma

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