Tumor Cell Heterogeneity in Multiple Myeloma: Antigenic, Morphologic, and Functional Studies of Cells From Blood and Bone Marrow

By Malcolm A. King and David S. Nelson

Tumor cells from six patients with immunoglobulin G (IgG) multiple myeloma were analyzed for surface antigens, cytoplasmic paraprotein, morphology, and response to various culture conditions. The tumor marker was the paraprotein idiotype. Low numbers of tumor cells were found in the blood of most of the patients. In some patients, the circulating tumor cells were solely B lymphocytes, whereas in other patients, they were lymphoid, lymphoplasmacytoid, and plasmacytoid. Dual surface antigen analysis of blood and bone marrow cells confirmed that the tumor may be composed of a spectrum of cell types. Thus, cells may range from surface-idiotype-CD19-CD20-, PCA-1-, cytoplasmic-idiotype- lymphocytes, to CD19+PCA-1+cytoplasmic-idiotype+ plasma cells that are surface-idiotype- or weakly surface-idiotype+. In one patient, some of the tumor cells co-expressed surface idiotype and CD10. The tumor B lymphocytes were activated in vitro to synthesize paraprotein by pokeweed mitogen (PWM), and by low molecular weight B cell growth factor (BCGF). In contrast, spontaneous synthesis of paraprotein by more mature tumor cells was inhibited by agents that also inhibit nonmyeloma plasma cells. These agents included PWM, gamma interferon, and phorbol ester. The results demonstrate that in multiple myeloma there exist different tumor cell types that are similar, by a variety of criteria, to normal B lineage cells at different stages of differentiation. Thus, further evidence is provided for the hypothesis of myeloma cell differentiation.

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marrow plasma cells to <10% of nucleated cells, plus a substantial clinical improvement. Blood and bone marrow samples were obtained as part of routine diagnostic procedures to which the patients had given informed consent according to practice in this hospital.

**Antibodies**

The isolation of the sheep anti-idiotype antibodies has been described previously. Each pure anti-idiotype antibody was tested for cross reaction with pooled normal human Ig and a series of unrelated IgG paraproteins by a modified radioimmunoassay (see below). For a given quantity of specific paraprotein, at least 20,000 times the quantity of polyclonal IgG or unrelated paraprotein was required to give the same reaction. These antibodies were also checked for possible cross reaction with surface antigens of normal and other patients’ cells, and with cytoplasmic antigens of other patients’ bone marrow mononuclear cells (BMMC). The antibodies showed negligible cross reaction in the rosette assay (see below), with the exception of anti-GM idiotype, which reacted with 1.7% of the peripheral blood lymphocytes (PBL) from one normal individual. Again, with the exception of anti-GM idiotype, there was negligible cross reaction on flow cytometry. There was no detectable cross reaction with the cytoplasm of other patients’ BMMC by immunofluorescence microscopy.

Where necessary, anti-idiotype antibodies were biotinylated. The following mouse monoclonal antibodies were used: OKM1 (Ortho Diagnostic Systems, Raritan, NJ), specific for human monocytes and ‘null’ cells (CD11b) but unreactive with B cells; anti-B1 (Coulter Immunology, Hialeah, FL), specific for human B cells; and some pre-B cells (CD20); anti-B4-RD1 (phycoerythrin conjugate; Coulter), specific for human B cells and all pre-B cells (CD19); anti-J5-FITC (fluorescein isothiocyanate conjugate; Coulter), specific for the common acute lymphoblastic leukemia antigen (CALLA) found on some* or perhaps all39 human pre-B cells and weakly expressed by some other normal cells including granulocytes and fibroblasts (CD10); anti-PCA-1 (Coulter), found on human plasma cells and weakly expressed by granulocytes and monocytes; anti-Leu-M3-PE (phycoerythrin conjugate), specific for human monocytes/macrophages (CD14), and anti-Leu-12-FITC (CD19), from Becton Dickinson (Mountain View, CA); anti-HuLyt 3 (New England Nuclear, Boston), specific for the E-rosette receptor on human T cells (CD2). The appropriate mouse Ig isotype controls were purchased from Coulter and Becton Dickinson. Streptavidin-PE was purchased from Becton Dickinson.

The following affinity isolated sheep antibodies were purchased from Silenus Laboratories, Hawthorn, Victoria, Australia: anti-human IgG (unconjugated and FITC conjugated), anti-human IgM, the F(ab')2 fraction of anti-mouse Ig (FITC conjugated), and whole anti-mouse Ig. The latter antibody preparation was subjected to immunoabsorption to remove antibodies cross reactive with human Ig. The FITC conjugated F(ab')2 fraction of affinity isolated rabbit anti-sheep IgG (H + L), absorbed to remove cross reaction with human serum proteins, was purchased from Jackson Immunoresearch Laboratories (West Grove, PA). The IgG fractions of goat anti-human kappa and anti-lambda were purchased from Atlantic Antibodies (Scarborough, ME).

**Mitogens and Cytokines**

Pokeweed mitogen (PWM) was from GIBCO (Grand Island, NY). Partially purified human B cell growth factor (BCGF) solution, and human gamma interferon (IFN-γ) purified from the supernatant of mitogen stimulated T lymphocytes, were from Cellular Products (Buffalo). BCGF was tested for reactivity in the Ig and idiotype radioimmunoassays and contained no detectable IgM or cross reactive idiotype but did contain some IgG (5% BCGF = 70 ng IgG/mL). This was allowed for when calculating the effect of BCGF on cells in culture. Recombinant (r) human IFN-γ (Escherichia coli) derived was a gift from Dr G. R. Adolff, Ernst-Boehringer Institute fur Arzneimittelforschung, Vienna, Austria. Phorbol 12-myristate 13-acetate (PMA) from Calbiochem-Behring (La Jolla, CA) was dissolved in dimethyl sulphoxide (1 mg/mL).

**Cells**

Whenever peripheral blood or bone marrow mononuclear cells (PBMC or BMMC) were taken from treated patients, at least 1 month had elapsed since the last course of chemotherapy. PBMC and BMMC were isolated by centrifuging heparinized blood or bone marrow aspirate over Lympho-Paque (Nyegaard, Oslo, Norway). The bone marrow aspirate was dispensed before centrifugation by passage through needles of decreasing diameter. If necessary, PBMC and BMMC were stored in liquid nitrogen then thawed when required.40

Before analyzing cell surface antigens, cells binding cytophilic paraprotein with high affinity were removed. Null cells and monocytes were removed by using the monoclonal antibody OKM1 (anti-CD11b) and rosetting or panning.41 Latterly, panning was used instead of rosetting to isolate the CD11b+ cells because the recovery was better (65% average compared with 41% with rosetting). Falcon (Becton Dickinson, Oxnard, CA) 25 cm² tissue culture flasks were coated with sheep anti-mouse Ig (50 μg/mL PBS, 3 mL, overnight, 4°C) then washed with PBS. Before use the flasks were left for one hour, at room temperature, containing PBS with 20% FCS. Twenty × 10⁶ OKM1 treated cells were added per flask in 2.5 mL and left undisturbed for one hour. Then the nonadherent cells were removed (the flask was washed several times), centrifuged, and panned a second time. Whether the rosette or panning method was used, the CD11b+ cells were >99% lymphocytes by morphology or by flow cytometry using anti-Leu-M3-PE.

Bovine aortic endothelial cells were included in some of the PBMC cultures, at a ratio of one bovine endothelial cell to 40 PBMC. The bovine endothelial cells were a gift from Wayne Riley, CSIRO Division of Biotechnology, North Ryde, NSW, Australia.

**Detection of Cell Surface Antigens by Rosetting**

This was performed as described previously.42 Cells were treated with human serum proteins, was purchased from Jackson Immunoresearch Laboratories (West Grove, PA). The IgG fractions of goat anti-human kappa and anti-lambda were purchased from Atlantic Antibodies (Scarborough, ME).

**Cell Preparation for Immunofluorescence**

Cells were prepared for cytoplasmic Ig staining by suspending in 50% FCS, centrifuging onto glass slides in a Shandon cytocentrifuge (Shandon Southern Products, Runcorn, England) at 600 rpm for five minutes, air drying, and fixing in 1% ethanol/5% acetic acid (15 minutes, −20°C). Slides were washed with PBS, incubated with sheep anti-human IgG-FITC (1/20 dilution) or sheep anti-idiotype (0.1 mg/mL) for 20 minutes at 37°C, and washed; the slides that had been incubated with anti-idiotype were then incubated with F(ab')2 rabbit anti-sheep IgG-FITC, and washed; all slides were mounted in DABCO (Sigma Chemical Co, St Louis). Cells were viewed with a Leitz Ortholux epi-illumination microscope.

Staining of cells for flow cytometry was performed in Minisorp tubes (Nunc, Roskilde, Denmark) at 0.2 to 1.0 × 10⁶ cells/100 μL. They were incubated with anti-idiotype (0.1 mg/mL) or monoclonal antibody (at the concentration recommended by the manufacturer), or both (for dual staining), in PBS/0.5% BSA/0.05% azide for 30
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...cold buffer. or 2 mL F(ab’)2 rabbit anti-sheep Ig-FITC, or 2 mL F(ab’)2 sheep anti-mouse Ig-FITC, as the case may be, per 100 mL of cells. Finally, the cells were fixed in 2% paraformaldehyde and stored in the dark on ice until analysis within the next two days.

Flow Cytometry

Cells were analyzed using the Coulter Epics V system. The argon laser was operated at 488 nm using 240 mW of power. Red and green dual fluorescence analysis was performed by splitting the beam with a 560 short pass dichroic mirror, with a 590 long pass filter in front of the green laser. Side (90°) light scatter and fluorescence signals were logarithmically amplified.

Cell Culture

The cells were cultured in 250 μL in round well 96-well culture plates as described before.44 When present, the concentrations of the mitogens and cytokines were as follows: PWM, 2 to 3 μL/well; BCGF, 12.5 to 20 μL/well; IFN-γ or r-IFN-γ, 50 to 250 U/well; PMA, 0.5 to 12.5 ng/well. The cultures were set up in quadruplicate or sextuplicate. The assays for paraprotein, total IgG and IgM were based on the test developed by Ceska and Lundkvist45 with the modifications described previously.46 An additional important modification to the paraprotein assay was that the radiolabeled antibody was anti-idiotype not anti-IgG. Also, the incubation buffer was 0.1 mol/L borate/0.5 mol/L NaCl/0.5% bovine serum albumin/0.5% Tween 20/0.05% azide, pH 8.0.

RESULTS

Examination of the Surface Antigens, Cytoplasmic Paraprotein, and Morphology of Patients’ Cells

Detection of Circulating Surface-Idiotype* Cells by Rosetting and Flow Cytometry

Table 1 shows small numbers of circulating surface-idiotype* lymphocytes in four patients at some stage of their

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Table 1. Surface-Idiotype (Id)* Blood Cells, by Rosetting and Flow Cytometry

<table>
<thead>
<tr>
<th>Patient</th>
<th>Treatment Status (Courses of Chemotherapy)</th>
<th>Assay System</th>
<th>CD11b* PBMC</th>
<th>CD11b* PBMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>LD</td>
<td>Diagnosis, 9/86 (0)</td>
<td>Flow</td>
<td>8 (CD19*)</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>Continuing, 1/87 (2)</td>
<td>Rosette</td>
<td>19 (lg*)</td>
<td>1.9 (small)</td>
</tr>
<tr>
<td></td>
<td>Continuing, 2/87 (2)</td>
<td>Rosette</td>
<td>63 (lg*)†</td>
<td>10.4 (small/medium)</td>
</tr>
<tr>
<td></td>
<td>Continuing, 9/87 (8)</td>
<td>Flow</td>
<td>33 (CD19*)</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>BM</td>
<td>Continuing, 3/87 (7)</td>
<td>Rosette</td>
<td>47 (lg*)</td>
<td>3.8 (small/medium)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>9.9 (medium)</td>
</tr>
<tr>
<td>GM</td>
<td>Continuing, 8/87 (21 since end of remission)</td>
<td>Flow</td>
<td>5 (CD19*)</td>
<td>?§</td>
</tr>
<tr>
<td>AS</td>
<td>Diagnosis, 8/86 (0)</td>
<td>Rosette</td>
<td>8 (lg*)</td>
<td>1.4 (small)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.9</td>
</tr>
<tr>
<td>JW</td>
<td>Remission, 7/86</td>
<td>Rosette</td>
<td>24 (lg*)†</td>
<td>0.6 (small)</td>
</tr>
<tr>
<td></td>
<td>Remission, 9/86</td>
<td>Flow</td>
<td>16 (CD19*)</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Remission, 3/87</td>
<td>Flow</td>
<td>44 (lg*)†</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>Relapse, 1/87 (3 since end of remission)</td>
<td>Flow</td>
<td>5 (CD20*)</td>
<td>0.7</td>
</tr>
</tbody>
</table>

*As estimated by light microscopy (during the rosette assay).
†During flow cytometry, cells were classified as lymphoid by light scatter.
‡These cells had also been partially T-cell-depleted, using anti-CD2 monoclonal antibody.
§The anti-idiotype antibody bound to some autologous T cells but its cross reaction with allogeneic normal PBL makes it very unlikely that this binding was tumor-specific.
†The batch of anti-Leu-12-FITC used in this experiment was faulty.
‡CD20* by the BRBC-Covasphere dual labeling technique.
Fig 1. Tumor cells in the blood of LD and BM. (A, B, and C) Surface-idiotype* cells forming rosettes with anti-idiotype coated BRBC. The PBMC are stained with toluidine blue. (A) A small/medium sized lymphocyte from LD, with scanty cytoplasm, forming a good, "tight" rosette. Note the prominent nucleolus. (B) A slightly larger lymphoplasmacytoid cell from LD, with more cytoplasm and a prominent nucleolus. The very loose structure of the rosette indicates weak expression of membrane idiotypic determinants. (C) An atypical medium sized lymphocyte from BM, with weak expression of surface idiotypic determinants and a kidney shaped nucleus. (D, E, and F) Cells that have been cytospun, fixed, and stained for paraprotein with sheep anti-idiotype then rabbit anti-sheep Ig-FITC. (D) Two lymphoplasmacytoid cells from LD, with differing amounts of cytoplasmic paraprotein. (E) A large plasma cell from LD with brightly stained cytoplasm. (F) A lymphoplasmacytoid/plasmacytoid cell, and a lymphoid cell, from BM.
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disease (including the patient in remission, JW). The tumor lymphocytes in patients AS and JW were CD19+ or CD20+ by dual surface antigen analysis. In patient AS, the surface-idiotype+ lymphocytes were CD10+, whereas in patient JW, most were CD10+. No PCA-1+ cells were detected in the blood of either AS or JW.

In one experiment, most of the polyclonal B cells (all kappa+ B cells) were removed from the CD11b+ PBMC of patient JW (who had IgGa myeloma) by means of rosetting with anti-kappa coated BRBC. This procedure did not remove any of the idiotype+ cells. Thus, the anti-JW-idiotype did not detect any determinants, either endogenous or cytophilic, on polyclonal (ie, nontumor) B cells.

In the blood of patients LD and BM there were two surface-idiotype+ cell types forming rosettes: small to medium sized lymphocytes (Fig 1A); and medium to large sized cells with increased cytoplasm, and decreased expression of surface idiotypic determinants (shown by the loose or incomplete structure of the rosette). In LD's PBMC, the nucleus of these latter cells contained a single prominent nucleolus on toluidine blue staining and the cells appeared lymphoplasmacytoid (Fig 1B). Some of LD's idiotype+ typically lymphoid cells also had the prominent nucleolus (Fig 1A). The medium sized, weakly surface-idiotype+ cells in BM's PBMC generally had kidney shaped nuclei (Fig 1C). In the cell populations from both LD and BM, there were also occasional nonrosetting cells resembling plasma cells.

Not shown in Table 1 is that, in LD's CD11b+ PBMC taken at diagnosis, 7.2% of the cells were surface-idiotype+ ,PCA-1+ ; presumably these were also tumor cells. None of the tumor cells were detectable in the blood of LD by September 1987, after continuing chemotherapy.

Examination of PBMC for Cytoplasmic Paraprotein

PBMC isolated from four patients were examined for cells containing intracellular idiotype and IgG. The PBMC were taken from LD, BM, and JW when they had circulating surface-idiotype+ cells, and from GM after substantial chemotherapy.

LD's and BM's PBMC contained lymphoplasmacytid and plasma cells whose cytoplasm reacted with anti-idiotype (Fig 1) and anti-IgG (not shown). The staining was brightest in the plasma cells (Fig 1E). Some lymphocytes from LD and BM also stained, either by virtue of a small amount of cytoplasmic paraprotein or because of membrane paraprotein (Fig 1F). GM's PBMC contained only rare cytoplasmic-idiotype+ plasma cells (0.05% to 0.1%), and JW's PBMC were negative for cells containing intracellular idiotype.

Flow Cytometric Analysis of Patients' BMMC

Samples of BMMC from two patients were subjected to dual surface antigen analysis. Both samples had been stored in liquid nitrogen and then thawed, a process that (it was discovered) damages the CD10 antigen (data not shown). Therefore, before staining for analysis, JW's cells were incubated overnight in phenol-red-free medium at 37°C to allow re-expression of CD10. Both cell samples were depleted of CD11b+ cells, and LD's cells were also depleted of T cells using the anti-CD2 monoclonal antibody and panning.

In both cell samples there were two distinct groups of cells by light scatter (Fig 2). Cells designated group 1 had light scatter characteristic of typical lymphocytes, while cells in group 2 had greater 90° scatter, and most had greater low angle scatter, which was indicative of larger cells. A gate was placed around each group and the fluorescent cells within were analyzed. On the assumption that there would be negligible normal plasma cells in these samples, surface-idiotype+ ,PCA-1+ cells were classified as tumor cells, ie, they were assumed to be cytoplasmic-paraprotein+ . Tumor cells, therefore, were defined by the presence of surface idiotype and/or PCA-1. In Table 2 the size of each tumor
Table 2. Subpopulations of Patients’ Bone Marrow Tumor Cells Defined by Light Scatter and Surface Antigens

<table>
<thead>
<tr>
<th>Cell Group*</th>
<th>Surface Antigens</th>
<th>% Total Marrow Tumor Cells† in Each Subpopulation, for Patient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>LD‡</td>
</tr>
<tr>
<td>1</td>
<td>Id+, PCA-1+</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Id-, PCA-1+</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Id+, PCA-1+</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Id+, CD19+</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Id+, CD10 (U5)</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Id+, PCA-1-</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>Id+, PCA-1-</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>Id-, CD19+</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Id-, CD10 (U5)</td>
<td>23</td>
</tr>
</tbody>
</table>

*Cell groups were defined by light scatter as in Fig 2.
†Tumor cells were defined as cells with surface idiotype (Id) and/or PCA-1.
‡Cells were isolated in 6/86, simultaneous with disease diagnosis. After CD2- and CD11b-depletion, 64% of the group 1 + group 2 cells were tumor cells.
§Cells were isolated in 6/87 when the patient had relapsed but before recommencement of treatment. After CD11b-depletion, 38% of the group 1 + group 2 cells were tumor cells.
¶These CD19+ cells were assayed using anti-B4-RD1.
||The real percentage is not known because of the poor reactivity of the batch of anti-Leu-12-FITC used in this experiment.

As with patient LD, JW’s BMMC tumor cells contained a subpopulation of B lymphocytes (group 1). Furthermore, within each morphologically distinct tumor cell population (ie, group 1 and group 2) there was a subpopulation of CD10+ cells (Table 2, Fig 3).

Culture Studies
Culturing Cells That Contained Lymphoplasmacytoid and/or Plasmacytoid Tumor Cells

Patients LD, BM, and GM had, at some stage in their disease, circulating cells that synthesized and secreted para-

Fig 3. Dual fluorescence analysis of JW’s CD11b+ BMMC in cell group 2. Surface idiotype and CD10 antigen were detected using biotinylated anti-idiotype then streptavidin-PE (red) and anti-JS-FITC (green). The low level green fluorescence of a group of cells in the control is probably a consequence of the overnight incubation of the cells before staining.
protein when their PBMC were cultured in medium alone, i.e., in the absence of PWM or exogenous cytokine. Such paraprotein synthesis is referred to herein as spontaneous. Table 3 shows that PWM inhibited that synthesis. These were the patients who had been found to have circulating plasmacytoid tumor cells.

Table 3 also shows that by September 1987, LD’s PBMC were no longer secreting paraprotein. The 16 ng of paraprotein present at the start of that culture, and then released into the supernatant, can be accounted for by serum paraprotein that was one of activation of Ig synthesis (Table 3).

The response of the nontumor B cells to PWM was assessed by measuring IgM secretion. If there was a surface-idiotype or PCA-1+ cells, presumably as a result of inhibition of paraprotein synthesis by PMA. There were 1 x 10⁵ and T cells: stimulation of polyclonal Ig synthesis and monocytes.

Table 3. PWM Induced Ig Response of PBMC,* Which Contained Cells Already Secreting Paraprotein

<table>
<thead>
<tr>
<th>Patient</th>
<th>Treatment Status (Courses of Chemotherapy)</th>
<th>Culture Period (days)</th>
<th>Cell-Associated Paraprotein at Day 0 (ng/well)</th>
<th>Supernatant Ig† ng/well</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM</td>
<td>Continuing, 8/86 (13 since end of remission)</td>
<td>15</td>
<td>NM</td>
<td>41 ± 9</td>
</tr>
<tr>
<td></td>
<td>Continuing, 8/87 (21)</td>
<td>3</td>
<td>&lt;10</td>
<td>29 ± 5</td>
</tr>
<tr>
<td>BM</td>
<td>Continuing, 8/87 (7)</td>
<td>3</td>
<td>10 ± 1</td>
<td>47 ± 7</td>
</tr>
<tr>
<td>LD</td>
<td>Diagnosis, 9/86 (0)</td>
<td>12</td>
<td>22 ± 7</td>
<td>52 ± 2</td>
</tr>
<tr>
<td></td>
<td>Continuing, 1/87 (2)</td>
<td>11</td>
<td>39 ± 7</td>
<td>75 ± 14</td>
</tr>
<tr>
<td></td>
<td>Continuing, 9/87 (8)</td>
<td>7</td>
<td>&lt;10</td>
<td>17 ± 1</td>
</tr>
</tbody>
</table>

Abbreviation: NM, not measured.

Data expressed as mean ± 1 SD, from quadruplicates or sextuplicates.

*Two x 10⁵ unfractionated PBMC/250 μL/well.

†At the end of the culture.

Bovine aortic endothelial cells, which have been shown to enhance PWM-stimulated polyclonal Ig production by normal PBMC,26 likewise enhanced PWM-stimulated polyclonal Ig synthesis by patients’ PBMC, but depressed spontaneous paraprotein synthesis (data not shown).

The effects of IFN-γ and r-IFN-γ were tested on LD’s September 1986 cells: 50 and 250 U of IFN-γ/well caused 40% and 60% inhibition, respectively, of spontaneous paraprotein synthesis by unfractionated PBMC (2 x 10⁵/well) during a nine-day culture; 50 and 250 U of r-IFN-γ/well, added to unfractionated BMMC (1 x 10⁵/well), caused 40% and 50% inhibition, respectively.

Figure 4 shows that the addition of PMA to purified CD2⁺CD11b⁺ PBMC from LD inhibited spontaneous paraprotein synthesis, while simultaneously stimulating polyclonal Ig synthesis. Note that in the absence of PMA, paraprotein was still being secreted after 31 days of culture. This figure also shows that the synthesis and secretion of paraprotein by these cultured cells was occurring from the earliest time the supernatant paraprotein was measured (ie, day 2). The most plausible explanation is that it was due to tumor cells that were secreting, or about to secrete, paraprotein at the time they were taken from the patient.

Response of PBMC That Contained Tumor B Lymphocytes in the Absence of Lymphoplasmacytoid or Plasmacytoid Tumor Cells

Experiments described in the first part of Results and in reference 26 demonstrated the existence of small numbers of surface-idiotype⁺, CD19⁺,CD20⁺ tumor B lymphocytes, and no other types of myeloma cells, in the blood of patients AS and FE. Unfractionated PBMC from those patients were cultured with PWM, BCGF, and endothelial cells, to assess the capacity of the myeloma and the nontumor B lymphocytes to differentiate.

At the time FE’s cells were taken for culture, 1.7% of the CD11b⁺ PBMC were surface-idiotype⁺ B cells.26 At diagnosis (August 1986), 1.4% to 1.9% of the CD11b⁺ PBMC from AS were myeloma B cells.

Figure 5 shows the polyclonal IgG response by cells from patient AS. It is typical of the polyclonal Ig response by PBMC from the patients and from normal individuals. The effect of PWM was enhanced by BCGF and by bovine endothelial cells, and the enhancing effects of the endothelial cells and the BCGF were additive.
Table 4 shows the IgM and, more importantly, the paraprotein response of PBMC from the two patients to PWM and BCGF. There was a small (but significant, by Student's t test) paraprotein response to each stimulus. When neither PWM nor BCGF were added, the paraprotein present at the end of the cultures was the same as that present at the beginning (day 0, cell-associated), showing that no paraprotein was synthesized in the absence of mitogen or cytokine. When BCGF and PWM were added together, the paraprotein response was no greater than when BCGF only was added, in contrast to the polyclonal Ig response. FE's cells gave a good polyclonal IgM response not only to PWM, but also to BCGF in the absence of PWM.

Bovine endothelial cells neither enhanced nor inhibited the synthesis of paraprotein due to these stimuli (data not shown).

The kinetics of the synthesis of paraprotein as a result of stimulation of AS's November 1986 cells are shown in Fig 6. When both the cell-associated paraprotein at the end of the culture (see values marked with asterisks) and the supernatant paraprotein were measured, it was clear that the cells could be stimulated by either PWM or BCGF, but the best response was to BCGF. The small amount released in the absence of stimulus was equal to the cell-associated paraprotein.

Table 4. Response of PBMC,* Which Contained Tumor B Cells,† to PWM and BCGF

<table>
<thead>
<tr>
<th>Patient</th>
<th>Culture Period (days)</th>
<th>IgM Present at End of Culture</th>
<th>Paraprotein</th>
</tr>
</thead>
<tbody>
<tr>
<td>FE§</td>
<td>7</td>
<td>-PWM, -BCGF</td>
<td>5.7 ± 0.2</td>
</tr>
<tr>
<td>AS‖</td>
<td>6</td>
<td>-PWM, -BCGF</td>
<td>13.5 ± 0.7</td>
</tr>
<tr>
<td>AS‖</td>
<td>9</td>
<td>+PWM, +BCGF</td>
<td>16.0 ± 0.3</td>
</tr>
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</table>

Abbreviations: ND, not done (PWM was not added to this culture); NM, not measured.

Data expressed as mean ± 1 SD, from quadruplicates.

*Two × 10⁶ unfractionated cells/250 µL/well.
†There were no detectable lymphoplasmacytoid or plasmacytoid tumor cells.
‡Supernatant Ig + cell associated Ig.
§Cells were taken 1 month after the first course of chemotherapy
‖Cells were taken at diagnosis.
¶Cells were taken 1 month after the third course of chemotherapy.
tein at day 0 and was obviously not synthesized during the course of the culture.

Samples of AS’s cultured cells were examined but contained no detectable cytoplasmic-idiotype* plasma cells, whether they had been stimulated or not.

**DISCUSSION**

The results confirm the presence of circulating surface-idiotype* B lineage cells in many multiple myeloma patients. Most importantly, however, the results illuminate the heterogeneity within the tumor cell population, by antigenic, morphologic, and functional criteria. It is clear that the tumor clone in many patients is composed of lymphoid, lymphoplasmacytoid, and plasmacytoid cells.

Could the cells identified as surface-idiotype* in these experiments really have been nontumor B cells bearing either cytophilic paraprotein or a cross reactive idiotype? We think this highly unlikely for the following reasons.

First, the experiments that showed that null cells and monocytes of myeloma patients may still bind detectable cytophilic paraprotein after washing and incubation at 37°C, also showed that the B cells did not.29

Second, over a period during which the patient received regular treatment, idiotype* cells disappeared from LD’s blood and yet substantial numbers of nontumor B cells were still detectable. The most reasonable explanation for this selective loss of circulating idiotype* cells is that the chemotherapy reduced the tumor to the extent that tumor cells ceased to enter the bloodstream. The alternative explanation, that the serum paraprotein level became so low that there was none available to bind to nontumor B cell Fe receptors, is not tenable: although 8.7 g/100 mL at diagnosis, it was still 2.7 g/100 mL at the time there were no longer any detectable idiotype* cells among the CD11b PBMC.

Third, some of the surface-idiotype* cells had an abnormal surface phenotype (ie, were CD10*).

Fourth, the anti-idiotype antibodies were highly specific. They were, for example, detecting only one of 20,000 molecules of normal, polyclonal IgG. This establishes their specificity for a private idiotype.46

Fifth, removal of most of the polyclonal B cells (ie, cells expressing the other Ig light chain isotype to that of the paraprotein) from JW’s CD11b PBMC, did not eliminate any of the CD20+ lymphocytes detected by the anti-idiotype antibody. This rules out cytophilic idiotype on polyclonal B cells. It is also inconsistent with the detection of cross-reactive idiotypes on nontumor B cells, unless it is envisaged that such determinants were, for some reason, confined to B cells expressing the same Ig light chain isotype as the myeloma paraprotein.

Tumor cells (idiotype*) have now been found in the blood of five of eight patients using this experimental approach (this report and reference 26). Each of the five patients had circulating tumor B lymphocytes (by morphology, surface paraprotein, and the presence of CD19 and CD20), and two of the five also had circulating lymphoplasmacytoid and plasmacytoid cells (by morphology, decreasing surface in conjunction with increasing cytoplasmic paraprotein, surface PCA-1, and active secretion of paraprotein). These circulating tumor cells could be responsible for the continuing dissemination of the disease. In one patient (LD) all the tumor cells (lymphoid, lymphoplasmacytoid, and plasmacytoid) disappeared from the blood after continuing chemotherapy. In another patient (JW) the tumor cells were still detectable in the blood throughout remission.

The circulating tumor B cells were a subpopulation of the total blood B cells (Table 1). Their numbers, even in newly diagnosed patients, were not sufficient to distort the K/A B cell ratio beyond the normal range (data not shown). This confirms the results of a minority of investigators in the field.21,24,26

The absence of such cells in treated patients29 can be explained as a consequence of the treatment (as seen with LD), but their absence in some newly diagnosed patients,30 is at variance with the results of other investigators who found large numbers in all newly diagnosed patients tested.17,18,20,22

This is hard to explain, unless the difference simply reflects different patients. The experimental approach to the problem of cytophilic paraprotein was different in that the other groups did not remove null cells and monocytes, but they did remove surface Ig by trypsin or anti-Ig and reported the regeneration of surface idiotype in culture.

A subpopulation of JW’s tumor B lymphocytes were aberrant in that they expressed CD10. The aberrant expression of CD10 on a fraction of plasmacytoid cells in some patients has been described before,47,51 and is considered to indicate a poor prognosis.50 Abnormal numbers of CD10+ lymphocytes in the blood of myeloma patients have also been described before.47,52 This report shows that at least some CD10+ lymphocytes in the blood and bone marrow of some patients are unquestionably tumor cells by their expression of surface idiotype and, indeed, are B lymphocytes with an aberrant phenotype. These cells are different from, but analogous to, the CD10+ lymphocytes established in culture from the marrows of three myeloma patients by Grogan et al.48 Those cells also had an aberrant phenotype, with antigenic features of pre-B lymphocytes and mature plasma cells, but they were surface-Ig*.

The culture experiments revealed that myeloma cells similar in surface and cytoplasmic antigens and morphology to normal plasma cells were in the process of synthesizing paraprotein, and that synthesis was inhibited by PWM, IFN-γ, endothelial cells, and PMA. Thus, these myeloma cells were inhibited by agents that inhibit nonmyeloma plasma cells: it is already known that normal (nontumor) plasma cells from the marrow of myeloma patients in remission50 and patients without lymphoid tumors53 are inhibited by PWM; likewise, PMA is known to be able to inhibit Ig production by mature B cell lines.54 and IFN-γ has been shown to inhibit polyclonal Ig production by PWM stimulated B cells55 and directly inhibit B cell activation due to soluble T cell helper factors.56 Regarding the inhibitory effect of the endothelial cells, it may be relevant that Zimpeli et al have reported that an endothelial–cell-like clone derived from mouse bone marrow stromal cells produced an inhibitor of the growth of mouse plasmacytoma cells.57

Finally, the culture experiments showed that the tumor B lymphocytes were activated to synthesize paraprotein by
agents (ie, PWM and BCGF) that activate some normal B lymphocytes to synthesize polyclonal Ig. This activation was limited and, unlike the stimulation of normal B lymphocytes in patients and healthy individuals, the effect of the PWM was not enhanced by endothelial cells or by BCGF. The low level of paraprotein production by the PWM- or BCGF-activated myeloma B lymphocytes indicates they did not differentiate in culture into plasma cells. This was proven by the absence of detectable idiotype* plasma cells at the end of the culture of AS’s PBMC. The limited nature of the response may be one reason why it has not been recognized before. It is also clear that some myeloma patients have small numbers of circulating tumor cells already synthesizing and secreting paraprotein. Since this stimulation is inhibited by PWM, a positive response to PWM by tumor B lymphocytes would be hidden in cultures of PBMC from those patients. The limited response of the myeloma B lymphocytes to the activating signals does not prove that they are incapable of differentiation to plasma cells but may reflect lack of suitable culture conditions. It is well known that normal hematopoiesis in the bone marrow requires the presence of an appropriate stromal cell environment, which is also required for the development of many leukemic cells.4 Investigations of the interactions of stromal cells and myeloma cells may lead to an understanding of how human myeloma cells develop, and why this tumor is predominantly located in the marrow.

The data in this study on the antigenic, morphologic, and functional intraclonal heterogeneity of myeloma cells contribute to an intriguing, complex picture of cellular heterogeneity in multiple myeloma. The evidence is consistent with myeloma cell differentiation, and suggests that it need not necessarily be grossly abnormal in its various features. The latter stages of the normal B lineage are characterized by acquisition of PCA-1, increasing cytoplasmic and decreasing surface Ig, and loss of CD19 and CD20.38,40 The antigenic and morphologic experiments described herein (with blood and bone marrow) revealed that a myeloma patient may have tumor cells ranging from surface-idiotype*,CD19*, CD20*,PCA-1*,cytoplasmic-idiotype* lymphocytes, to CD19*,PCA-1*,cytoplasmic-idiotype* plasma cells that are surface-idiotype* or weakly surface-idiotype*. However, the abnormal phenotype of some of the cells found in this study (surface-idiotype*,CD10*), and the cells described by Caligaris-Cappio et al (CD10*,CD19*,CD20*,surface- and cytoplasmic-Ig*),47 and by Grogan et al (CD10*,CD19*,CD20*,PCA-1*,cytoplasmic-μ*,surface-Ig*),48 support the hypothesis that, if myeloma cell differentiation is indeed occurring, it may not be reflected in exactly the same sequence or pattern of surface antigens as is to be found in normal B lineage cell differentiation.48

ACKNOWLEDGMENT

Many thanks go to Scott Andrew for showing us how to operate the flow cytometer, to Dr J.V. Wells for his critical reading of the manuscript, to Professor A. Basten for some helpful suggestions, to Justine Roberts for her perseverance in typing the Tables, and to the Royal North Shore Hospital Staff Specialists Trust Funds for financial assistance in the purchase of materials.

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