Activated Idiotype-Reactive Cells in Suppressor/Cytotoxic Subpopulations of Monoclonal Gammopathies: Correlation With Diagnosis and Disease Status

By Umberto Dianzani, Alessandro Pilieri, Mario Boccadoro, Antonio Palumbo, Paola Pioppo, Alberto Bianchi, Alessandro Camponi, Giorgio Fossati, Silvano Battaglio, and Massimo Massaia

The phenotypic pattern of peripheral blood T (PBT) lymphocytes was correlated with diagnosis and clinical status in 63 patients with monoclonal gammopathies (MGs). The numbers of lymphocytes expressing activation and CD11 determinants were significantly increased in suppressor/cytotoxic and helper/inducer subpopulations of patients with multiple myeloma (MM) and MG of undetermined significance (MGUS). The number of activated suppressor/cytotoxic cells was closely correlated with diagnosis and disease status. These cells were significantly higher in MM patients with monoclonal gammopathies (MGs) suggesting their central role in the immune dysregulation of these diseases. In multiple myeloma (MM), T lymphocytes displaying the suppressor phenotype (CD11+, ecto-5'-nucleotidase) and granular morphology are expanded in suppressor/cytotoxic subpopulations. In normal subjects, these lymphocytes suppress proliferation and differentiation of B lymphocytes into immunoglobulin-secreting cells. Their expansion in MM may contribute to the arrest in terminal B-cell maturation and polyclonal hypergammaglobulinemia. Lymphocytes carrying CD11 determinants also appear in helper/inducer subpopulations of MM patients. So far, no function has been attributed to these cells, which are rarely found in normal peripheral blood and normally populate reactive lymph nodes. Finally, cells expressing activation markers (HLA-DR+) have been detected in helper, suppressor, and cytotoxic precursor subsets of MM, indicating their functional activation. Patterns of phenotypic changes suggest a single coordinated T-cell deregulation in MM.

ALTERATIONS in peripheral blood T lymphocytes of patients with monoclonal gammopathies (MGs) suggest their central role in the immune dysregulation of these diseases. In multiple myeloma (MM), T lymphocytes displaying the suppressor phenotype (CD11+, ecto-5'-nucleotidase) and granular morphology are expanded in suppressor/cytotoxic subpopulations. In normal subjects, these lymphocytes suppress proliferation and differentiation of B lymphocytes into immunoglobulin-secreting cells. Their expansion in MM may contribute to the arrest in terminal B-cell maturation and polyclonal hypergammaglobulinemia. Lymphocytes carrying CD11 determinants also appear in helper/inducer subpopulations of MM patients. So far, no function has been attributed to these cells, which are rarely found in normal peripheral blood and normally populate reactive lymph nodes. Finally, cells expressing activation markers (HLA-DR+) have been detected in helper, suppressor, and cytotoxic precursor subsets of MM, indicating their functional activation. Patterns of phenotypic changes suggest a single coordinated T-cell deregulation in MM.

The wide range of alterations described is in keeping with the heterogeneity of clinical presentation and progression of MG, which ranges from aggressive, chemoresistant MM to smouldering MM and MG of undetermined significance (MGUS). Features intrinsic to the neoplastic cells, such as proliferative activity or plasmablastic morphology, mainly determine the progress of the disease. However, monocytoid and T lymphocytes may regulate the neoplastic clone. We studied the phenotypic pattern of peripheral blood T (PBT) lymphocytes in 63 patients with MG and correlated the findings with clinical disease status. In a subset of patients with IgG MM, we examined the reactivity of the suppressor/cytotoxic subpopulations with the patients' own M-protein to better understand their relation to the neoplastic subset.

MATERIALS AND METHODS

Patients. Sixty-three patients with MG were studied; 38 had MM, 25 had MGUS. Diagnosis was determined as previously reported. Those with MM were further divided according to clinical data and some of them were evaluated more than once when they shifted from one group to another during the follow-up. Thirteen MM patients were at diagnosis (MMd: one stage IA, two stage II, ten stage IIIA). Twenty-one MM patients were on chemotherapy (MM: three stage IIA, 16 stage IIIA, two stage IIIB). Of these, 11 were on first choice chemotherapy, according to the MS3 Italian Multiple Myeloma Study Group protocol (VMCP/VBAP, n = 6; MP, n = 5), and ten patients were on salvage chemotherapy (high doses cyclophosphamide [CTX], n = 7; melphalan/prednisone [MP] intravenous [IV], n = 3). All patients were studied at least 3 weeks after the last day of chemotherapy. Twelve MM patients were in “unmaintained” stable remission phase (MMr: two stage IA, two stage IIA, eight stage IIIA). They obtained a >50% reduction of the M-component and had been off therapy for at least 5 months (mean ± SD: 8 ± 5; range, 3 to 14). Eighteen MM patients had tumor progression (MMtp: two stage I, two stage II, 12 stage IIIA, two stage IIIB). Of these, four were in relapse, and 14 presented response failure or progression under chemotherapy (four on first choice therapy, ten on salvage chemotherapy).

Patients were not on antibiotics, did not have infections, and had not received transfusions for at least 2 weeks before the study.

Cell preparation. Current denomination, cluster designation (CD), and monoclonal antibody (MoAb) reactivity of lymphocyte subsets investigated in this study are listed in Table 1. Monocytes were removed by plastic adherence, and T lymphocytes were isolated by E-rosette formation with sheep erythrocytes treated with 2-aminoethylisothiouronium bromide and centrifugation on Ficoll-Hypaque gradients. The purity of T-cell preparations, checked by OKT1 (CD2) MoAb, was always >95%. B-cell and monocyte contamination, checked by indirect immunofluorescence (IF) using B1 (CD19), B4 (CD20) (Coulter Clone, Hialeah, FL), and LeuM1 (CD15) (Becton Dickinson, Mountain View, CA) MoAbs (Table 1) was always <2%.

In some experiments, suppressor/cytotoxic subpopulations were isolated from T-cell preparations by using the panning technique, as

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**ACTIVATED IDIOTYPE-REACTIVE T CELLS IN MG**

**Table 1. Cluster Designation and MoAb Reactivity of T-Lymphocyte Subsets Investigated in This Study**

<table>
<thead>
<tr>
<th>CD</th>
<th>Antibodies</th>
<th>T-Lymphocyte Subsets*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+</td>
<td>Leu3+</td>
<td>Helper/inducer cells</td>
</tr>
<tr>
<td>CD8+</td>
<td>Leu2+</td>
<td>Suppressor/cytotoxic cells</td>
</tr>
<tr>
<td>CD8+ CD11+</td>
<td>Leu2+ Leu15+</td>
<td>Suppressor cells</td>
</tr>
<tr>
<td>CD8+ CD11-</td>
<td>Leu2+ Leu15-</td>
<td>Cytotoxic precursor cells</td>
</tr>
<tr>
<td>CD8+ HLA-DR+</td>
<td>Leu2+ anti-HLA-DR+</td>
<td>Activated suppr./cytot. cells</td>
</tr>
<tr>
<td>CD4+ CD11+</td>
<td>Leu3+ Leu15+</td>
<td>Helper/CD11+ cells†</td>
</tr>
<tr>
<td>CD4+ HLA-DR+</td>
<td>Leu3+ anti-HLA-DR+</td>
<td>Activated help./ind. cells</td>
</tr>
</tbody>
</table>

Abbreviation: CD, Cluster designation.

*Proposed functional capability.
†No definite function.

Previously reported.12 The purity of cell preparations was checked by direct IF using phycoerythrin (PE)-conjugated Leu2 MoAb (Becton Dickinson). Purity was always >80%. Cell viability was always higher than 95% by the Trypan-blue exclusion.

**Immunofluorescence studies.** Cell preparations were analyzed by double-color IF using Leu2, Leu3, Leu15, and anti-HLA-DR MoAbs (Becton Dickinson) (Table 1). Direct IF was performed when FITC-conjugated and PE-conjugated MoAbs were available. When no FITC-conjugated MoAb was available, an indirect IF was performed with unconjugated MoAb and goat anti-mouse immunoglobulins as the second layer; cells were then incubated for 30 minutes with 5 μL of purified mouse immunoglobulins (Coulter) to saturate free binding sites of the second layer, and the appropriate PE-conjugated MoAb was added. A Leitz Orthoplan microscope was used to score the percentage of positive cells.

**M-protein purification.** Immunoglobulins were isolated from serum by precipitation in ammonium sulfate 45% and dialyzed against 0.015 mol/L phosphate buffer, pH 8. Immunoglobulins were applied to columns of DEAE cellulose (De52; Whatmann, Inc, Clifton, NY), and eluted with buffers of increasing molarity (0.015 to 0.3, pH 8). The first peak, containing monoclonal IgG, was indicated. Results are expressed as means ± SD.

**DNA extraction and Southern blot analysis.** Purified DNA was transferred to a nitrocellulose filter. The blot was hybridized, washed, and autoradiographed as previously described.22 The T-beta probe was a cDNA T-beta clone (YT35) kindly provided by Dr C.M. Croce. To generate probes representative for the C region, the insert of YT35 plasmid was digested with HincII and the fragment corresponding to the C region purified by preparative agarose gel electrophoresis. For use as probes, DNA fragments were P32-labeled by nick-translation.22

**Statistical analysis.** Differences were analyzed using the Student’s t test or the Mann-Whitney U test for paired samples, as indicated. Results are expressed as means ± SD.

Clinical and immunological analysis were performed by two independent, experienced teams to exclude misleading data interpretation.

**Table 2. Immune Phenotype of Peripheral Blood T Cells From Normal Controls and Different Groups of MG Patients**

<table>
<thead>
<tr>
<th>Subpopulation</th>
<th>CD4:CD8 Ratio</th>
<th>Controls (n = 24)</th>
<th>MGUS (n = 25)</th>
<th>MMd (n = 13)</th>
<th>MMt (n = 21)</th>
<th>MMr (n = 12)</th>
<th>MMtp (n = 18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suppressor/Activ</td>
<td>2 ± 0.7</td>
<td>196 ± 69</td>
<td>63 ± 50</td>
<td>228 ± 145</td>
<td>250 ± 196</td>
<td>120 ± 58</td>
<td></td>
</tr>
<tr>
<td>Cells Cells</td>
<td></td>
<td>19 ± 25</td>
<td>41 ± 31</td>
<td>(.001)†</td>
<td>.01)†</td>
<td>.05)†</td>
<td></td>
</tr>
<tr>
<td>CD11+ Cells</td>
<td></td>
<td></td>
<td>55 ± 34</td>
<td>.001)†</td>
<td>116 ± 85</td>
<td>120 ± 58</td>
<td></td>
</tr>
<tr>
<td>Activated Cells</td>
<td></td>
<td></td>
<td>60 ± 35</td>
<td>(.001)†</td>
<td>36 ± 31</td>
<td>.05)†</td>
<td></td>
</tr>
</tbody>
</table>

*Results are expressed as mean cell numbers per microliter ± SD.
†Significantly different from the controls; the Student’s t test was used.
‡Significantly different from MMd patients.
§Significantly different from MGUS patients.
‡‡Significantly different from MMr patients.
Table 3. Depletion of Activated and Suppressor Cells in Suppressor/Cytotoxic Subpopulations of IgG MM Patients After Panning of PBT Lymphocytes on Related and Unrelated M-Protein Coated Dishes

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Starting Cell Prep Phenotype</th>
<th>Related M-Protein</th>
<th>Unrelated M-Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Activated</td>
<td>Suppressor</td>
<td>Activated</td>
</tr>
<tr>
<td>1a</td>
<td>44</td>
<td>52</td>
<td>12 (72)</td>
</tr>
<tr>
<td>1b</td>
<td>45</td>
<td>60</td>
<td>22 (51)</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
<td>90</td>
<td>27 (55)</td>
</tr>
<tr>
<td>3</td>
<td>56</td>
<td>68</td>
<td>34 (39)</td>
</tr>
<tr>
<td>4a</td>
<td>64</td>
<td>90</td>
<td>34 (47)</td>
</tr>
<tr>
<td>4b</td>
<td>40</td>
<td>88</td>
<td>23 (43)</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>36</td>
<td>4 (60)</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>34</td>
<td>0 (100)</td>
</tr>
<tr>
<td>7</td>
<td>68</td>
<td>84</td>
<td>16 (77)</td>
</tr>
<tr>
<td>8</td>
<td>15</td>
<td>58</td>
<td>14 (7)</td>
</tr>
<tr>
<td>9</td>
<td>9</td>
<td>56</td>
<td>8 (11)</td>
</tr>
</tbody>
</table>

P values: <.001† <.001† NS† NS† <.02‡ <.02‡

The percentage of depletion is given in parentheses.
Abbreviations: ND, not done; NS, not significant.

*Results are expressed as percentages of cells positive for the second MoAb (activated cells, anti-HLA-DR PE; suppressor cells, Leu15 PE) in suppressor/cytotoxic subpopulations defined by Leu2-FITC.
†P values for differences with starting cell preparations. A Mann-Whitney U test for paired samples was used.
‡P values for differences with cells panned on related M-protein.

RESULTS

PBT cell phenotypes. The number of activated, suppressor, and helper/CD11+ cells was significantly increased in MGUS and MMd patients compared with the controls (Table 2). The number of activated cells in suppressor/cytotoxic subpopulations was significantly higher in MMd than MGUS patients and decreased significantly in MMr compared with MMd patients (Table 2). Activated cells remained significantly higher in suppressor/cytotoxic subpopulations of MMtp compared with MMr patients (Table 2).

The number of suppressor cells was significantly lower in MMr than MGUS, MMd, and MMtp patients (Table 2). The number of helper/CD11+ cells was significantly lower in MMr than MGUS and MMd patients (Table 2). The CD4/CD8 ratio and the number of suppressor and helper/CD11+ cells were significantly lower in MMt than MMd patients (Table 2).

A subset of suppressor/cytotoxic cells adhere to M-protein–coated plates. T cells from nine IgG MM patients were plated on dishes coated with the related M-protein (Table 3). This resulted in a significant depletion of activated and suppressor cells in suppressor/cytotoxic subpopulation. Differences were not significant after panning on unrelated M-protein–coated dishes. Helper/inducer subpopulations showed no changes (data not shown). There was a direct correlation in individual patients between the numbers of activated and idiotype-reactive adherent cells (r = 0.95, P < .001).

In some experiments, enriched suppressor/cytotoxic subpopulations were plated and then analyzed by double-color IF: depletion of activated cells occurred in both suppressor and precursor cytotoxic subsets (Table 4).

T-beta gene rearrangement analysis of PBT lymphocytes. Organization of the T-beta gene locus was investigated in the total of PBT cells and enriched suppressor/cytotoxic subpopulations of two MM patients displaying a high expansion of activated cells. Neither case displayed monoclonal T-beta gene rearrangement (data not shown).

DISCUSSION

In this report, the expansion of activated, suppressor, and helper/CD11+ lymphocytes was correlated with clinical findings in a large series of MG patients. The number of activated cells in the suppressor/cytotoxic subpopulations was closely related to the disease status. These cells increased significantly from MGUS to MMd and remained greatly expanded in MMt patients. Their number declined to the MGUS values in the event of remission, whereas no decrease was observed in MMtp patients.

The number of suppressor cells was weakly related to the disease status: they were lower in MMr patients than MMd and MMtp patients, but their number was similar in MGUS and MMd. Phenotypic changes in helper/inducer subpopulations did not show clinical correlations.

The expansion of activated cells in suppressor/cytotoxic subpopulations was not directly influenced by chemotherapy or supportive care. The number of activated cells was significantly higher in MMd than MGUS patients, both groups being neither treated nor transfused. The negligible effect of chemotherapy was also confirmed by the observation of similar counts in MMd and MMtp patients, whereas suppressor and helper/CD11+ cell counts were significantly lower in MMt patients.

No correlation was found with serum M-protein levels,
and high counts of activated cells were detected in suppressor/cytotoxic subpopulations of nonsecretory (n = 1) and micromolecular MM (n = 3) as well. No relationship could be defined with renal failure, because only two patients had stage B disease.

A proportion of activated cells in suppressor/cytotoxic subpopulations specifically adhered to related M-protein coated dishes. In individual patients, the greater the number of activated cells, the higher was the binding of idiotype-reactive cells. Binding was idiotype- and not isotype-specific, ruling out T-gamma cells, whose expansion has been reported in IgG MM.25 No monoclonal T-beta gene rearrangement was detected in PBT and suppressor/cytotoxic subpopulations from two patients with a large proportion of activated cells, as recently reported.26 The presence of a polyclonal rather than a monoclonal expansion is not in conflict with the panning data. Heterogeneity of idiotype-reacting cells has been previously reported,27 and is suggested in this study by their variable phenotype (both suppressor and cytotoxic precursor).

It is interesting to observe that the expansion of idiotype-reactive, activated lymphocytes was associated with tumor progression. In mice, anti-tumor effector cells belong to cytotoxic and suppressor subsets and both types may display anti-idiotypic determinants.29-33 However, these cells are induced late in the course of tumor growth30 and their expansion may be too late to be effective. It is possible that part of the activated suppressor/cytotoxic cells in MM are the human counterpart of murine anti-tumor effector cells. They seem to produce an ineffective immune response to the tumor, because their counts increase as the disease progresses. Our follow-up data are in keeping with this conclusion: 13 patients died (11 MMtp, two MMd) in the year since the study ended. The mean number of activated cells in these patients was significantly higher than MMr patients (251 ± 194 v 58 ± 53; P < .01).

In conclusion, our MGUS and MM patients displayed different numbers of activated cells in suppressor/cytotoxic subpopulations. In MM patients, there was a significant relationship with the disease status: low counts in patients in stable remission and high counts in patients with tumor progression or response failure. Part of these cells are a polyclonal idiotype-reactive population. Future studies should be directed to the functional analysis of these cells.

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