Detection of Hyperreactive T Cells in Multiple Myeloma by Multivalent Cross-Linking of the CD3/TCR Complex

By Massimo Massaia, Alberto Bianchi, Carmela Attisano, Silvia Peola, Valter Redoglia, Umberto Dianzani, and Alessandro Pileri

Cellular immunity was investigated in 43 patients with multiple myeloma (MM) by assessing \(^{3}H\)Tdr uptake induced by monocyte-dependent [CD3 monoclonal antibodies (MoAbs), phytohemagglutinin (PHA)] and monocyte-independent (CD2 MoAbs, ionomycin + phorbolester) stimulations. The former were evaluated in peripheral blood mononuclear cells (PBMC) and purified T cells; the latter were evaluated in purified T-cell preparations only. MM showed significantly lower PBMC responses to PHA \((P < .001)\), soluble OKT3 (CD3) \((P = .01)\), and immobilized OKT3 MoAbs \((P = .01)\). On purification of T cells, MM responses were still defective to soluble T11\(_1\) + T11\(_2\) (CD2) MoAbs \((P = .004)\), phorbol myristate acetate (PMA) plus ionomycin \((P < .001)\), but significantly higher to plastic-immobilized OKT3 \((P < .001)\). In some MM, \(^{3}H\)Tdr uptake, interleukin-2 (IL-2) receptor (CD25) expression, and IL-2 production were as high on stimulation with plastic-immobilized OKT3 as that observed in normal subjects under optimal conditions (ie, plastic-immobilized OKT3 plus accessory signals). CD3 hyperreactivity correlated with the number of CD8\(^+\) HLA-DR\(^+\) cells in MM T-cell preparations. MM patients with more than 10% CD8\(^+\) HLA-DR\(^+\) cells had significantly higher responses to immobilized OKT3 \((P < .001)\), but lower responses to T11\(_1\) plus T11\(_2\) \((P = .01)\), and PMA plus ionomycin \((P = .03)\) than patients with less than 10% CD8\(^+\) HLA-DR\(^+\) cells. Phenotyping of CD45RA (naive) and CD45RO (memory) expressions in resting MM T cells showed a lower ratio of CD45RA to CD45RO in both CD4 \((P < .06)\) and CD8 \((P < .01)\) subpopulations. These data indicate that (a) some MM T cells require significantly fewer accessory signals (if any) to express the IL-2 receptor fully, secrete IL-2, and proliferate on multivalent cross-linking of the CD3/TCR complex, and (b) this peculiar state of activation is associated with high HLA-DR expression in CD8\(^+\) lymphocytes.

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A VARIETY OF phenotypic alterations have been demonstrated in T cells of patients with multiple myeloma (MM) based on the original finding that expression of the purine degradative enzyme ecto-5'-nucleotidase (now referred to as the differentiation lymphocyte antigen CD73) is defective in their CD8\(^+\) subpopulations.\(^1\) Activated (HLA-DR\(^+\)) cells have been reported in CD4\(^+\) and CD8\(^+\) subpopulations,\(^2\) and unbalanced proportions of CD8\(^+\) CD11b\(^-\) and CD8\(^+\) CD11b\(^+\) subsets, respectively, have been demonstrated in CD8\(^+\) subpopulations.\(^3,4\) Because precursors of alloreactive cytotoxic T cells (CTL) and lymphokine-activated killer (LAK) normally belong to the CD8\(^+\) CD11b\(^-\) and CD8\(^+\) CD11b\(^+\) subsets, respectively, CTL and LAK functions have also been investigated in MM patients. Both functions were defective,\(^5,6\) and correlations have been demonstrated with the disease status.\(^7,8\) Other studies have shown that MM-derived T-cell subpopulations display impaired responses to mitogens,\(^9\) abnormal immunoregulatory functions,\(^10,12\) and decreased cloning efficiency.\(^13\) Together, these data indicate that T-cell immunity is affected in MM patients, although T cells do not belong to the malignant clone.\(^14\)

Alternative approaches were recently developed to investigate T-cell functions. Surface molecules involved in T-cell activation and their natural ligands have been identified, and the cascade of metabolic events subsequent to this interaction has been characterized. The cross-linking of surface molecules by monoclonal antibodies (MoAbs) mimics naturally occurring interactions and can be used as a model system to investigate T-cell activation. A prominent role is played by the CD3 molecule, which transmits activation signals on binding of the T-cell receptor (TCR) to the Ag-major histocompatibility complex (MHC).\(^15\) Valency of CD3 cross-linking regulates the requirements of accessory signals to initiate T-cell proliferation. Interaction of CD3 with soluble MoAbs leads to generation of activation signals\(^16\) but is insufficient to initiate T-cell proliferation. Additional signals delivered by monocytes are required to augment CD3 stimulation and cause proliferation. The requirement for monocytes can be circumvented with phorbol myristate acetate (PMA)\(^17\) or CD2 MoAbs immobilized on Sepharose beads plus exogenous lymphokines.\(^18-20\) Only multivalent CD3 cross-linking by MoAbs immobilized on plastic initiates T-cell proliferation in the absence of additional signals however, provided that the density of MoAbs exceeds a critical threshold.\(^21\) An alternative activation pathway is mediated by the 50-Kd CD2 molecule,\(^22\) whose natural ligand is LFA-3.\(^23\) CD2/LFA-3 interactions play an important role in facilitating intercellular adhesion and regulating CD3-mediated stimulation.\(^24,25\) The appropriate pair of soluble CD2 MoAbs initiates T-cell proliferation in the absence of monocytes or other accessory signals.\(^2,7,26\) Unlike CD3 stimulation, plastic immobilization of CD2 MoAbs completely abrogates their stimulatory activity.\(^27\)

Metabolic events occurring in T cells on CD3 and CD2 stimulation have been characterized. Phosphorylation and dephosphorylation on tyrosine residues represent a very
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early event regulating G protein activation.\(^{28,29}\) Phospholipase C is then activated, and phosphatidylinositol breakdown is initiated. 1,2-Diacyl-glycerol and 1,4,5-inositoltriphosphate are produced: the former activates protein kinase C (PKC), while the latter increases free intracellular Ca\(^{2+}\) concentrations.\(^{16,30}\) All steps preceding PKC activation and Ca\(^{2+}\) mobilization can be circumvented by using PMA and Ca\(^{2+}\) ionophores, such as ionomycin.\(^{31}\) When T cell responses are abnormal, stimulation with PMA and ionomycin may be useful to locate the possible sites of defective activation down the T-cell signaling pathway.

In this study, T-cell functions were evaluated in 43 patients with MM by investigating their responses to CD3, CD2, and PMA plus ionomycin. The CD3 MoAb OKT3 was used in soluble and plastic-immobilized forms; the appropriate combination of CD2 MoAbs was used in the soluble form only. The panel thus consisted of monocyte-dependent (soluble OKT3, immobilized OKT3) and monocyte-independent (CD2, PMA plus ionomycin) stimulations.

We wished first to evaluate the ability of MM T cells to be activated by signals delivered through CD3/TCR and CD2 molecules, and second to define the accessory signals required to initiate T-cell proliferation fully on CD3/TCR stimulation.

MATERIALS AND METHODS

Patients. Forty-three MM patients entered this study from November 1989 to April 1991. MM was diagnosed as previously reported.\(^{32}\) According to the Durie and Salmon staging system,\(^{33}\) 6 patients were classified as stage II and 37 were stage III; 5 were stage IV. Twenty-nine were IgG, 9 were IgA, and 5 had Bence-Jones myeloma. Thirteen patients were evaluated at diagnosis; 22 were received first-line chemotherapy according to the Italian Myeloma Study Group protocols. All patients receiving treatment were studied at least 3 weeks after the last day of chemotherapy. Twelve patients were on maintenance therapy with recombinant interferon-\(\alpha\) (rIFN-\(\alpha\)).\(^{34}\) Two patients were in “unmaintained” stable remission phase,\(^{35}\) and 3 patients were in relapse. Sixty-two functional analyses were performed because some MM patients were evaluated more than once when they shifted from one group to another (ie, from diagnosis to chemotherapy). Patients were not receiving antibiotics, did not have infections, and had not received transfusions for at least 10 days before the study.

Controls were 32 normal blood donors aged >50 provided by the local Blood Bank and matched for sex and age. Thirty-two experiments were performed, each individual experiment including both normal and MM sample(s).

Lymphocyte isolation. The standard medium was RPMI 1640 containing 10% fetal calf serum (FCS, Gibco, Milano, Italy), 2 mmol/L glutamine, penicillin (100 U/mL), streptomycin (100 \(\mu\)g/mL), and amphotericin B (0.25 \(\mu\)g/mL). Peripheral blood mononuclear cells (PBMCN) were obtained by density-gradient centrifugation (Ficoll-Hypaque) of heparinized venous blood.\(^{36}\) Monocytes were removed by the carbonyl-iron method.\(^{37}\) The lysosomotropic compound L-leucine methyl ester (Leu-Ome) was used to deplete monocytes further.\(^{38}\) PBMCN depleted of monocytes by carbonyl-iron treatment were incubated for 45 minutes at room temperature with 5 mmol/L Leu-Ome, pH 7.4, in standard medium without FCS. T lymphocytes were isolated by rosetting with sheep erythrocytes at 29°C for 1 hour to exclude most CD3\(^+\), CD2\(^+\) rosette-forming cells.\(^{39}\) Cells forming rosettes (T lymphocytes) were isolated from nonrosetting cells on a Ficoll-Hypaque density gradient. Purity of T-cell preparations was 90% to 95%, as shown by cytofluorometric analysis with Leu-4-FITC (Becton Dickinson, Mountain View, CA). Contaminating monocytes were less than 0.1% to less than 0.5% in both MM-derived and normal T-cell preparations, as shown by the nonspecific esterase staining\(^{40}\) and cytofluorometric analysis with LeuM3-FITC MoAb (Becton Dickinson).

In a limited series of experiments, MM-derived T cells were further depleted of HLA-DR\(^+\) cells by an indirect panning technique using anti-HLA-DR MoAb (Becton Dickinson) and rabbit anti-mouse immunoglobulins (Dako, Milano, Italy), as previously reported.\(^{41}\)

In some experiments, T-cell preparations were depleted of monocytes by the plastic adherence method followed by carbonyl-iron and Leu-Ome treatments; 5 \(\times\) 10\(^5\) PBMCN/mL were incubated in RPMI + 20% FCS for 1 hour at 37°C in a humidified atmosphere of 5% CO\(_2\) in air. When monocytes were required as a source of accessory cells, adherent cells (AC) were gently recovered with a cell scraper. AC were 85% to 95% pure, as shown by the nonspecific esterase staining. Cell preparations were counted microscopically, and their viability as determined with the trypan blue exclusion dye test was always greater than 98%.

MoAbs. Various MoAbs were used for T-cell stimulation and phenotyping, including OKT3 (CD3, IgG2a) (American Tissue Culture Collection, [ATCC], Bethesda, MD); T11 (CD2, IgG\(_2\)) and T11 (CD2, IgG\(_2\)) (gifts from Dr Ellis Reinherz); UCHL1 (CD55R0, IgG\(_2\)) (Dako Immunoglobulins), 2H4 (CD45RA) (IgG\(_4\)) (Coulter Clone), Leu2 (CD8, IgG\(_1\)) (Becton Dickinson), Leu3 (CD4, IgG\(_1\)) (Becton Dickinson), anti-HLA-DR (IgG\(_2\)) (Becton Dickinson), and anti-IL-2 receptor (CD25, IgG\(_1\)) (Becton Dickinson). For proliferation studies, OKT3 was purified by affinity chromatography with Protein A-Sepharose (Pharmacia, Piscataway, NJ); T11 and T11, ascites were used at the appropriate dilutions as indicated. The appropriate FITC- or phycoerythrin (PE) conjugated MoAbs were used for cytofluorometric analysis.

Reagents. Stock solutions of PMA (Sigma, St Louis, MO) and ionomycin (Calbiochem, La Jolla, CA) were prepared in dimethylsulfoxide (DMSO) and stored at −70°C until use. PHA was obtained from Sigma. Recombinant human IL-2 (rhIL-2 3 \(\times\) 10\(^5\) NIH Biological Response Modifiers Program (BRMP) U per milligram of specific activity) was from Genzyme (Boston, MA) and was stored concentrated at −70°C and diluted just before addition to cultures.

Adherence of MoAbs to plastic. MoAbs were bound to plastic using a previously reported minor modification\(^{17}\) of the method of Geppert and Lipsky.\(^{42}\) MoAbs were diluted in phosphate-buffered saline (PBS), and 200 \(\mu\)L of the appropriate concentration was dispensed into wells of 96-well flat-bottomed microtiter plates (Costar, Cambridge, MA). After 2-hour incubation at room temperature, wells were washed twice with 200 \(\mu\)L standard medium. Residual binding sites were saturated by 1-hour incubation at room temperature with 200 \(\mu\)L standard medium (containing 10% FCS). In wells to which only soluble reagents were added, a parallel pretreatment was performed with standard medium only.

Proliferation assay. T lymphocytes 2 \(\times\) 10\(^5\) were cultured in a final volume of 200 \(\mu\)L per flat-bottomed microtiter plate in triplicate wells in complete media at 37°C in a humidified atmosphere of 5% CO\(_2\) in air. On day 3, cultures were pulsed with 1 \(\mu\)C \(\text{HTdR} (60 \text{Ci/mmol specific activity, ICN Radiochemicals, Irvine, CA})\) per well and harvested 4 hours later with a semi-automated sample harvester. The filters were counted in a liquid scintillation counter.

Measurement of IL-2 secretion. The concentrations of IL-2 secreted into the supernatants by cultured normal and MM-
derived T cells on stimulation with plastic-immobilized OKT3 were determined in a proliferative assay with the IL-2-dependent murine T-cell line CTLL. One thousand CTLL cells were cultured in a final volume of 100 μL per flat-bottomed microtiter plate in duplicate wells with serial dilutions of T-cell supernatants (1:2 to 1:6,250). After 24-hour incubation at 37°C in 5% CO2, cells were pulsed with 1 μCi 3H-TdR per well and harvested 4 hours later as described above. rhIL-2 was used as the standard.

Flow cytometry. T-Cell surface antigens were detected by flow cytometry using a FACSCAN (Becton Dickinson). Two-color cytofluorometric analysis was performed on purified T-cell preparations with Leu2-FITC, Leu3-FITC, Leu2-PE, Leu3-PE, anti-HLA-DR-PE, anti-IL-2R-PE, UCHL1-FITC, and 2H4-FITC MoAbs. FITC- and PE-conjugated mouse myeloma proteins of the appropriate subclasses were used as negative controls. Ten thousand events were accumulated and analyzed for fluorescence. On the basis of the control sample, contour plots were divided into quadrants to identify unstained cells (lower left quadrant), cells stained with one MoAb (upper left quadrant), cells stained with two MoAbs (upper right quadrant), and cells stained by a single MoAb only (upper left and lower right quadrants).

Statistical analysis. Results are mean ± SD or SE as indicated. Differences between means were evaluated with Student's t-test or the Wilcoxon test for unpaired samples as indicated. Significance of correlations was calculated by linear regression analysis. P < .05 was considered significant.

RESULTS

Defective responses of MM-derived PBMC to PHA and CD3 MaAbs. PBMC were stimulated with optimal concentrations of PHA, soluble OKT3, and plastic-immobilized OKT3 (ads) (Table 1). On the average, all responses were significantly lower in MM-derived PBMC, but because the percentage of monocytes and T cells was not determined, differences may have been due to a lower proportion of T cells in MM-derived PBMC preparations.

Enhanced response of MM-derived PBT to immobilized OKT3. Monocytes were removed by carbonyl-iron and Leu-OMe treatments. Residual monocytes were comparable (≤0.1%) in MM-derived and normal T-cell preparations, as shown by nonspecific esterase staining.60 In our experiments, this degree of monocyte depletion yields T-cell preparations totally unresponsive to soluble OKT3 and concanavalin A (ConA) (data not shown), partially responsive to suboptimal concentrations of plastic-immobilized OKT3 (25 to 50 ng/well) and fully responsive to monocyte-independent stimulations (CD2, PMA plus ionomycin).61 Responses were highly heterogeneous among MM patients, but, on the average, CD2 and PMA plus ionomycin stimulations were significantly lower whereas, unexpectedly, responses to plastic-immobilized OKT3 were significantly higher than those of controls (Table 2).

Because monocyte contamination may critically influence CD3-induced activation, we performed additional experiments to investigate further any regulatory role of MM monocytes on CD3 hyperreactivity. Enriched populations of autologous monocytes were prepared by recovering AC and were added back to T cells in variable proportions (from 20% to 0.1%) (Fig 1). Autologous AC clearly enhanced CD3-stimulated T cells from normoreactive (MM-NV) and controls. In contrast, AC had no effect when added back to CD3-hyperreactive MM (MM-AV). We performed a limited series of experiments in which T cells were extensively depleted of monocytes by plastic-adherence, carbonyl-iron, and Leu-OMe treatments (Table 3). Although the degree of monocyte depletion was comparable to that observed in routinely monocyte-depleted T-cell preparations (<0.1% by nonspecific esterase staining, <0.5% by cytofluorometric analysis with LeuM3-FITC), defective PHA responses demonstrated a very effective monocyte depletion. Again autologous AC (from 10% to 20%) had no effect on OKT3- and PMA-induced proliferations of hyperreactive MM-derived T cells.

Several data argue against the possibility that CD3 hyperreactivity of MM was induced by chemotherapy. First,
ZL-2 was observed in treated patients whatever their therapy, was supplemented with 50- to 100-U/mL concentrations of rIFN-α to rule out any immunomodulatory role of rIFN-α on CD3 responses, some experiments in MM-derived and normal T-cell responses to plastic-immobilized OKT3. Purified T cells were incubated on plastic-immobilized OKT3 (25 ng/well) and variable proportions of autologous adherent cells (AC, containing 85% to 95% monocytes, as described in the Material and Methods section) were added back. Results are from one of three experiments, each performed in triplicate: (●) control, (○) normoreactive MM (MM-VA), and (△) hyperreactive MM (MM-AV).

Response to plastic-immobilized OKT3 was significantly higher in 13 MM patients at diagnosis than in relevant controls (n = 10) (MM: 98,022 ± 19,663; controls: 41,250 ± 7,907) (P = .02). Second, CD3 hyperreactivity was observed in treated patients whatever their therapy, including 12 patients on maintenance therapy with rIFN-α. To rule out any immunomodulatory role of rIFN-α on CD3 responses, some experiments in MM-derived and normal T cells were performed, in which plastic-immobilized OKT3 was supplemented with 50- to 100-U/mL concentrations of rIFN-α. No synergistic effect was observed (data not shown).

CD3-hyperreactive MM-derived T cells produce and secrete IL-2. Normal T cells stimulated by suboptimal concentrations of immobilized OKT3 can be driven to optimal proliferation when exogenous rIL-2 or rIL-1 is given, or accessory signalling is provided by costimulatory molecules such as CD28, CD73, or PMA. To investigate the role of IL-2 production in MM patients displaying CD3-induced hyperreactivity, we performed some experiments with immobilized OKT3 and rIL-2 added. Two representative experiments are shown in Table 4. Hyperreactive MM patients (C.H., G.A., T.U.) showed no further increase on costimulation with rIL-2. In contrast, HTdR uptake of the controls and the normoreactive MM patient (M.A.) improved when CD3 stimulation was supplemented with exogenous rIL-2.

To explore the role of IL-2 in CD3 hyperreactivity of MM further, we evaluated IL-2R expression (CD25) and IL-2 production in resting and CD3-stimulated T cells. A few MM-derived CD4+ cells (3% to 8%) constitutively expressed CD25, as previously reported in normal CD4+ cells, whereas no expression was observed in MM-derived and normal CD8+ lymphocytes. Even MM-derived T-cell preparations with high proportions of HLA-DR+ CD8+ cells (Fig 2A and B) did not express CD25 (Fig 2C and D). When CD25 expression was evaluated in T cells after 3-day stimulation with plastic-immobilized OKT3 (Fig 3, right), however, more than 60% to 70% of T cells expressed CD25 in CD3-hyperreactive MM patients (Fig 3F), whereas lower values were observed in normoreactive MM patients (Fig 3D) and controls (Figure 3B). HLA-DR+ cells promptly acquired CD25 expression on effective CD3 stimulation (Fig 3F, upper right quadrant). CD25 expression was induced in both CD4 and CD8 subpopulations (data not shown).

We noted that CD3-hyperreactive MM patients, in keeping with CD25 expression data, secreted significant amounts of IL-2 in the supernatants on stimulation with plastic-immobilized OKT3 (Fig 4).

Increased number of CD8+ HLA-DR+ cells in MM T-cell preparations hyperreactive to immobilized OKT3. Although T cells of MM patients showed defective PBMC responses and T-cell hyperreactivity to immobilized OKT3 on the average, very different patterns were observed in individual patients. Other T-cell functions have previously been shown to be heterogeneously impaired in MM and to correlate with the proportion of activated cells (HLA-DR+ ) in the CD8+ subpopulations. HLA-DR+ expression was thus evaluated in CD8+ cells from 35 MM T-cell preparations, and its high heterogeneity was confirmed (mean ± SD

Table 3. MM Hyperreactivity to Plastic-Im mobilized OKT3 (ads) is Associated With PHA Hyporeactivity

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control (cpm/10^5 cells)</th>
<th>MM-VA</th>
<th>MM-AV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>96 ± 3</td>
<td>71 ± 28</td>
<td>123 ± 43</td>
</tr>
<tr>
<td>OKT3 (ads)†</td>
<td>6,510 ± 532</td>
<td>20,287 ± 1,388</td>
<td>48,897 ± 1,157</td>
</tr>
<tr>
<td>OKT3 (ads) + 20% AC#</td>
<td>61,372 ± 2,149</td>
<td>27,736 ± 5,188</td>
<td>52,269 ± 713</td>
</tr>
<tr>
<td>OKT3 (ads) + 10% AC</td>
<td>45,106 ± 7,403</td>
<td>27,736 ± 2,708</td>
<td>51,201 ± 1,269</td>
</tr>
<tr>
<td>PHA§</td>
<td>19,286 ± 1,500</td>
<td>7,842 ± 821</td>
<td>5,192 ± 1,607</td>
</tr>
<tr>
<td>PHA plus 20% AC#</td>
<td>60,926 ± 2,586</td>
<td>9,961 ± 594</td>
<td>3,406 ± 106</td>
</tr>
<tr>
<td>PHA plus 10% AC</td>
<td>45,424 ± 1,896</td>
<td>9,083 ± 850</td>
<td>3,467 ± 167</td>
</tr>
</tbody>
</table>

Monocytes were removed by plastic adherence, carbonyl-iron and Leu-OMe treatments.

*Results are mean ± SD. Each experiment was performed in triplicate. Data are from one of three representative experiments.
†OKT3 was immobilized on plastic at 25 ng/well.
‡Variable concentrations of autologous adherent cells (AC), containing 85% to 95% monocytes, were added back to monocyte-depleted T cells.
§Final concentration 5 μg/mL.
Table 4. Exogenous rIL-2 Synergizes With Suboptimal Concentrations of Plastic-Immobilized OKT3 (ads) in Normoreactive MM T Cells and Controls But Not in Hyperreactive MM T Cells

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Medium</th>
<th>rIL-2 (50 U/mL)</th>
<th>OKT3 (ads) 25 ng/well</th>
<th>OKT3 (ads) 25 ng/well Plus rIL-2 (50 U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>128 ± 12</td>
<td>712 ± 60</td>
<td>8,619 ± 1,425</td>
</tr>
<tr>
<td>MM-CH</td>
<td></td>
<td>181 ± 23</td>
<td>5,335 ± 467</td>
<td>98,747 ± 8,635</td>
</tr>
<tr>
<td>MM-MA</td>
<td></td>
<td>168 ± 13</td>
<td>2,748 ± 275</td>
<td>8,735 ± 531</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>151 ± 37</td>
<td>1,710 ± 83</td>
<td>13,418 ± 1,909</td>
</tr>
<tr>
<td>MM-GA</td>
<td></td>
<td>50 ± 12</td>
<td>2,156 ± 27</td>
<td>171,956 ± 2,842</td>
</tr>
<tr>
<td>MM-TU</td>
<td></td>
<td>45 ± 7</td>
<td>3,124 ± 62</td>
<td>160,051 ± 2,593</td>
</tr>
</tbody>
</table>

* T cells were depleted of monocytes by carbonyl-iron and Leu-OMe treatments. Results are mean ± SD from each experiment performed in triplicate and are from two of five representative experiments.

12% ± 9%, range 1% to 51%). When patients were divided according to their percentage of CD8+ HLA-DR+ cells (≤ 10%, a cutoff value reported to discriminate monoclonal gammopathy of undetermined significance (MGUS) and MM), MM patients with more than 10% CD8+ HLA-DR+ cells had higher CD3 responses (P < .001) but lower responses to PMA plus ionomycin (P = .03) and T11, plus T11, (P = .01) than MM patients with less than 10% CD8+ HLA-DR+ cells (Fig 5). In individual patients, a positive correlation between the proportion of HLA-DR+ CD8+ cells and CD3 hyperreactivity (expressed as percentage of the related control) (Fig 6, left) together with a negative correlation between CD8+ HLA-DR+ cells and defective responses to PMA plus ionomycin (Fig 6, right) were noted.

To investigate the role of HLA-DR+ cells in MM CD3 hyperreactivity directly, experiments were performed in which purified MM T cells were further depleted of HLA-DR+ cells by the panning technique. Cytofluorometric analyses showed that depletion of HLA-DR+ cells ranged from 50% to 60% owing to weak expression of HLA-DR+ in CD8+ lymphocytes. Nevertheless, HLA-DR depletion paralleled the decrease in CD3 hyperreactivity, indicating that CD8+ HLA-DR+ cells directly contributed to CD3 hyperreactivity (Fig 7). Pretreatment with anti-HLA-DR MoAb had no effect on CD3 hyperreactivity when T cells were poured on plastic dishes not coated with polyclonal rabbit anti-mouse Ig (data not shown).

Unbalanced proportions of naive and memory T cells in CD4 and CD8 MM subpopulations. MM CD3 hyperreactivity was reminiscent of the normal memory (CD45RO+) T cell response to CD3 cross-linking. The distribution of naive (CD45RA+) and memory (CD45RO+) cells was thus investigated in a limited number of CD4+ and CD8+ subpopulations of MM-derived T-cell preparations by two-color cytofluorometric analysis. MM patients showed a decreased percentage of CD4+ CD45RA+ cells and an increase in CD8+ CD45RO+ cells (Table 5), but the differences were not statistically significant (presumably because of the small number of patients). When ratios of CD45RA to CD45RO ratios were compared, however, MM-derived T cells were depleted of monocytes by carbonyl-iron and Leu-OMe treatments. Results are mean ± SD from each experiment performed in triplicate and are from two of five representative experiments.
Fig 3. CD25 and HLA-DR expression in resting (left) and CD3-stimulated T cells (right) from a representative control (A,B), a CD3-normoreactive patient (C,D), and a CD3-hyperreactive MM patient (E,F). Two-color flow cytograms using FITC-anti-HLA-DR and PE-anti-IL-2R MoAbs. Stimulated T cells were stained after 3-day incubation with plastic-immobilized OKT3.

Fig 4. IL-2 secretion by MM and normal T cells activated by plastic-immobilized OKT3 MoAb. Culture supernatants were collected at the indicated times and assessed for their ability to support proliferation of the murine IL-2-dependent cell line CTLL. Results are expressed as mean cpm 3H-TdR uptake/1 x 10^6 CTLL cells and refer to the final dilution of 1:2 only: (▲) control; (■) MM-TE; (□) MM-RE. 3H-TdR uptake of OKT3-stimulated T cells was 13,231 ± 231, 14,657 ± 567, and 73,778 ± 3,123 for control, MM-RE, and MM-TE, respectively. Inset: CD25 expression on day 3 in control (▲), MM-TE (■), and MM-RE (□). With human rIL-2 as standard, 50,000 cpm in this experiment equals approximately 8.7 U/mL.
Fig 5. Proliferation induced by PHA, soluble OKT3 + PMA, soluble T11 + T11, PMA + ionomycin, and plastic-immobilized OKT3 in MM T cells divided in function of HLA-DR expression in CD8 subpopulations (≤10%). Results are mean ± SE of triplicate experiments. Statistical significance of differences between means was calculated with Student's t test. The proportion of CD8' HLA-DR' cells was evaluated by cytofluorimetric analysis using FITC-Leu2 and PE-anti-HLA-DR MoAbs. The mean proportions of CD8' HLA-DR' cells were 35 ± 12% and 8% ± 2% in the groups with more than 10% and less than 10% groups, respectively; <10% CD8' HLA-DR' (●); >10% CD8' HLA-DR' (□).

Fig 6. Correlations in individual patients between the number of CD8' HLA-DR' cells in MM T-cell preparations and responses to plastic-immobilized OKT3 (left) and PMA plus ionomycin (right). MM-derived T-cell proliferations are expressed as percentage of the relative controls. The number of CD8' HLA-DR' cells was determined by cytofluorimetric analysis of purified T-cell preparations using FITC-Leu2 and PE-anti-HLA-DR MoAbs.

Fig 7. CD3 hyperreactivity of MM T cells was partially abrogated by depletion of HLA-DR' cells. T Cells were depleted of HLA-DR' cells by an indirect panning technique using anti-HLA-DR MoAbs and rabbit anti-mouse precoated plastic dishes. Percentages of CD8' HLA-DR' cells were determined by cytofluorimetric analyses using PE-anti-HLA-DR and FITC-Leu2 MoAbs before panning; and (a) after panning, FITC-GAM F(ab')2, (b) anti-HLA-DR plus FITC-GAM F(ab')2, and (c) PE-anti-HLA-DR and FITC-Leu2 MoAbs. The percentage of CD8' HLA-DR' cells in T and T (HLA-DR-) cells were MM-RI 26% and 13%, MM-TR 22% and 9%, and MM-CR 6% and 2%. The data are from one of three representative experiments.

DISCUSSION

Multivalent cross-linking of the CD3/TCR complex by suboptimal concentrations of plastic-immobilized OKT3 was very effective in initiating cell proliferation in purified T cells of MM patients. Proliferation was significantly higher in MM than controls, but this hyperreactivity was observed only in purified MM T-cell preparations.

T-cell proliferation, CD25 expression, and IL-2 production in some CD3-hyperreactive MM patients equaled the values observed in normal subjects under optimal conditions of CD3 stimulation. In the latter, suboptimal CD3 stimulation (like that induced by 25 ng per well of plastic-immobilized OKT3) yields to maximal proliferation only if associated with costimulatory signals, such as exogenous rIL-2 or rIL-1.18-20 PMA,17 or signals delivered by surface accessory molecules such as CD2842 and CD73.27 These data indicate that some MM T cells have a less stringent need for accessory signals to initiate cell activation fully through the CD3/TCR complex than do normal T cells: the former need only CD3 stimulation, the latter require agonistic signals.

Monocytes are among the cells that can influence mostly CD3-induced activation, but some data contradict a role for contaminating monocytes in CD3 hyperreactivity of MM T cells. First, residual monocytes were comparable in MM patients and controls, as shown by nonspecific esterase staining and cytofluorimetric analyses. Second, from 0.1%
### Table 5. Unbalanced Distribution of CD45RA and CD45RO Expression in MM T-Cell Preparations as Compared With Normal Subjects

<table>
<thead>
<tr>
<th>Expression</th>
<th>Percentage of Positive Cells in Purified T-Cell Preparations</th>
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<tbody>
<tr>
<td></td>
<td>MM</td>
</tr>
<tr>
<td>CD8⁺ CD45RO⁺</td>
<td>22 ± 7 (8)</td>
</tr>
<tr>
<td>CD8⁺ CD45RA⁺</td>
<td>24 ± 9 (12)</td>
</tr>
<tr>
<td>CD4⁺ CD45RO⁺</td>
<td>30 ± 11 (8)</td>
</tr>
<tr>
<td>CD4⁺ CD45RA⁺</td>
<td>16 ± 12 (12)</td>
</tr>
<tr>
<td>CD8⁺ (CD45RA⁺:CD45RO⁺)*</td>
<td>0.99 ± 0.37 (8)</td>
</tr>
<tr>
<td>CD4⁺ (CD45RA⁺:CD45RO⁺)*</td>
<td>0.60 ± 0.88 (8)</td>
</tr>
</tbody>
</table>

Results are mean ± SD. Statistical significance was calculated using the Wilcoxon test for unpaired samples: NS, not significant. Number of patients is shown in parentheses.

*CD45RA:CD45RO ratio was calculated by dividing the proportion of CD45RA⁺ by the proportion of CD45RO⁺ cells in each patient.

To 20% of autologous AC (containing 85% to 95% monocytes) did not influence CD3-induced responses of hyperreactive MM, whereas those of normoreactive MM and controls were appropriately enhanced. Third, PHA responses (highly monocyte sensitive) were low or negligible in CD3-hyperreactive MM.

Previous reports have been contradictory with regard to the regulatory role of MM monocytes. Either enhanced suppressor or impaired helper activity has been reported on B- and T-cell responses. In this study, monocytes exerted the appropriate agonistic signaling on CD3 stimulation of normoreactive MM, whereas they had no effect on CD3 and PHA stimulations of hyperreactive MM.

The finding that MM T cells strongly proliferate to plastic-immobilized OKT3 was unexpected, because MM T-cell dysfunctions have previously been associated with defective rather than increased responses. Paradoxically, defective responses have mainly been described in patients with increased numbers of HLA-DR⁺ cells in CD8 subpopulations. To explain the discrepancy, MM T cells have been proposed to be already activated by a so-far-unknown tumor-related stimulus (indeed, idioype-reactive cells have been reported among CD8⁺ HLA-DR⁺ cells), and this functional commitment makes their activation state undetectable unless an appropriate assay is used. If this assay is assumed to be the one that uses the antigen(s) by which MM T cells have already been activated in vivo, it is not surprising that they are hyperreactive in vitro to MoAbs against the CD3/TCR complex (as a surrogate for the natural antigen stimulation). Evidence supporting this hypothesis was provided by the direct correlation between the proportion of CD8⁺ HLA-DR⁺ cells and CD3 hyperactivity (the higher the former, the higher the latter), and by the decrease in CD3 hyperreactivity that occurred with partial depletion of HLA-DR⁺ cells. These data indicate that HLA-DR expression in MM T cells reflects a peculiar state of activation characterized by the ability to produce and secrete IL-2 optimally and to proliferate on suboptimal multivalent cross-linking of the CD3/TCR complex. Whether MM T cells occasionally can be exposed in vivo to

![Fig 8. Most resting HLA-DR⁺ T cells belong to the memory subset (CD45RO⁺) of T cells. A representative normoreactive (A,B) and a hyperreactive (C,D) MM patient are shown. Two-color flow cytograms using UCHL1-FITC and anti-HLA-PE MoAbs (A,C); 2H4-FITC and anti-HLA-DR-PE MoAbs (B,D).](image-url)
triggers events like that induced by plastic-immobilized OKT3 in vitro is not yet known.

Previous studies have shown that the number of CD8+ HLA-DR+ cells is correlated with diagnosis and disease status. It is higher in MM at diagnosis than in MGUS patients and decreases to MGUS levels in MM with stable remission but not in MM with tumor progression. These data were recently extended to 32 MM patients at diagnosis: Increased numbers of circulating CD38+ lymphocytes, highly expressed by activated T cells, are indicators of poor survival. Because of the correlation between activated T cells, defective T-cell functions, and tumor evolution, the emergence of CD8+ HLA-DR+ T cells has been interpreted as indicative of a progressive deterioration of cell-mediated immunity, which in turn facilitates tumor growth. We offer new data suggesting that tumor progression in MM patients is associated instead with a peculiar state of T-cell activation.

Atypical T-cell activation has also been described in certain viral infections, after allogeneic BM transplantation, and in AIDS. These conditions share several of the T-cell abnormalities observed in MM, such as emergence of HLA-DR+ CD25+ cells in CD8+ subpopulations and defective responses to various stimuli. CD3 reactivity has been investigated only in AIDS, in which it was shown to be defective. Whether this difference reflects intrinsic features of AIDS or is due to technical reasons is not known, because AIDS T cells were stimulated with OKT3 bound to magnetic beads rather than being immobilized on plastic. We observed CD3 hyperreactivity in patients with autoimmune diseases and a high number of CD8+ HLA-DR+ cells (M. Massaia, unpublished observations, 1990), suggesting that this assay is worth investigating in diseases other than MM.

CD3 hyperreactivity (and CD2 hyporeactivity) of MM T cells was reminiscent of the functional behavior of normal memory T cells. Normal T cells can be divided by their expression of different CD45 isoforms into naive and memory T cells: T cells expressing high-molecular-weight (mol wt) isoforms (defined by the CD45RA MoAb 2H4) are naive or virgin T cells, whereas T cells expressing low-mol-wt isoforms (defined by the CD45RA MoAb UCHL1) are memory T cells. This dichotomy being more evident in CD4+ than CD8+ cells. Memory T cells hyperrespond to CD3/TCR cross-linking, whereas conflicting data have been reported about CD2 reactivity. Phenotyping of a limited number of MM T-cell preparations confirmed the altered distribution of CD45RO and CD45RA subsets already reported in MM CD4 and CD8 subpopulations. A diminished ratio of CD45RA to CD45RO was observed in both subpopulations, but in CD4 cells this was mainly due to a decrease in CD45RA- cells, whereas in CD8 it was mainly due to an increase in CD45RO+ cells. Two-color cytofluorometric analysis showed that the majority (70% to 80%) of HLA-DR+ cells in MM patients belong to the CD45RO subset, as previously reported in normal T cells. Whether the relative expansion of CD45RO+ T cells is enough to account fully for the CD3 hyperreactivity of MM T cells is not known. Future experiments are needed on isolated T-cell subsets to characterize the contribution of each single subset to MM T-cell CD3 hyperreactivity.

We conclude (a) that T cells are indeed activated in some MM patients, but an appropriate assay (ie, multivalent CD3/TCR cross-linking) on purified T cells is required to detect their state of activation; (b) that CD3 hyperreactivity is not due to contaminating monocytes, indicating that the need for accessory signals to initiate cell proliferation fully on CD3/TCR stimulation is less stringent in some MM; and (c) that these features are mostly evident in T-cell preparations with high proportions of CD8+ HLA-DR+ cells (accordingly, removal of HLA-DR+ cells induces a parallel decrease in CD3 hyperreactivity).

The in vivo importance of these features has not yet been explained. The clinical data suggest that expansion of T cells with such a peculiar state of activation is not a favorable event in the evolution of the disease: Patients with many activated cells are expected to have a poor prognosis. Studies are currently in progress to identify cytokines involved in MM T-cell hyperreactivity, the molecular basis of their enhanced production, and the tumor-derived factors that induce and maintain this state of activation. Whether any T-cell derived cytokine(s) exert a paracrine effect on tumor cell growth also remains to be elucidated.

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Detection of hyperreactive T cells in multiple myeloma by multivalent cross-linking of the CD3/TCR complex [see comments]

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