Rapid Generation of Antiplasma Cell Activity in the Bone Marrow of Myeloma Patients by CD3-Activated T Cells

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We have recently shown that peripheral blood T cells of multiple myeloma (MM) patients are very susceptible to stimulation of the T-cell receptor/CD3 complex with anti-CD3 monoclonal antibodies (MoAbs). CD3 stimulation is currently under clinical investigation as a nonspecific approach to boost antitumor effector mechanisms. The aim of this study was to determine whether the hyperresponsiveness of MM T cells to CD3 stimulation could be exploited to generate antitumor activity. Bone marrow mononuclear cells (BMMCs) from 65 MM patients were stimulated with the anti-CD3 MoAb OKT3, and the effect of this stimulation on autologous T cells and plasma cells was evaluated. The number of CD3+ CD25+ cells on day 6 was significantly higher in MM than the controls (30 normal individuals) (P = .001). Kinetic studies showed that 3H-thymidine incorporation peaked on day 3 and that the T-cell expansion peaked on days 5 and 6. In MM, T-cell activation markedly affected the survival of autologous plasma cells; their number in OKT3-treated cultures was significantly lower than in unstimulated cultures (P < .0001). T-cell activation and plasma cell decrease were not observed when T cells were removed from BMMCs preparations. MM produced significantly higher levels of interferon-γ (P = .005) and tumor necrosis factor-β (P = .001), but lower levels of tumor necrosis factor-α (P < .001) than normal individuals. Interferon-γ only was partially involved in CD3-induced plasma cell killing. Transwell cultures showed that the main mechanism by which CD3+ CD25+ cells affected plasma cells was direct cell-to-cell contact rather than cytokines. In conclusion, T cells in MM BMMCs possess distinct features in terms of susceptibility to CD3 stimulation and cytokine production compared with normal bone marrow T cells that can be exploited to generate antiplasma cell activity.

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SEVERAL PHENOTYPIC and functional alterations have been described in peripheral blood T cells of multiple myeloma (MM) patients. Some of them are correlated with prognosis and disease status, suggesting a progressive deterioration of T-cell immunity, which, in turn, may facilitate tumor growth. However, it is surprising that such deterioration is accompanied by the emergence of activated T cells in the peripheral blood. Indeed, many malignant tumors trigger a specific antitumor response that is not strong enough to reverse their progression. Evidence for specific interactions in MM includes the presence of idiotypic-reactive T cells (mainly in CD8+ HLA-DR+ subpopulations) and the biased usage of T-cell receptor (TCR) Vα and Vβ gene segments.

To further investigate the status of T-cell immunity in MM, we have recently evaluated the ability of T cells to be activated by anti-CD3 monoclonal antibodies (MoAbs). We used CD3 stimulation with plastic-immobilized OKT3 as a surrogate for antigen-specific stimulation. Unexpectedly, T-cell proliferation, CD25 expression, and interleukin-2 (IL-2) production in some MM patients reached values only observed in normal subjects in the presence of accessory signals. CD3 hyperreactivity correlated with the number of CD8+ HLA-DR+ cells; the higher the number of CD8+ HLA-DR+ cells, the higher the activation induced by OKT3. Activation of antitumor effector mechanisms by CD3 stimulation in a number of murine models has led to its clinical investigation as a nonspecific approach to the generation of T-cell–mediated antitumor activity in cancer patients. To determine whether the particular susceptibility of MM T cells to CD3 stimulation could be exploited to generate antitumor activity, it was decided to investigate the effect of CD3-activated T cells on autologous malignant plasma cells. Activated T cells produce a number of cytokines, including IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and IL-6, which have the potential to directly or indirectly stimulate plasma cells.

In normal individuals, CD3-induced T-cell activation has also been reported to drive the plasma cell differentiation of normal B cells.

The aim of this work was thus to investigate whether CD3-activated T cells have a positive or negative effect on autologous malignant plasma cells in MM patients. Results indicate that T cells display distinct features of CD3 reactivity and cytokine production that can be exploited to generate an effective antiplasma cell activity.

MATERIALS AND METHODS

Patients. Sixty-five MM patients entered this study from November 1990 to March 1993. MM was diagnosed as previously reported. According to the Durie and Salmon staging system, 12 patients were classified as stage I, and 53 were stage II; 11 were substage B. Forty patients were IgG, 15 were IgA, and 8 were Bence Jones MM. Twenty-four patients were evaluated at diagnosis; 33 were on first-line chemotherapy according to the Italian Myeloma Study Group protocols. All patients receiving treatment were stud-
ied at least 3 weeks after the last day of chemotherapy; 8 patients were in relapse. Patients were not on antibiotics, did not have infections, and had not received transfusions for at least 10 days before the study.

Normal bone marrow (BM) samples were obtained from 30 individuals during orthopedic surgery or thoracotomy for cardiovalvular surgery. The control group was matched in age and sex.

Isolation of BM mononuclear cells (BMMCs). The standard medium was Iscove's modified Dulbecco's medium (IMDM) containing 10% fetal calf serum (FCS; GIBCO, Milano, Italy), 2 mmol/L glutamine, penicillin (100 U/mL), streptomycin (100 μg/mL), and amphotericin B (0.25 μg/mL). BM samples were aspirated after informed consent and pre-examined for suitability by May-Grunwald-Giemsa staining. After passages through needles of decreasing gauge, BMMCs were obtained by density gradient centrifugation (Ficoll-Hypaque).

In some experiments, T lymphocytes were removed by rosetting with sheep erythrocytes at 29°C for 1 hour, whereas phagocytic cells were removed by the carbonyl-iron method. Cells were counted microscopically and their viability as determined with the trypan blue exclusion dye test was always greater than 98%.

BMMC stimulation. For culture studies, OKT3 (CD3, IgG2a; American Type Culture Collection, Rockville, MD) was purified by affinity chromatography with protein A-Sepharose (Pharmacia, Piscataway, NJ). OKT3 was routinely used after immobilization on plastic to minimize the role of accessory and Fc-bearing cells, depending on their proportion, greatly influence T-cell responses to soluble OKT3. OKT3 was bound to plastic in the bottom half as described above.

Recombinant human IL-2 (18 × 10^6 International Units/mg specific activity) was from Roche (Milano, Italy). For neutralization experiments, mouse antihuman IFN-α MoAb was from Genzyme Corp (Omnia Res, Milano, Italy); mouse antihuman TNF-α MoAb was from Boehringer Mannheim (Milano, Italy); rabbit antihuman TNF-β was from Genzyme Corp.

Proliferation assay. BMMCs were cultured at 1 × 10^6/mL in IMDM + 10% FCS in a flat-bottomed microtitre plate at 37°C in a humidified atmosphere of 5% CO2 in air. Cells were counted and their viability was determined with the trypsin blue exclusion dye test. They were then divided into aliquots for subsequent analysis. Total counts were determined by dividing viable cell counts by the percentage of activated T cells identified by flow cytometry (see below). Supernatants were collected and stored at -70°C until assessed for Ig or cytokine contents.

OKT3-induced proliferation in BMMC cultures was also assessed by pulsing 200 μL of cells with 0.5 μCi of 3H-thymidine (60 Ci/mmol specific activity; Amersham, Milano, Italy) and harvesting 4 hours later with a semiautomated sample harvester. The filters were counted in a liquid scintillation counter.

Cytofluorometric analyses. Fluorescein isothiocyanate (FITC)-conjugated Leu4 (CD3, IgG1) and phycoerythrin (PE)-conjugated anti-IL-2 receptor (CD25, IgG1; Becton Dickinson, Milano, Italy) were used to identify activated T cells (CD3+ CD25+) by two-color flow cytometry with a FACSScan (Becton Dickinson). FITC- and PE-conjugated mouse myeloma proteins of the appropriate subclasses were used as negative controls. Five thousand events were accumulated and analyzed for fluorescence. The control sample was used as the reference to divide contour plots into quadrants for identification of unstained cells (lower left quadrant), cells stained...
Fig 2. Kinetics of T-cell activation induced by CD3 stimulation: daily 3H-thymidine incorporation in BMMCs from MM (A) and the controls (B); daily counts of viable CD3+ CD25+ cells in BMMCs from MM (C) and the controls (D). Each point represents the mean value ± SE from four to six experiments in MM and from 6 to 10 experiments in the controls. (●) OKT3; (○) IMDM.

by both MoAbs (upper right), and cells stained by only one MoAb (upper left and lower right). Percentages of positive cells in gated files always referred to the total number of events.

Cytoplasmic immunofluorescence (CyIF). Cytospin smears were prepared in duplicate with a Shandon cytocentrifuge. The slides were fixed in 95% ethanol and 5% acetic acid for 15 minutes at −20°C. Plasma cells were stained for 40 minutes with an FITC-conjugated rabbit antihuman Ig (Dako Immunoglobulins, Glostrup, Denmark) at the appropriate dilution and washed several times in cold phosphate-buffered saline (PBS) for at least 48 hours. CyIF was evaluated in a Leitz Orthoplan microscope (Leitz, Milan, Italy) with a Ploemopack vertical illuminator and a 100 W mercury lamp. At least 500 cells were scored at 50× magnification on duplicate slides. Total counts of viable plasma cells were determined by dividing viable cell counts by the percentage of plasma cells.

Quantification of Ig, TNF-α, TNF-β, and IFN-γ in the supernatants. Production and release of Ig in the supernatants was assessed with isotype-specific enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well, flat-bottomed microtiter plates were incubated overnight at 4°C with the appropriate dilution of affinity purified goat antihuman serum IgA or IgG (Cappel Organon Teknika Co, West Chester, PA). Wells were washed with PBS + 0.02% Tween 20 and residual plastic binding sites were saturated with PBS + 2% bovine serum albumin (BSA). Culture supernatants from IgG MM were tested undiluted, whereas IgA MM supernatants were diluted with PBS + 0.5% Tween 20. After 1 hour of incubation at room temperature (RT), bound Ig was detected with specific alkaline phosphatase-conjugated goat antihuman IgG or IgA serum (Cappel Organon Teknika). The phosphatase substrate was p-nitrophenyl phosphate disodium (Sigma Chemical Co, St Louis, MO). Absorbance was determined at a wavelength of 405 nm on a Multiskan MK II reader (Titertek, Gruppo Flow spa, Milano, Italy). Human serum protein calibrator (Dakopatts, Glostrup, Denmark) was used as a standard.

Quantification of TNF-α, TNF-β, and IFN-γ contents in supernatants was assessed using commercially available ELISA kits from Genzyme Corp (TNF-α and IFN-γ) and from R&D Systems (Minneapolis, MN) (TNF-β). The sensitivity of these assays is 12 pg/mL TNF-α, 100 pg/mL IFN-γ, and 7 pg/mL TNF-β, respectively. According to the manufacturer’s instructions, these assays do not show any detectable cross-reactivity with any other cytokine. Absorbance was determined at a wavelength of 492 nm (TNF-α and IFN-γ) or 450 nm (TNF-β) on a Multiskan Plus MK II reader (Titertek).

Cytotoxicity assay. On day 6, cultures were scored and cellularity equalized. The natural killer (NK)-resistant Daudi, NK-sensitive K562, and the myeloma-derived cell line LP-1 were used as target cells. A standard 4-hour ⁵¹Cr release assay was performed as
previously reported. Briefly, $^{31}$Cr-labeled target cells (5 x 10$^5$ cells in 0.1 mL) were mixed in triplicate wells with the effector cells (cultured BMMCs in 0.1 mL) at effector to target cell ratios of 20:1, 10:1, and 5:1 in round-bottomed microtiter plates. The percentage of specific $^{31}$Cr release was calculated from the expression:

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\% \text{ } ^{31}\text{Cr Release} = \frac{\text{Experimental} - \text{Spontaneous}^{31}\text{Cr Released}}{\text{Total Releasable} - \text{Spontaneous}^{31}\text{Cr Released}} \times 100
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where the spontaneous $^{31}$Cr released represents the amount of $^{31}$Cr released by target cells incubated without effector cells. Total releasable $^{31}$Cr is that released by target cells treated with 10 N NaOH.

**Fig 4.** Comparison between the capacity of plastic immobilized OKT3 [OKT3(ads)] and soluble OKT3 [OKT3(sol)] to induce T-cell activation and plasma cell decrease in BMMCs of MM patients. Each bar represents the mean value ± SE of 20 patients, each single patient being studied side-by-side with immobilized and soluble OKT3 (1 μg/mL final concentration).

**RESULTS**

**Comparison between the response of myeloma and normal BMMC T cells to CD3 stimulation.** CD3 stimulation induced the emergence of cells cytofluorometrically located as a distinct subpopulation by plotting the forward scatter (FSC), a function primarily of cell size, versus the orthogonal scatter (SSC), a function partially dependent on cell granularity. The dot plot and two-color cytofluorometric analyses of BMMCs from a representative MM patient after 6 days of incubation with IMDM and OKT3 are shown in Fig 1A and B and Fig 1C and D, respectively. The gated file was mostly composed of activated (CD25$^+$) CD3$^+$ T cells. The number of viable CD3$^+$ CD25$^+$ cells was used to evaluate the magnitude of CD3-induced T cell activation. Figure 1E shows the number of CD3$^+$ CD25$^+$ cells induced on day 6 by CD3 stimulation in MM and the controls. Values in OKT3-treated cultures were significantly higher than IMDM cultures in both MM and the controls ($P < .0001$), but the number of CD3$^+$ CD25$^+$ cells in MM was significantly higher than in the controls ($P = .002$).

In three experiments, the number of NK cells as evaluated with FITC-Leu7 MoAb was not influenced by CD3 stimulation.

**Kinetics of T-cell expansion.** Kinetics of T-cell activation and expansion were evaluated by daily measuring $^3$H-thymidine incorporation and total counts of viable CD3$^+$ CD25$^+$ cells. $^3$H-thymidine incorporation in IMDM cultures showed the same pattern in MM and the controls. $^3$H-thymidine incorporation in OKT3-treated MM BMMCs (Fig 2A) peaked on day 3 and was significantly higher than the controls (Fig 2B) on day 3 ($P = .002$) and day 6 ($P = .006$). Total counts of CD3$^+$ CD25$^+$ cells in OKT3-treated MM BMMC (Fig 2C) peaked on days 5 and 6.

**Statistical analysis.** Results are means ± SD or SE as indicated. Differences between means were evaluated with the two-tailed Student's $t$-test. $P < .05$ was considered significant.
CD3-INDUCED ANTIPLASMA CELL ACTIVITY

Fig 5. Kinetics of CD3-induced plasma cell decrease and CD3 priming requirement in BMMCs of MM patients. (A) Results are expressed as percent plasma cell decrease according to the formula:

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\text{Plasma Cell Counts in OKT3-Treated Cultures} - \text{Plasma Cell Counts in IMDM Cultures}
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where the IMDM cultures considered are those from the corresponding day. Each point represents the mean value ± SE from 4 to 8 patients. (B) Requirement of CD3 priming by MM BMMCs to generate antiplasma cell activity. Aliquots of BMMCs were removed after 24, 48, and 72 hours from OKT3-coated wells, poured into uncoated wells, and cultured until day 6 in the presence or absence of exogenous human IL-2 (50 U/mL final concentration). Results are expressed as percent plasma cell decrease calculated as reported above. Each bar represents the mean ± SE of 4 patients.

and were significantly higher compared with normal BMMCs (Fig 2D) on day 3 (P = .02), day 4 (P = .01), day 5 (P < .0001), and day 6 (P = .004). In 4 patients, the number of CD3+ CD25+ cells was also determined on day 10; T-cell activation had almost completely regressed and CD3+ CD25+ values were comparable to day 1 values.

Effect of T-cell activation on BM plasma cells. The effect of CD3+ CD25+ cells on autologous plasma cells was determined by counting the total number of viable plasma cells on day 6. The number of plasma cells in OKT3-treated cultures was significantly lower than in IMDM cultures (P < .0001) (Fig 3A). The range of this decrease was very wide, but there was no correlation with the expansion of CD3+ CD25+ cells (data not shown). Plasma cell killing was independent of the initial number of plasma cells and observed in MM with very low and very high BM plasma cell infiltration.

The amount of Ig secreted in the supernatants of OKT3 and control IMDM cultures was compared in 25 MM (15 IgA and 10 IgG) (Fig 3B). CD3 stimulation induced a statistically significant isotype-specific Ig decrease (IgA, P = .001; IgG, P = .03).

To rule out the possibility that plasma cell disappearance was rather associated with the expansion of monoclonal B lymphocytes, evidence of possible restricted light-chain isotype expression was sought cytofluorometrically in four experiments using FITC-anti-κ and PE-anti-λ MoAbs. No emergence of κ+ or λ+ cells was observed upon CD3 stimulation.

Comparison between plastic immobilized and soluble OKT3. In 20 patients, CD3 stimulation was performed with both soluble OKT3 and plastic immobilized OKT3 (Fig 4). On day 6, the number of CD3+ CD25+ cells in cultures stimulated with soluble OKT3 was slightly lower (P > .05). However, staining with FITC-goat antimouse alone showed that BMMC cultured with soluble OKT3 had 15% to 30% of T cells with the CD3 molecule still occupied by OKT3. Two-color staining with FITC-Leu4 and PE-anti-IL-2 receptor confirmed that CD25+ cells were more than CD3+ CD25+ cells, indicating that the total number of activated T cells was underestimated in soluble OKT3 cultures. The plasma cell decrease was the same with plastic immobilized and soluble OKT3 (Fig 4).

Kinetics of plasma cell killing. Total counts of viable plasma cells were assessed daily in OKT3 and IMDM cultures to determine how long CD3 stimulation was required for effective T-cell activation and plasma cell killing. Plasma cells began to decrease after 48 hours (Fig 5A). To further dissect the CD3 priming requirement of MM BM T cells, aliquots of BMMCs were removed after 24, 48, and 72 hours from OKT3-coated wells, poured into uncoated wells, and cultured until day 6 in the presence or absence of exogenous human IL-2 (50 U/mL final concentration) (Fig 5B). The generation of antiplasma cell activity was signifi-
cultured without further processing under standard conditions (not shown); after depletion of T cells by rosetting with SRBC all conditions.

Distinct pattern of cytokine production in MM and normal BMMCs. CD3 stimulation induces the release of a variety of cytokines from T cells, including TNF-α, TNF-β, and IFN-γ, which have a central role in the antitumor activity of LAK and TIL cells. The production of these cytokines was thus investigated daily in OKT3 and IMDM cultures from MM and the controls. Cytokine concentrations in IMDM cultures were almost undetectable in MM and the controls (data not shown). By contrast, significant production with a distinct pattern was detected in OKT3-stimulated cultures: IFN-γ concentrations were significantly higher in MM supernatants on day 2 (P = .008), day 3 (P = .03), and day 4 (P = .005) (Fig 7A); TNF-β was higher on day 4 (P = .01), day 5 (P = .001), and day 6 (P = .04) (Fig 7B); whereas TNF-α was lower on day 1 (P = .03), day 2 (P = .02), day 3 (P = .0009), day 4 (P = .0001), day 5 (P < .0001), and day 6 (P = .0001) (Fig 7C).

The role of these cytokines in CD3-generated antitumor activity was investigated by adding neutralizing antibodies to OKT3-stimulated cultures (Fig 8). Concentrations of antibodies were well above the peak concentrations of cytokines in the supernatants. Anti-IFN-γ partially blocked plasma cell killing (OKT3, 83% ± 12%; OKT3 + anti-IFN-γ, 58% ± 21%; P < .05), whereas anti-TNF-α and anti-TNF-β had no effect. No synergistic effect was observed when the antibodies were added together.

Direct cell-to-cell contact is required for CD3-induced plasma cell killing. To investigate the role of cell-to-cell contact rather than soluble factors in CD3-induced plasma cell killing, some experiments were performed using Transwell culture plates in which the top and bottom halves are separated by a 0.4-μm membrane allowing the diffusion of soluble factors only. BMMCs were stimulated with immobilized OKT3 in the bottom half, while an aliquot of the same BMMCs was left unstimulated in the top half after depletion of T cells. TNF-β concentrations in the supernatants from the bottom and the top halves were also determined. Standard cultures with aliquots of BMMCs and T-cell–depleted BMMCs were set up in regular plates as a control. T-cell activation, plasma cell decrease, and TNF-β production in the bottom half were comparable to those observed in standard BMMC cultures (Fig 9, left). The top half showed no T-cell activation, a smaller plasma cell decrease (20% to 30% compared with the standard T-cell–depleted BMMCs), and high TNF-β concentrations similar to those in the bottom half (Fig 9, right). These data indicate that cell-to-cell contact is the main mechanism by which CD3-activated T cells act on plasma cells and confirm the complementary role of soluble factors such as IFN-γ.

CD3-induced antiplasma cell activity is not dependent on significantly lower in the absence of exogenous IL-2 (P < .05) in 24-hour primed cultures only. This difference was not detected anymore in 48- and 72-hour primed cultures, showing that full activation of MM T cells requires an exposure of at least 48 hours to endogenous cytokines (or exogenous IL-2) other than OKT3.

The generation of CD3-induced antitumor activity is T-cell dependent. To determine the role of T cells in CD3-induced antiplasma cell activity, three BMMC aliquots were cultured without further processing under standard conditions (data not shown), after depletion of T cells (Fig 6A) or depletion of phagocytic cells (Fig 6B). T-cell depletion almost completely abrogated CD3-induced T-cell activation and plasma cell killing, whereas phagocytic cell depletion had no effect. In addition, when unrelated MoAbs or polyclonal mouse Igs other than OKT3 were immobilized, no effect was observed on T cells or plasma cells (data not shown).
promiscuous LAK activity. MM T cells release up to 8 to 10 U/mL IL-2 when stimulated with OKT3. To investigate whether CD3-induced antiplasma cell activity simply reflected the generation of promiscuous LAK activity, plasma cell killing induced by OKT3 and by 50 U/mL IL-2 was compared in 20 patients (Fig 10A). Unlike OKT3, IL-2 did not induce any evident expansion of CD3+ CD25+ cells, and generated significantly lower plasma cell killing \( (P < .001) \). Generation of promiscuous LAK activity was further investigated by comparing the cytotoxicity generated by OKT3 and IL-2 (50 U/mL) in a 4-hour standard \(^{51} \)Cr release assay against unrelated targets such as the K562, Daudi, and the myeloma-derived cell line LP-1. Results are shown in Fig 10B. The cytotoxicity observed in OKT3-treated cultures was not different from IMDM cultures (always \( P > .05) \), whereas that induced by IL-2 was significantly higher (always \( P < .05) \).

**Fig 7.** Kinetics of IFN-\( \gamma \) (A), TNF-\( \beta \) (B), and TNF-\( \alpha \) (C) production in the supernatants of OKT3-stimulated BMMC cultures of (•) MM and (○) the controls. At the indicated times, supernatants were collected and stored at \(-70^\circ \text{C}\) until assessed for cytokine contents. All patients and the controls had T-cell activation upon CD3 stimulation. Each point represents the mean value ± SE of four to six experiments (IFN-\( \gamma \)), four to eight experiments (TNF-\( \beta \)), and three to five experiments (TNF-\( \alpha \)).

**Fig 8.** Effect of neutralizing anti-TNF-\( \alpha \), anti-IFN-\( \gamma \), anti-TNF-\( \beta \), and their combination on CD3-induced plasma cell decrease. BMMCs were incubated for 6 days with the indicated reagents at the final concentrations of 40 U/mL anti-TNF-\( \alpha \) (2,000 pg/mL final neutralizing capacity), 800 U/mL anti-IFN-\( \gamma \) (32,400 pg/mL final neutralizing capacity), and 10 ng/mL anti-TNF-\( \beta \) (10 ng/mL final neutralizing capacity). Each bar represents the mean value ± SE from 6 to 9 patients. Results are expressed as percent plasma cell decrease according to the formula:

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\frac{\text{Plasma Cell Counts in OKT3 ± Antibody-Treated Cultures}}{\text{Plasma Cell Counts in IMDM Cultures}} \times 100
\]

**Fig 9.** Role of direct cell-to-cell contact versus soluble factors in CD3-induced plasma cell killing. BMMCs were stimulated with OKT3 in the bottom half of Transwell plates, whereas a second aliquot was depleted of T cells and left unstimulated in the top half. The top and bottom halves are separated by a membrane that allows the diffusion of soluble factors only. (Left) CD3+ CD25+ cell expansion, plasma cell decrease, and TNF-\( \beta \) concentration in the bottom half; (right) CD3+ CD25+ cell expansion, plasma cell decrease, and TNF-\( \beta \) concentration in the top half. Each bar represents the mean value ± SE of four experiments.
DISCUSSION

This study shows that T cells in the BM of MM patients are highly susceptible to CD3 stimulation and their activation is associated with a marked decrease in the number of autologous malignant plasma cells. The expansion of MM CD3+ CD25+ cells displays distinct features in terms of kinetics and magnitude compared with T cells in normal BM: the former are more reactive to OKT3 and their number increases more rapidly than normal T cells. The CD3 hyper-reactivity of MM BM T cells was reminiscent of that of peripheral blood T cells. The plasma cell decrease was at least threefold in 40%, 10-fold in 25%, and more than 100-fold in 20% of MM patients. It did not merely reflect plasma cell dilution among proliferating T cells: first, there was no correlation between the magnitude of T-cell expansion and that of plasma cell killing; second, the decrease was already detectable on day 2, when the number of CD3+ CD25+ cells had not yet increased significantly. Only 13% of MM showed a CD3-induced expansion of CD3+ CD25+ T cells without any effect on plasma cells. Whether these patients represent an MM subgroup whose BM plasma cells are less susceptible to T-cell control is currently under investigation.

Depletion experiments have shown that the CD3-induced antiplasma cell activity is mediated by T cells. Removal of phagocytic cells has no effect, and unrelated MoAbs or control mouse myeloma proteins do not activate T cells or decrease plasma cells, further confirming that OKT3 does not act by simply eliciting nonspecific antibody-dependent cell cytotoxicity (ADCC) or nonspecific activation of FcR-bearing cells. CD3+ CD25+ cells may have exerted their antiplasma cell activity through two mechanisms: first, by secreting an excess of cytokines with antiplasma cell activity; second, via direct cell contact and lysis. Studies on TIL and LAK cells have not clarified yet which mechanism is predominant. When cytokine production was investigated, MM BMMCs showed a distinct pattern compared with normal BM T cells, i.e., higher IFN-γ and TNF-β, but lower TNF-α production. Whether this pattern reflects a peculiar state of functional competence specific to MM T cells deserves further investigation. IFN-γ only was found to play a role, but limited to a 20% to 30% of CD3-induced plasma cell killing. Transwell cultures showed that direct cell-to-cell contact was the main mechanism by which OKT3-activated T cells exert antiplasma cell activity. In these experiments, T-cell-depleted BMMC were used in the top half to minimize the possibility that OKT3 accidentally unbound from the bottom half crossed the membrane and activated T cells in the top half. In addition, TNF-β concentrations were determined in the supernatants from the bottom and the top halves to verify the free diffusion of cytokines across the membrane. Transwell cultures also showed that the mixture of cytokines produced by CD3-activated T cells (very likely several more than those measured, including IL-6 and GM-CSF, which can be stimulatory on plasma cells15,17) did not promote plasma cell growth as the final result; secondly, the antiplasma cell activity of IFN-γ was direct and not mediated by the amplification of T-cell effector mechanisms.30

The relevance of cell-to-cell contact and the lack of significant TNF-α production suggest that the effector mechanisms used by CD3-activated T cells are different from those used by TIL cells, which mediate tumor regression mainly through the production of IFN-γ and TNF-α. The CD3-induced antiplasma cell activity also differed from LAK activity, which was not generated by OKT3 stim-
ulation; side-by-side experiments showed that IL-2 is less effective than OKT3 and did not induce a comparable expansion of CD3⁺ CD25⁺ cells, as previously reported. Secondly, unlike the much higher number of CD3⁺ CD25⁺ cells, OKT3 did not generate cytotoxicity against a variety of unrelated targets, including a myeloma-derived cell line. Although these data do not show that the cytotoxicity generated by CD3 stimulation was plasma cell restricted, several reports indicate that CD3 stimulation, compared with IL-2, acts preferentially on tumor-sensitized T cells and even small numbers of memory-tumor-specific T cells may very rapidly be amplified in resting populations.

Triggering of antitumor effector mechanisms by CD3 stimulation can be achieved under in vivo or ex vivo conditions. The former approach, requiring soluble OKT3, has been used in mice and humans and has clearly been shown to induce T-cell activation. Immunostimulation or immunosuppression as the final result depends mainly on the dosage. However, even when used to suppress T-cell responses, the first shot of OKT3 induces transient T-cell activation and cytokine release, which subside in about 24 hours, when further administrations of high doses of OKT3 lead to permanent downregulation of cell surface CD3; in the absence of CD3, OKT3 cannot further trigger T cells. Few data are available to predict how long CD3-induced T-cell activation may last after a single shot of OKT3. Data in mice have shown that a single dose may significantly protect against a subsequent tumor challenge up to 60 days later and may prevent the growth of progressive tumors for 3 weeks. Data in humans are not available. The only study reporting OKT3 treatment in vivo as an immunostimulatory regimen for cancer patients was based on multiple injection of low doses of OKT3 (from 1 to 100 μg) and did not show any clear evidence of T-cell activation. Our data indicate that T-cell activation may last up to 10 days in vitro. Conditions similar to those established for culture are hardly likely to be maintained for so long in vivo. CD3-activated T cells require the costimulatory signalling of endogenous cytokines for at least 48 hours. This is unlikely to happen in vivo, due to the transient CD3-induced cytokine release. Our data and published work indicate that exogenous IL-2 may be a substitute for endogenous cytokines as early as 24 hours after CD3 stimulation even if OKT3 has been removed. This is due to the very rapid CD3-induced CD25 upregulation that makes T cells very sensitive to IL-2. Full T-cell activation upon CD3 stimulation is thus dependent on adequate exposure to cytokines other than OKT3. The experiments in mice have recently shown in two hematologic patients that a single injection of soluble OKT3 followed by continuous infusion of IL-2 induces detectable expansion of CD3⁺ CD25⁺ cells in the peripheral blood.

T-cell activation can also be achieved by stimulating T cells ex vivo before autologous adoptive transfer. Both soluble and immobilized anti-CD3 MoAbs have been used in murine models, but the latter may be one up on the former: immobilized MoAbs are unparalleled to induce the expansion of unprimed or tumor-sensitized T cells in 7 to 14 days and are effective with isolated T-cell subsets or in the absence of accessory cells. By preventing CD3 downmodulation and being absent from the surface of T cells after stimulation, they may also decrease the risk of inadvertent immunosuppression associated with soluble MoAbs. The T-cell activation induced by ex vivo CD3 stimulation can be amplified and prolonged in vivo by subsequent IL-2 infusion. This approach has very recently been reported in cancer patients.

A variety of treatments, including autologous and allogeneic BM transplantation, are currently under investigation to improve long-term survival in myeloma patients. Activation of host antiplasma cell effector mechanisms may be favorably considered once the tumor mass has been reduced. The hyperreactivity of myeloma T cells to CD3 stimulation may offer a chance to rapidly activate T-cell-mediated effector mechanisms. Both soluble and plastic immobilized OKT3 could be used in MM, because T-cell activation and antiplasma cell activity are generated under both conditions. A current pilot study at our institution in chemoreduced high-risk myeloma patients is based on the sequential administration of soluble OKT3 on day 1 (60 μg/mq) followed by continuous infusion of IL-2 for 6 days at doses decreasing from 12 × 10⁶ UI/mq to 6 × 10⁶ UI/mq. The regimen has been proved safe and well tolerated. Five courses have so far been delivered to 3 patients with clear evidence of T-cell activation in vivo; the number of CD3⁺ CD25⁺ cells in the peripheral blood has increased as high as 9,000/μL. It is encouraging that CD3 stimulation followed by IL-2 infusion induces significant T-cell activation in vivo in MM. A number of strategies can be adopted to further improve the efficacy of CD3 stimulation, such as more appropriate timing and dosage of OKT3 and IL-2; the use of liposomal-encapsulated IL-2, which is more potent and less toxic than unencapsulated IL-2; the use of bispecific antibodies to specifically target CD3⁺ CD25⁺ cells on plasma cells. Further in vitro investigation and the clinical results of ongoing trials in cancer patients will help to refine these strategies and determine whether CD3 stimulation could really be the source of alternative immunotherapeutic options.

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Rapid generation of antiplasma cell activity in the bone marrow of myeloma patients by CD3-activated T cells

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