INTERLEUKIN 3 AND INTERLEUKIN 6 SYNERGISTICALLY PROMOTE THE PROLIFERATION AND DIFFERENTIATION OF MALIGNANT PLASMA CELL PRECURSORS IN MULTIPLE MYELOMA

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Multiple myeloma (MM) is a human B cell malignancy characterized by the bone marrow (BM) accumulation of plasma cells that secrete monoclonal Ig and cause typical osteolytic lesions. By the time the disease is detected, it is already widespread throughout the axial skeleton. Still, few plasma cells are seen in the circulation and only at the terminal phase of the disease, suggesting that MM may be disseminated to the BM by circulating precursors. Transplantation experiments have shown that the clonogenic cells are circulating in murine plasmacytoma. Four different lines of evidence favor a similar possibility in humans. First, peripheral B lymphocytes expressing the relevant idiotype have been described. Second, DNA-aneuploid cells have been detected in peripheral blood (PB) samples. Third, plasma cell colonies may be grown from PB. Finally, PBMC may show a monoclonal Ig gene rearrangement.

We reasoned that if precursors are circulating they will be revealed as monoclonal plasma cells upon in vitro exposure to the proper growth and differentiation factors. The present work shows that PBMC from patients with MM, cultured in presence of IL-3 and IL-6 for 6 d, give origin to a population of actively proliferating B blasts that differentiate into monoclonal plasma cells expressing, in each individual case, the same L and H chain produced by BM malignant plasma cells. We conclude that plasma cell precursors circulate in the peripheral blood of MM patients and that their growth and differentiation are under the synergistic control of IL-3 and IL-6.

Materials and Methods

Cells. PBMC were obtained by Ficoll-Hypaque gradient separation from 11 patients with MM at diagnosis, 5 men and 6 women, and from 4 normal subjects matched for age and sex. Seven patients were in stage I-II and four were in stage III according to Durie and Salmon.

Cytokines. rIL-1α (No. A-IL-1; Genzyme, Boston, MA), rIL-1β (No. B-IL-1), rIL-3 (No. 87.01207.44); Genzyme, Boston, MA), rIL-1β (No. B-IL-1).

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H-IL-3), r-IL-4 (No. H-IL-4) and r-IL-6 (No. H-IL-6, all from Genzyme) were used either alone or in various combinations. The following concentrations were used: IL-1α + β, 25–50 + 25–50 U/ml; IL-3, 5–15 U/ml; IL-4, 500–1,000 U/ml; IL-6, 200–1,000 U/ml.

Antibodies. Polyclonal antibodies (Abs) to μ, δ, γ, α, λ, and κ chains directly conjugated with tetraethylrhodamine (TRITC) were from Dakopatts, Glostrup, Denmark (No. R-152, R-153, R-151, R-153, R-154, R-155). The following mAbs were used: anti-HLA-DR (No. 7360; Becton Dickinson & Co., Mountain View, CA); CD3 (Leu 4, No. 7340); CD10 (anti-CALLA, No. 7500; both Becton Dickinson & Co.); CD14 (MO1, No. 6602147; Coulter Immunology, Hialeah, FL); CD19 (B4, No. 6603024; Coulter Immunology); CD38 (A10, reference 9), PCA-1 (No. 6603471, Coulter Immunology).

Cell Cultures. PBMC cells (2 x 10⁶/ml) were cultured in 24 Limbro plates (Flow Laboratories, Irvine, CA) for up to 6 d at 37°C and 5% CO₂ either alone or in presence of different combinations of cytokines. Cell samples were harvested at days 3 and 6 and analyzed for the morphology, DNA synthesis, and phenotype. The morphology was evaluated with conventional May-Grünwald-Giemsa (MGG) staining on cytocentrifuge slides. The proportion of cells in the S phase of the cell cycle was evaluated by adding bromodeoxyuridine (BrdU, No. B-5002; Sigma Chemical Co., St. Louis, MO) at the final concentration of 10 µM to 10⁶ cells resuspended in RPMI medium. The cells were incubated for 1 h, washed, fixed (10), and cytospins were obtained. Cells incorporating Brdu were identified in indirect immunofluorescence (IF) by means of a specific anti-BrdU mAb (No. 7580, Becton Dickinson & Co.). The phenotype was defined by double staining IF on cytospins air dried and fixed with cold acetone (4°C). Polyclonal Abs were used in direct IF at 1:50 final dilution. mAbs were used in indirect IF either as culture supernatants in 1:5 final dilution or as purified Ig at final dilution of 2.5–µg/ml and revealed by rabbit (R)-anti-mouse (M)-Ig labeled with FITC (No. F-232, Dakopatts) and used at 1:10 dilution. A Zeiss epifluorescent microscope equipped with a planapochromat 63 x /1.4 oil immersion lens was used.

Results

MGG staining (Fig. 1A) and IF studies with anti-Ig isotypes established that the percentage of plasma cells in the PBMC seeded at day 0 was between 0.01 and 0.2%. B cells (CD19⁺ cells) were 10.2 ± 2.8%, T lymphocytes (CD3⁺ cells) were 63 ± 10%, and monocytes (CD14⁺ cells) were 15 ± 5%. The proportion of DNA-synthesizing (BrdU⁺) cells was 0.13 ± 0.06%. Preliminary experiments were performed on these cultured MM PBMC to determine the optimal combinations and concentrations of cytokines using two parameters to assess the cytokine activity: (a) the proportion of Brdu⁺ cells after 3 d of culture and (b) the appearance of plasma cells after 6 d of culture. These experiments unequivocally showed that the combination of IL-3 (15 U/ml) + IL-6 (1,000 U/ml) concomitantly added to the cultures at day 0 was strikingly active on MM PBMC cells (Table I). IL-1, IL-3, IL-4, and IL-6 used alone as single stimuli and the various combinations of IL-1 + IL-3, IL-1 + IL-4, IL-1 + IL-6, IL-3 + IL-4, IL-4 + IL-6 concomitantly added at day 0 were instead ineffective. Likewise inefficient was the priming of MM PBMC with IL-3 (or IL-6) at day 0 followed by the addition of IL-6 (or IL-3) at day 3. The simultaneous addition to the cultures at day 0 of IL-3 (15 U/ml) + IL-6 (1,000 U/ml) was therefore used throughout the experiments. IL-3; IL-6-induced long-term cultures were not attempted.

**MM PBMC Cultured for 3 d in the Presence of IL-3 + IL-6 Give Origin to Proliferating Immunoblast-like B Cells.** After 3 d of culture in presence of IL-3 + IL-6, a population of immunoblast-like cells (Fig. 1B) appeared in all the MM samples studied (35 ± 12.6%, range 20–62%, Table I). These large blasts were actively proliferating (8.5 ± 2.5% Brdu⁺ cells, Table I) and belonged to the B cell lineage as they were HLA-
FIGURE 1. MM PBMC (A) were cultured in vitro in presence of IL-3 + IL-6. After 3 d, large immunoblast-like cells appeared (B, arrows). After 6 d, a population of plasma cells was morphologically evident (C). These plasma cells were sometimes binucleated (D, arrows) and expressed monoclonal Ig light and heavy chains (anti-k-TRITC; E). PBMC from normal donors were unaffected by the IL-3 + IL-6 combination (F).

DR+, uniformly expressed the B cell–specific CD19 and CD20 antigens (Table II), while they did not react with the T cell–specific CD3 and the monocyte–specific CD14 mAbs (<1%). Also, these cells were CD38+, but no Ig molecules could be observed on their surface nor in the cytoplasm (Table II). In 3 of 11 cases, the vast majority

| Case | Isotype | Stage | Plasma cells | Brdu+ cells | Blasts | Brdu+ blasts | Plasma cells | Brdu+ plasma cells |
|------|---------|-------|--------------|-------------|--------|--------------|--------------|-------------------|
| 1    | γκ      | I     | 0.01         | 0.1         | 25 (2)*| 8.1 (0.1)*   | 40 (2)*      | 1.0               |
| 2    | γκ      | I     | 0.01         | 0.1         | 23 (1) | 6.0 (0.2)    | 35 (2)       | 1.0               |
| 3    | ακ      | II    | 0.05         | 0.1         | 20 (3) | 7.5 (0.1)    | 38 (1)       | 0.1               |
| 4    | ακ      | II    | 0.03         | 0.1         | 35 (4) | 8.3 (0.2)    | 42 (3)       | 0.1               |
| 5    | γλ      | II    | 0.03         | 0.1         | 30 (3) | 9.0 (0.3)    | 30 (2)       | 0.1               |
| 6    | γλ      | II    | 0.05         | 0.1         | 30 (5) | 9.3 (0.1)    | 30 (2)       | 0.1               |
| 7    | γκ      | II    | 0.1          | 0.1         | 30 (2) | 10 (0.2)     | 35 (3)       | 0.1               |
| 8    | λκ      | III   | 0.1          | 0.2         | 35 (4) | 5.7 (0.3)    | 40 (3)       | 0.1               |
| 9    | γκ      | III   | 0.2          | 0.3         | 62 (7) | 8.1 (0.5)    | 45 (5)       | 0.2               |
| 10   | γκ      | III   | 0.1          | 0.1         | 50 (4) | 15 (1.0)     | 35 (1)       | 0.5               |
| 11   | γκ      | III   | 0.1          | 0.2         | 45 (5) | 6.6 (1.0)    | 50 (4)       | 0.2               |
| Mean |         |      | 0.81 ± 0.13  | 35 ± 12.6   | 8.5 ± 2.5 | 38.2 ± 6.09 | 0.3 ± 0.3    |
| ± SD |         |      | (0.1 ± 0.06) | (3.6 ± 1.7) | (0.4 ± 0.3) | (2.5 ± 1.2) |               |

Normal donors (four samples)

<0.001 <0.1 0.5 ± 0.3 <0.5 0.6 ± 0.3

* Control culture data in parentheses.
of blasts (78, 85, 90%, respectively) expressed the CD10 antigen (Table II). These three patients (Nos. 8, 9, 10; Table I) had an aggressive stage III MM and their PBMC produced high numbers of proliferating immunoblast-like cells. The percentage of B blasts was 3.6 ± 1.7% in MM control culture cells and <1% in samples from normal donors (Table I).

**MM PBMC Cultured for 6 d in Presence of IL-3 + IL-6 Give Origin to Monoclonal Plasma Cells.** After 6 d of culture in the presence of IL-3 + IL-6, a population of morphologically evident plasma cells (Fig. 1 C), sometimes binucleated (Fig. 1 D), became apparent in all the MM samples studied (38.2 ± 6.09%, range 30–50%; Table I). Some large blast-like cells were still present (15.5 ± 4.7%) and these were incorporating BrdU, while the proportion of BrdU+ plasma cells was 0.3 ± 0.3% (Table I). The plasma cells were CD10+, weakly CD19+, strongly CD38+, PCA-1+ (Table II). In 3 of 11 cases, the plasma cells expressed in the cytoplasm the same light and heavy chain produced by the BM myeloma cells (Fig. 1 E): these were the three cases who had produced CD10+ B blasts after 3 d of culture (Table I). Cytoplasmic monoclonal light and heavy chains were expressed by the plasma cells of all the remaining eight cases, when the initial signal provided by IL-3 + IL-6 was followed by a refeeding of IL-6 (1,000 U/ml) at day 3. The proportion of morphologically evident plasma cells was 2.5 ± 1.2% in MM control cultures and 0.6 ± 0.3 (Table I) in normal subjects (Fig. 1 F).

**Discussion**

In this paper we show that PBMC obtained from MM patients harbor a population of cells that, after exposure to IL-3 and IL-6, develop into proliferating immunoblast-like B cells (Fig. 1 B) and subsequently differentiate into monoclonal plasma cells (Fig. 1, C–E). Our findings demonstrate that malignant plasma cell precursors circulate in the PB of MM patients and establish that their terminal differentiation is promoted by the concerted action of IL-3 and IL-6. IL-3 supports the proliferation of B cell precursors in the murine lymphohemopoietic system (11, 12) and has been recently shown to be active also on human acute lymphoblastic leukemia B blasts (13). IL-6 promotes the differentiation of activated B cells (14, 15), and in con-

**Table II**

|                      | Day 3 blasts |          | Day 6 plasma cells |          |
|----------------------|--------------|----------|--------------------|----------|
|                      | Positive/tested | Percent | Positive/tested | Percent |
| Surface Ig           | 0/11         | <1      | 0/11              | <1      |
| Cytoplasmic Ig       | 0/11         | <1      | 11/11             | >95     |
| HLA-DR               | 11/11        | 87 ± 12 | 3/11              | 65 ± 11*|
| CD 10                | 3/11         | 84 ± 5* | 0/11              | 3 ± 2   |
| CD 19                | 11/11        | 90 ± 7  | 10/11             | 90 ± 51 |
| CD 38                | 11/11        | 92 ± 7  | 11/11             | 91 ± 6  |
| PCA-1                | 4/11         | 75 ± 9  | 11/11             | 85 ± 6  |

* Mean ± SD of positive cases.
† Weak reactivity.
§ Same light and heavy chain produced, in each case, by bone marrow plasma cells.
cert with IL-3, may also effect multipotential stem cells (16). Further, IL-6 receptors are expressed on the surface of MM plasma cells (17) and indeed IL-6 not only controls the growth of murine malignant plasma cells (18) but also influences the proliferation of human MM cells (17, 19). It is unlikely that MM precursors have a plasma cell morphology since, in our culture system, high numbers of monoclonal plasma cells were obtained at day 6 even when the plasma cells seeded at day 0 were 0.01%. Likewise, CD10+ cells are not "the" clonogenic cells of MM but appear to represent a hyperactive transition compartment of proliferating pre-plasma cells (20). This observation provides a biological explanation for the highly aggressive clinical behavior of CD10+ MM, as are defined the cases with a high proportion of BM CD10+ B cells (21) that may eventually spill over in the PB (22). The IL-3/IL-6 responsiveness of MM circulating plasma cell precursors favors the view that the transforming events of MM occur at an early stage of hematopoiesis (2, 5, 20).

Our findings suggest the likely dynamics of precursor-plasma cell transition within the MM malignant clone. MM plasma cell precursors have IL-3 and IL-6 receptors, circulate and find the appropriate microenvironment enriched in IL-3 + IL-6 in the BM, where they first transform into actively proliferating blast-like cells possibly expressing the CD10 antigen, and then they develop into monoclonal plasma cells. This model indicates that a close cooperation between transformed cells and cytokine-producing cells may be a major mechanism in the progression of MM (2) and also has two apparent clinical implications. First, the culture of PBMC in the presence of IL-3 + IL-6 may be a simple assay to monitor the patient's response to the treatment and to assess a complete remission. Second, the burst of proliferative activity observed after 3 d of culture of PBMC in presence of IL-3 + IL-6 may be applied to the cytogenetic investigation of MM patients that is usually performed on BM samples and provides unsatisfactory results because of the very low fraction of cells in DNA synthesis (23).

Summary

PBMC from 11 patients with multiple myeloma (MM) were cultured in vitro in presence of IL-3 and IL-6. After 3 d, actively proliferating immunoblast-like B cells (20-62%) were apparent. After 6 d, a population of morphologically evident plasma cells was observed (30-50%) that expressed, in each individual case, the same light and heavy chain produced by bone marrow malignant plasma cells. We conclude that in MM the malignant plasma cell precursors are circulating and their growth and terminal differentiation are under the synergistic control of IL-3 and IL-6.

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