Primary Tumor Cells of Myeloma Patients Induce Interleukin-6 Secretion in Long-Term Bone Marrow Cultures

By Henk M. Lokhorst, Tanja Lamme, Martin de Smet, Siegrid Klein, Roel A. de Weger, Rien van Oers, and Andries C. Bloem

Long-term bone marrow cultures (LTBMC) from patients with multiple myeloma (MM) and normal donors were analyzed for immunophenotype and cytokine production. Both LTBMC adherent cells from myeloma and normal donor origin expressed CD10, CD13, the adhesion molecules CD44, CD54, vascular cell adhesion molecule 1, very late antigen 2 (VLA-2), and VLA-5, and were positive for extracellular matrix components fibronectin, laminin, and collagen types 3 and 4. LTBMC from myeloma patients and normal donors spontaneously secreted interleukin-6 (IL-6). However, levels of IL-6 correlated with the stage of disease; highest levels of IL-6 were found in LTBMC from patients with active myeloma. To identify the origin of IL-6 production, LTBMC from MM patients and normal donors were cocultured with BM-derived myeloma cells and cells from myeloma cell lines. IL-6 was induced by plasma cell lines that adhered to LTBMC such as ARH-77 and RPMI-8226, but not by nonadhering cell lines U266 and FRADVEL. Myeloma cells strongly stimulated IL-6 secretion in cocultures with LTBMC adherent cells from normal donors and myeloma patients. When direct cellular contact between LTBMC and plasma cells was prevented by tissue-culture inserts, no IL-6 production was induced. This implies that intimate cell-cell contact is a prerequisite for IL-6 induction. Binding of purified myeloma cells to LTBMC adherent cells was partly inhibited by monoclonal antibodies against adhesion molecules VLA-4, CD44, and lymphocyte function-associated antigen 1 (LFA-1) present on the plasma cell. Antibodies against VLA-4, CD29, and LFA-1 also inhibited the induced IL-6 secretion in plasma cell-LTBMC cocultures. In situ hybridization studies performed before and after coculture with plasma cells indicated that LTBMC adherent cells produce the IL-6. These results suggest that the high levels of IL-6 found in LTBMC of MM patients with active disease are a reflection of their previous contact with tumor cells in vivo. These results provide a new perspective on tumor growth in MM and emphasize the importance of plasma cell-LTBMC interaction in the pathophysiology of MM.

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MATERIALS AND METHODS

Patients

BM samples were obtained from normal individuals that were donors for an allogeneic BM transplant. All MM patients were in stage 3 according to the criteria of the Southwest Oncology Group. Patients with nonactive disease had no or mild clinical symptoms and their M protein remained stable for at least 3 months before the time of analysis. Patients with active disease showed symptoms, including bone pain, and their M protein increased for at least 25% in the 1 months before the analysis.

LTBMC

BM mononuclear cells (BMMC) were isolated by Ficoll-Isopaque (Pharmacia, Uppsala, Sweden) density centrifugation of BM aspirates.
Tables and Figures

Table 1. Characterization of LTBMC-Adherent Cells

<table>
<thead>
<tr>
<th>MoAb</th>
<th>Mean % of Positive Cells (n = 6)*</th>
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<tbody>
<tr>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td>CD10</td>
<td>45 (13-78)†</td>
</tr>
<tr>
<td>CD13</td>
<td>43 (9-90)</td>
</tr>
<tr>
<td>CD54</td>
<td>39 (18-67)</td>
</tr>
<tr>
<td>CD95</td>
<td>95 (82-100)</td>
</tr>
<tr>
<td>CD29</td>
<td>94 (88-99)</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>56 (12-80)</td>
</tr>
<tr>
<td>VLA-2</td>
<td>29 (12-65)</td>
</tr>
<tr>
<td>VLA-5</td>
<td>85 (59-100)</td>
</tr>
<tr>
<td>Collagen type III</td>
<td>+‡</td>
</tr>
<tr>
<td>Collagen type IV</td>
<td>+</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>+</td>
</tr>
<tr>
<td>Laminin</td>
<td>+</td>
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</tbody>
</table>

* Analyzed by flow cytometry.
† Brackets represent range in percentage positive cells.
‡ Cytoplasmic analysis by immunofluorescence microscopy; ±, weakly positive; +, positive; ++, strongly positive.

Fig 2. Adhesion of myeloma cell lines to LTBMC adherent cells (second passage) from two patients with multiple myeloma (MM 1, [●]; MM 2, [●]) and two normal donors (norm. donor, [●] and [●]) was evaluated in a static adhesion assay. The adhesion ratios were determined after 1 hour by flow cytometry as described in Materials and Methods.

Cell Lines and Purification of Myeloma Cells From BM Aspirates

The human myeloma cell lines ARH-77, RPMI-8226, U-266, and Fravel were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in RPMI 1640 with 5% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin (GIBCO). BMMC from aspirates from MM patients were isolated by Ficoll-Hypaque density centrifugation. Myeloma plasma cells were purified by high-resolution cell sorting on the basis of CD38 expression and forward and orthogonal light-scatter properties. The sorted cells were cultured, and the CD38+ cell population was assessed for adhesion to LTBMC adherent cells by flow cytometry. The CD38+ population was then analyzed for expression of other markers such as CD138, CD118, VCAM-1, and CD15, as well as for the production of cytokines such as IL-6 and IL-8. The results showed that the CD38+ cell population expressed high levels of CD138 and VCAM-1, and produced significant amounts of IL-6, suggesting that these cells were actively producing cytokines and therefore were likely to be myeloma plasma cells. The purified cells were then used in experiments to study their interaction with LTBMC adherent cells, as described in the Materials and Methods section.
consisted of 100% myeloma plasma cells as determined by morphologic analysis and analysis of the Ig content (heavy and light chain) of the isolated cells by cytoplasmic immunofluorescence microscopy.25,26

Cytokine Production

The following cytokines were quantified by enzyme-linked immunosorbent assay (ELISA) according to instructions of the manufacturer: IL-1α, IL-1β, IL-6, tumor necrosis factor α (TNFα), and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Med Genix, Flemur, Belgium), and IL-6 (CLB).

Adhesion of Plasma Cells and Myeloma Cell Lines

The binding of 18M plasma cells from MM patients and of cells from myeloma cell lines to LTBMc adherent cells was measured by direct counting and/or by a flow cytometric adhesion assay (FCAA) as described previously.22 LTBMc adherent cells (second passage) from normal donors and from MM patients were isolated as described above and plated in 24-well culture plates in a concentration of 50,000 cells/well. Using this concentration, a nearly confluent monolayer of LTBMc adherent cells is obtained after binding of the cells to the plastic (4 hours). As a source for plasma cells in the different experiments, either (1) cells from myeloma cell lines, (2) thawed BM aspirate cells from selected MM patients containing more than 95% CD38+ plasma cells, or (3) myeloma plasma cells purified from BM aspirates with a fluorescence-activated cell sorter (FACS) (see above) were added to the wells (2 × 10^6 cells/well). The cells were allowed to adhere to the LTBMc adherent cells under static conditions for 1 hour at 37°C. Nonadherent cells were removed by washing and quantitated by microscopic counting. The absolute number of bound plasma cells was calculated by subtracting the number of nonbound cells from the number of input cells. For the flow cytometric quantitation of plasma cell adhesion, a single cell suspension was made of LTBMc adherent cells and bound plasma cells by trypsinization, which was stained for CD38 expression and analyzed by flow cytometry. Plasma cells were distinguished from LTBMc adherent cells by morphologic criteria (forward scatter and side scatter) and CD38 expression.22 Within each sample, 10,000 cells were analyzed and an adhesion ratio was calculated, dividing the number of CD38+ myeloma cells by the number of LTBMc adherent cells. Similar adhesion experiments were performed with cells from the RPMI-8226 cell line that were mildly fixed for 10 minutes in 1% paraformaldehyde (PFA).

Binding inhibition studies were performed as described before.22,25 In short, confluent monolayers of LTBMc adherent cells were prepared as described above and FACS-purified myeloma cells (2 × 10^5/well, in triplicate) were allowed to adhere for 1 hour in presence of antibodies (10 μg/mL) directed against VLA-4 (HP1/7),29 CD44,30 CD11a (F8.8.2),31 or isotype- and subclass-matched mouse MoAbs. Plasma cell-LTBMc adhesion was quantitated by the FCAA described above.

Induction of IL-6 Production in LTBMc Adherent Cells

LTBMc adherent cells (second passage) were grown until confluency in 24-well culture plates as described above and cocultured for 48 hours with 2 × 10^5 cells from different myeloma cell lines, thawed BM aspirate cells of MM patients containing more than 95% plasma cells or FACS-purified myeloma plasma cells. Supernatants were harvested and analyzed for IL-6 content by ELISA. Similar experiments were performed with PFA-treated RPMI-8226 cells. Tissue-culture inserts (Falcon, Lincoln Park, NJ) were used to coculture RPMI-8226 cells and LTBMc adherent cells without allowing physical contact between cell populations. All experiments were performed in triplicate. The mean of the IL-6 levels in the individual wells was calculated. The standard deviation (SD) was always less than 10%.

In Situ Hybridization for Detection of IL-6 mRNA

Oligonucleotide primers. Oligonucleotide primers for IL-6 were synthesized in a DNA/RNA Synthesizer (Applied Biosystems, Foster City, CA). 5′ and 3′ primers (5′ ATGTAAGGCUCCCACACAGA 3′ and 5′ CATCCACCTTTTTCAGCCAT 3′, respectively) were chosen in different exons to avoid positive signals caused by contaminating DNA. The cytokine polymerase chain reaction (PCR) product generated with these primers was controlled by hybridization with an oligonucleotide complementary to internal sequences.

Digoxigenin (dig) labeling. Products from PCR reactions were used in a repeated PCR with the same cytokine-specific primers under the following conditions: 5 μL of PCR product, 2 mmol/L MgCl2, 0.5 μg 5′ and 3′ primer, 2 U Taq polymerase (GIBCO), 2 mmol/L of each deoxynucleotide (deoxyadenosine triphosphate, deoxyctydilne triphosphate, deoxyguanosine triphosphate, deoxythymidine triphosphate), and, in addition, 3.5 μg dig-labeled deoxyuridine triphosphate (dig-11-UTP), Boehringer Mannheim (Mannheim, Germany) in a volume of 50 μL. The reactions were performed in triplicate plus a control without dig-11-UTP. The resulting dig-labeled DNA was analyzed on 2% agarose gels and isolated by ethanol precipitation.

Hybridization. Hybridization was performed as described before.11,22 LTBMc adherent cells were grown on glass slides and cocultured for 24 hours with and without cells from the plasma cell line ARH-77. In the cocultures, the nonadherent plasma cells were removed by carefully rinsing the glass slides. The LTBMc adherent cells and bound plasma cells were fixed in 6% formalin, treated with proteinase K (Boehringer/ Mannheim), fixed again, permeabilized by 0.01% Triton-X 100, dehydrated with ethanol, and air dried. The glass slides were incubated with the hybridization mix, containing the dig-labeled IL-6 probe, at 42°C for 10 minutes and then at 37°C for 16 hours, washed and incubated with mouse-anti-dig antibodies (1 hour at room temperature, 100 μg/mL, Boehringer). After washing, the glass slides were subsequently incubated with rabbit-anti-mouse antibodies coupled to peroxidase (30 minutes at room temper-
podia was present in the MGG-stained preparations. Hemo-
poietic cells, including plasma cells and macrophages, were
CD10, CD13, and the adhesion molecules CD11a, CD54, 
broblast-like cells with basophilic cytoplasm with pseudo-
Churcitzulion
the LTBMC adherent cells (Table 1) showed positivity for 
adherent cells by immunofluorescent microscopy (Table 
laminin could be detected in the cytoplasm of the LTBMC 
CD3, vWF (cytoplasmic), and collagen type I (cytoplasmic) 
CD19, CD38, CD45, CD56 (N-CAM), CD18, CD11a (LFA-
integrins). Collagen types 3 and 4, as well as fibronectin and 
VCAM-1, VLA-2, VLA-5, and CD29 (P-chain of the 4-
positive cells were not identified, indicating the absence of 
hemopoietic and endothelial cells in the cultures. No appar-
onds with hematolin, rinsed with water, dehydrated with ethanol, 
and embedded in DPX (Klinipath, Duiven, NL).

ature, 1:100 dilution in phosphate-buffered saline (10% normal 
human serum, Dakopatts, Glostrup, Denmark) and swine-antirabbit 
Ig coupled to peroxidase (Dakopatts). Stained cells were visualized with 0.06% 
3,3 diaminobenzidine-tetrahydro-chloride (DAB; Sigma 
Chemical Co, St Louis, MO). Cells were counterstained for 30 sec-
onds with hemalum, rinsed with water, dehydrated with ethanol, 
and embedded in DPX (Klinipath, Duiven, NL).

RESULTS

Characterization of LTBMC Adherent Cells.

A homogeneous population of large spindle-shaped fi-
broblast-like cells with basophilic cytoplasm with pseudo-
podia, including plasma cells and macrophages, were 
scarce; usually less than 1%. Flow cytometric analysis of 
the LTBMC adherent cells (Table 1) showed positivity for 
CD10, CD13, and the adhesion molecules CD44, CD54, 
VCAM-1, VLA-2, VLA-5, and CD29 (β-chain of the β1-
integrins). Collagen types 3 and 4, as well as fibronectin and 
laminin could be detected in the cytoplasm of the LTBMC 
adherent cells by immunofluorescent microscopy (Table 1). 
CD19, CD38, CD45, CD56 (N-CAM), CD18, CD11a (LFA-
1), CD62, E-Selectin, CD49d/CD29 (VLA-3), CD38, CD14, 
CD3, vWF (cytoplasmic), and collagen type 1 (cytoplasmic) 
positive cells were not identified, indicating the absence of 
hemopoietic and endothelial cells in the cultures. No appar-
ent differences in antigenic profile between identical cultured 
LTBMC adherent cells from normal donors and MM patients 
were observed.

Cytokine Production by LTBMC Adherent Cells

The presence of several cytokines was assessed in super-
natants of the LTBMC. IL-1α, IL-1β, IL-3, TNFα and GM-
CSF could not be detected at any moment during culture 
(data not shown). IL-6 was consistently present in superna-
tants of LTBMC of MM patients and normal donors (Fig 1). 
Lowest levels were detected in LTBMC of normal donors 
(mean, 1,068; range, 480 to 1,980). In LTBMC of myeloma 
patients, IL-6 levels correlated with clinical stage of the 
patients (nonactive myeloma: mean, 1,702 U/mL [range, 870 
to 2,930]; and active myeloma: mean, 4,460 U/mL [range, 
3,160 to 7,230]).

Quantitative Binding of Myeloma Cell Lines to LTBMC 
Adherent Cells

For these experiments, LTBMC adherent cells from two 
MM patients and two normal donors and the cell lines ARH-
77, RPMI-8226, U266, and Fravel were used. Plasma cells 
were allowed to adhere to LTBMC adherent cells for 1 hour 
in static adhesion assays. Adhesion was quantified by flow 
cytometric analysis (Fig 2). ARH-77 and RPMI-8226 gave 
the best binding results; U266 and Fravel bound less effi-
ciently under these conditions. Quantification of binding by 
counting nonadherent cells (see Materials and Methods) gave 
similar results; 85% (range, 43% to 95%) of the ARH-77 
cells, 64% (39% to 80%) of the RPMI-8226 cells, 17% (5% 
to 28%) of the U266 cells, and 6% (0% to 18%) of the 
Fravel cells bound to the LTBMC adherent cells. There 
were no differences in quantitative binding of the plasma 
cells to normal and myeloma LTBMC adherent cells (data not 
shown).

IL-6 Secretion Is Induced After Coculture of Myeloma Cell 
Lines and Malignant Plasma Cells With LTBMC Adherent 
Cells

Cells from myeloma cell lines were cocultured for 48 
hours with LTBMC adherent cells from three MM patients 
and from one normal donor. In Fig 3, results from representa-
tive experiments are shown, indicating that IL-6 is produced

Fig 4. IL-6 present in supernatants of cocultures (48 hours) of 
LTBMC adherent cells from six MM patients (MM stroma, P1-6) and 
three normal donors (donor stroma, D1-3) with myeloma cell line 
ARH-77 (□) and BMMC containing more than 95% tumor cells of two 
myeloma patients (MM 1, □□□; and MM 2, □□□). Mean IL-6 levels (SD < 
10%) of triplicate cultures are shown. Control (○): IL-6 levels in 
supernatants of LTBMC cultured without plasma cells.

Fig 5. (A) PFA treatment of RPMI-8226 does not interfere in the 
ability to induce enhanced IL-6 secretion in LTBMC cocultures. Con-
trol: spontaneous IL-6 secretion in LTBMC, RPMI-8226: IL-6 levels 
found after coculture of RPMI-8226 and LTBMC adherent cells; RPMI-
8226 PFA: IL-6 levels found after coculture of PFA fixed RPMI-8226 
cells and LTBMC adherent cells. (B) Direct cell-cell contact is neces-
sary for induction of IL-6 secretion in cocultures of LTBMC adherent 
cells and RPMI-8226. Control: spontaneous IL-6 secretion in LTBMC; 
RPMI-8226: IL-6 levels in supernatants of cocultures of 
RPMI-8226 cells and LTBMC adherent cells without using a tissue-culture 
insert (TCI); RPMI-8226 + TCI: IL-6 levels in supernatants of cocultures of 
RPMI-8226 cells and LTBMC adherent cells using a tissue-culture 
insert to prevent plasma cell-LTBMC adherent cell contact. (C) Instead 
of RPMI-8226 cells (B), BM aspirate cells from a MM patient (MM) 
containing more than 95% tumor cells were used. Results of a repre-
sentative experiment of three independent experiments are shown. All 
cultures were performed in quadruplicate and supernatants were 
pooled and analyzed for IL-6 content.
during coculture of LTBMC adherent cells and plasma cell lines RPMI-8226 and ARH-77. No major differences were observed when LTBMC adherent cells from normal donors or myeloma patients were used. Spontaneous IL-6 secretion by the LTBMC adherent cells in these experiments ranged from 1,500 to 4,100 U/mL, depending on the origin of the LTBMC (normal versus myeloma). U266 and Fravel did not or only marginally enhanced IL-6 secretion during coculture with LTBMC adherent cells.

When LTBMC adherent cells, either from myeloma patients or normal donors, were cocultured with thawed BM aspirate cells from two MM patients containing more than 95% plasma cells, high levels of IL-6 could be measured in the supernatants of these cultures (Fig 4). In all experiments, coculture of LTBMC adherent cells with myeloma tumor cells generated significantly higher levels of IL-6 as compared with coculture of the same LTBMC adherent cells with ARH-77 cells (ANOVA: F[1,17] = 6.2; P < .05) or culture of LTBMC adherent cells alone (ANOVA: F[1,17] = 12.8; P < .002). No IL-1α, IL-1β, IL-3, TNFα, and GM-CSF were detectable in supernatants of plasma cell-LTBMC cocultures (T. Lamme et al, unpublished results, October 1993). In the supernatants of myeloma cell lines and of BM myeloma cells cultured without stromal cells no IL-6 was detected (data not shown).

**Direct Cell-Cell Contact Between Plasma Cell and LTBMC Adherent Cell Is Necessary for Induction of IL-6**

When direct cell-cell contact between plasma cells, either from cell lines or myeloma patients, and LTBMC adherent cells was prevented by using a tissue-culture insert, no enhanced IL-6 production was observed (Fig 5). Also, coculture of RPMI-8226 cells, which were mildly fixated by PFA treatment, and LTBMC adherent cells resulted in increased IL-6 levels in the supernatant (Fig 5). This suggests that the LTBMC adherent cells are responsible for the IL-6 production.

**MoAbs Directed Against Adhesion Molecules Involved in Plasma Cell—LTBMC Interaction Partially Inhibit Induced IL-6 Secretion**

Plasma cells from BM aspirates of three MM patients with active and two MM patients with nonactive disease were analyzed for VLA-4, CD44, and LFA-1 expression using flow cytometry and purified by FACS sorting. Sorted cells, containing 100% myeloma plasma cells, were cocultured with LTBMC for 1 hour, their binding to LTBMC adherent cells was quantitated and the involvement of VLA-4, CD44, and LFA-1 in the binding process was evaluated (Table 2). No major differences in the expression of VLA-4, CD44, and LFA-1 on plasma cells of MM patients with active and nonactive disease were found. With the exception of plasma cells of patient MM6 that were CD44+LFA-1+, low levels of VLA-4 could not be inhibited by CD11a MoAbs despite the presence of LFA-1 on the tumor cells.

Results in Fig 6 show that IL-6 secretion by LTBMC adherent cells induced by myeloma cells from three MM patients with active disease and only 31% and 35% of the plasma cells from two MM patients with nonactive disease adhered to LTBMC after 1 hour of in vitro coculture. Blocking antibodies directed against VLA-4, CD44, and LFA-1 partially inhibited the binding of plasma cells of the active and nonactive MM patients to LTBMC. The binding of myeloma cells of patient MM 4 to LTBMC could not be inhibited by CD11a MoAbs despite the presence of LFA-1 on the tumor cells.

**In Situ Hybridization Studies**

LTBMC adherent cells with low levels of spontaneous IL-6 secretion (<900 U/mL) were cultured on glass slides
and cocultured with or without ARH-77 cells. After 24 hours, the nonbound plasma cells were removed by rinsing and the remaining adherent cells were hybridized for IL-6 mRNA using a dig-labeled probe as described in Materials and Methods. As shown in Fig 7A, LTBMc adherent cells cultured without ARH-77 cells do not hybridize with the IL-6 probe, whereas coculture with ARH-77 cells (Fig 7B) induces IL-6 mRNA in the adherent cells from the LTBMc. In these cocultures, ARH-77 cells are easily distinguishable from the LTBMc by the shape of the cell and the nucleus. In these experiments, no mRNA could be detected in ARH-77 cells.

**DISCUSSION**

The role of IL-6 as the major myeloma growth factor in vivo and in vitro has been established. IL-6 is a pleiotropic cytokine that can be produced by a variety of cells, including fibroblasts, endothelial cells, monocytes and T cells. Its expression at RNA and protein level is induced by viral infection, lipopolysaccharide, and proinflammatory mediators like IL-1, TNFα, and interferon γ. In the present study, we have shown that myeloma tumor cells and myeloma cell lines induce or enhance IL-6 production at mRNA and protein levels in LTBMc adherent cells from myeloma patients and normal donors. Direct binding of plasma cells to the LTBMc adherent cells is a prerequisite for the induction of IL-6.

Morphologic and surface-antigen analysis did not show differences between LTBMc adherent cells from myeloma patients and normal donors. In addition, no differences were observed in quantitative binding of cells from plasma cell lines to LTBMc adherent cells from myeloma and normal donor origin. However, LTBMc from normal donors and myeloma patients differed in their spontaneous secretion of IL-6. LTBMc derived from patients with active disease produced more IL-6 than LTBMc from myeloma patients with nonactive disease or normal donors. These results agree with findings published by other investigators. Interestingly, in long-term myeloma cultures, in the absence of plasma cells, a continued (>3 months) production of IL-6 was observed (data not shown). This suggests the presence of a yet-undefined, autocrine stimulation pathway resulting in a prolonged IL-6 secretion in vivo. If these in vitro findings reflect in vivo behavior, a continued production of IL-6, even during disease remission, might provide optimal conditions for proliferation and/or differentiation of residual tumor cells or their precursors in the affected BM.

IL-6 was detected in the supernatants of all LTBMc tested; the concentration depended on the origin of the adherent cells (see above). Coculture of LTBMc adherent cells from myeloma or normal donor origin with plasma cells increased, in most cases, the concentration of IL-6 measured in the supernatants. These results agree with recently published data by Uchiyama et al. When plasma cells were separated from the stroma by a tissue-culture insert, which prevents direct cellular contact, but allows the exchange of soluble factors, no IL-6 secretion was induced. Plasma cells are capable of producing IL-6, but the experimental data support the idea that the IL-6 detected in the supernatants of the plasma cell-LTBMc cocultures is of LTBMc adherent cell origin. Firstly, IL-6 mRNA seems to be localized in the LTBMc adherent cells and not in plasma cells after coculture. Secondly, mildly fixed primary tumor cells or cells from plasma cell lines also induce IL-6 secretion in LTBMc adherent cells. Because plasma cells are able to induce IL-6 production and secretion in normal LTBMc adherent cells in vitro, the spontaneous IL-6 secretion found in LTBMc of myeloma patients might be a reflection of their previous contact with tumor cells in vivo.

Plasma cells are not unique in their ability to induce IL-6 secretion in LTBMc in vitro. Also Epstein-Barr virus-transformed cell lines with a B- or pre-B-cell phenotype are potent IL-6 inducers (M. de Smit, unpublished observations, February 1993). Therefore, the selective growth of aberrant plasma cells in the BM might be explained by preferential homing of myeloma tumor (precursor) cells to the BM, combined with a susceptibility of such cells for IL-6 as a proliferation/differentiation-inducing cytokine. The importance of IL-6 in the pathophysiology of myeloma might reflect the role of IL-6 in normal B-cell physiology. Recent data imply that IL-6 and binding of VLA-4 to fibronectin are essential for terminal B-cell differentiation. Experiments performed with fibroblast-like cells from tissues other than BM may give clues as to whether the processes described here are unique for adherent cells from the BM. If so, the BM environment might also contribute to the localization of the myeloma tumor. Current experiments are directed to elucidate these questions.

The data show that physical interaction of plasma cells and LTBMc adherent cells trigger de novo IL-6 synthesis in LTBMc adherent cells from myeloma patients and normal donors. The secreted IL-6 is functional as plasma cell prolif-
MYELOMA CELLS INDUCE IL-6 IN STROMA CELLS

Fig 7. In situ hybridization for IL-6 mRNA in: (A) LTBMC adherent cells and (B) the same LTBMC adherent cells cocultured for 24 hours (see Material and Methods) with ARH-77 cells. Brown cytoplasmic staining identifies IL-6 mRNA present in LTBMC adherent cells. Based on a dense nuclear hematoxylin-eosin staining (indented nucleus), plasma cells can readily be distinguished from LTBMC adherent cells.

...eration is further stimulated after adhesion to LTBMC. Little is known about adhesion molecules used by primary tumor cells for their interaction with LTBMC adherent cells. Adhesion molecules possibly involved in plasma cell binding to LTBMC and their matrix components, include VLA-4, CD44, syndecan, and LFA-1. Adhesion of purified myeloma tumor cells to LTBMC could be partially inhibited by antibodies to VLA-4, CD44, or LFA-1, depending on the presence of these adhesion molecules on the tumor cells. No major qualitative differences were observed in the use of different adhesion pathways in LTBMC binding between tumor cells of patients with aggressive versus indolent myeloma. However, although the number of patients studied is relatively small, the data suggest that tumor cells from patients with aggressive forms of disease, quantitatively adhere better as compared with patients with mild forms of disease. These results might relate to the affinity of the adhesion molecules expressed on the plasma cells for their respective ligands. It is well established that the affinity of VLA-4, LFA-1, and CD44 for their ligands is subject to regulation. It is conceivable that in patients with aggressive forms of disease, locally produced cytokines positively effect the adheriveness of myeloma tumor cells. Alternatively, genetic aberrancies accumulated during the disease might af-
fect the adhesive behavior of the tumor cell. We know the purification method did not influence the experimental data because identical adhesion results were obtained when a flow cytometric adhesion assay was used that allows quantification of adhesion of plasma cells within BMMC (data not shown).

The prevention of plasma cell-LTBMC binding, either by tissue-culture inserts or the use of antibodies against VLA-4, CD29, or LFA-1, inhibited the plasma cell-induced IL-6 secretion by the LTBMC. These results stress the importance of physical interaction between plasma cells and LTBMC for the induction of IL-6 production. However, in addition to physical binding, qualitative aspects are also important for the IL-6 induction in LTBMC adherent cells. This is illustrated by the varied IL-6 production by different LTBMC after binding of the same plasma cell line or primary tumor cells and the ability of primary tumor cells to induce higher levels of IL-6 than ARH-77 cells (Fig 4). This, irrespective of the adhesion pathways used for LTBMC interaction, which seem to be identical for myeloma cells and different plasma cell lines.40

Different signaling pathways have been described for the regulation of IL-6 production in fibroblasts.41,42 Through different transcription factors,43-46 several inflammatory mediators induce a rapid and transient activation of the IL-6 promoter, which results in gene transcription and cytokine production. The mechanism involved in the plasma cell-induced IL-6 secretion in LTBMC adherent cells is unknown at present. Because plasma cells can produce IL-1,47 TNFβ,48 and tumor growth factor β,49 one could speculate that close proximity of plasma cells and stroma mediated through adhesion molecules create favorable conditions for growth factor receptor signaling. Alternatively, intimate cellular contact might allow signaling and IL-6 induction through adhesion molecules. Evidence is growing that members of different adhesion families, including the integrins, are involved in transmembrane signaling processes in a variety of cells.50 Because myeloma tumor cells or plasma cell lines, lacking VLA-4, CD44, or LFA-1, are capable of inducing LTBMC IL-6 secretion,49 it seems unlikely that these adhesion molecules are directly involved in signaling processes essential for the regulation of IL-6 production.

In conclusion, our findings show that primary tumor cells, via direct interaction, induce or enhance IL-6 production by LTBMC adherent cells from myeloma patients and normal donors. Adhesion molecules involved in tumor cell binding include VLA-4, CD44, and LFA-1. Interference in binding results in an inhibition of IL-6 production by the LTBMC. These results support the concept that tumor growth in myeloma is regulated by the direct interaction of the tumor cells with their BM stromal environment.

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Primary tumor cells of myeloma patients induce interleukin-6 secretion in long-term bone marrow cultures

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