Multiple Myeloma: Circulating Lymphocytes That Express Plasma Cell Antigens

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The bone marrow and peripheral blood of 14 patients with multiple myeloma were studied with murine monoclonal antibodies that identify antigens on plasma cells (R1–3 and OKT10). Peripheral blood lymphocytes expressing plasma cell antigens were found in six cases. Five of these cases expressed the same antigens that were present on the plasma cells in the bone marrow. Patients that showed such peripheral blood involvement were found to have a larger tumor burden and higher bone marrow plasma cell proliferative activity. In some patients, antigens normally found at earlier stages of B cell differentiation (B1, B2, and J5) were expressed by peripheral blood lymphocytes and/or bone marrow plasma cells.

CURRENT CONCEPTS of tumor development are based on the malignant transformation of a single cell that proliferates to generate a malignant clone. The malignant cells may retain the ability of the normal cells to differentiate into various stages of functional maturity[1] or may be arrested in an immature stage of differentiation.[2]

Multiple myeloma (MM) has been regarded as a tumor of differentiated immunoglobulin (Ig) producing plasma cells and was one of the first human neoplasms proposed to be monoclonal.[3] Accumulated evidence now suggests that some lymphocytes in the peripheral blood may share common characteristics with myeloma cells in the marrow. Lymphocytes expressing M-protein idiotype have been identified in the peripheral blood (PB) of patients with MM; in about half of the untreated cases, there is evidence of peripheral blood (PB) involvement.[4] These monoclonal lymphocytes appear more frequently in the blood of MM patients with a large tumor burden and may reflect tumor overload in the bone marrow, as they usually become undetectable in remission.[5] The analysis of the surface Ig light chain type on the surface of the PB lymphocytes in MM patients ("kappa-lambda" analysis for clonal excess) has also indicated circulating monoclonal B lymphocytes in some cases;[6] in addition, in MM patients, the DNA synthetic activity of the BM plasma cells is similar to that of the PB B lymphocytes.[7] Thus it seems likely that the malignant plasma cell clone also includes B lymphocytes in the peripheral blood.

Hybridoma technology has been used to raise specific reagents to identify cell surface differentiation antigens. Monoclonal antibodies identifying antigens on human plasma cells have been described by us[8] and others;[9,10] some of them recognize lymphocytes and granulocytes as well. In order to further elucidate the relationship between circulating peripheral blood cells that might participate in the malignant process and myeloma cells in the bone marrow, we used monoclonal antibodies to identify plasma cells and B cells at various differentiation stages in 14 MM patients. We analyzed simultaneously the antigenic expression of the mononuclear cells of the bone marrow (BM) as compared to that of the PB lymphocytes and correlated these results with tumor burden and BM plasma cell DNA synthetic activity.

MATERIALS AND METHODS

Patients

Diagnosis of MM was made according to previously described criteria.[11] Tumor burden was assessed at the time of sampling and was based on the level of M-protein in the serum and urine, hemoglobin level, serum creatinine and calcium levels, and the degree of bone disease.[12] The untreated patients were studied at the time of diagnosis, immediately before institution of therapy. Treated and untreated cases were included. The DNA labeling index of the plasma cells was performed as previously described.[13] Bone marrow was separated on Ficoll-Hypaque gradients within one hour of sampling, then incubated with high specific activity tritiated thymidine. Cytospin slides were prepared and autoradiographed. The percent plasma cells incorporating tritiated thymidine was recorded as the plasma cell labeling index. Using this method to assess proliferative activity in patients with myeloma, low values range from 0.0% to 0.2%, intermediate values are from 0.4% to 0.8%, and high values are greater than 1.0%.

Cell Collection

Heparinized PB and BM samples were diluted 1:3 in RPMI 1640, 10% fetal calf serum (S-10), and after centrifugation for 30 minutes at 400 g over Ficoll-Hypaque cushions, the mononuclear (MNC)

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cells were harvested, washed three times with S-10, and the cell concentration adjusted to 10^7/mL.

Monoclonal Antibodies

R1-3 has been previously described.[8] The R1-3 antigen has been detected on some human plasma cells and bone marrow lymphocytes; it has not been detected on T lymphocyte cell lines, thymus cells, granulocytes, on circulating T lymphocytes, and is present on less than 3% of circulating, T-depleted, mononuclear cells. OKT10 and OKIa*$_1$ were purchased from Ortho Diagnostic Systems (Raritan, NJ). The monoclonal antibodies to human common acute lymphoblastic leukemia antigen (CALLA) (J5), to human B cell antigen (B1), and human B cell subset antigen (B2) were purchased from Coulter Immunology (Hialeah, Fla). FITC-labeled Fab fragment anti-human immunoglobulin (TAGO, Burlingame, Calif) was used to measure surface immunoglobulin (SIg) bearing MNC, and FITC-labeled goat anti-mouse IgG and IgM (affinity-purified, TAGO) was used as the second antibody in a 1:200 dilution, as previously determined.[8]

Fluorescein-Activated Cell Sorter (FACS IV) Immunofluorometric Assay

Cell samples (0.1 mL) were incubated with 0.01 mL of each antibody; after incubation for 30 minutes on wet ice, cells were washed twice with PBS, 0.1 mL of diluted anti-mouse conjugate added, and incubated again for 30 minutes on wet ice. After two washes, cells were resuspended in 1 mL of PBS prior to analysis in the FACS IV. In the PB samples, the cell number enumeration was restricted to the lymphocyte population, defined by using forward *v* right-angle scatter display; whereas in the BM samples, the analysis included all but the red blood cells as defined by the same cell scatter display. A control specimen containing the FITC-coupled antibody without the initial monoclonal antibody was included for each patient sample; this value, expressed as percent positive cells, was defined as background fluorescence and subsequently subtracted from the percent value of each sample. Samples with final percent values $\leq$ 5% were considered as negative, those between 5% and 15% as low positive (+), and those above 15% as high positive (++).

RESULTS

Patients

Table 1 shows the salient clinical features of the patients studied, divided according to tumor burden. Table 1 also lists the diagnosis, tumor burden, M-protein, therapy at time of sampling, and clinical follow-up. All patients, except patient 1, received therapy after the blood and marrow samples were obtained. Patient 1 had smoldering multiple myeloma and has remained stable for three months without treatment.

Bone Marrow Plasma Cell Phenotype

Table 2 lists the patients according to tumor burden and contains the bone marrow plasma cell labeling

Table 1. Multiple Myeloma, Circulating Lymphocytes That Express Plasma Cell Antigens: Clinical Parameters

Case No.	Diagnosis	Tumor Burden	M-Protein Serum (Urine)	Treatment (Rx) at Time of Study	Follow-up
1	SMM,* new diagnosis	Low	Gκ (κ)	0	Alive at 3 months (no treatment)
2	MM† in PR‡	Low	λ (λ)	M + P‖	Alive at 2 months (stable on M + P)
3	MM, new diagnosis	Intermediate	Gκ (κ)	0	Alive at 4 months (PR to M + P)
4	MM, new diagnosis	Intermediate	Gκ (neg)	0	Alive at 3 months (PR to M + P)
5	MM, slowly progressive	Intermediate	Neg (κ)	C + P¶	Alive at 3 months (slowly progressive on C + P)
6	MM, early relapse	Intermediate	Gλ (λ)	Stopped Rx for 1 year	Alive at 4 months (relapse)
7	MM, relapse	Intermediate	κ (κ)	M + P	Alive at 4 months (PR to VBAP)
8	MM, new diagnosis	High	Aλ (λ)	0	Dead at 4 months (from MM)
9	MM, new diagnosis	High	κ (κ)	0	Alive at 2 months (NR§ to M + P)
10	MM, relapse	High	Gκ (κ)	VBAP**	Dead at 5 months (active MM)
11	MM, progressive	High	Gκ (κ)	M + P	Dead at 2 months (from MM)
12	MM, relapse	High	Neg (κ)	M + P	Dead at 2 months (from MM)
13	MM, relapse	High	Gλ (λ)	VBAP	Dead at 2 months (from MM)
14	MM, relapse	High	Gλ (λ)	M + P	Dead at 1 month (from progressive leukemic phase)

*SMM, smoldering multiple myeloma.

†MM, multiple myeloma.

‡PR, partial response (>25% but < 50% reduction in serum and urine M-protein).

§NR, no response (<25% reduction in serum or urine M-protein).

‖M + P, melphalan and prednisone.

¶C + P, cyclophosphamide and prednisone.

**VBAP, vincristine, BCNU, Adriamycin, and prednisone.

Table 2. Multiple Myeloma, Circulating Lymphocytes That Express Plasma Cell Antigens: Laboratory Studies

Tumor Burden	Case No.	Plasma Cell Labeling Index (%)	Percent Plasma Cells in Bone Marrow*	Antigen-Positive Cells:† Peripheral Blood (Bone Marrow) R1-3	OKT10	J5: CALLA
Low						
	1	0.4	25	– (–)	– (–)	– (–)
	2	0.0	20	– (–)	– (–)	– (–)
Intermediate						
	3	0.0	20	– (+)	– (+)	– (–)
	4	0.0	20	– (–)	– (+)	– (–)
	5	0.0	75	– (–)	– (++)	– (–)
	6	1.4	10	– (+)	– (–)	– (–)
	7	0.4	20	– (+)	– (–)	– (–)
High						
	8	0.6	100	+ (+)	– (+)	– (–)
	9	1.4	45	– (+)	+ (++)	– (–)
	10	1.4	40	+ (–)	+ (–)	– (–)
	11	5.8	90	+ (++)	+ (++)	– (++)
	12	1.8	95	++ (++)	– (++)	– (–)
	13	2.2	90	– (++)	– (++)	– (++)
	14	21.8	60	+ (+)	++ (++)	+ (–)

*Percent plasma cells by morphology in Ficoll-Hypaque-separated bone marrow.

†(–) <5% reactive cells; (+) 5%-15% reactive cells; (++) >15% reactive cells.

index, the percent plasma cells in the bone marrow, and the reactivity of peripheral blood and bone marrow cells with R1-3, OKT10, and J5 (CALLA) antibodies. In normal bone marrow, the number of R1-3$^+$, OKT10$^+$, or J5$^+$ cells is below 5%. R1-3$^+$ bone marrow cells were found in nine cases, T10$^+$ in nine and J5$^+$ in two (Table 2). The two patients with low tumor burden did not express either R1-3 or OKT10 on their bone marrow cells. All five patients with intermediate tumor burden expressed either R1-3 or OKT10, and six of the seven high tumor burden patients' bone marrow cells expressed both R1-3 and OKT10. Patients 11 and 13 were found to have greater than 90% plasma cells in the bone marrow that bore early and late B cell antigens (J5, T-10, and R1-3); the plasma cells of these patients were also positive for other antigens expressed at early stages of B cell differentiation: SIg, Ia, B1, and B2 (data not shown).

Peripheral Blood Lymphocytes Phenotype

In normal peripheral blood, the number of R1-3$^+$ and OKT10$^+$ lymphocytes is below 5%.[8,14] The reactivity pattern of the PB lymphocytes was abnormal in six of 14 MM cases (Table 2), where the number of R1-3$^+$ or OKT10$^+$ cells was abnormally high (over 5% and up to 51%). The abnormal expression of antigens on PB lymphocytes correlated with antigens expressed by bone marrow plasma cells in five cases. In one case (patient 10), an abnormally high number of OKT10$^+$ and R1-3$^+$ lymphocytes in the PB was found despite the absence of R1-3$^+$ and OKT10$^+$ cells in the bone marrow. The peripheral blood of patient 14 reacted with R1-3, OKT10, and J5. This patient had 40% "large unstained cells" in the peripheral blood, using the peroxidase channel of the Technicon-Hemalog cell counter, and they were initially classified as monocytes (Fig 1). However, the cells were negative for butyrate esterase and peroxidase.[16] The T cell antigens (Leu-2, Leu-3, and Leu-4)[17] were negative, but the cells were positive for both surface and cytoplasmic immunoglobulin and for CALLA (Table 2, Fig 1). This patient progressed to overt plasma cell leukemia. No other patient in this study had morphologically identifiable plasma cells in the peripheral blood.

Tumor burden, plasma cell labeling index, and the presence of circulating cells expressing R1-3 or OKT10 are all independent parameters that have a positive correlation. Of the seven patients with a low or intermediate tumor burden, none expressed R1-3 or OKT10 on circulating cells, only one had a labeling index greater than 1.0, and all seven are alive at this short follow-up period. Of the seven patients with high tumor burden, six express R1-3 or OKT10 on circulating cells, six had a labeling index greater than 1.0, and six are dead from multiple myeloma.

DISCUSSION

Our data show that myeloma patients with a high tumor burden and a high plasma cell labeling index are likely to have significant numbers of circulating cells that express plasma cell antigens (Table 2). There are a number of monoclonal antibodies that identify antigens on human plasma cells.[18] These include R1-3,[8] which also binds to late B cells; OKT10,[10] which binds

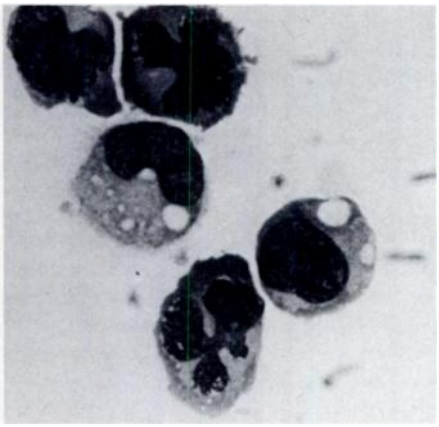

Fig 1. Wright's stained peripheral blood smear of the Ficoll-Hypaque isolated mononuclear cells from patient 3 (1,600×). These large cells were identified by immunofluorescence as R1-3$^+$, T10$^+$, cytoplasmic Ig$^+$, Leu-4$^-$, Leu-3$^-$, Leu-2$^-$, and OKM1$^-$. The phenotype is similar to that of the malignant plasma cells in the bone marrow.

to early thymocytes; and PCA1 and PCA2,[9] which cross-react with granulocytes. We have used R1-3 and OKT10 as plasma cell markers in this study. Because morphologically identifiable plasma cells were found in only one patient (case 14), we interpret these observations to show that in myeloma, the peripheral blood may contain cells from the malignant clone.

We have found that malignant plasma cells from patients with MM may express antigens normally present in earlier B cells: CALLA was identified in 16% and 41% of the plasma cells in the bone marrow of two patients (Nos. 11 and 13, respectively). The third patient with a leukemic phase of multiple myeloma (patient 14) had CALLA-positive cells of the peripheral blood but not in the bone marrow. To our knowledge, this is the first description of CALLA-positive plasma cells in multiple myeloma. Greaves et al[19] did not find any CALLA-positive cells in a group of nine MM patients. These patients' bone marrow plasma cells (Nos. 11 and 13) were reactive with all the B cell markers studied, including Ia, SIg, B1 and B2. The bone marrows of the other patients in this study contain less than 5% reactive cells when analyzed with antibodies to Ia, B1, or B2. Patient 14 had large bizarre mononuclear cells in the PB (Fig 1) that were identified as belonging to the B cell lineage by means of reactivity with all the B cell markers studied: 45% of the mononuclear cells in this case were positive with the plasma cell marker OKT10 and 50% of them showed cytoplasmic immunoglobulin. Again, these abnormal B cells expressed antigens present in different stages of the normal B cell differentiation pathways, including CALLA. However, in the absence of double marker studies, we cannot conclude with certainty that a single cell expresses both early and late B lineage antigens or whether this is a population phenomenon. One can speculate that myeloma cells expressing early B cell antigens represent progenitor cells that are important in maintaining and expanding tumor burden. More sensitive techniques to detect such cells, such as clonogenic assays, must be investigated to determine whether such cells are also present at lower tumor burden, or whether they arise during expansion of the malignant clone.

Six of 14 multiple myeloma patients (43%) had abnormally high numbers of peripheral blood lymphocytes expressing plasma cell antigens. This figure is similar to that found by Smith et al[6] using the kappa-lambda ratio of the surface Ig expressed by the peripheral blood B lymphocytes, but lower than that reported using other approaches.[4] However, our interpretation is the same: some patients with multiple myeloma show peripheral blood involvement by the malignancy and not necessarily in the classical plasma cell leukemia fashion. The presence of lymphocytes expressing plasma cell antigens in peripheral blood of multiple myeloma patients was more frequent in those with a high tumor burden and may reflect bone marrow "spillage" into the peripheral blood. In one case studied before and after treatment (patient 8), the bone marrow antigen expression was initially OKT10$^+$ and R1-3$^+$, with PB lymphocytes expressing R1-3; after one course of chemotherapy, the R1-3–positive PB cells disappeared, while the bone marrow cells still expressed R1-3.

It seems clear that there is a positive correlation in myeloma patients between the presence of circulating lymphocytes expressing plasma cell antigens and the high plasma cell labeling index and tumor burden (Table 2). We interpret these observations as suggestive of PB involvement by more immature cells (as defined by morphology and antigen expression) in the malignant clonal process. The possibility of explaining a high number of T10$^+$ and R1-3$^+$ PB lymphocytes on the basis of B cell "activation," such as a viral infection, cannot be ruled out completely; however, no patient showed a clinical picture of viral infection and only one[11] showed a high number of Ia$^+$ lymphocytes in the peripheral blood, another "stimulated cell" marker.[20] It has also been suggested that the idiotype-bearing lymphocytes in myeloma patients reflect an expansion of T cell subsets expressing Fc receptors for the M-protein.[21] Except for patient 14, we have not performed double marker studies to rule out the coexpression of R1-3 or T10 with mature T cell markers in these patients. We have not, however, previously

detected R1-3 on T cells or thymus cells. The cells from patient 14 that were positive for the expression of R1-3 and OKT10 were also positive for cytoplasmic Ig and were negative for Leu-2, Leu-3, and Leu-4.

Our findings support the concept of peripheral blood involvement in multiple myeloma patients, especially those with a high tumor burden and high DNA synthetic rate. The relationship between these findings and the presence of idiotype-bearing lymphocytes and/or clonal excess cells remains to be studied.

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