1985.004

Pre-B Cells in Peripheral Blood of Multiple Myeloma Patients

By Linda M. Pilarski, Michael J. Mant. and Bernard A. Ruether

Although multiple myeloma is a disease of plasma cells, abnormalities have been detected in both B and T lymphocytes in peripheral blood. Although multiple myeloma patients are deficient in surface Ig (sIg)-positive B lymphocytes, analysis of lymphocytes present in blood indicates an abnormally large pool of circulating pre-B cells. These pre-B cells express BA-1, do not bear sIg, and contain cytoplasmic μ chains. High numbers of pre-B cells occur in 88% of individuals with frank myeloma and in 44% of individuals with monoclonal gammopathy of undetermined significance. Pre-B cells bearing BA-1 differ between patients in their expression of HLA-DR and receptors for peanut agglutinin (PNA). Those pre-B cells in myeloma patients are either BA-1$^+$ PNA$^-$ HLA-DR$^+$ (54% of patients) or BA-1$^+$ PNA$^+$ HLA-DR- (30% of patients), or a mixture of phenotypes (14% of patients). Pre-B cells of the PNA$^-$ phenotype are almost always HLA-DR$^+$, and PNA$^+$ pre-B cells are HLA-DR-. Within the same patient, the pre-B cell population varies by both quantitative and qualitative definitions. The number of pre-B cells may increase 460-fold and temporal shifts of surface phenotype BA-1$^+$ PNA$^-$ to BA-1$^+$ PNA$^+$ or vice versa have been detected. These observations indicate an abnormality in the B lymphocyte differentiation pathway leading to cells in the periphery that vary in number and cell surface phenotype, and that are unable to express sIg.

THE MAJORITY of patients with multiple myeloma are deficient in their ability to mount a humoral immune response[1-5] and in secretion of serum immunoglobulin (Ig) other than the monoclonal protein! In addition, they show severely reduced numbers of B cells in peripheral blood as defined by their expression of surface immunoglobulin (sIg), HLA-DR, and an antigen uniquely expressed on sIg$^+$ B cells (41H.16).[7] It seems likely that the inability to manifest humoral immunity'" and susceptibility to infections* are direct results of the deficiency in sIg$^+$ B lymphocytes.

The reasons for the decreased numbers of sIg$^+$ B cells are unknown, but could result from a shift in differentiation toward plasma cells, a block in B cell development at the stem cell stage, or a maturation block at some point after commitment to the B cell lineage.[7,9-11] In this study we demonstrate that in multiple myeloma patients, differentiation of sIg$^-$ pre-B cells to sIg$^+$ B cells is blocked, and pre-B cells appear in the peripheral blood. Although the majority of the 47 patients studied have decreased numbers of sIg$^+$ cells among peripheral blood lymphocytes (PBLs), they exhibit normal numbers of BA-I+ cells. BA-I is a marker that defines mainly sIg$^+$ B cells in normal donors, although a small subset of sIg$^-$ BA-I$_+$ lymphocytes is detectable." Gathings et al[13] have described sIg$^-$ cells with low amounts of cytoplasmic μ chains as pre-B cells. We present evidence here to show that the sIg-BA-I$^+$ subset of cells in patients' PBLs represents cμ pre-B cells.

MATERIALS AND METHODS

Fifty-five patients with multiple myeloma (nine with light chain, two with nonsecretory, three with IgA, and 41 with IgG M-components), 20 patients with monoclonal gammopathy of undetermined significance (MGUS), and 23 normal donors (age 40 to 60) were studied. Informed consent was obtained prior to participation in the study. Of the multiple myeloma patients, 13 were untreated, 36 were receiving intermittent chemotherapy, and six had received no chemotherapy treatment for at least six months before the time of sampling.

Purification of PBLs. Heparinized blood samples were centrifuged and the buffy coat was collected. This was diluted eightfold in commercially prepared RPMI growth medium (GIBCO, Grand Island, NY) combined with 20% fetal calf serum, and layered over Isolymph (Gallard-Schlesinger, Carle Place, NY) followed by centrifugation. The white cells on the RPMI/Isolymph interface were collected, washed three times in the RPMI growth medium, and subjected to a viable count and a differential count of May-Grunwald-Giemsa–stained smears to determine the percentage of lymphocytes in the sample. Recovery of lymphocytes after purification was routinely 90% to 100%. In addition, all immunofluorescence (IF) assays included aliquots of cells incubated with anti-granulocyte or anti-monocyte monoclonal antibodies to assess the purity of PBLs actually screened by IF. Most preparations had <5% granulocytes and/or monocytes. Plasmacytoid cells were only rarely seen in cytospin preparations.

Immunofluorescence. For this study the assay method was an indirect IF assay. Every set of assays included a normal PBL sample as a control to ensure that B cells were detectable with the reagents used on a particular day. Purified PBLs (5×10^5 to 10^6) were aliquoted into v-bottomed microtiter wells, pelleted, and resuspended in 50 μL of the test murine anti-human antibody diluted in normal saline to an appropriate concentration, and incubated for 60 minutes at 4 °C. Plates were centrifuged and washed twice in saline, and the cell pellet was resuspended in 50 μL of a 1/20 dilution of F(ab)$_2$ fragments of sheep anti-mouse Ig labeled with fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (RITC) (Tago, Burlingame, Calif), incubated for 60 minutes at 4°C, and washed twice. When indicated, cells were resuspended in warm saline and incubated for ten minutes at 37 °C to allow cap formation, followed by fixation of cells in 1% formalin. If the capping step was not to be included, cells were kept in 0.02'6 sodium azide throughout the procedure and fixed in 1% formalin after the final wash.

IF was examined using a Zeiss microscope with fluorescence epi-illumination, and selective filters. Cell samples were counted for total cells in the field and for total number of cells with capped or ring fluorescence using a hemacytometer A capped cell was defined as one having a polar aggregation of fluorescence and at least one third of the cell lacking fluorescence. Caps were generally distinct, covered approximately one third of the cell surface, and were very

From the Departments of Immunology and Medicine. University of Alberta, Edmonton. and the Department of Medicine, University of Calgary, Alberta. Canada.

Supported by a grant from the National Cancer Institute of Canada (L.M.P.).

Submitted Sept 10, 1984; accepted Feb 19, 1985.

Address reprint requests to Dr L.M. Pilarski, Department of Immunology, University of Alberta. Edmonton. Alberta. Canada T6G 2H7.

0006–4971/85/6602–0031$03.00/0

bright. The percentage of positive cells was calculated after screening a minimum of 1,000 cells. In samples with less than 0.1% of caps or rings, a minimum of 5,000 cells were screened. Assays for detection of sIg involved enumeration of capped cells as has been described.' Assays for BA-1 involved ring fluorescence. For the $c\mu$ chain determinations, a modification of described procedures was used.[13] Cytospin preparations of PBLs were fixed in carbon tetrachloride followed by washing and then treatment with FITC–sheep anti-human IgM (Tago), or RITC rabbit anti-p (Jackson Immunochemicals, Avondale, Pa); comparable results were obtained with both reagents. Negative controls were always done on patient samples using FITC–sheep anti-mouse Ig, and every test included slides of Nalm-6, a $c\mu^+$ pre-B cell line, as a positive control on the reagents used; these controls were uniformly completely negative or completely positive, respectively. We found that reproducible results were obtained only with the carbon tetrachloride fixation procedure; use of acid-ethanol, when it was succesful, gave comparable intensity and quantity of $c\mu^+$ cells, but frequently no fluorescence was observed even for the Nalm-6 control slides.

The number of BA-I+ cells per milliliter of blood was calculated as (fraction of BA-I+ cells in purified PBLs) x (fraction of lymphocytes in WBCs) x (number of WBCs per milliliter of blood) = number of BA-1$^+$ cells per milliliter of blood.

Double IF (dIF). Aliquots of PBLs were tested for expression of two different markers by either indirect IF or a combination of indirect and direct IF. Cells were incubated in sodium azide with BA-I antibody or with saline, washed, and treated with RITC-$F(ab)_2$ fragment of sheep anti-mouse Ig. After washing again, cells were treated with the indicated second antibody or saline, washed, and incubated with an FITC-$F(ab)_2$ fragment of sheep anti-mouse Ig. For the controls in which saline was used instead of a second antibody, no double-labeled cells were detected. To determine the number bearing both BA-I and PNA receptors, cells were treated sequentially with BA-I, RITC-$F(ab)_2$ sheep anti-mouse Ig, and finally PNA-FITC. Cells exhibiting both red and green fluorescence were enumerated. Every test included a negative control in which saline was substituted for the BA-I antibody. These did not show red fluorescence.

dIF to detect $c\mu$ chains was done using suspension of PBLs. Cells were tested sequentially as follows: BA-I, RITC-$F(ab)_2$ sheep anti-mouse Ig, fixation in ethanol, and finally FITC-sheep anti-human IgM (Tago).[14] Cells with red ring fluorescence and intracellular green fluorescence were considered to be BA-1$^+$ cIg^+. Every assay included a negative control with saline instead of the BA-I antibody.

Antibodies. BA-1 is a monoclonal antibody specific for a marker expressed on sIg^+ B cells and some granulocytes'' (Hybritech, San Diego). Anti-κ and anti-λ were monoclonal reagents from BRL (Gaithersburg, Md). OKT3, OKT8, and OKTlO were from Ortho Pharmaceutical Corp (Raritan, NJ). 80H-3 and 80H-5[15] are monoclonal antibodies specific for granulocytes, and 82H-3[16] is specific for monocytes; these were a gift from Dr Patrice Mannoni. University of Alberta. 7H-3 is a monoclonal antibody specific for monomorphic determinants on HLA-DR,[17] provided by Dr BM Longenecker, University of Alberta. PNA-FITC was from Sigma (St Louis).

RESULTS

Expression of BA-1 on PBLs. A series of 47 patients with multiple myeloma and nine patients with MGUS were assessed for expression of BA-1 and sIg on their PBLs (Fig 1).[7] Although the majority of multiple myeloma patients with IgG, IgA, or light chain disease had reduced numbers of sIg^+ cells, the number of BA-1$^+$ lymphocytes was within tlie normal range (Fig 1 and Table 1). In 78% of the patients, tlie ratio of BA-I+ to sIg^+ cells was significantly higher than observed in normal individuals where Ba-1$^+$ and sIg^+ levels were comparable (Fig 1 and Table 1).

The discrepancy between the number of BA-1$^+$ cells and sIg^+ cells did not correlate with treatment status (Table 1), and was not restricted to frank myeloma, as 44% of patients with MGUS also exhibited an elevated BA-1$^+$:sIg^+ ratio. Abnormally high ratios of Ba-1$^+$ relative to sIg^+ cells were seen in 50% of patients with light chain disease, and in two patients with nonsecretory myeloma, (mean, 4.9% ± 1% BA-1$^+$ cells), indicating that the high levels of BA-I+ cells are not due simply to the presence of large amounts of M-component in serum. Usually, in patients with MGUS, the number of BA-1 cells in PBLs, and the number of BA-I$^+$ cells per milliliter of blood were within the normal range (Table 1), although values from myeloma patients varied considerably more than those from normal donors or patients with MGUS (Fig 1). The presence or quantity of BA-1$^+$ sIg^- cells showed no detectable correlation with type of monoclonal protein expressed or with its location in serum or urine (data not shown).

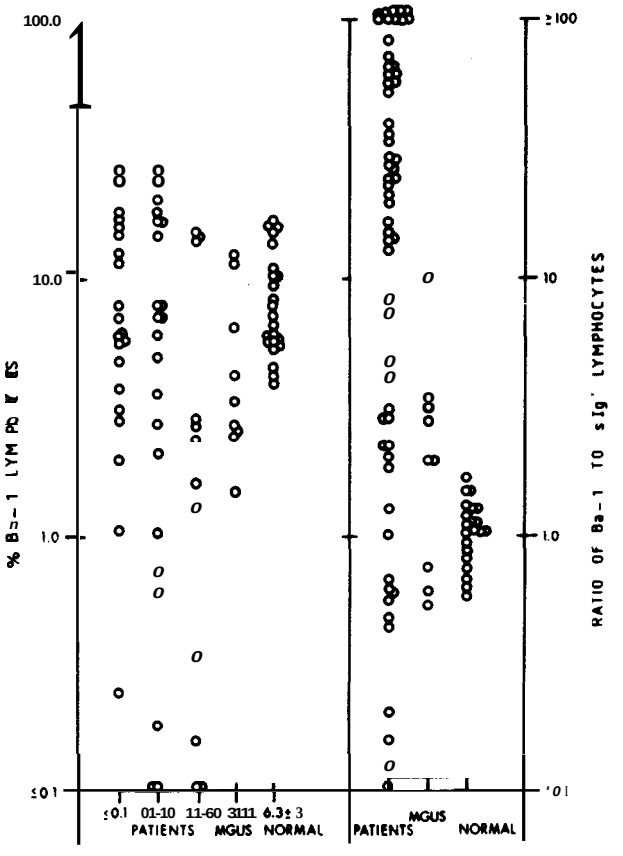

Fig 1. Frequency of Ba-1$^+$ cells and the ratio of Ba-1$^+$ to sIg^+ cells in individual samples of PBIs. sIg^+ cells were defined as the number of cells able to cap on binding a mixture of anti-κ/anti-λ antibodies or anti-human gamma globulin (HGG) as previously described.' Mean normal ratio, 1.1 ± 0.36 SD.

Table 1. BA-1+ Cells in Patients Classified According to Treatment Status

Patients	% BA-1* Cells in PBLs (No. of Samples)	% sIg+ Cells in PBLs	No. of Samples With Abnormal BA-1+:sIg+	BA-1 Cells x 10^{-6}/mL of Blood
Untreated	4.4 ± 1.9 (12)	0.3 ± 0.13	8/12	11 ± 7
Intermittent chemotherapy	8.6 ± 1.2 (64)	0.3 ± 0.4	50/64	9.9 ± 3.8
Off treatment	5.9 ± 2.5 (6)	1.7 ± 0.6	5/6	—
MGUS	5.5 ± 1.3 (9)	3.1 ± 1	4/9	8.2 ± 2.4
Normal	9.7 ± 1 (23)	6.34 ± 0.5	0/23	6.8 ± 4

Values are the means ± SE. An abnormal ratio of BA-1+ to sIg was defined as one exceeding the mean of the ratio in normal donors by more than 3 SD (22.2). The number of BA-1+ cells in blood was calculated from the WBC and differential counts on the blood to be purified, or on a differential and peripheral blood counts done on a separate tube of blood taken at the same time.

Variation in BA-I+ levels. A series of patients was assessed for the number of BA-1+ cells in PBLs at regular intervals for a nine-month period (Table 2). Considerable variation was observed in absolute numbers of BA-I+ and in the ratio of BA-I+ to sIg+ cells. Data from case No. 086 suggests possible cyclical variation.

Other phenotypic markers on BA-I+ cells. dIF studies with BA-1 and a panel of antibodies directed to other B and pre-B cell markers were performed to identify the BA-I+ subpopulation of PBLs (Table 3).

Pre-B cells have been shown to be sIgM− and cμ.[13,18] BA-I+ cells from case No. 086 were 95% sIgM− as defined by a lack of ring fluorescence with the FITC–anti-IgM on RITC/BA-1-labeled cells. However, 88% of the BA-1+ cells were weakly positive for cIgM in double IF determinations. The majority of BA-I+ cells did not bear markers expressed on granulocytes or monocytes and were negative for expression of OKTIO, or receptors for PNA. In a series of double IF tests on PBLs from different individuals, each of three patients had BA-1+ sIgM− cells. Ten of 12 patients had 3% to 20% BA-1+ cells but <0.6% OKT10+ cells. In a series of cμ determinations, 18 of 22 myeloma patients whose PBLs expressed low numbers of sIg+ cells had, on average, 22-fold higher numbers of cμ+, and twofold more cμ+ cells than BA-1+ cells (Table 4). Patients with MGUS and normal donors had numbers of cμ+ cells that correlated with the number of sIg+ cells and, on average, twofold more BA-I+ cells than cμ+ cells. Thus, the pattern in patients with MGUS and normal donors was considerably different from that of myeloma patients (Table 4). Preliminary data suggest that the BA-1− sIg− cμ+ population seen in some of the myeloma patients represents a very early stage of pre-B cell development (L.M.P., unpublished observations, 1984). The majority of the cIgM+ cells had a large, sometimes convoluted, nucleus and a thin rim of cIgM+ cytoplasm. IF with the anti-p or anti-IgM reagents was dim to moderate, although in two patients a very low number of cells with the bright IF and morphology characteristic of plasma cells was observed. In six of eleven patients analyzed, 1–5% of cμ+ cells were positive for the expression of cytoplasmic light chains.

Heterogeneity in the expression of receptors for PNA and of HLA-DR on BA-I+ cells from patients. Using dIF, the BA-1+ cells in a number of patients were screened for simultaneous expression of HLA-DR and receptors for PNA (Table 5). Although the BA-I+ cells within PBLs of a given individual were in most cases homogeneous, heterogeneity in the expression of PNA receptors was seen among the patients. For multiple myeloma, we detected either BA-1+ PNA+ or BA-1+ PNA− cells. Normal donors and those with MGUS had mainly BA-1+ PNA− cells. In all categories we detected some patients with a mixture of BA-1+ PNA+ and BA-1+ PNA− cells; most of the multiple myeloma patients had BA-I+ PNA+ with a small subset of BA-1+ PNA− cells, while normal donors and MGUS patients with mixed populations had the reverse pattern. Heterogeneity among patients was also observed in the expression of HLA-DR. BA-1+ PNA− cells in myeloma patients, MGUS patients, and normal donors were HLA-DR+ (Table 5). In all cases where BA-I+ HLA-DR− cells were seen, those correlated very closely with the number of BA-I+ PNA+ cells in aliquots of

Table 2. Temporal Variation in the Number of EA-1+ sIg− Cells in PBLs of Multiple Myeloma Patients

	Percentage of BA-1+/sIg+ in Individual Samples of PBLs							
No.	July	Aug	Sept	Oct	Nov	Dec	Feb	March
086	510.3	1510.05	1710.08	2810.02	5.8/0.02	22/0.02	2410.07	610.02
013	1.6/2.4	0.0311.6	0.34/1.5	14/1.9	—	—	—	—
075	3.0*/0.32	—	—	1510.07	—	—	2.4/0.09	26/0.06
109	—	—	—	—	20*/0.75	—	4.0/0.7	1.9/0.3
082	8/0.4	—	19/0.06	—	—	—	—	—
093	—	5.9/0.33	—	—	1210.03	—	—	1/0.09
087	—	—	18.3/0.08	2.0/0.08	—	—	—	—
097	—	1610.28	7.6/0.11	2.910.15	—	7.2/0.1	—	2.3/0.7
101	2.8/0.2	—	0.6/0.16	13.5/0.11	—	—	—	0.16/0.2
080	710.9	2.1/0.7	—	—	—	4.3/0.9	—	13.4/0.9

*PBLs taken from patients while still untreated. Patient No. 075 had light chain disease, and patient No. 080 had an IgA M-component; the rest all had IgG M-components.

Table 3. Phenotypic Markers on BA-1$^+$ Cells

Marker	Double-Labeled Cells/No. of BA-1$^+$ Cells (%)	Phenotype of Major BA-1$^+$ Subset
Double immunofluorescence		
sIgM	10/195 (5.1)	sIgM$^-$
cμ	43/49 (88)	cμ
PNA receptor	2/132 (1.5)	PNA$^-$
80H.3 (granulocyte)	79/299 (26)	80H.3$^-$
80H.5 (granulocyte/myeloid))	16/101 (16)	80H.5$^-$
82H.3 (monocyte)	115/381 (30)	82H.3$^-$
Single immunofluorescence		
OKT10:BA-1	0:250 (<0.04)	OKT10$^-$

PBLs were from patient No. 086 (IgG-κ; on treatment) and were 70% lymphocytes and 30% polymorphs or monocytes by Giemsa staining. Of to al PBLs, 22% were BA-1$^+$. For all markers except OKT10, double immunofluorescence, performed at 4 °C in the presence of sodium azide, was used :o define the BA-1$^+$ population. For OKT10, separate aliquots of cells were tested which yielded 22% BA-1$^+$ cells and <0.05% OKT10$^+$ cells, makiig double fluorescence studies unnecessary.

the same PBL preparations. In the case of normal donors, MGUS patients, and four myeloma patients, BA-I+ cells that appeared to be sIg$^+$ B cells were also PNA$^-$.

Temporal variation in the PNA/HLA-DR phenotype of BA-1$^+$ cells from individual patients. In addition to quantitative temporal variations in BA-1$^+$ cells (Table 2), the sIg$^-$ BA-I$^+$ population in individual patients exhibited qualitative shifts in the PNA/HLA-DR phenotype over time (Table 6). During a seven-month period, patient No. 086 exhibited a shift from a PNA$^-$ to a PNA$^+$ BA-I$^+$ population as defined by dIF studies. Both populations were sIg$^-$ cIgM$^+$. In PBLs from patient No. 075, BA-I$^+$ cells showed a transition from a PNA$^+$ to a PNA$^-$ phenotype. Patient No. 097 maintained a stable BA-1$^+$ phenotype.

The level of sIg$^-$ BA-1$^+$ cells in PBLs does not correlate with T cell subset distribution. Since patients have a skewed ratio of OKT4- to OKT8-bearing T cells[19,20] that involves reduced numbers of OKT4$^+$ cells coupled with normal numbers of OKT8$^+$ cells, we searched for a relationship between the fluctuation in BA-1$^+$ cells and the T cell subset distribution. The majority of patients studied here had reduced numbers of OKT4 and normal numbers of OKT8 cells; the total number of T cells (OKT3$^+$) varied considerably.[21] We observed no correlation between the absolute number of BA-1$^+$ cells or the ratio of BA-1$^+$:sIg$^-$ to absolute numbers of OKT4$^+$ or OKT8$^+$ cells (data not shown), and no correlation between BA-1$^+$:sIg$^-$ and the proportion of total T cells that are OKT8$^+$ (OKT8:OKT3, Fig 2).

The level of sIg$^-$ BA-1$^+$ cells does not correlate with the concentration of polyclonal Ig in serum. Since B cell differentiation is arrested in most myeloma patients, t seemed possible that the level of sIg$^-$ BA-1$^+$ cells migl t correlate with the concentration of normal serum Ig. The amount of serum IgM, as the uninvolved class for IgG, IgA, and light chain disease, was quantitated and compared to the percentage of BA-1$^+$ cells. There was no correlation between number or PNA$^{+/-}$ phenotype and reductions in the concer - tration of serum IgM (Table 7).

DISCUSSION

Lymphocytes from multiple myeloma patients appeared to be frozen in their differentiation from sIg$^-$ pre-B cells to sIg$^+$ B cells, and pre-B cells were abnormally located in th: peripheral blood. We detected high numbers of BA-1$^+$ sIg$^-$ cμ lymphocytes in the PBLs of myeloma patients and in 44% of patients with MGUS. We also detected temporal fluctuation in BA-1$^+$ levels of up to 460-fold in some patients. Thes: observations suggest that the B lymphocyte lineage of patients bears superficial similarity to the developmental

Table 4. cμ$^+$ Cells in Multiple Myeloma. MGUS. and Normal Donors

	No. of Samples	Percentage of PBLs (Range) cμ$^+$	sIg$^+$	BA-1$^+$
Multiple myeloma	18	15.6 ± 4.3 (2–80)	0.7 c 0.4	7.0 ± 1.4
MGUS	4	c ı	0.19 ± 0.1	0.67 ± 0.3
	9	3.3 ± 0.7 (<1–7)	3.1 ± 1	6.2 ± 1.2
Normal	3	6.3 ± 1.2 (4–10)	4.4 ± 0.5	11.9 ± 0.8

The mean values reported above accurately reflect the pattern of marker expression in individual samples. Marker expression was determined by IF on three aliquots of each individual PBL sample.

Table 5. Heterogeneity in Expression of PNA and HLA-DR Markers on BA-1$^+$ Cells From Different Individuals: Double Immunofluorescence Studies

	No. of Individuals With		
	BA-1$^+$ PNA$^+$	BA-1$^+$ PNA'	Mixed.
Multiple myeloma	14/52	29/52	9/52
MGUS	5/20	12/20	3/20
Normal	—	15/16	1/16
	BA-1$^+$ HLA-DR$^+$†	BA-1$^+$ HLA-DR$^-$‡	Mixed.
Multiple myeloma	10/26	8/26	8/26
MGUS	5/8	1/8	2/8
Normal	5/6	—	1/6

A population of SA-1$^+$ cells was considered to be mixed if the relevant subsets exceeded 20% of the total BA-1$^+$ cells.

*Patients with a mixture of PNA$^{+/-}$ phenotypes had a mixture of HLA-DR$^{+/-}$.

†All patients with BA-1$^+$ HLA-DR$^+$ phenotype also expressed the BA-1$^+$ PNA$^-$ phenotype.

‡All patients with BA-1$^+$ HLA-DR- were also BA-1$^+$ PNA$^+$.

Table 6. Temporal Stability of Surface Phenotype in Some but not all Patients

Patient No.	Date	PNA BA-1+ Cells (%) +	−	Predominant Phenotype
097	Dec 1983	26	74	PNA−
	Feb 1984	14	86	PNA−
	March 1984	34	66	PNA−
086	Oct 1983	18	82	PNA−
	Nov 1983	10	90	PNA−
	Feb 1984	75	25	PNA+
	March 1984	81	19	PNA+
	April 1984	75	25	PNA+
075	Oct 1983	86	14	PNA+
	Feb 1984	69	31	PNA+
	March 1984	5	95	PNA−

All determinations of PNA binding were done in double IF with BA-1+ cells. Patient No. 097 had 3% to 25% BA-1+ cells, patient No. 086 had 2% to 28% BA-1+, and patient No. 095 had 3% to 26% BA-1+ cells over the course of these assays (see Table 2).

stage of an early fetus when pre-B cells predominate and sIg+ B cells are low or undetectable.[13,18]

BA-1 is a monoclonal antibody generated in response to a pre-B ALL which expresses the sIg− cμ+ phenotype.[12] The BA-I antigen is expressed on sIg+ B cells and is lost on terminal differentiation to a plasma cell. PBLs from normal donors included 6% to 10% BA-1+ sIg+ cells and 0% to 1% BA-1+ sIg− cells." Our results confirmed that BA-1+ cells from normal donors were sIg+ B cells. Finally, normal and chronic lymphocytic leukemia progenitor cells able to produce B lymphocyte colonies in vitro bore BA-1.[22]

To further identify the BA-I+ cells in patients, we used dIF. Since patients are deficient in sIg+ cells: BA-1+ cells do not have a high density of sIg. However, it was possible that they represented immature B cells with a low density of sIg, or B cells able to rapidly shed sIg.[20,23] We performed dIF in sodium azide to prevent shedding of sIg and found very few BA-I' cells, if any, that were sIgM+. They did, however, exhibit moderately positive intracytoplasmic staining with FITC–sheep anti-IgM (Table 3). Also consistent with a

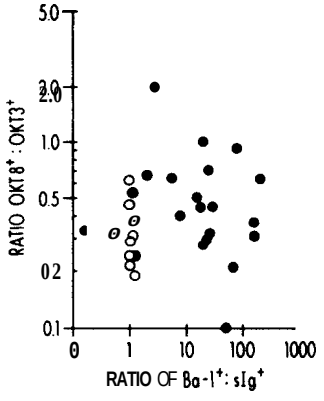

Fig 2. Numbers of Ba-1+ cells do not correlate with the number of OKT8+ cells. Closed symbols represent valuer in individual patients; open symbols represent values in normal patients.

Table 7. Lack of Correlation Between Percentage of BA-1+ Cells and Serum IgM Levels

Serum IgM (mg/mL)	Mean % of EA-1+ Cells (No. of Samples)	Range (%)	BA-1 phenotype (No. of Samples)
<0.18	6.1 ± 0.9 (44)	0.13–26	PNA+ (7) PNA− (13)
0.19–0.39	6.6 ± 0.8 (17)	0.5–18.8	PNA+ (4) PNA− (11)
0.4–2.6	6.4 ± 1.0(54)	0.31–28	PNA+ (14) PNA− (17)

Serum IgM levels were quantitated by nephelometry (Beahring Diagnostics of Canadian Hoechst Ltd, Montreal). BA-1+ cells and serum IgM were always quantitated at the same time point. The normal range of serum IgM is 0.4 to 2.6 (mean ± 2 SD). Values for BA-1+ cells are the means ± SE.

definition of the BA-I+ cells as pre-B cells in many individual patients was their expression of a low density of HLA-DR (non-capping subset).[7,18]

Although OKT10 is expressed on sIg+ B cells in bone marrow,[24] we were unable to detect expression of OKT10 on the BA-I+ PBLs observed here, indicating that they are not equivalent to immature bone marrow B cells. In contrast, BA-1+ PBLs from some patients expressed receptors for the lectin PNA, which in normal patients occur only on B cells of bone marrow origin.[18,25] In a series of studies using a murine model, Osmond and his colleagues[26–29] have shown that the B cell maturation pathway includes large PNA+ pre-B cells which give rise to small PNA+ pre-B cells, followed by differentiation to PNA− B cells. The subpopulations of pre-B cells in the PBLs of multiple myeloma patients appeared to parallel the murine pattern. The PNA− pre-B cells observed may represent an intermediate stage between the PNA+ pre-B cell and a PNA− B cell. The multiple myeloma patients exhibited heterogeneity in that some had BA-1+ PNA− cells and others had BA-1+ PNA+ cells. MGUS and normal donors had all or mainly PNA− BA-1+ cells which in normal and most MGUS patients were sIg+ B cells.

HLA-DR expression on the BA-1+ population differed between individual patients (Table 5). BA-I+ PNA− cells were always HLA-DR+, while BA-1+ PNA+ cells were HLA-DR−. Both phenotypic sets were cμ+ sIg−. The phenotypic patterns seen in patients and normal donors are summarized in Table 8. In two patients sequentially typed over a six- to seven-month period, we observed a shift in the BA-1+ phenotype from PNA− to PNA+ in patient No. 086, and from PNA+ to PNA− in patient No. 095. It seems likely that such temporal shifts in phenotype account for the mixed PNA+/− HLA-DR+/− populations we observed in some patients. The functional significance of the various BA-1+ populations and the acquisition or loss of markers is currently being investigated. They may reflect stages of developmental blockade, and suggest that the arrest in differentiation is a dynamic process.

Results analogous to or consistent with our own have been obtained in other systems. A developmental arrest in the expression of sIg by pre-B cells has been shown in congenital agammaglobulinemia,[30,31] with pre-B cells located in the periphery." sIg− PBLs expressing a B cell antigen were

Table 8. Phenotype of BA-1$^+$ Cells in Myeloma, MGUS, and Normal Donors

Category	BA-1	PNA	HLA-DR	sIgM	No. of Individuals (Type of BA-1$^+$ Cells)
Myeloma	+	+	−	−	14 (Pre-B)
	+	−	+	−	25 (Pre-B)
	+	±	+	−	7 (Pre-B)
	+	− or ±	− or ±	+	6 (B cells)
MGUS	+	−	+	+	5 (B cells)
	+	−	+	−	12 (Pre-B)
	+	+	+	+ or −	1 (Pre-B)/2 (B cells)
Normal	+	−	+	+	16 (B cells)

±, mixed population.

described in one myeloma patient by Turesson et al[32] as reflecting a lower degree of B cell maturity. Mayumi et al[33] have described an unusual population of bone marrow pre-B cells in leukemia patients. The apparent cycling in PBLs of the BA-1$^+$ cells seen in patient No. 086, which appears to correlate with shifts in the PNA-binding phenotype, is similar to that reported for bone marrow pre-B cells in a patient with cyclic neutropenia.[11]

Reports that B cells from myeloma patients showed enhanced sensitivity to T cell suppression[35] prompted us to search for correlations between the fluctuation pattern of BA-1$^+$ cells and OKT4$^+$ or OKT8$^+$ cells. No such correlations were detected. Neither could we detect a correlation between number or phenotype of pre-B cells and the degree of reduction of serum IgM.

It is not clear whether the Ba-1$^+$ pre-B cells have entered the peripheral circulation as a result of disordered differentiation or immunoregulatory influences, or if it is a consequence of extensive and diffuse marrow infiltration by the Plasma cell neoplasm. The existence of pre-B cells in the periphery of some patients with MGUS, and patients in very early stages of the disease, both of which have very few plasma cells in the marrow, suggests that tumor cell infiltration of the intramedullary space does not play a role in the exodus of pre-B cells from the bone marrow. In addition, the number or phenotype of pre-B cells in circulation does not correlate in any obvious way with the stage of disease. Experiments are in progress to explore whether these abnormally located cells are pre-malignant by determining the monoclonal or polyclonal nature of the pre-B cell population and to determine the reasons for the existence of pre-B cells in the periphery.

ACKNOWLEDGMENT

We thank Dr Edna Rapp for providing access to her patient!. Excellent technical assistance was provided by Donna Jean Gibney, Margret Krezolak, Parviz Somji, and Gaila Hinz. Invaluable help in patient accrual came from Maggi Staworski and Pauline Ashley. We are particularly grateful to Dr Ted Zipf for providing the resources of his lab to facilitate transport of samples.

REFERENCES

1. Zinneman HH, Hall WH: Recurrent pneumonia in multiple myeloma and some observations on immunologic response. Ann Intern Med 41:1152, 1954

2. Cone L, Uhr JW: Immunological deficiency disorders associated with chronic lymphocytic leukemia and multiple myeloma. J Clin Invest 43:2241, 1964

3. Fahey JL, Scoggins R, Utz JP, Szwed C F Infection, antibody response and gamma globulin components in multiple myeloma and macroglobulinemia. Am J Med 35:698, 1963

4. Heath RB, Hamilton Fairley G, Malpas JS: Production of antibodies against viruses in leukaemia and related diseases. Br J Haematol 10:365, 1964

5. Harris J, Alexanian R, Hersh E, Migliore P: Immune function in multiple myeloma: Impaired responsiveness to keyhole limpet hemocyanin. Can Med Assoc J 104:389, 1971

6. Pruzanski W, Gidon MS, Roy A: Suppression of polyclonal immunoglobulins in multiple myeloma: Relationship to the staging and other manifestations at diagnosis. Clin Immunol Immunopathol 17:280, 1980

7. Pilarski LM, Mant MJ, Ruether BA, Belch A: Severe deficiency of a mature B cell subset in peripheral blood from multiple myeloma patients. J Clin Invest 74:1301, 1984

8. Cohnen HJ, Rundles RW: Managing the complications of plasma cell myeloma. Arch Intern Med 135:177, 1975

9. Ullrich S, Zolla-Pazner S: Immunoregulatory circuits in myeloma. Clin Haematol 11:87, 1975

10. Lindstrom FD, Hardy WR, Eberle BJ, Williams RC: Multiple myeloma and benign monoclonal gammopathy: Differentiation by immunofluorescence of lymphocytes. Ann Intern Med 78:837 1973

11. Chen Y, Bhoopalam N, Yakulis V, Heller P Changes in lymphocyte surface immunoglobulins in myeloma and the effect of an RNA-containing plasma factor. Ann Intern Med 83:625, 1975

12. Abramson CS, Kersey JH, LeBein TW: A monoclonal antibody (BA-I) reactive with cells of human B lymphocyte lineage.. Immunol 126:83, 1981

13. Gathings WE, Lawton AR, Cooper MD: Immunofluorescent studies of the development of pre-B cells, B lymphocytes and immunoglobulin isotype diversity in humans. Eur J Immunol 7:804 1977

14. Zipf T: (submitted for publication)

15. Mannoni P, Janowska A, Turner AR, McGann L, Turc JM Monoclonal antibodies to human myeloid antigens. Hum Immunol 5:309, 1982

16. Mannoni P, Janowska A, Fromont P, Weiblen B, Turner AR. Turc JM: Myeloid differentiation studied by monoclonal antibodies, in Dausset J, Milstein C, Schlossman S (eds): Proceedings of the First International Symposium on Leukocyte Differentiation Antigens. Paris, Karger, 1983, p 410

17. MacLean GD, Mosmann RT, Akabutu JJ, Longenecker BM: Preference of the early murine immune response for polymorphic determinants on human lymphoid-leukemia cells and the potential

use of monoclonal antibodies to these determinants in leukemia-typing panel. Oncodev Biol Med 3:223, 1982

18. Cooper MD: Pre-B cells: Normal and abnormal development. J Clin Immunol 1:81, 1981

19. Mills KH, Cawley JC: Abnormal monoclonal antibody defined helper/suppressor T-cell subpopulations in multiple myeloma: Relationship to treatment and clinical state. Br J Haematol 53:271, 1983

20. Mellstedt H, Holm G, Pettersson D. Bjorkholm M, Johansson B, Lindemalm C, Peest D, Ahre A: T cells in monoclonal gammopathies. Scand J Haematol 29:57, 1982

21. Pilarski LM, Mant MJ, Ruether BA, Carayanniotis G, Otto D. Krowka J F (submitted for publication)

22. Perri RT, Royston I, LeBein TW, Kay NE: Chronic lymphocytic leukemia progenitor cells carry the antigens T65, BA-I, and Ia. Blood 61:871, 1983

23. Melchers F, von Boehmer H, Phillips RA: B-lymphocyte subpopulations in the mouse. Transplant Rev 25:26, 1975

24. Janossy G. Tidman N, Papageorgious ES, Kung PC, Goldstein G: Distribution of T lymphocyte subsets in the human bone marrow and thymus: An analysis with monoclonal antibodies. J Immunol 126:1608, 1981

25. Gobbi M, Caligaris-Cappio F, Janossy G: Normal equivalent cells of B cell malignancies: Analysis with monoclonal antibodies. Br J Haematol 54:393, 1983

26. Osmond DG: Production and differentiation of B lymphocytes in the bone marrow; in Battisto JR, Knight KL (eds): Immunoglobulin Genes and B Cell Differentiation. New York, Elsevier/North Holland, 1980. p 135

27. Osmond DG, Owen JJT Pre-B cells in bone marrow: Size distribution profile, proliferation capacity and peanut agglutinin binding of cytoplasmic p-chain bearing cell populations in normal and regenerating bone marrow. Immunology 51:333, 1984

28. Osmond DG, Melchers F. Paige CJ: Pre-B cells in mouse bone marrow: In vitro maturation of peanut agglutinin binding B lymphocyte precursors separated from bone marrow by fluorescence-activated cell sorting. J Immunol 133:86, 1984

29. Osmond DG: Pre B cells in bone marrow: Peanut agglutinin binding and separation of cytoplasmic μ chain-bearing cell populations in normal, post-irradiation and polycythemic mice using fluorescence-activated cell sorting. Eur J Immunol 14495,1984

30. Pearl ER, Vogler LB, Okos AJ, Crist WM, Lawton AR, Cooper MD: B lymphocyte precursors in human bone marrow An analysis of normal individuals and patients with antibody deficiency states. J Immunol 120:1169, 1978

31. Dosch HM, Percy ME, Gelfand EW: Functional differentiation of B lymphocytes in congenital ag immaglobulinemia: Generation of hemolytic plaque-forming cells. J Immunol 119:1959, 1977

32. Turesson I, Berntorp E, Zettervall O The expression of human B-lymphocyte antigen and surface membrane immunoglobulin by lymphoid cells from patients with lymphocytic lymphoma, multiple myeloma and benign monoclonal gammapathy. Acta Med Scand 206:37, 1979

33. Mayumi H, Kubagawa H, Omura GA, Gathings WE, Kearney JF, Cooper MD: Studies on the clonal origin of human B cell leukemia using monoclonal anti-idiotype antibodies. J Immunol 129:904, I982

34. Engelhard D, Landreth KS. Kapoor N, Kincade PW, De Bault LE, Theodore A, Good RA: Cycling of peripheral blood and marrow lymphocytes in cyclic neutropenia. Proc Natl Acad Sci USA 80:5734, 1983

35. Perri RT, Oken MM, Kay N E Enhanced T cell suppression is directed towards sensitive circulating 3 cells in multiple myeloma. J Lab Clin Med 99512, 1982