Abnormal Function of B Lymphocytes from Peripheral Blood of Multiple Myeloma Patients

Lack of Correlation between the Number of Cells Potentially Able to Secrete Immunoglobulin M and Serum Immunoglobulin M Levels

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Abstract

Multiple myeloma patients are deficient in normal polyclonal serum immunoglobulins. To determine the reasons for this decrease, we quantitated and compared the number of surface IgM$^+$ B lymphocytes, and the number of B cells susceptible to transformation by Epstein-Barr virus (EBV) with the concentration of IgM in serum. Serum IgM levels varied considerably in individual patients, temporally shifting from undetectable to normal amounts and then dropping again to undetectable levels. A transient rise to normal serum IgM concentrations was seen in 42% of patients assessed at two or more time points.

Of 44 patients, 52% showed a lack of correlation between the number of surface IgM$^+$ (sIgM$^+$) B cells and serum IgM concentration. One subset of patients (25%) had detectable to normal numbers of sIgM$^+$ B cells in blood but undetectable levels of serum IgM. Transformation of B cells from these patients indicated a block in IgM secretion that was extrinsic to the B cells that were fully able to transcribe, translate, and secrete IgM after EBV transformation. A second subset of patients (27%) had undetectable numbers of sIgM$^+$ B cells but near normal levels of serum IgM, suggesting abundant secretion by few clones of B cells. Of 18 patients with monoclonal gammopathy of undetermined significance (MGUS), 26% showed a lack of correlation between the numbers of sIgM$^+$ B cells and serum IgM concentration. We suggest that in patients with multiple myeloma, and in some with MGUS, there exists a mechanism(s) extrinsic to the B cell that mediates an arrest in terminal B lymphocyte maturation.

Introduction

A frequent finding in multiple myeloma is a decrease in normal serum Ig levels that correlates with disease severity (1). The underlying mechanism is sufficiently powerful to suppress even monoclonal gammopathies unrelated to the myeloma (2, 3). Although most multiple myeloma patients have greatly reduced numbers of B lymphocytes (4–7), it is not clear whether the decreased serum polyclonal Ig is due to lack of B lymphocytes or to defective maturation of B cells potentially able to secrete Ig. Broder et al. (8) have suggested that suppressive effects partially prevent polyclonal Ig synthesis but this conclusion remains controversial (9). To explore the reasons for decreased serum Ig, we have quantitated and compared the number of surface IgM$^+$ (sIgM$^+$)[1] B cells in peripheral blood, the frequency with which B cells from myeloma patients are transformed by Epstein-Barr virus (EBV) to produce IgM-secreting clones in vitro (10–13), and serum IgM levels. We find that a substantial subset of myeloma patients and some individuals with monoclonal gammopathy of undetermined significance (MGUS) have a normal quantity of sIgM$^+$ B cells fully able to secrete IgM after EBV transformation in vitro but unable to differentiate to IgM secretors in vivo as measured by serum IgM levels.

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Methods

44 multiple myeloma patients and 18 individuals with monoclonal gammopathy of undetermined significance (MGUS) were studied after informed consent had been obtained. With one exception, the myeloma patients had a monoclonal component (M component), plasma cell infiltration of the bone marrow, multiple skeletal lytic lesions, or generalized osteoporosis, and reduced polyclonal Ig levels. Patient 132 was a nonsecretor of M component. Patients with MGUS had an M component that was stable in concentration over time and had no other evidence of multiple myeloma. Except where indicated, blood samples were taken at least 4 wk after chemotherapy. Patients described as off treatment received their last cycle of chemotherapy at least 6 mo before participating in this study. Normal donors were randomly chosen and supplied by the Red Cross Blood Transfusion Service.

Peripheral blood lymphocytes (PBL) were purified by centrifugation over Ficoll-paque (Pharmacia Fine Chemicals, Piscataway, NJ), and the composition of the resulting cell population was determined by a differential count using Giemsa stain. Aliquots were characterized for number of sIg$^+$ and sIgM$^+$ B cells by indirect immunofluorescence (IF) (4). A second aliquot of cells was subjected to separation by rosetting with 2-amino-ethylisothiouronium bromide hydrobromide-treated sheep erythrocytes to obtain an E$^-$ population for use in EBV transformation procedures (10). Briefly, purified PBL (5×10^5–10^6) were aliquoted into v-bottom microtiter wells, and were resuspended in 50 μl of the test murine anti-human antibody and incubated for 60 min at 4°C. After washing, 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) (Tago Inc., Burlingame, CA), incubated for 60 min at 4°C, and washed twice. Cells were then resuspended in warm saline and incubated for 10 min at 37°C to allow cap formation (4) followed by fixation of cells in 1% formalin.

1. *Abbreviations used in this paper:* EBV, Epstein-Barr virus; IF, immunofluorescence; M component, monoclonal component; MGUS, monoclonal gammopathy of undetermined significance; PBL, peripheral blood lymphocyte; sIg, surface Ig.

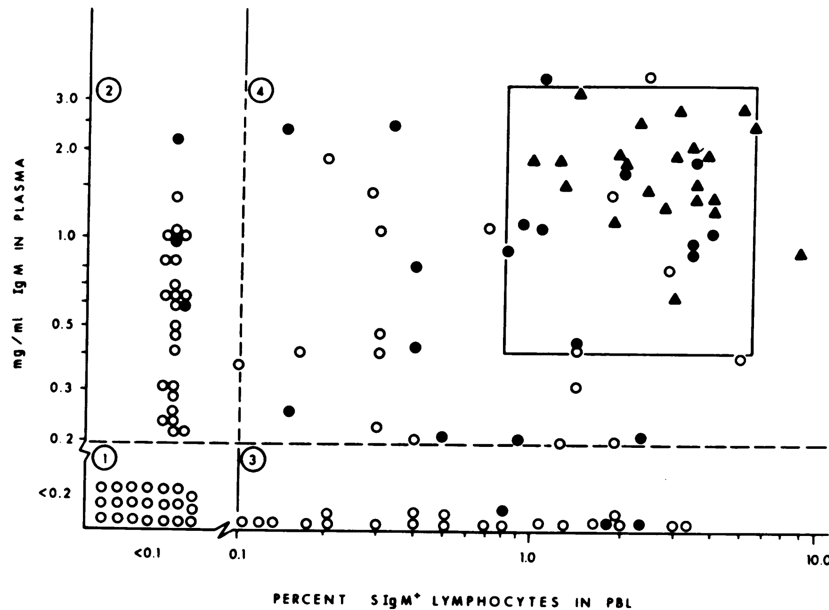

Figure 1. Assessment of serum IgM and percent $sIgM^+$ PBL in individual blood samples from normal, MGUS, and multiple myeloma donors. Values of <0.2 mg/ml serum IgM and <0.1% $sIgM^+$ were below the limits of reliable detection. The boxed area represents the mean±2 SD of normal values. Points represent individual samples. ○, multiple myeloma; ●, MGUS; ▲, normal.

Previous experiments have shown that in both patients and normal donors, the number of $sIgM^+$ cells detected by the capping technique was the same as that when IF was done in azide and ring fluorescence was assessed (4). In no case were rings seen in patient or normal samples where IF was done under capping conditions, indicating that all cells able to bind the anti-IgM, were able to cap.

IF was examined using a Zeiss microscope (Carl Zeiss, Inc., Thornwood, NY) with fluorescence epi-illumination and selective filters. Cell samples were counted for total cells in the field and for total number of capped cells using a hemocytometer. 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 bright. The percent capped cells was calculated after screening a minimum of 1,000 cells. In samples with <0.1% of caps, a minimum of 5,000 cells was screened. Cells with ring fluorescence were not seen in these samples.

The patients analyzed for this study include the set of patients analyzed previously (4) where B cells were defined by their expression of HLA·DR, and of 41H·16, a marker for mature B cells (14). The number of B cells defined by these two markers was within the same range as those defined by their ability to bind anti-IgM.

Plasma IgM levels were determined by nephlometry (Behring Diagnostics, American Hoechst Corp., San Diego, CA). Plasma samples were taken from the same tubes of blood used for purification of PBL.

EBV-transformation. The techniques of Winger et al. (10) were used to infect the E^- cells with EBV followed by their distribution into microtiter wells (Flow Laboratories, Inc., McLean, VA), containing 10^5 E^- irradiated feeder cells (1,500 rads) from normal fresh buffy coat. EBV-infected cells were plated at 3×10^3 to one cell per well in a decreasing threefold dilution series in RPMI (Gibco Canada, Burlington, Ontario) plus 20% fetal calf serum (Flow Laboratories, Inc.). Supernatants were collected at 3 wk and tested for IgM activity as described below. The frequency of transformed cells within an EBV-infected E^- population was defined as the reciprocal of the cell number at which 37% of the microculture wells in the limiting dilution series were negative; on average each well receives one transformed B cell at this cell concentration (11–13).

The concentration of IgM in supernatants was determined by a reverse enzyme-linked immunosorbent assay, in which plates were coated with monoclonal mouse anti-human IgM (Bethesda Research Laboratories, Gaithersburg, MD); IgM bound from added supernatants was detected with alkaline phosphatase-coupled, goat anti-human IgM (Sigma Chemical Co., St. Louis, MO). Every assay plate included a set of wells containing known concentrations of purified human IgM (gift from Dr. T. Zipf, University of Calgary, Alberta, Canada) for constructing a standard concentration curve.

Results

Serum IgM and $sIgM^+$ B cells. In a series of 44 multiple myeloma patients, 18 individuals with MGUS, and 19 normal donors, we have quantitated serum IgM and numbers of $sIgM^+$ B cells in peripheral blood. In a plot of the serum IgM concentration versus the percent $sIgM^+$ B cells, the distribution of individual myeloma samples differed significantly from that of normal donors. Four broadly defined patterns are distinguished in Fig. 1 and Table I: (*a*) undetectable or exceedingly low serum IgM and $sIgM^+$ B cells (quadrant 1, Fig. 1; and line 1, Table I), (*b*) detectable serum IgM and undetectable

Table I. Lack of Correlation between Concentration of Serum IgM and Percent of $sIgM^+$ PBL in Multiple Myeloma

Serum IgM	Percent $sIgM^+$ B cells	Percent of samples: Normal	MGUS	Multiple myeloma
mg/ml				
<0.20	<0.10	None	None	26.0
>0.2	<0.10	None	9.3	27.0
<0.2	>0.1	None	16.6	25.0
>0.2	>0.1	100	75.0	22.0
No. of samples		21	24	71
No. of individuals		19	18	44
Percent of samples outside normal range*		4.8	57.1	95.8

* Mean±SD of serum IgM = 1.9±0.72 mg/ml; mean percent ±SD of $sIgM^+$ cells in PBL = 3.2±1.7; normal range is considered to be mean±2 SD. (Boxed area in Fig. 1.)

Table II. Aberrant B Cell Differentiation as a Function of Treatment Status

Serum IgM	sIgM+	Percent of samples Untreated	Treated	Off treatment
<0.2	<0.10	30	28	17
>0.2	<0.1	10	31	30
<0.2	>0.1	30	19	30
>0.2	>0.1	30	24	17
No. of samples		9	56	6
No. of individuals		8	30	6
Percent of samples outside the normal range		91	96	100

$sIgM^+$ B cells (quadrant 2 and line 2), (*c*) undetectable serum IgM and detectable $sIgM^+$ B cells (quadrant 3 and line 3), and (*d*) detectable levels of both serum IgM and $sIgM^+$ B cells (quadrant 4 and line 4). Of normal donors, 100% fell into category 4. For multiple myeloma only 22% fell into this category (Table I). 96% of myeloma samples were outside the range of normal values. Unlike myeloma patients, and like normal donors, most individuals with MGUS were in category 4 (75%) (Table I), although 57% of MGUS samples were outside the normal range (Fig. 1). For 15 of the individuals falling into quadrants 1 and 2 of Fig. 1, the number of B cells in the sample was also quantitated by IF with anti-HLA · DR and 41H · 16, a marker for mature B cells (14) as previously described (4). Although these two markers will define B cells expressing any class of sIg, the number of total B cells was either equivalent to the number of $sIgM^+$ B cells or within two- to threefold of that value (data not shown).

The lack of correlation between serum IgM levels and $sIgM^+$ B cells, in particular those individuals with undetectable or very low levels of serum IgM and detectable to normal numbers of $sIgM^+$ B cells (quadrant 3, Fig. 1; and line 3, Table I), or low $sIgM^+$ B cells and normal serum IgM (quadrant 2, Fig. 1; and line 2, Table I), present a paradox. In the individuals shown in quadrant 3, apparently normal numbers of $sIgM^+$ cells are unable to secrete IgM themselves, or to differentiate to IgM-secreting cells in vivo. This situation is not an artefact of chemotherapeutic treatment, as 30% of untreated patients and of patients off therapy exhibited the aberrant pattern; 91% (untreated) to 100% (off therapy) of such patients had serum IgM/$sIgM^+$ levels outside the normal range (Table II). In contrast, an equally large number of patients had undetectable numbers of $sIgM^+$ B cells and near normal levels of serum IgM (quadrant 2). The patients described in quadrants 1 and 4 represent expected phenotypes where the number of B cells correlates with the concentration of serum IgM.

Temporal variation in serum IgM levels. Serum IgM levels in individual myeloma patients were monitored for up to 1 yr. Although the concentration of IgM remained uniformly low for some, up to 10-fold variations were observed in other myeloma patients (Fig. 2). In two patients normal concentrations were observed, which subsequently fell to very low or

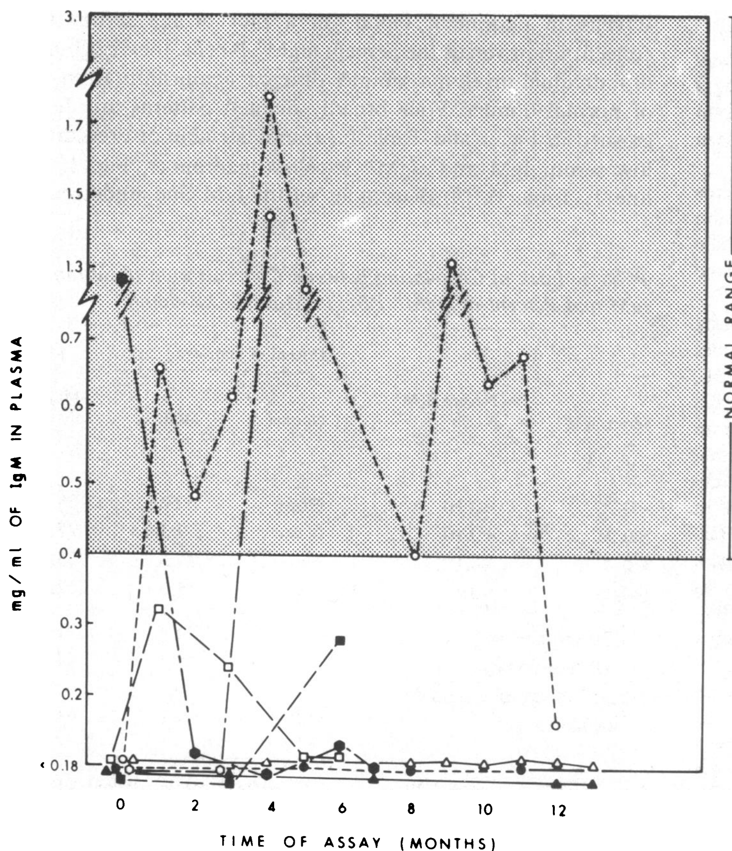

Figure 2. Temporal variation of serum IgM levels in individual patients. Hatched area represents the range of normal values (mean±2 SD). Serum IgM levels in normal individuals are relatively constant over time (not shown). ○, 086, IgGK on treatment; △, 075, IgGK untreated on month 0, on chemotherapy thereafter; ●, 097, IgGK on chemotherapy; □, 101, IgGK on chemotherapy; ●, 066, IgGL untreated at month 0, on chemotherapy thereafter; ▲, 073, IgGK untreated, transition from MGUS to early myeloma between month 0 and month 7; ■, 106, IgGK, untreated MGUS; o, 109, IgGK, untreated on month 0, on chemotherapy thereafter.

undetectable levels. Periodic increases in serum IgM to normal or nearly normal values were observed in 42% of patients (8/19) assessed at two or more time points.

$sIgM^+$ B cells secrete IgM in vitro. B cells from peripheral blood of 12 myeloma patients, two MGUS, and six normal donors were subjected to transformation with EBV to determine their potential for IgM secretion (Table III). Infection with EBV results in B cells able to proliferate autonomously and secrete IgM. In normal donors 20–100% of the $sIgM^+$ B cells gave rise to an IgM-secreting clone as defined after culture at limiting dilution. For myeloma patients the frequency of transformation ranged from 10% to >100%. Values above 100% may reflect transformation of B cells with a level of sIgM below the limits of detection in our IF assay.

In four myeloma patients and one individual with MGUS, substantial numbers of IgM-secreting B cells were detected in vitro by EBV transformation even though in vivo, these apparently normal numbers of $sIgM^+$ cells gave rise to only very low or undetectable levels of serum IgM (Table III, patients 007, 047, 073, 096, and 102).

Discussion

The immunodeficiency in multiple myeloma patients is characterized by a pronounced decrease in serum levels of normal Ig, particularly IgM. The mechanism effecting this decrease is unknown. In this study we show that in a substantial subset of myeloma patients, near normal numbers of $sIgM^+$ B cells are unable to either secrete IgM themselves or to differentiate to IgM-secreting cells. These $sIgM^+$ B cells are potentially able to differentiate to IgM secretors as defined by their ability to secrete normal amounts of IgM upon transformation with EBV in vitro. To accurately quantitate the number of B cells able to secrete Ig in vitro, a stimulating agent is required that allows the B cell to proliferate and secrete Ig independently of any other positive or negative regulatory signals. Many systems using mitogenic substances to stimulate B cells involve T cell-dependent activation, concurrently induce suppressor T cells, or are susceptible to inhibitory influences particularly in bulk cultures (15, 16–20). In our experiments the use of EBV transformants allowed us to measure capability for IgM secretion independently of any requirements for regulatory activation signals from mitogens or T cells, and the implementation of limiting dilution methodology allowed the transformed B cells to escape any inhibitory influences.

The lack of correlation between $sIgM^+$ B cells and serum IgM concentration could be a result of reduced production of IgM in vivo or alternatively, a reduced half-life of normal IgM in serum. Since no evidence exists to indicate the latter, it seems possible that an arrest in terminal differentiation of receptor-bearing B cells to IgM-secreting plasma cells occurs in multiple myeloma that is remarkably similar to the immunodeficiency observed in agammaglobulinemic individuals (21–27). However, unlike some (15, 26–29), but like other cases of agammaglobulinemia (15, 29–31), the defect in myeloma patients is extrinsic to the B cell rather than an intrinsic genetic block, since these B cells are fully able to transcribe and translate genes for IgM and to secrete IgM after EBV transfor-

Table III. Lack of Correlation between IgM and the Number of B Cells Able to Secrete IgM after EBV Transformation

Patients	EBV transformants per 10^5 PBL*	$sIgM^+$ cells per 10^5 PBL‡	Efficiency of transformation§	Serum IgM
				mg/ml
Myeloma				
007‖	403	1,200	0.33	<0.18
047‖	266	300	0.89	<0.18
050	17	70	0.24	0.42
071	137	1,400	0.10	0.40
073¶	277	1,100	0.25	<0.18
096	342	1,800	0.19	0.19
099‖	55	30	1.83	1.81
101	18	210	0.86	<0.18
107	24	70	0.34	0.63
108	49	20	2.45	<0.18
132**	43	150	0.29	0.39
137	69	170	0.41	0.39
mean±SE	142±57	543±260		
MGUS				
102¶	559	400	1.39	0.79
106¶	917	1,900	0.48	<0.18
Normal mean±SE	2,656±323	2,900±500	0.2–1.0	1.9±0.16
No. of individuals	6	34		24

* Based on the frequency of IgM-secreting transformants calculated from a plot of percent negative wells in a limiting dilution series (11, 12). Positive wells were defined as those contaiing >5 ng of secreted IgM. ‡ Determined by immunofluorescence (4). § (Number of IgM-secreting transformants) ÷ (number of $sIgM^+$ cells). ‖ Patients off chemotherapy. ¶ Untreated. ** Nonsecretory myeloma. Clones of IgM secretors from myeloma or MGUS patients produced amounts of IgM equivalent to those of EBV-transformed clones from normal B cells.

mation in vitro. We speculate that terminal differentiation of B cells in these patients is being blocked by a suppressor T cell able to deliver negative regulatory signals to the B cell, and suggest that such a T cell might in fact be autoimmune with a receptor specificity directed to a determinant (sIg?) on autologous B cells. Broder et al. (8) have described inhibitory cells in PBL from myeloma patients that block Ig synthesis by normal B cells, but removal of these cells resulted in only very limited restoration of Ig synthesis by myeloma B cells. Peest and co-workers (32) have reported normal polyclonal Ig synthesis by fractionated and reconstituted myeloma PBL in vitro but did not quantitate the number of B cell precursors giving rise to the Ig secretors.

A second group of myeloma patients exhibited a pronounced reduction in numbers of $sIgM^+$ B cells but had normal or near normal serum IgM levels. A similar phenomenon has been observed in agammaglobulinemic individuals and has been attributed to abundant numbers of plasma cells derived from very few B cells (33), based on similar findings in bursectomized chickens (34). This suggests that the specificity repertoire of the serum IgM may be considerably less diverse than in normal donors. Preliminary experiments indicate this is in fact the case (L. M. Pilarski, unpublished data). Alternatively, patients may have a population of noncirculating B cells although the work of Turesson (35) argues against this. Work is in progress to determine if the IgM in these patients is oligoclonal or polyclonal in nature.

Those myeloma patients with few B cells and very low or undetectable serum IgM would seem to have a straight forward correlation between the number of B cells and serum IgM, although it is noteworthy that individual patients exhibit temporal variation in both serum IgM concentration and $sIgM^+$ B cells (Fig. 2; and [4]). Over time, some patients shift into different categories of serum/surface IgM correlates, indicating that the mechanism(s) mediating arrested differentiation may act differentially on selected subpopulations of B cells and perhaps at more than one stage of differentiation.

An important question in multiple myeloma concerns the relationship between acquired immunodeficiency and progression of the plasma cell neoplasm. If immunodeficiency correlates with aggressive neoplastic disease, as has been suggested for other cancers (36, 37) and in murine model systems (38), its reversal might lead to a better prognosis for myeloma patients. Experiments are in progress to determine the mechanism(s) mediating B lineage differentiation arrest in myeloma, with particular emphasis on a search for suppressor T cells.

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