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Blood Lymphocytes in Myeloma Patients; High Percentage of Complement Receptor Bearing Cells is Accompanied by Decreased Anti-Immunoglobulin (Ig) Binding Capacity*

O. WETTER and K.-H. LINDER

Innere Universitätsklinik (Tumorforschung), Westdeutsches Tumorzentrum, Hufelandstraße 55, *D*-4300 *Essen, Germany*

Abstract—*Receptor functions of peripheral blood lymphocytes of* 60 *myeloma patients have been studied. Cell surface immunoglobulin, spontaneous rosette formation with SRBC, complement receptors using EAC rosette formation and uptake of radioiodinated antibodies against human Ig have been examined and compared to the results obtained with cells from normal individuals. Also the response to polyclonal B cell activators has been studied. Significant differences between normal individuals and myeloma patients have been found for the numbers of EAC rosette forming cells. The binding capacity for radiolabelled anti-Ig antibodies showed a strong tendency to low values in the patient group. Our results are compatible with the idea that cells with an exclusive capacity for complement binding are increased in myeloma patients. A high number of EAC binding cells has been found also in bone marrow aspirates of myeloma patients showing infiltration by tumor cells. Therefore, it seems possible that the site primarily affected from disease is the main source of complement receptor bearing cells. No correlation has been seen between the response to polyclonal B cell activators or the concentration of normal serum Ig to the frequency of EAC binding cells in individual patients* (r_s 0.07 and 0.11, respectively).

INTRODUCTION

ABNORMALITIES of lymphocyte functions in myeloma patients have been reported by several authors [1–4]. It has been found that synthesis of polyclonal, that is, normal Ig is depressed [1, 4] and that activation by PBA as measured by 3H-thymidine incorporation into DNA of lymphocytes is diminished when compared to normals [2, 3]. These results can be explained partly by the finding of a population of PBL with abnormal binding capacity for PBA and/or metabolic defects. The aim of our experiments was to search for cell surface receptor properties which might provide additional information as to lymphocyte function in myeloma of plasma cell type. It has been tested also if a relationship exists between certain receptor functions and overall immune functions in myeloma patients.

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Abbreviations used: EAC, erythrocyte antibody complement; Ig, immunoglobulin; S.Ig, surface Ig; B cell, bursa-equivalent dependant lymphocyte; T cell, thymus dependant lymphocyte; PBL, peripheral blood lymphocyte(s); FITC, fluorescein isothiocyanate; HBSS, Hanks' balanced salt solution; PBA, polyclonal B cell activators; CLL, chronic lymphatic leukemia; s.d., standard deviation; The patients were numbered according to their entry into this study. Numbers given in Table 2 are used throughout the text.

MATERIALS AND METHODS

Patients

The diagnosis of plasma cell myeloma has been made in 60 patients by bone marrow aspiration cytology and histological examination of biopsies from the iliacal crest (Prof. Dr. L. D. Leder, Essen). Monoclonal proteins were present in all cases as shown by electrophoresis and immunoelectrophoresis of the serum and/or the urine. One patient (No. 44) had large extramedullary plasma cell tumors and bone marrow infiltration. Quantitation of Ig has been done by radial immunodiffusion (Partigen®, Behring-Werke, Marburg).

Immunofluorescence studies

Venous blood was drawn in heparin (Liquemin®, Roche, 20,000 i.u./ml, 0.4 ml/20 ml blood). Density gradient centrifugation was performed using Ficoll–Isopaque (Lymphoprep®, Nyegaard & Co., Oslo) according to Bøyum [5] at 1400 g plastic tubes of 16 mm diameter have been used. S.Ig positive cells were counted after staining with FITC-conjugated polyvalent anti-Ig antiserum from the goat (Meloy Lab., Springfield, USA) using a Leitz Orthoplan microscope equipped with a Philipps CS 100 W mercury lamp and an Opak Fluor vertical illuminator. An excitation filter KP 490 and a barrier filter K 510 were used. Redistribution of S.Ig (cap formation) by polyvalent anti-Ig antiserum has been done according to Huber *et al.* [6]. Results were given as percentage of capped cells on the basis of S.Ig positive cells.

Controls have been done using FITC-conjugated antialbumin antisera (Medac, Hamburg) and commercial monospecific antisera absorbed on appropriate myeloma proteins prepared in our lab. Conjugation of albumin with FITC (Merck, Darmstadt) has been done according to Hijmans *et al.* [7]. Antisera were filtered through Millipora® filters, type AAWP, pore size 0.45 μm (Millipore® S.A., Molsheim, France) before use.

Rosette experiments

Rosette formation (E rosettes) was performed according to Weiner *et al.* [8]. The same sample has been used for the enumeration of S.Ig positive cells as described by Kaplan *et al.* [9] using the direct immunofluorescence technique. The results are given as "corrected" percentage numbers of T and B cells on the basis of lymphocytes/100 cells present in each preparation from the ficoll gradient. Identification of cells was done by conventional staining according to Wright after cytocentrifugation (Cytospin, Shandon Instr. Ltd, G.B.).

EAC rosettes were prepared according to Huber *et al.* [10]. Fresh human serum was used as source of complement. Amboceptor (titer 1:4000) was purchased from Behring-Werke, Marburg), erythrocytes were supplied by Oxoid Deutschland GmbH, Wesel.

Radiolabelling experiments

Antibodies against human Ig (polyvalent anti-Ig antiserum from the goat, Meloy Lab., Springfield, USA) were labelled according to Bolton and Hunter [11]. Labelled protein (5 μg) was added to 2 ml antiserum. From this mixture 0.1 ml were incubated with 5×10^6 cells suspended in 1 ml HBSS for 30 min at 4°C. After three washings in HBSS and centrifugation at 700 g the radioactivity in the sediment was measured in a Siemens gamma counter. Binding capacity is expressed as pg antibody protein/S.Ig positive cell according to formula A.

$$\text{pg/cell} = \text{bound activity (CPM)} \times \frac{\text{mg protein}}{\text{ml antiserum}} \times 100$$

divided by

$$\frac{\text{CPM}}{\text{ml antiserum}} \times 5 \cdot 10^6 \times \%\text{S.Ig pos. cells.}$$

Stimulation experiments with PBA

Cells were prepared as described in the section "immunofluorescence studies". Cultures and activation were done according to DuBois *et al.* [12]. Briefly, cells (3×10^5/ml) were cultured in medium 199 with Hanks' salts (Seromed, München) fortified with 20% fetal calf serum. In some experiments 10^6 cells/ml were used. For measurements of 3H-thymidine incorporation cells were washed on Millipore® filters type AA 0.8 μm pore width in a sampling manifold device (Millipore Corp., Bedford, Mass. USA). Radioactivity was measured in a TriCarb liquid scintillation counter (Packard, Frankfurt) using Unisolve® (Fa. Zinsser, Frankfurt) as scintillation liquid.

For lymphocyte activation commercial reagents (PHA-P Difco, Detroit USA), PWM from Grand Island, USA, and Con A from Serva, Heidelberg) were used. Bone marrow cells from the iliacal crest were taken by needle aspiration. Specimens were centrifuged in a ficoll gradient as described before.

Mathematical tests

Comparison of mean values has been done using the U test according to Mann and Whitney [13]. The accociation of values for EAC binding cells, polyclonal Ig concentration of serum and activation index of PBL has been tested according to Spearman [14].

RESULTS

Lymphocyte activation in 19 myeloma patients with various doses of PHA and a single dose of PWM and Con A has been expressed

as activation index, that is the proportion of 3H-thymidine incorporation rates of activated patient cells to activated normal cells. Patients showing an index smaller than 1 are listed in Table 1 (a), those with an index greater than 1 are listed in Table 1 (b). The numbers in table 1 show that from 19 patients tested with 20 μg PHA 9 patients showed an impaired response. From the figures given in Table 1 (b) it follows that cells from 7 patients responded to a higher degree than normal lymphocytes at this PHA dose and 3 patients had a response of the same magnitude as normal individuals. The grade of the alteration of responsiveness at each PHA concentration is expressed in percent of normal response. Using Con A and PWM in doses which have been found to induce maximal activation with normal PBL 14 patients showed an impaired response and one patient had an increased response with Con A, 4 patients showed a response of the same order as normals. Using PWM in 5 patients, normal responsiveness has been found.

Table 1. *Response of* 19 *myeloma patients to PHA at various doses and to Con A and PWM at a single dose*

(a)	10	20	30	40	50	60	70	80	90	Percent of normal response	S
PHA (μg) 5		2		1			1			4	
10	1		2		2	1				6	
20*	1	2	1	1	1	1		1	1	9	
30	2		2	1	1	2				8	
40	3	1	1	1	1	1	1			9	
60	2					1	1			4	
80		2	1	1			2			6	
Index < 1 As average of all PHA concentrations tested in 10 patients	1	2	1				2			6	
At maximal activating PHA concentrations	1	2	2	2		1				8	
Con A* (4μg)										14	
PWM * (10μg)										14	

(b)	10	20	30	40	50	60	70	80	90	Percent of normal response	S
PHA (μg) 5				1		1			1	3	
10			1						2	3	
20†	2	1		1	1				2	7	
30									2	2	
40										0	
60									3	3	
80	1	1			1				2	5	
Index > 1 As average of all PHA concentrations tested in 10 patients						1			2	3	
At maximal activating PHA concentrations										0	
Con A† (4 μg)						1				1	
PWM† (10 μg)											

*$n = 20$.
†$\mu = 20$.
The numbers indicate patients who show an activation index 1 (part a) and 1 (part b) at a given PHA concentration. Patients with an index of 1 are not shown. "Percent of normal response" is the range of 3H-thymidine incorporation when the rate of normal cells is 100. For further explanations see text.

Table 2. Results in 21 representative cases of myeloma

Patient No.	PBL/cu. mm $\times 10^6$	E-rosettes %	EAC-rosettes %	S.Ig pos. cells %	Cap formation†	^{125}I-labelled	Antibody/cells (pg)
14	1.860	72	38	17	n.d.	0.3627	0.3144
15	1.770	86	n.d.‡	7	37	n.d.	
16	1.400	78	41	10	38	1.1797	
17*	700	33	n.d.	43	33	n.d.	
18	1.020	81	n.d.	n.d.	22	n.d.	
19	1.500	80	22	20	24	n.d.	
20*	2.400	86	44	12	22	0.2868	
21*	2.000	66	37	19	27	n.d.	
22	670	53	23	32	15	0.0875	
23	600	72	41	19	25	0.3560	
24	2.200	19	35	33	33	0.3235	
25	1.530	99	28	25	24	0.1121	
26	1.680	53	39	28	38	0.0498	
27	1.640	89	61	33	8	0.1260	
28	2.475	81	34	10	43	0.3254	
29*	800	89	21	13	43	0.4885	0.4858
30*	1.240	64	31	38	27	0.2725	
31	2.090	75	19	18	37	0.2865	
32	1.300	82	26; 37	24	57	0.3310	
33	2.600	88	17	6	49	0.3417	0.4465
34	1.300	44	21	23	44	0.2314	
35	1.180	73	57	18	n.d.	3.7040	4.8402; 1.0530

*Treated by cytostatic agents or radiotherapy.
†Percentage of capped cells taken total S.Ig pos. cells as 100.
‡Not done.

Table 2 shows the numbers of PBL, E rosettes, S.Ig positive cells, capped cells, EAC rosettes and the amount of anti-Ig antibody/cell of 21 representative myeloma patients. Out of a total of 65 patients 55 were untreated. Ten patients were treated by cytostic agents or radiotherapy. The mean $\pm$s.d. of the lymphocyte count/mm^3 in the peripheral blood was 1505 ± 563 in the patient group and 1618 ± 370 in the normal group ($n = 13$). Statistically no difference exists between both groups. The mean value of E rosettes was 71 ± 19 and the mean value of S.Ig positive cells was 16 ± 9 in the patient group. Statistical evaluation of these figures revealed no significant differences between the patient group and the normal group for E rosettes ($P < 0.2$) and a slight difference for S.Ig positive cells ($P < 0.025$).

In 9 patients EAC binding cells have been counted in the peripheral blood and in the bone marrow (Table 3). Compared to the number of EAC positive cells in the bone marrow from 3 normal individuals a tendency to increase complement receptor bearing cells is evident. In most cases the frequency of EAC positive cells in the bone marrow is higher or equal to that found with PBL of the same individual. Repated tests were performed in one case (No. 2a, b, c) at intervals of 3 months. The first experiment (a) has been done under cytostatic treatment which had been finished when the other tests (b, c) were done. Also given in Table 3 are the numbers of lymphocytes, myeloma cells of plasma cell type and precursor cells of granulopoiesis and erythropoiesis.

Figure 1 shows the results of cap formation by PBL induced by anti-Ig antiserum in normal individuals and myeloma patients. Capped cells were found in a significant lower frequency in myeloma patients ($P < 0.0001$).

Figure 2 shows the binding of ^{125}I-labelled anti-Ig antibodies to PBL of 32 myeloma patients and 22 normal individuals. The mean value of pg antibody bound per cell was 0.693 ± 1.385 in the patient group and 0.958 ± 0.783 in the normal group. The difference between the two groups was of statistical significance ($P < 0.001$). Repeated tests were done in 4 patients (Nos. 14, 29, 33, 35) several weeks or months apart. The results were 0.3627 and 0.3144 pg/cell (No. 14), 0.4885 and 0.4858 pg/cell (No. 29), 0.3417 and 0.4465 (No. 33) and 4.8402 pg, 3.7040 pg and 1.0530 pg per cell (No. 35) respectively.

Figure 3 shows the numbers of EAC binding cells in the peripheral blood of 60 myeloma patients and 42 normal individuals. The frequency of EAC positive cells was signi-

Table 3. Percentage numbers of EAC binding cells in the bone marrow and the peripheral blood of 9 myeloma patients and 3 normal individuals. Numbers of lymphocytes, plasmocytes and hemopoietic precursor cells are also given. For further explanations see text

Patient No.	Peripheral blood EAC (%)	Bone marrow EAC (%)	Lymphocytes %	Plasmocytes %	hemopoietic precursor cells %
1	18	11	32	12	56
2a	53	20	33	40	27
b	49	50	20	52	28
c	45	48	n.d.	n.d.	n.d.
3	20	35	55	5	40
4	31	31	29	4	67
5	33	54	54	7	39
6	26	29	31	14	55
7	21	30	38	7	55
8	10	14	27	4	69
9	34	21	38	4	58
Normal individuals					
1	20	16	27	0	73
2	17	16	58	1	41
3	17	10	24	2	74

ficantly higher in the patient group when compared to normals ($P \sim 0.0001$).

We tested if a correlation exists between the number of EAC binding cells in the peripheral blood of 17 myeloma patients with monoclonal IgG protein and the concentration of polyclonal IgM and IgA in the serum of these patients. No correlation has been found between EAC rosettes and concentration of IgM or IgA, respectively ($r_s = 0.042$ for IgM, $r_s = 0.073$ for IgA). Also it was tested if a correlation could be seen between the response to PBA and the fre-

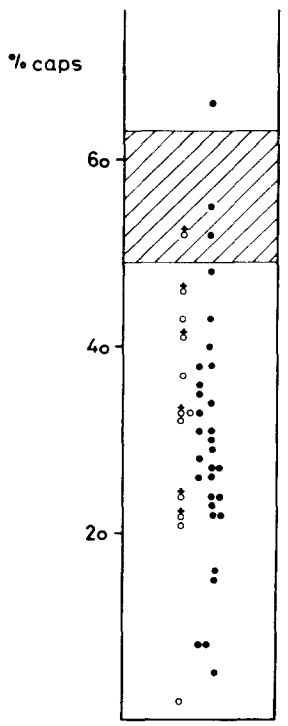

Fig. 1. Frequency of cells showing polar redistribution (capping phenomenon) after incubation of isolated PBL in the presence of polyvalent FITC-conjugated anti-Ig antibody. Patients under treatment (+) and patients producing light chain paraprotein (0) are marked. Normal range is given by the hatched area.

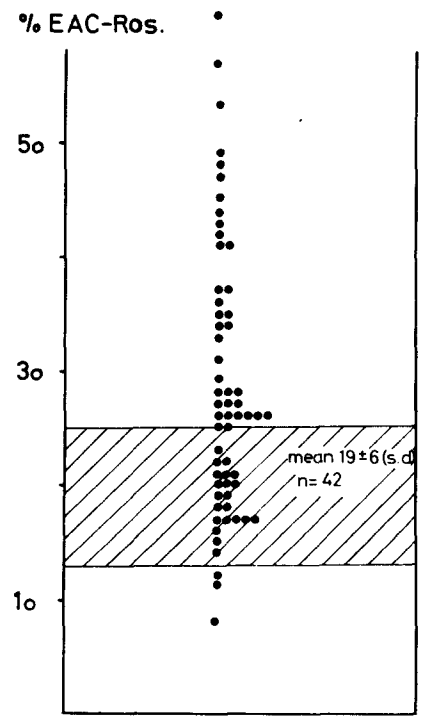

Fig. 2. Frequency of EAC rosette forming cells in isolated PBL of 60 myeloma patients. Normal range is given by the hatched area.

quency of EAC cells of 10 myeloma patients. No correlation has been found ($r_s = 0.00$).

DISCUSSION

The total number of lymphocytes in untreated myeloma patients does not differ from that of healthy donors ($P > 0.4$). No difference was observed in the number of E rosettes which generally are accepted as a measure of thymus-dependent cells.

The frequency of S.Ig positive cells which account for the major part of B cells in the peripheral blood was not significantly different in both groups. The increase of percentage numbers of complement receptor bearing cells in the patient group, therefore, seems to indicate an increase of the absolute count of these cells. We cannot confirm the finding of another author [15] who reported that the number of S.Ig positive cells in myeloma patients closely follows the number of EAC binding cells. Slightly decreased numbers of S.Ig positive cells have been reported in myeloma patients [16, 17]. The experiments using ^{125}I-labelled anti-Ig antibodies reported here reveal a significantly lower binding capacity in most cases within the myeloma group. We do not know if this is due to a decrease of the total number of cells with binding capacity or to a partial loss of binding sites on the individual cell or to a combination effect. It has been excluded that differences of cell size exist between normal individuals and myeloma patients by measuring the diameter of 100 cells using a calibrated ocular micrometer. The mean diameter was 15 μm in both groups.

As shown in Fig. 3 there is a considerable overlapping of binding values in the normal and the patient group. Compared to normal individuals cells from myeloma patients show a smaller variation of the amount of bound radiolabel. The reason for this difference remains obscure for the moment as well as the meaning of runaway points occurring in both groups of Fig. 3. Repeated tests gave similar results in three patients, with the fourth patient (No, 35 in Table 2) a decrease from high initial values to a moderate elevated level within 6 months has been observed. During this time the patient had a relapse of disease. Corticoids, cytostatic agents or radiotherapy were not given to the patient.

Specificity of binding of ^{125}I-labelled antibodies has been tested by blocking experiments (results not reported here). Pretreatment of cells with unlabelled antibodies inhibits the uptake of radiolabelled antibodies. We found an inhibition in the range of 75% of the binding shown by untreated cells. This inhibition is of the same order as observed with mouse spleen cells by other authors [18]. We observed a regain of 85% of the binding capacity of untreated cells when cells used in a blocking experiment were incubated for 15 hr at 37°C. This time interval for regrowing of S.Ig receptors corresponds to that found by other authors [19]. According to their results re-expression of S.Ig is accomplished within 12–24 hr. The result of the blocking experiment also shows that the binding activity of anti-Ig antibodies used in our experiments is not reduced significantly by the radiolabelling procedure.

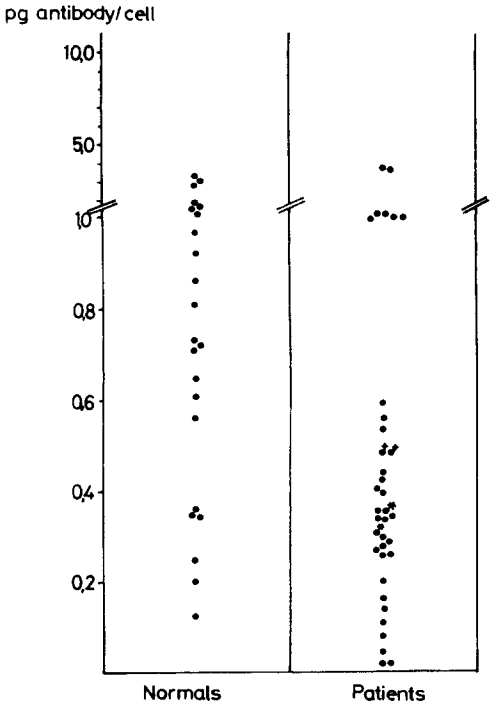

*Fig. 3. Uptake of ^{125}I-labelled anti-Ig antibody (pg antibody/cell) by isolated PBL of 34 myeloma patients and 23 normal individuals. Two patients (+, *) have been tested on two occasions.*

The problem of formation of immune complexes which bind to cells via F_c receptors is inherent to binding experiments of the kind reported here. Filtration of the antiserum through suitable millipore filters possibly does not prevent formation of new complexes on the cell surface. The formation of such complexes cannot be excluded with certainty by blocking experiments [20]. We do not believe, however, that this event has great influence upon our results as another author [17] using antiserum of the same source and gel filtration for elimination of immune complexes has

found essentially the same numbers of S.Ig positive cells in normals and myeloma patients as we did. Moreover, in our hands, samples of lymphocytes stained by FITC-conjugated anti-Ig antibodies and incubated at 37°C overnight did not show a significant decrease of the number of stained cells. This result is not compatible with the presence of a significant amount of immune complexes on the cell surface [21].

Receptor mobility as tested by polar redistribution of surface Ig under capping conditions has been found significantly reduced in myeloma patients when compared to normal individuals. It has been tested if a correlation exists between the number of capped cells and the amount of monoclonal protein in the serum. The result was negative. Also no correlation has been found to the number of perpheral lymphocytes and to the type of monoclonal protein, respectively. Cap formation of PBL of four individuals with polyclonal hypergammaglobulinemia was in the normal range. It has been tested if cap formation of normal PBL is influenced by incubation in plasma of myeloma patients and it was found that the number of capped cells did not change after 1 hr of incubation when compared to control. It does not seem, therefore, that alterations of cap formation observed with PBL of myeloma patients is caused by an exogenous factor. Intracellular changes leading to altered redistribution of surface components of normal and transformed cells are known [22, 23].

With respect to receptor functions in myeloma patients the finding of a significant increase of EAC binding cells in the bone marrow and in the peripheral blood is of special interest. Increased EAC counts in the peripheral blood of myeloma patients also have been found by other authors [15, 17] and from changes with cytostatic treatment it has been concluded that this population is involved in tumor proliferation [17]. The finding of a high number of EAC binding cells in the bone marrow of some of our patients is compatible with this assumption. Criticism has to be applied with concern to the extent of contamination of aspirated material from the bone marrow with peripheral blood. It seems possible to avoid this difficulty by using a method for the quantification of EAC positive cells in sections of biopsies [24].

As far as it can be judged from the number of hemopoietic precursor cells in the aspirated material from bone marrow (Table 3) the number of EAC positive cells cannot be attributed to contamination by peripheral blood. The meaning of the high frequency of EAC cells in myeloma patients remains to be clarified. It is generally accepted that EAC cells play a role in immune recognition functions of lymphocytes [25]. In CLL patients the observation of a decrease of complement receptors has been made [26]. The suggestion has been made by these authors [26] that this decrease contributes to the immunodeficiency known from CLL patients. From our results the impact of the frequency of EAC cells on the overall immune functions does not seem to be very strong as myeloma patients show immune defects very much like CLL patients. It is known, for example, that PBL of CLL [27, 28] and myeloma patients (table 1) show an impaired response to PBA almost regularly and that in both diseases a decrease of polyclonal Ig is one of the most constant findings. The difference of EAC receptor function in both diseases, therefore, may be of discriminating value with respect to the quantitative expression of B cell functions. This conclusion is confirmed by our finding of a significant decrease of the binding capacity for anti-Ig antibodies by PBL of myeloma patients. Using peroxydase-labelled antibodies Ternynck *et al.* [29] calculated that CLL cells bear only 10% of light chain determinants present on normal lymphocytes.

It has been tested by us if a correlation exists between the number of EAC cells in the peripheral blood of myeloma patients and the concentration of polyclonal Ig and the response to PBA, respectively. The result was negative. Also, no correlation has been found for the frequency of EAC cells and the amount of monoclonal protein in the serum of myeloma patients which in most cases is thought to be in direct proportion to tumor mass.

As the role of EAC cells in myeloma patients in terms of tumor progression and host defense mechanisms remains unsolved other aspects should be considered. It has been shown that in mice regeneration of complement receptor bearing lymphocytes in lymph nodes of adult thymectomized whole-body X-irradiated animals is accomplished by the reconstitution with semi-allogeneic bone marrow cells [30]. It seems possible that the homing of EAC cells in myeloma patients has been changed profoundly due to heavy bone marrow infiltration by tumor cells. Following this reasoning the effect of cytostatic treatment on the number of EAC cells shown in Table 3 (patient No. 2) may indicate the restoration of

homing capacity. It may be relevant to this discussion that by rosette inhibition experiments with antilymphocyte globulin T cells of low resistance to inhibition of rosette formation have been found increased in the peripheral blood of myeloma patients [31]. Cells of such binding properties have been found to prevail in normal bone marrow [32]. We concluded from this finding on an increased influx of T cells from the bone marrow of myeloma patients into a compartment which rapidly exchanges with peripheral blood circulation [31].

A comment has to be given on conflicting results of reactivity of PBl of myeloma patients to PBA by various authors [2, 3, 15, 17, 33]. The reason for this is not obvious. It follows from the results given in Table 1 that a detailed analysis of lymphocyte activation by different doses of PHA discloses hyporesponsiveness of varying degree in individual cases. Some of the conflicting results may be caused by inappropriate experimental conditions as not enough activator concentrations have been tried.

Finally, it has to be discussed if monocytes may account for the high frequency of EAC rosettes in cell preparations from the ficoll gradient in myeloma patients. It has been reported on the basis of electron microscopic studies that up to 44% of mononuclear cells are monocytes when ficoll gradient centrifugation was performed on normal peripheral blood [34]. We examined cell preparations from three patients by staining with naphthyl-AS-D-acetate esterase and by their ability to ingest Ig coated SRBC. A good correlation between the two methods has been found when smears of cell preparations were compared. The number of monocytes range from 8 to 23%. This result is in agreement with the number of monocytes in preparations of mononuclear cells from normal individuals [35] but significantly lower than that obtained by the cited author [34]. It has to be mentioned here that in the sample of one patient 40% monocytes were found when the cells were brought into the slide by cytocentrifugation before the staining procedure. The reason for this discrepancy between results obtained by smear and cytocentrifugation technique for enumeration of cells remains unknown at this moment.

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