ABSTRACT

Blood monocytes were analyzed in 28 patients with chronic lymphocytic leukemia without previous cytotoxic therapy and without recent infection. Using monoclonal antibodies and flow cytometry, monocytes identified by LeuM3 or My4 were low in percentage (2.3%), but absolute numbers were increased in many patients with values exceeding the normal range (120 to 510/μl) in seven of 28 patients. Monocytosis was more prominent in patients with high leukemic counts, but there was no correlation to clinical stages. Monocytopenia was evident with less than 50 LeuM3* cells/μl in three patients.

Two-color fluorescence was used for the analysis of cell surface expression of major histocompatibility complex (MHC) Class II molecules, complement receptors, and Fc receptors on the LeuM3* monocytes. Compared to cells from control donors, there was an increase for MHC Class II antigens, complement receptors, and Fc receptors on the monocytes in chronic lymphocytic leukemia, in terms of both the percentage of positive cells among the LeuM3* monocytes and of fluorescence intensity. This increase was not restricted to patients with monocytosis nor were the molecules always upregulated concomitantly. The increase of antigen expression on LeuM3* monocytes was more than 50% (1.5-fold) in seven of 22 patients for MHC Class II antigens, in seven of 16 patients for complement receptor and in six of 12 patients for Fc receptor. A similar decrease of antigen expression was observed only in one patient for MHC Class II and in one patient for complement receptor expression.

Monocytosis and increased expression of monocyte cell surface antigens described for a large portion of patients might be causally involved in the immunodeficiency in chronic lymphocytic leukemia.

INTRODUCTION

Multiple abnormalities of the immune system have been detected in CLL. Among these are antibody deficiency (1–4), alterations in T-cell subsets (5–9), defective primary responses of both T- and B-cells (10, 11), and defective NK cell activity (12, 13) going along with the absence of CD16+ lymphocytes (14). These abnormalities substantially contribute to the clinical problems of CLL patient, including frequent infections (15–17) and secondary malignancies (18–20). Whether these abnormalities are governed by several independent events or by a single element which influences T-cells, B-cells, and NK cells has not been determined. Monocytes with their variety of regulatory functions (for review, see Ref. 21) could form such an element.

In CLL, however, little is known about monocytes, probably because of the difficulty of identifying and of purifying these cells in the presence of overwhelming leukemic cell counts. Hence we determined monocyte numbers and relevant cell surface molecules in a group of previously untreated patients for complement receptor and in six of 12 patients for Fc receptor. This approach we could demonstrate numeric and phenotypic alterations in T-cell subsets (5–9), defective primary responses from the same company were used. For the preparation of HIGG, 100 mg of human IgG (Miles Laboratories, Uppsala, Sweden) were treated at 62°C for 30 min and afterwards applied to a Sepharose 4B column (Pharmacia, Freiburg, Federal Republic of Germany). Fractions containing oligomers of IgG were pooled, vacuum dialyzed, and titrated for their ability to inhibit Fc receptor-mediated nonspecific binding. MAB L243, M522, HIGG, and their isotype controls were biotinylated with biotin-N-hydroxysuccinimide ester (Boehringer Mannheim, Federal Republic of Germany).

Materials and Methods

Patient Populations. The study included 28 patients, 18 men and 10 women, with an mean age of 63.4 yr (range, 48 to 82 yr), with B-CLL as assessed by clinical, morphological, and immunological criteria (Ba-1, B1, Leu-1, H6d, surface immunoglobulin). Twenty patients never received any chemotherapy or cortisone, and the remaining 8 patients had not been treated for at least 12 wk prior to this study. No patient had an infection at the time of study. Staging (Table 1) was performed according to Binet et al. (22). Briefly, Stages A through C are all characterized by lymphocytosis in both blood and bone marrow; in addition, Stage A includes lymphadenopathy of up to 2 lymph node areas; Stage B, lymphadenopathy of 3 or more lymph node areas; and Stage C patients exhibit anemia and/or thrombocytopenia. Control donors were healthy volunteers between the ages of 52 and 76 (mean, 62.4 yr).

Cell Separation and Storage. Peripheral blood mononuclear cells were separated from heparinized blood by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient sedimentation, according to routine procedures (23). Aliquots of cells were stored in liquid nitrogen after controlled freezing in the presence of 10% dimethyl sulfoxide and 10% heat-inactivated fetal calf serum. Previous experiments had shown that these procedures do not result in change of cellular properties. Directly before use, mononuclear cells were rapidly thawed in a 37°C water bath and washed twice.

Monoclonal Antibodies. MAB LeuM3-FITC (Ref. 24; purchased from Becton-Dickinson), My4 (Ref. 25; purchased from Coulter Immunology), and M42 (kindly provided by Professor E. P. Rieber) identify the majority of monocytes. MAB L243 recognizes HLA-DR Class II MHC antigens (26), MAB M522 recognizes the C3bi receptor (CR3) on monocytes (27, 28), and for identification of the Fc receptor, HIGG was used. Immunoglobulin on monocytes was determined with a biotinylated mouse anti-human immunoglobulin MAB (Tago, Burlingame, CA). As isotype controls, MABs of the same isotype purchased from the same company were used. For the preparation of HIGG, 100 mg of human IgG (Miles Laboratories, Uppsala, Sweden) were treated at 62°C for 30 min and afterwards applied to a Sepharose 4B column (Pharmacia, Freiburg, Federal Republic of Germany). Fractions containing oligomers of IgG were pooled, vacuum dialyzed, and titrated for their ability to inhibit Fc receptor-mediated nonspecific binding. MAB L243, M522, HIGG, and their isotype controls were biotinylated with b-ibio-N-hydroxysuccinimide ester (Boehringer Mannheim, Federal Republic of Germany).

Immunofluorescence. In order to inhibit nonspecific binding of the MAB to the monocyte surface, 1 × 10^6 PBMC were preincubated with 25 μl of HIGG for 30 min. For two-color immunofluorescence, an appropriate dilution of LeuM3-FITC (25 μl) and of a biotinylated MAB (25 μl) was added, followed by incubation for 30 min on ice. After washing with PBS/2.5% fetal calf serum/0.02% NaN₃ the cells were incubated with 50 μl of avidin phycoerythrin (1:3; Becton Dickinson) for development of the biotinylated MAB. MAB My4 used for single-color fluorescence was developed in the second incubation with goat anti-mouse FITC (Tago, Burlingame, CA). The cells were fixed with 1% paraformaldehyde dissolved in PBS and analyzed with an EPICS V flow cytometer (Coulter Electronics) with 488-nm excitation wavelength and photomultiplier tubes at 900 to 1250 V as described (29). At least 200,000 cells were analyzed for both specific MAB and isotype control. Data analysis was performed with the MDADS software of the EPICS V. For estimation of changes in cell size, we used forward-angle light scatter signals. Standard beads of 7.5 and 9.99 μm in diameter (Flow Cytometry Standard Corporation and Coulter Electronics, respectively), i.e., with roughly a 2-fold difference in cell surface area, gave a 6-channel difference on the 64-channel linear scale. For light scatter analysis of monocytes, the control and CLL samples were
ABNORMAL BLOOD MONOCYTES IN CLL

1. Absolute monocyte numbers in CLL patients. Given is the number of LeuM3-positive cells/µl of peripheral blood. The open box indicates the control range (±1 SD). Results were obtained by fluorescence-activated cell sorter analysis of at least 200,000 cells per sample with high leukocyte counts.

studied side by side with the identical excitation conditions and the identical gain for the scatter detector. The absolute number of monocytes is expressed as LeuM3+ cells recovered after isolation and storage by using the formula: % of LeuM3+ cells x mononuclear cells recovered from 1 µl of blood/100. Statistics. For statistical evaluation Student’s t test was used.

RESULTS

In peripheral blood of patients with CLL, percentages of LeuM3+ monocytes are low (Table 1). Absolute numbers expressed in cells per µl of blood are, however, increased in many patients with values of up to 1200 LeuM3+ monocytes/µl. Numbers of monocytes exceeding the control range were found in 7 of 28 CLL samples (Fig. 1). Monocytosis was more evident in patients with high leukemic cell counts. Patients with more than 50,000 WBC/µl had in average 672 LeuM3+ cells/µl (P < 0.01). Further monocytosis was also more prominent in later stages (cf. Table 1). Eighteen patients had monocyte counts within the control range, 4 patients had moderately decreased monocyte numbers, and 3 patients (Patients D., E., M., and N. J.) had clear-cut monocytopenia with less than 50 LeuM3+ cells/µl.

When using two other monoclonal antibodies for definition of monocytes (My4 and M42), very similar monocyte numbers were determined (Table 1).

In order to study functionally relevant cell surface structures on monocytes in CLL, we used two-color immunofluorescence analysis. Monocytes were defined with the fluorescein-conjugated LeuM3 MAB, and MHC Class II antigens, C3bi receptors (CR3), and Fc receptors were identified with biotinylated reagents that were visualized with avidin phycoerythrin.

An example of the flow cytometry analysis for MHC Class II antigens on 400,000 cells of a CLL sample is shown in Fig. 2C compared with a control sample (Fig. 2A). The population of green fluorescent LeuM3+ cells on the x-axis is strongly positive for yellow fluorescent MHC Class II antigens on the y-axis (Fig. 2C). In Fig. 2, B and D, the low background staining of LeuM3+ cells with a 2a isotype control is depicted. The

Table 1 Immunofluorescence data of CLL patients compared to controls

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Therapy*</th>
<th>Stage*</th>
<th>Immunoglobulin levels*</th>
<th>PBM*</th>
<th>Monocytes*</th>
<th>LeuM3</th>
<th>My4</th>
<th>M42</th>
<th>L243</th>
<th>M522</th>
<th>HIGG</th>
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<td>A. N.</td>
<td>59</td>
<td>M</td>
<td>(n)</td>
<td>B</td>
<td>d</td>
<td>8.1</td>
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<td>4.2</td>
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<td>d</td>
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<td>A</td>
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<td>n</td>
<td>B</td>
<td>n/d</td>
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<td>B</td>
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<td>C</td>
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<td>M</td>
<td>A</td>
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<td>2.1</td>
<td>1.5</td>
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</table>

Control donors: n = 12; 8 M; 4 F

Mean (range): 62.4 (52–76)

* Therapy: n, none; (n), none for at least 12 wk prior to investigation.
* Staging according to J. L. Binet, 1981.
* Immunoglobulin levels: n, normal; d, decreased.
* PBM x 10^3/µl separated from 1 ml of blood.
* Monocytes (LeuM3+ cells)/µl of blood.
* —, not done.

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ABNORMAL BLOOD MONOCYTES IN CLL

Leu M 3

Fig. 2. Expression of Class II MHC antigens on monocytes in CLL. Two-color immunofluorescence for LeuM3/L243 (A, C) compared to LeuM3/UPC (B, D) as isotype control. A and B, control donor; C and D, CLL Patient H. R. Results for the CLL sample were derived from fluorescence-activated cell sorter analysis of 400,000 cells.

projections of yellow fluorescence gated on the LeuM3+ cells are used for further evaluation.

Fig. 3A compares specific staining for MHC Class II antigens on LeuM3+ monocytes in the CLL patient and the control donor depicted in Fig. 2. Percentages of positive cells were higher in this CLL patient compared to control (cf. legend to Fig. 3). More importantly, mean specific fluorescence intensity for the CLL patient was 9.7 channels higher in this log scale display. Compared to control, this reflects a 3-fold-higher antigen expression on the CLL monocytes. Fig. 3C shows the specific staining for C3bi receptor (CR3) on LeuM3+ monocytes in another CLL patient and a control. The monocytes of this patient express 2-fold more C3bi receptor compared to control. Histograms of the isotype controls for MABs L243 and M522 (Fig. 3, B and D, respectively) show an identical staining pattern in CLL and control donors.

Additional data, including expression of Fc receptors, are given in Table 1. Fluorescence intensity was 1.5-fold (50%) higher than in controls in 7 of 22 CLL patients for MHC Class II antigen expression \( (P < 0.01) \), in 7 of 16 CLL patients for C3bi receptor expression \( (P < 0.01) \), and in 6 of 12 CLL patients for Fc receptors \( (P < 0.01) \). The higher signal for Fc receptors on CLL monocytes was not due to a decreased endogenous loading of Fc receptors by serum antibody, since expression of opsonized immunoglobulin on monocytes was also increased in many CLL patients (data not shown).

The expression of cell surface molecules on CLL monocytes was not always upregulated together. Patient H. H., for instance, expressed only increased MHC Class II antigen, and Patient S. R., only increased C3bi receptor. An increased expression of at least one of the cell surface molecules studied was found in 14 of 22 patients.

Increased expression of cell surface molecules was found in patients with normal and with increased monocyte numbers (e.g., Patient A. N. and Patient H. R.). Further monocytosis was not always correlated with upregulated cell surface molecules (Patient W. L.), indicating that the enhanced expression of Class II molecules, complement, and Fc receptors is not directly related to monocyte number.

An estimation of size of LeuM3+ monocytes with forward-angle light scatter signals (30, 31) revealed no difference between monocytes of CLL patients and controls with a mean channel of \( 32.4 \pm 4.6 \) for CLL monocytes and \( 32.2 \pm 2.6 \) for control monocytes \( (n = 10) \). Since forward-angle light scatter correlates with cell size, this indicates that the increased antigen expression per cell might be due to an increased antigen density on the cell surface.
DISCUSSION

Analysis of monocytosis in CLL is difficult, since in the presence of high numbers of leukemic B-cells, these cells form only a few percentages of the mononuclear cells. In one previous study, Zeya et al. (32, 33) found a decreased cellular enzyme content of monocytes in CLL after density gradient separation using Percoll. The study, however, is difficult to interpret, because of the problem of clearly identifying and purifying the monocytes. Contaminating leukemic lymphocytes, which also have decreased enzyme activities in CLL, could account for the observed deficiency.

For our analysis we used MABs like LeuM3 and M42, which to our knowledge exclusively react with monocytes. The MAB M7, however, does react with CLL B-cells (Ref. 34; Footnote 5). The specific fluorescence intensity, however, is approximately 30-fold lower on CLL B-cells compared to the monocytes (data not shown). Hence, clear identification of the intensely stained monocytes was possible in every instance.

In order to reliably analyze the small percentage of monocytes within the leukemic sample, we used immunofluorescence and flow cytometry and could analyze more than 200,000 cells per sample (the same number of cells was also analyzed in the isotype control samples). Since no purification procedures involving 37°C incubations are required in our procedure, artificial alterations of cell surface molecules can be excluded.

Using this approach in our studies, monocytosis was evident in 7 of 28 patients. This increase of monocytes in peripheral blood could either be a reaction in response to increased cellular decay that requires removal of debris, or it could be the result of frequent infections. In a retrospective analysis, however, we could not detect an increased report of infectious disease in patients with and without monocytosis. Whatever the reason for the monocytosis might be, these cells could have regulatory effects on other cells of the immune system in CLL. Hence, to learn more about the functional status of these monocytes, we performed two-color immunofluorescence analysis in flow cytometry.

Irrespective of the presence of monocytosis we found an increased expression of MHC Class II antigens, of C3bi receptors, and of Fc receptors on monocytes in CLL. The 3 patients with the most elevated Class II antigen expression on monocytes (A., H. B., H. R.; cf. Table 1) all exhibited hypogamaglobulinemia. Furthermore, there was a positive correlation between C3bi receptor expression and stage of disease (P < 0.01). This points to a possible clinical relevance of these findings. The increase of Fc and C3bi receptor expression might hint towards a higher phagocytic capacity for opsonized infectious agents. In light of the prevalent antibody deficiency in CLL (1—4), such a higher receptor expression might at least partially compensate for the reduced protective capacity due to the low antibody level.

Expression of MHC Class II antigens together with IL-1 secretion is a critical element in antigen presentation to T-cells. Cell-mediated immune response, when looking at skin reactivity to neoeantigens, like dinitrofluorobenzene, is, however, strongly reduced in CLL (10). This might indicate that other components of this reaction are defective. To elucidate the mechanisms involved in this process in CLL, additional studies on monocyte-derived mediators like prostaglandin E2, IL-1, and TNF are required.

In the parallel study on TNF (which in many instances is concomitantly regulated with IL-1) we found defective production of TNF in many CLL samples.6 Such functional studies together with the phenotypic analysis reported herein might help in answering the question of whether monocytes form a central regulatory element in the immunodeficiency in CLL.

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6 Unpublished observations.

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