Monocyte and Granulocyte Defect in Chronic Lymphocytic Leukemia

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Enzymatically homogeneous populations of lymphocytes, monocytes, and neutrophils were isolated by zonal centrifugation from 5 untreated patients with chronic lymphocytic leukemia (CLL) and 2 patients with CLL in full remission. The cells were then quantitatively analyzed for six leukocytic enzymes and compared with cells from normal subjects. CLL monocytes were deficient in β-glucuronidase (0.06 units; normal, 0.16), myeloperoxidase (0.07 mg; normal, 0.5 mg), and lysozyme (0.7 mg; normal, 3.3 mg). In 2 cases, CLL neutrophils were severely deficient in lysozyme (1 to 2 mg; normal, 7 mg) and myeloperoxidase (2 to 3 mg; normal, 7 mg). Neutrophil alkaline phosphatase and neutral protease were unaffected. CLL lymphocytes shared with the monocytes the deficiency of β-glucuronidase (0.03 units; normal, 0.09 units). The 2 CLL patients in full remission carried normal enzyme levels in leukocytes of all three cell lines. The CLL lymphocytes of untreated patients were unresponsive to mitogens but became responsive in remission. The CLL monocytes from both untreated and treated patients transformed into macrophages. The pattern of shared enzyme deficiency among lymphocytes, monocytes, and neutrophils of CLL patients and its normalization in all three cell types under remission suggest that the differentiation of the three leukocytic cell lines may be an enzymatically interlinked process and that the deficiency of these enzymes in leukemia may reflect an interrelated aberrant differentiation of the leukemic cells. (Am J Pathol 95:43–54, 1979)

LEUKEMIC LYMPHOCYTES of patients with chronic lymphocytic leukemia (CLL) have been reported to be deficient in the enzymes β-glucuronidase and, possibly, acid phosphatase.[1-3] The significance of this defect is unknown.

It is also not known if the enzyme deficiency in CLL is confined to the lymphocytes or involves the monocytes and granulocytes as well. This knowledge would be of interest in studying the developmental interrelationships among lymphocytes, monocytes, and granulocytes. Indications of such an interrelationship, at least at the early stages of leukocyte development, have come from several directions. Suggestions that lymphocytes may possess attributes of stem cell populations have been made by a number of investigators.[4-6] A lymphocyte-like cell is thought to be the precursor of monocytes.[7] Monocytes, in turn, are considered to share a common progenitor cell with the granulocytes.[8-10]

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Work with enzymatically pure, normal human lymphocytes, monocytes, and granulocytes has indicated that the three cell types may be linked enzymatically in a differentiation sequence through increasing sets of shared enzymes as well as by groups of enzymes specific for each cell line.[11]

Should the leukocytic enzymes provide developmental links between the lymphocytoid and the monocytoid cells and between the monocytoid and the granulocytoid cells as part of a differentiation sequence, then it would be expected that an enzyme defect in the lymphocytes (such as the one in the lymphocytes of CLL patients) may also involve the monocytes and that the defect may even extend to the granulocytes of these patients. Such a possibility has been tested with the isolated lymphocytes, monocytes, and granulocytes of CLL patients. We report a hitherto unrecognized deficiency of multiple enzymes in monocytes and granulocytes of patients with CLL. The anomaly was not present in leukocytes of CLL patients in full remission.

Materials and Methods

For normal subjects, healthy volunteers with no recent history of respiratory infection and with low basophil and eosinophil counts were selected. The 5 CLL cases were diagnosed by clinical, peripheral blood, and bone marrow findings and were untreated because of lack of any constitutional signs or symptoms. Their peripheral blood counts ranged between 30,000 and 70,000 leukocytes/cu mm and their neutrophil counts ranged between 10 and 30%. The smears of untreated CLL were strikingly deficient in large mature monocytes. The 2 CLL patients in full remission carried normal total and differential counts. Mature monocytes were in their smears.

Separation of Leukocytes

Cell separation of 30 to 50 ml of heparinized peripheral blood was carried out by zonal centrifugation on a linear density gradient of sucrose, as detailed elsewhere.[11] The method involved the following steps: a) withdrawal of 30 to 50 ml of heparinized blood from peripheral circulation of the patient and the normal subjects; b) removal of bulk of red cells by plasma gel sedimentation; c) passage of leukocyte-rich fraction through a linear density gradient of 30 to 50% (w/v) sucrose (at 2000 rpm for 10 minutes); d) recovery and enumeration of completely separated leukocytic components of lymphocyte, monocyte, and neutrophil bands; e) quantitative determination of six hydrolytic enzymes: acid and alkaline phosphatases, β-glucuronidase, lysozyme, myeloperoxidase, and neutral protease.[11]

For normal human blood, a characteristic gradient profile of the separated cells is obtained routinely.[11] The profile consists (in the order of increasing density) of platelets (Band 1), red cells (Band 2), lymphocytes (Band 3), monocytes (Band 4), and neutrophils (Band 5). In the gradient the red cells (Band 2) behave as being of lighter density than the leukocytes and, as such, stay 0.5 cm above the lymphocyte band. The monocytes (Band 4) occupy a midway place, 0.5 cm below the lymphocyte band and 0.5 cm above the neutrophil band. The separation of red cells as a sharply defined red band above the three white leukocyte bands is a prerequisite for a complete separation of lymphocytes, monocytes, and granulocytes. Such a separation of the red cells and the leukocytes is achieved because the cells in the layered sample and in the individual bands are kept monodisperse. The red cells separate above the leukocyte bands because, as single cells and cells without a nucleus, they are of lighter density than the nucleated lymphocytes, monocytes, or

neutrophils. The red cells settle below the leukocyte bands only when they are undergoing aggregation or rouleau formation; as such, it is their fall in the gradient that causes the intermixing of the leukocyte bands, with the resulting overlap of the enzyme activities. It is emphasized that for a complete separation of lymphocytes, monocytes, and neutrophils, it is imperative that the red cells stay as a sharply defined band, above the leukocyte bands, not below them, and that the white leukocyte bands should not show even a tinge of red. The aggregation of red cells, platelets, and leukocytes is prevented by adequate heparinization of blood (most important); removal of bulk of red cells by plasma-gel sedimentation; use of freshly made sucrose solutions; and monodispersion of cells (21-gauge needle) just prior to layering of the sample.

To prevent overloading, not more than 2 to 3 $\times$ 10^7 cells are layered on the gradient. Seventy to eighty percent of the layered leukocytes are recovered in the three leukocyte bands: the neutrophil band contains 60 to 70% of the cells; the lymphocyte band constitutes 20 to 30%; and the monocyte band contains 5 to 10% of the leukocytes. The enzyme recovery studies show that, although the neutrophils are not viable, their granule-associated enzymatic apparatus is more or less undamaged and intact. The enzymes in purified neutrophils are 1.5- to 2-fold higher than the enzymes in the neutrophils of the uncentrifuged mixed cell sample, which usually contains 70% neutrophils and 30% mononuclear cells.[11]

The homogeneity of the lymphocytic, monocytic, and neutrophilic fractions is determined mainly by a combination of multiple enzyme assays and through morphologic identification of the cells by light and electron microscopy. Complete separation of individual bands, as seen in the gradient, correlates well with the lack of enzymatic overlap between the bands in the biochemical assays. The lymphocyte fraction, with a morphology of small cells having condensed nuclei and small cytoplasm, is characterized biochemically by the presence of specific amounts of β-glucuronidase, acid phosphatase, and protein and by the absence of four enzymes, including lysozyme and myeloperoxidase, the enzymes that are present in the lower monocyte band (4). The monocyte fraction is identified morphologically by cells with large, finely granular cytoplasm and horseshoe-shaped nuclei and biochemically by the presence of four enzymes, including lysozyme and myeloperoxidase, and by the absence of two enzymes, ie, neutral protease and alkaline phosphatase. The neutrophil band contains morphologically the most homogeneous group of cells and carries all six enzymes in characteristically high amounts. The amounts of enzymes in lymphocytes, monocytes, and neutrophils of normal human subjects have been studied by us for the past 2 years and have remained, with some variation, consistently similar. It is in comparison with these studies of the leukocytes of normal human subjects that we can detect with some confidence the presence of an extensive biochemical defect in multiple cell lines of various leukemias.

PHA and PWM responsiveness of CLL lymphocytes was tested in autologous plasma at 3-day incubation time. For macrophage transformation, the cells were cultured into autologous plasma and were compared at 8 days and 15 days with macrophage transformation of normal human cells.

Results

The gradient profile of CLL patients, untreated and treated, is compared with that of normal subjects in Figure 1.

Normal Leukocytes

Gradient Profile

As detailed in the previous paper,[11] the gradient system completely separated the five major elements of normal human blood, ie, platelets, red cells, lymphocytes, monocytes, and PMN, into five distinct bands. The

routinely reproducible pattern consisted of the lightest platelets (Band 1), red cells (Band 2), below which were aligned three white bands of lymphocytes (Band 3), monocytes (Band 4), and neutrophils (Band 5).

The monocytes in the normal gradient occupied a characteristic position that was 0.5 cm midway between the upper lymphocyte band and the lowest neutrophil band. This position of the normal monocytes was seldom altered in normal gradients.

Enzymatic Profile

As shown in Table 1, the normal lymphocytes (Band 3) contained two of the six enzymes: β-glucuronidase and acid phosphatase. The β-glucuronidase content of the normal lymphocytes was approximately 0.08 to 0.1 units, whereas the acid phosphatase averaged 0.5 mg. The normal monocytes (Band 4) contained, in addition to β-glucuronidase and acid phosphatase, two other enzymes, ie, lysozyme and myeloperoxidase, but were devoid of neutral protease and alkaline phosphatase (the PMN enzymes). Lysozyme content of the normal monocytes averaged 3.5 mg, whereas that of myeloperoxidase averaged 0.5 mg. The monocytes contained 1.5 to 2 times more acid phosphatase (1 to 1.5 mg) and β-glucuronidase (0.15 units) compared with the level of the enzymes in normal lymphocytes. All six enzymes were present in the normal neutrophils (Band 5) with characteristically high amounts. Alkaline phosphatase content of the human neutrophils was approximately 0.5 mg; that of the neutral protease averaged 45 units. Both myeloperoxidase and lysozyme averaged 6 to 7 mg, whereas β-glucuronidase and acid phosphatase content of the neutrophils was twice as high as in the monocytes.

CLL Leukocytes

Gradient Profile

As shown in Figure 1, one of the most regular features of the untreated CLL gradient was that the position of the normal monocyte band was shifted upward closer to the lymphocyte band, leaving a wider gap between the neutrophils and monocytes. In some CLL gradients, a normal monocyte band seemed to be missing from the gradient, leaving an empty space between the lymphocyte band and the neutrophils. However, on careful examination, a faint band of lighter density was discernible close below the lymphocyte band. The cells collected from the gap and the lighter monocyte band constituted 1 to 6% of the gradient leukocytes. Morphologically the cells appeared small, with kidney-shaped nuclei. There was a scarcity of large mature monocytes in untreated CLL

Table 1—Enzymatic Levels of Lymphocytes, Monocytes, and Neutrophils in Chronic Lymphocytic Leukemia (Activity per 10^8 Cells)

Hydrolases	Lymphocytes			Monocytes			Neutrophils		
	Normal	Untreated	Remission	Normal	Untreated	Remission	Normal	Untreated	Remission
Alkaline phosphatase (mg)*	0	0	0	0	0	0	0.53 ± 0.09	0.67 ± 0.05	0.85 ± 0.35
Neutral protease (units)†	0	0	0	0	0	0	43 ± 4.9	39 ± 6	42 ± 3.5
Peroxidase (mg)*	0	0	0	0.5 ± 0.06	*0.07 ± 0.03*	0.45 ± 0.005	7 ± 0.25	*4 ± 1.4*	5.5 ± 1.5
Lysozyme (mg)*	0	0	0	3.3 ± 0.3	*0.7 ± 0.2*	3.1 ± 0.4	6.5 ± 0.46	*3.3 ± 0.8*	5.1 ± 0.1
β-Glucuronidase (units)‡	0.09 ± 0.008	*0.03 ± 0.01*	0.07 ± 0.005	0.16 ± 0.013	*0.06 ± 0.005*	0.13 ± 0.005	0.25 ± 0.014	0.18 ± 0.04	0.24 ± 0.005
Acid Phosphatase (mg)*	0.6 ± 0.04	1.5 ± 0.25	0.5 ± 0.1	1.3 ± 0.12	0.82 ± 0.08	1.0	2.9 ± 0.18	1.6 ± 0.3	2.3 ± 0.05
Protein (mg)*	45 ± 1	39 ± 2	39 ± 1.5	59 ± 1.2	49 ± 1.8	58 ± 2.5	87 ± 3	73 ± 1.8	79 ± 2.5

This study involved 5 normal subjects, 5 untreated patients, and 2 patients in remission.
Values are expressed as mean ± SEM.
* Milligram equivalent of commercial enzymes[11]
† 1 unit = OD of 1.0/hr at 280 mμ
‡ 1 unit = 1 μmole of phenolphthalein liberated/min
Values in italics indicate enzyme deficiencies.

compared with normal and treated CLL gradients. It was difficult at times to distinguish morphologically whether the cells in the untreated CLL gradient were small monocytes or large lymphocytes. However, the enzymatic profile of the cells (described later) left little doubt that the cells were monocytic in origin. In CLL under remission, a normally positioned monocyte band appeared in the gradient.

The gradient positions of the CLL lymphocytes and neutrophils were relatively unaltered (Figure 1). The leukemic lymphocytes, as a thick band, occupied their normal position completely separate and below the red cell band in the gradient. The lymphocyte band constituted 60 to 70% of the cells in the gradient and, despite the close proximity, was totally uncontaminated with the monocytes, as shown enzymatically. The neutrophil band constituted 20 to 30% of the gradient leukocytes and contained PMN in 95 to 98% purity with less than 0.2% contamination with mononuclear cells. Morphologically the neutrophil band was the most homogeneous of the three bands. The lymphocyte band showed preponderance of small lymphocytes with condensed nuclei and scanty cytoplasm. However, 10 to 20% of the cells possessed varying degrees of larger cytoplasmic content.

The untreated CLL lymphocytes were unresponsive to PHA on 3-day culture; the CLL lymphocytes in remission patients were fully responsive to mitogen. Monocytes of both untreated and treated CLL transformed into macrophages and giant cells within 8 to 12 days of the culture. In some cases, monocytes from untreated CLL showed a tendency to form macrophage colonies between Day 3 and 5 of the culture. This was seen in 2 of 5 CLL cases.

Enzymatic Profile

Enzymatic analysis and the comparison of the enzyme levels of cells from untreated CLL with cells of CLL patient in remission and with leukocytes of normal subjects are shown in Table 1.

The untreated CLL lymphocytes showed a deficiency of β-glucuronidase. In CLL lymphocytes, the enzyme was present in amounts only one third of that found in normal lymphocytes. However, the acid phosphatase level of the CLL lymphocytes was three times higher than the level in the normal cells. Neither the deficiency of β-glucuronidase nor the increase of acid phosphatase was seen in CLL lymphocytes of patients in full remission. The lymphocyte band was devoid of lysozyme and myeloperoxidase, indicating that monocytic cells were not present in this band.

The lighter density monocyte band of untreated CLL contained lysozyme and myeloperoxidase in addition to β-glucuronidase and acid

phosphatase but was devoid of neutral protease and alkaline phosphatase (the PMN enzymes). The levels of β-glucuronidase, lysozyme, and myeloperoxidase were considerably reduced in these cells. The CLL monocytes carried one third to one half the amount of β-glucuronidase, one fourth the amount of lysozyme, and one seventh the amount of myeloproxidase in the normal monocytes. The protein content of the CLL monocytes was also diminished conspicuously. In remission, normal levels of these constituents were found in the patients' monocytes.

The untreated CLL neutrophils showed the deficiency of lysozyme and myeloperoxidase and, to a certain extent, also of β-glucuronidase, but their neutral protease and alkaline phosphatase levels were normal. Two of five untreated CLL patients exhibited a severe deficiency of lysozyme in their neutrophils, with less than 1 mg of lysozyme in one patient and 1.5 mg in another. Along with lysozyme, myeloperoxidase was also reduced to one fourth to one third the normal level in these patients' PMN, but the alkaline phosphatase and neutral protease levels of the PMN were normal. CLL patients in full remission carried normal levels of the enzymes in their neutrophils.

Discussion

The results indicate that extensive deficiencies of digestive enzymes occur in lymphocytes, monocytes, and neutrophils of the patients with chronic lymphocytic leukemia. Tested for the levels of six hydrolytic enzymes, the CLL lymphocytes were deficient in β-glucuronidase; the CLL monocytes were deficient in β-glucuronidase, lysozyme, and myeloperoxidase; and CLL neutrophils were deficient in lysozyme and myeloperoxidase (with normal levels of neutral protease and alkaline phosphatase). In CLL patients under full remission, the enzymatic defect appeared to be normalized in all three cell lines.

Among the three cell lines, the cell most seriously affected by the enzyme deficiency appeared to be the patients' monocytes. The CLL monocyte seemed to be altered both biophysically and biochemically, if not so clearly morphologically.

Biophysically, the most consistent change seen in the CLL monocytes was their altered behavior in the gradient. Whereas the normal monocytes routinely separated as a distinct band staying midway between the lymphocyte and the neutrophil bands, the CLL monocytes moved upward in the gradient as a band of lighter density and aligned closer to the lymphocyte band, leaving a characteristic wide gap between the lymphocytes and the neutrophils. In CLL patients under full remission, the position of the monocyte band reverted to normal. It seemed unlikely that the apparent

absence of the normal monocyte band in CLL gradient was an artifact created by large numbers of leukemic lymphocytes. It was evident from the gradient profile of the treated CLL (Figure 1) that an equally large number of lymphocytes in the thick lymphocyte band gave rise to an equally thick normal monocyte band that still occupied its normal position in the gradient. Most likely the finding indicates that there may be a real paucity in CLL of monocytes with normal size, maturity, and density.

Morphologically the CLL monocyte appeared to be smaller, with preponderance of kidney-shaped nuclei. A decrease of large mature monocytes with horseshoe-shaped nuclei was seen in the blood smears of the patients with untreated CLL. This was in marked contrast to the smears of the CLL patients under full remission, which contained 10 to 12% of large mature monocytes. Although it was difficult to distinguish whether the untreated CLL cells were "large lymphocytes" or "small monocytes" morphologically, the identity of these cells as monocytes was fully confirmed enzymatically.

Enzymatically the cells of the lighter Band 4 were of monocytic origin because they carried lysozyme and myeloperoxidase but were totally devoid of neutral protease and alkaline phosphatase, the two PMN enzymes that were present in normal strength in the lower neutrophil band. Enzymatically it was clear that the monocytic cells were confined to the band despite its altered position in the gradient, since no lysozyme and myeloperoxidase carrying cells were present in the lymphocyte band, despite its proximity, and since no more than 0.2% of monocytes was present in the neutrophil band seen microscopically. Although on the basis of the enzyme assays it could be stated with confidence that no contamination of the lymphocyte band with cells of the monocyte band occurred, it could not be proved that the monocyte band was not contaminated with cells of the lymphocyte band. However, considering the relatively small number of the cells involved, combined with such sharply defined separation of cells, both biophysically and biochemically, as well as the presence of a characteristic gradient profile, it is less likely that the altered monocyte band in CLL was derived from the lymphocytes. Most likely these were either smaller immature monocytes or monocytes with some form of a maturation defect which expressed itself in the form of multiple enzyme deficiencies and perhaps also imparted to the cell a tendency to proliferate.

Despite the enzyme deficiency, the CLL monocytes were able to transform regularly into macrophages in 8- to 12-day plasma cultures. However, in some cases a proliferative tendency leading to the formation of macrophage colonies was seen between Days 3 and 5 of the culture. There are some initial indications that the transformed macrophages of CLL pa-

tients may also be enzyme-deficient. The macrophage transformation of the CLL monocyte is particularly significant since in a case of hairy cell leukemia, the similarly enzyme-deficient monocytes were unable to transform into macrophages.[12]

The value of analyzing multiple cell lines of CLL patients with a large group of six hydrolytic enzymes was seen when it became apparent that the monocytic deficiency of the digestive enzymes seemed to be shared by the patients' lymphocytes and by the neutrophils in a selective manner.

The β-glucuronidase deficiency of the monocytes appeared to be shared by the CLL lymphocytes. CLL lymphocytes, through cytochemical studies, are reported to be deficient in β-glucuronidase.[1-3] However, the results show that the deficiency of β-glucuronidase was accompanied by a 3-fold rise of another enzyme acid phosphatase in the CLL lymphocytes. A similar deficiency of β-glucuronidase coupled with increase of acid phosphatase was found in the lymphocytes of a patient with hairy cell leukemia.[12] This selective depletion of one enzyme accompanied by the increase of another enzyme was also seen in monocytes of the monocytic leukemia (ML) patients. The ML monocytes were deficient in myeloperoxidase but contained increased amounts of lysozyme.[13]

The lysozyme and myeloperoxidase deficiency of the CLL monocytes appeared to be shared by the patients' neutrophils. The patients' neutrophils were selectively deficient in lysozyme and myeloperoxidase since their levels of two other PMN enzymes, ie, neutral protease and alkaline phosphatase, were unaltered. Although on the average the lysozyme and myeloperoxidase content of CLL neutrophils appeared to be reduced only by half, 2 of the 5 patients carried only 1 to 2 mg of lysozyme and myeloperoxidase in their neutrophils. Since these findings were compiled, studies of other CLL patients have indicated that in nearly 30% of untreated CLL patients the neutrophils have serious deficiency of lysozyme and myeloperoxidase. A similar, although not as severe, deficiency of lysozyme and myeloperoxidase was seen in the neutrophils of a patient with hairy cell leukemia.[12] However, in this patient the deficiency also involved two other PMN enzymes, ie, neutral protease and alkaline phosphatase; the two enzymes were totally absent from the patient's neutrophils.[12]

Although it is too early to be sure, there are some indications that the deficiency of lysozyme in the monocytes and the neutrophils of the patients may be a specific feature of lymphocytic leukemias. Other leukemias may have a different pattern of enzyme deficiencies. Initial testing of the leukocytes of a few ML patients [13] indicated that lysozyme is increased in both monocytes and the neutrophils of the ML patients. Yet both the monocytes and the neutrophils of the patient are deficient in myelo-

peroxidase. In addition, the ML neutrophils are severely depleted of neutral protease, with no change in alkaline phosphatase. Leukemic monocytes in ML have high lysozyme content;[14] cytochemical indications of the deficiency of myeloperoxidase in ML monocytes have been reported.[15,16]

The pattern of shared enzyme deficiency between the lymphocytes and the monocytes and between the monocytes and the neutrophils, along with its normalization in all three cell lines under remission, implies that developmental interrelationships may exist among the leukocytes of the three cell lines. The findings raise the possibility that the enzymes may be involved in the differentiation of the leukocytes and that the deficiencies of these factors may reflect an aberrant differentiation of the leukemic cells.

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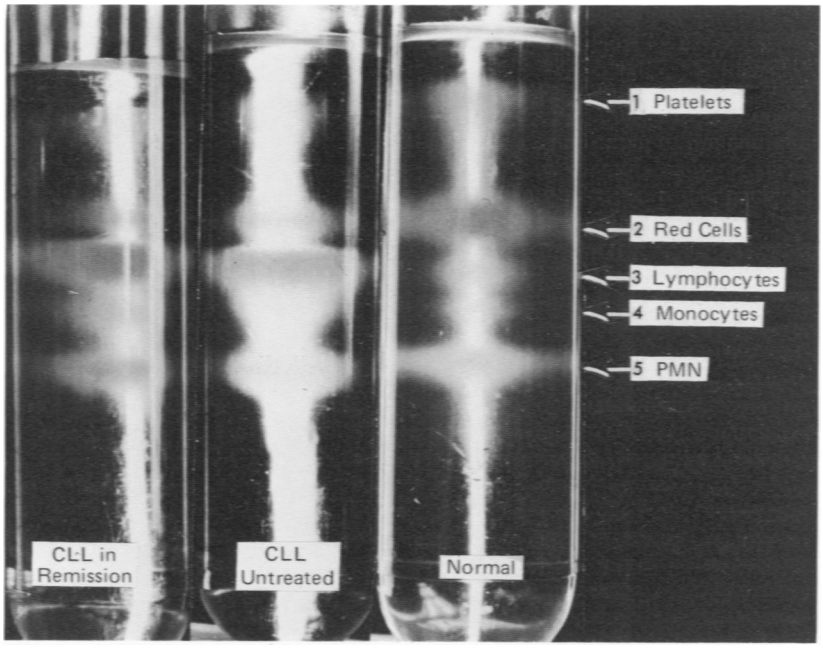

Figure 1—Sucrose density gradient centrifugation of cellular elements of human blood from normal subjects, patients with untreated CLL, and CLL patients in remission. Centrifugation: 2000 rev/min; 20 minutes; rotor, JS-13; Beckman J-12B centrifuge. The untreated CLL gradient shows deficiency of normal monocyte band (4); the band reappeared in CLL remission.

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