Abnormal Expansions of Polyclonal Large to Small Size Granular Lymphocytes: Reactive or Neoplastic Process?

By G. Semenzato, G. Pizzolo, A. Ranucci, C. Agostini, M. Chilosi, I. Quinti, G. De Sanctis, B. Vercelli, and F. Pandolfi

MUCH ATTENTION has been focused recently on natural killer (NK) cells by the finding that NK activity may be important in immune surveillance, in tumor immunity, and in host defense mechanisms. In humans, NK cells represent a population of Fc-γ receptor-positive, nonphagocytic, nonadherent cytotoxic effector cells. In addition, evidence has been provided that a close relationship exists between NK activity and a subpopulation of peripheral blood mononuclear cells, usually referred to as large granular lymphocytes (LGL), which are characterized by the presence in their cytoplasm of azurophilic granules. Although the cellular lineage of NK cells is yet a matter of speculation, with some authors favoring the hypothesis that these cells belong to a separate lineage of natural killer cells and T lymphocytes, it is widely assumed that LGL share determinants with T lymphocytes. More recently, monoclonal antibodies have been produced that have been claimed to recognize cells with NK activity.

In the last few years, T cell chronic lymphocytic leukemias (T-CLL), characterized by the expansion of lymphocytes with azurophilic granules in their cytoplasm, and proliferations of LGL have been described. Although these patients showed peripheral blood and bone marrow lymphocytosis compatible with a diagnosis of CLL, most cases presented relatively low numbers of peripheral lymphocytes and a rather favorable clinical course, which remained stable for years without any treatment. Because monoclonality is difficult to demonstrate on non-B expanded cell subpopulations, until more convincing evidence of malignancy is available, these patients have been referred to with the noncontroversial terms of chronic T cell lymphocytosis, abnormal expansions of LGL, or T gamma cell proliferations.

In the present article, we describe two patients with persistent blood expansions of granular lymphocytes (GL). In both of them, clinical and laboratory data suggest that the increased numbers of these cells may be secondary to an underlying disease, rather than the primary cause of the disease. These observations cast serious doubts about the neoplastic nature of some "chronic lymphoproliferative disorders" and raise the challenge of seeking a better definition of these cases.

CASE REPORTS

Patient 1

In March 1978, a 42-yr-old male came to our attention at the Department of Hematology, University of Padua at Verona. He had been splenectomized at the age of 20 as a consequence of a road accident. Since that time, routine laboratory tests had been performed. At that time, while he was asymptomatic, tests showed an elevated white cell count (20,500/cu mm). Detailed history revealed no obvious etiologic factors, and physical examination was normal, except for mild hepatomegaly. A complete blood count showed hemoglobin 16.7 g/dl, WBC 20,400/cu mm with 86% lymphocytes, and platelet count of 240 x 10^9/liter. The peripheral blood smear demonstrated a predominance of large granular lymphocytes (78%) of both small and larger cells, and contained sparse intracytoplasmic azurophilic granules. Bone marrow aspiration and trephine biopsy revealed increased numbers of lymphocytes, comprising approximately 40% of nucleated elements. Routine blood chemistries showed the following data: bilirubin 1.8 mg/dl (0.6 mg/dl of direct bilirubin), SGOT 64 mU/ml (normal <30), alkaline phosphatase 155 mU/ml (normal <85), γ-GT 250 mU/ml (normal <35). Albumin, cholinesterase, and hepato Quick values were normal, as well as copper and ceruloplasmin serum levels. Serum antibodies against rheumatoid arthritis nuclear antigen and rheumatoid factor titer were all negative. Polyclonal hypergammaglobulinemia was demonstrated (IgG 2,000 mg/dl; IgA 500 mg/dl; IgM 150 mg/dl); no traces of monoclonal Ig were found in serum or urine. Liver biopsy showed a microscopic picture consistent with the diagnosis of chronic hepatitis.
Since initial presentation and during a follow-up of more than 4 yr, the patient has remained asymptomatic, while laboratory findings of hepatic damage have remained virtually unchanged. He has never received any treatment. Total peripheral lymphocyte counts have fluctuated from 14,000 to 21,000/cu mm. Serologic tests were presumptive of primary infection by Epstein-Barr virus (EBV). Antibodies to EBV capsid antigen (VCA), early natigen (EA), and Epstein-Barr nuclear antigen (EBNA), as detected by indirect immunofluorescence techniques, showed the following titers: VCA >640, >640, and 320; EA 10, <5, <5; EBNA >20, 20, 20, at different bleedings within 1 yr and during the presence of the abnormal lymphocytosis. Hepatitis B surface antigen (HBsAg) and anti-HBsAg antibodies were negative. The presence of serum antibodies against the structural core protein (p24) of a new human type C retrovirus, called human leukemia/lymphoma virus (HTLV), were kindly investigated by Dr. R. C. Gallo in Bethesda, MD, and found to be negative. Karyotype analysis with G-banding techniques of peripheral blood mononuclear cells (PBMC) after phytohemagglutinin stimulation revealed a normal 46,XY pattern.

Patient 2

A 62-yr-old man came to our attention at the Department of Clinical Medicine of Padua University in November 1982 for further assessment because of an atypical lymphocytosis. Two years earlier (December 1980), he had been admitted to another hospital with abdominal pain, diarrhea, and fever. The spleen and liver were moderately enlarged. At that time, a complete blood count showed WBC 5,700/cu mm, with 68% segmented forms and 32% lymphocytes, hemoglobin 15.3 g/dl, and platelets 247 x 10^9/liter. Widal test against Paratyphus B was positive at 1:320 dilution. Bilirubin was 2.30 mg/dl (0.7 mg/dl of direct bilirubin). Other routine blood chemistries, including SGOT/SGPT, were normal, but the patient was HBsAg positive. A diagnosis of Salmonella paratyphi infection in a HBsAg-positive normal carrier was made. After treatment with antibiotics, the symptomatology subsided and agglutination titers diminished (1:160). During the follow-up, the patient regularly underwent laboratory tests, which confirmed HBsAg positivity, slight increase of bilirubin (1.87–2.43 mg/dl), without other laboratory findings of hepatic damage. Since May 1981, however, increased percentage and absolute numbers of peripheral blood lymphocytes were observed, ranging from 2,400/cu mm (May 1981) to 5,100/cu mm (January 1982), to 7,600/cu mm (September 1982). Other blood parameters were normal, except for HBsAg positivity.

In November 1982, physical examination was normal, apart from a moderate enlargement of the liver. Investigations were as follows: Hb 14.2 g/dl; platelets 207 x 10^9/liter; WBC 9,500/cu mm, with 88% lymphocytes, 84% of them showing few sparse azurophilic granules in their cytoplasm; IgG 1,210 mg/dl, IgA 243 mg/dl, IgM 516 mg/dl (polyclonal); direct and indirect Coombs tests were negative. Bone marrow was hypercellular with a moderate lympho-histiocytic infiltration (21%), with no other abnormalities. A liver biopsy showed moderate infiltration of lymphocytes in the sinusoids. In immunohistologic analysis, most of these cells displayed HNK-1 positivity. At this time, HBsAg was negative and Widal test titer against Paratyphus B was positive, 1:80. The patient has remained asymptomatic and he is not currently receiving any treatment. His lymphocyte count shows mild variations, and he is in good physical condition. Serum antibodies against structural core protein (p24) of HTLV were found to be negative. Serologic investigations for EBV, CMV, and herpes simplex virus infections showed either absence or low titers of antibodies. Karyotype analysis with G-banding technique of peripheral blood mononuclear cells after phytohemagglutinin stimulation revealed a normal 46,XY pattern.

MATERIALS AND METHODS

Surface Markers

Isolation of peripheral blood mononuclear cells (PBMC) from freshly drawn heparinized blood from both patients and normal donors was performed by Ficoll-Hypaque gradient centrifugation. Sheep rosette-forming cells (SRFC), surface membrane immunoglobulin (Ig) bearing PBMC, mouse rosette-forming cells (MRFC), and cells positive for FcγR were determined as previously described.17 PBMC were studied with a panel of monoclonal antibodies: OKT6 reacts with mature thymocytes;24 OKT3 and OKT1 react with mature T cells;24 OKT11 binds to sheep erythrocyte receptors;24 OKT4 and OKT8 selectively bind to lymphocyte subsets containing cells with helper and cytotoxic/suppressor activity, respectively;24 OKM1 identifies an antigen expressed on cells from the myelomonocytic lineage and on some LGL;24 helper and ratio. To suppressor T cells;6,24 anti-Tac monoclonal antibody recognizes the receptor for human T cell growth factor;25 OKI1 reacts with Ia-like antigens;24 OKT10 reacts with the majority of thymocytes and about 15% of bone marrow cells and has been reported to recognize some LGL;24 and HNK-1 reacts with LGL and with a few feral bone marrow and peripheral blood non-LGL cells.8,12 Antibodies were used in an indirect immunofluorescence test as previously described.17

Electron Microscopic Analysis and Cytchemistry

PBMC isolated from peripheral blood and washed in phosphate-buffered saline (PBS) were fixed for 1 hr at 4°C in osmium tetroxide, dehydrated, and embedded in Epon 812. Thin sections cut with an ultramicrotome were stained with uranyl acetate and lead citrate and examined with a Philips EM300 transmission electron microscope (TEM).17

Cytocentrifuged preparations of PBMC were stained for peroxidase acid phosphatase (ACP), o-naphthyl acetate acid esterase (ANAE), and beta-glucuronidase (β-gluc), as previously described in detail.27

Functional Activities

Responses to phytohemagglutinin (PHA) were assessed in triplicate on PBMC, using purified PHA-M (Difco, Detroit, MI), as previously reported.17,20 Antibody-dependent cellular cytotoxicity (ADCC) and natural killer activity (NK) were studied as previously reported in detail.7,17,23 In brief, ADCC against 51Cr-labeled chicken erythrocytes coated with anti-chicken erythrocyte IgG was measured by counting the 51Cr release after 20 hr incubation of the erythrocytes and PBMC. NK activity was assessed against the K562 cell line. Effector PBMC were depleted of monocytes with carbonyl iron at 37°C, and then incubated with target cells at different effector target cell ratios. The plates were incubated for 18 hr at 37°C in a 5% CO2 atmosphere, then 50 μl of each supernatant was removed and counted in a gamma spectrophotometer. The percentage of specific lysis was calculated as already reported.22 The two patients and a normal donor were run simultaneously in the same experiments.

In vitro phagocytosis of PBMC was determined as a percentage of PBMC ingesting two or more particles of zymosan opsonized with fresh normal human serum.

RESULTS

The morphology of proliferating cells in Giemsa-stained smears is strikingly similar in the two cases. The frequency of granular lymphocytes, evaluated in different bleedings at different times, accounted for...
67%–82% of PBMC in patient 1 and 74%–90% in patient 2. As shown in Fig. 1 (A, B, and C for patient 1 and D, E, and F for patient 2) with light microscopy, cellular sizes are quite heterogeneous, including both large lymphocytes, 15–17 μm in diameter (about 30% and 20% in patients 1 and 2, respectively), with abundant cytoplasm and numerous azurophilic granules (LGL), and small-to-medium size lymphocytes (about 45% and 65% in patients 1 and 2, respectively) 7–12 μm in diameter, with a narrow rim of cytoplasm and sparse granules (SLG). Nuclei are more frequently convoluted in LGL and round in SLG, with a coarse chromatin pattern; nucleoli rarely can be observed. The number of intracytoplasmic granules is variable from cell to cell.

At the ultrastructural level (Fig. 2), low magnifica-

![Image of blood smear and electron micrograph]
tion electron micrographs confirm the heterogeneity of cellular sizes. LGL (Fig. 2A) usually present an irregularly round nucleus with marginated heterochromatin and occasional nucleoli, abundant cytoplasm with a distinct Golgi apparatus, vesicles, and several scattered membrane-bound granules containing an electron-dense matrix. In SGL (Fig. 2, B and C), the nucleus is membrane-bound granules containing an electron-dense matrix. In SGL (Fig. 2, B and C), the nucleus is membrane-bound electron-dense granules. Parallel tubular arrays (PTA) were not observed.

Cytochemical analysis in both patients shows over 75% of the lymphocytes to be strongly positive for AcP (tartarate sensitive), ANAE, and β-glucuronidase.

Immunologic characterization of PBMC (Table 1) in both cases shows low percentages of cells identified by B-related markers (slg and MRFC). SRFC are found to be normal in percentage; however, considering the absolute number of these cells, their number appears highly increased (12,900/cu mm and 5,300/cu mm in cases 1 and 2, respectively, versus 1,200/cu mm in controls). It is interesting to note that, in both patients, most of the cells bear FcγR, and proliferative responses to PHA were defective.

Table 1. Immunologic Characterization of PBMC From Patients 1 and 2

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Patient 1 (%) Positive Cells</th>
<th>Patient 2 (%) Positive Cells</th>
<th>PBMC From Normal Donors (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E rosettes</td>
<td>91 61</td>
<td>78 ± 10</td>
<td></td>
</tr>
<tr>
<td>slg</td>
<td>5 8</td>
<td>6 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>MRFC</td>
<td>2 5</td>
<td>9 ± 2.7</td>
<td></td>
</tr>
<tr>
<td>FcγR</td>
<td>76 90</td>
<td>12 ± 2.2*</td>
<td></td>
</tr>
<tr>
<td>FcμR</td>
<td>14 9</td>
<td>58 ± 12*</td>
<td></td>
</tr>
<tr>
<td>Response to PHA (cpm, 3H-Thy incorporation)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>background</td>
<td>162 184</td>
<td>204 ± 96</td>
<td></td>
</tr>
<tr>
<td>0.3 μg</td>
<td>273 349</td>
<td>9,172 ± 4,034</td>
<td></td>
</tr>
<tr>
<td>1.5 μg</td>
<td>449 872</td>
<td>24,246 ± 9,321</td>
<td></td>
</tr>
<tr>
<td>6 μg</td>
<td>659 840</td>
<td>18,751 ± 7,193</td>
<td></td>
</tr>
<tr>
<td>Percent killing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADCC effector: target ratio</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:1</td>
<td>66 ND</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>10:1</td>
<td>71 71.6</td>
<td>78</td>
<td></td>
</tr>
<tr>
<td>5:1</td>
<td>74 60.1</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>2.5:1</td>
<td>64 51.0</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>1:2:1</td>
<td>68 50.9</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>100:1</td>
<td>59 39.6</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>NK effector: target ratio</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50:1</td>
<td>52.9 28.6</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>25:1</td>
<td>52.9 14.5</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>12.5:1</td>
<td>31.5 11.4</td>
<td>19</td>
<td></td>
</tr>
</tbody>
</table>

*Evaluated in the purified T cell fraction.
ND, not determined.

Table 2. Reactivity With Monoclonal Antibodies of PBMC From Patients 1 and 2

<table>
<thead>
<tr>
<th>Monoclonal Antibody</th>
<th>Patient 1 (% Positive Cells)</th>
<th>Patient 2 (% Positive Cells)</th>
<th>PBMC From Normal Donors (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OKT6</td>
<td>1 2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>OKT11</td>
<td>78 55</td>
<td>69 ± 6</td>
<td></td>
</tr>
<tr>
<td>OKT1</td>
<td>12 ND</td>
<td>66 ± 7</td>
<td></td>
</tr>
<tr>
<td>OKT3</td>
<td>36 30</td>
<td>63 ± 6</td>
<td></td>
</tr>
<tr>
<td>OKT4</td>
<td>15 18</td>
<td>44 ± 4</td>
<td></td>
</tr>
<tr>
<td>OKT8</td>
<td>33 14</td>
<td>26 ± 4</td>
<td></td>
</tr>
<tr>
<td>3A1</td>
<td>58 40</td>
<td>48 ± 3</td>
<td></td>
</tr>
<tr>
<td>OKT10</td>
<td>5 4</td>
<td>7 ± 2</td>
<td></td>
</tr>
<tr>
<td>OKM1</td>
<td>44 16</td>
<td>14 ± 3</td>
<td></td>
</tr>
<tr>
<td>OK11</td>
<td>9 12</td>
<td>10 ± 2</td>
<td></td>
</tr>
<tr>
<td>a-Tac</td>
<td>3 14</td>
<td>&lt;2</td>
<td></td>
</tr>
<tr>
<td>HNK-1</td>
<td>40 58</td>
<td>14 ± 4</td>
<td></td>
</tr>
</tbody>
</table>

ND, not determined.

Both patients, most of the cells bear FcγR, and proliferative responses to PHA were defective.

Table 2 reports the data obtained with a panel of monoclonal antibodies. The majority of PBMC were OKT11-positive but OKT10-negative in both patients. Further characterization showed heterogeneous expression of OKT3 (36% and 30%), 3A1 (58% and 40%), OKT4 (15% and 18%), OKT8 (33% and 14%), OKT11 (78% and 55%) markers, respectively. In addition, data obtained with HNK-1 (40% and 58%) and OKM1 (44% and 16%) showed a relevant proportion of positivity, even if not all GL are recognized by the above reagents. This statement is based on the observation that if all the GL were HNK-1, similar percentages of HNK-1+ and GL should have been detected, whereas GL cells represented, at the time of these studies, 80% and 86% of PBMC, respectively, and were therefore higher than the proportion of HNK-1+ cells. Considering the increase in the number of lymphocytes in patient 2 during the last year (see Case Reports), deviations from the normal range are present when considering the absolute numbers of different subpopulations. However, results expressed as percentages were similar at the different times of observation.

Results of ADCC and NK activity at different target:effector ratios are reported in Table 1. PBMC from both patients were capable of strong ADCC. NK activity showed increased levels in patient 1 and values in the normal ranges in patient 2.

DISCUSSION

In this article, we have reported two patients with chronic lymphocytosis sustained by an expansion of GL. The majority of PBMC in these cases displayed a...
morphological pattern characterized by the presence in their cytoplasm of azurophilic granules typical for NK cells. Because these granules have been previously described only in lymphocytes with an abundant cytoplasm, it is noteworthy that here, at both the light and ultrastructural level, not only large granular lymphocytes were observed, but also a consistent proportion of small-to-medium-sized granular lymphocytes. The above heterogeneity probably reflects a heterogeneity within the expanded GL population in our patients. This is further confirmed by immunologic evaluation, as GL from our patients failed to display a homogeneous cellular membrane phenotype. In fact, whereas up to 82% and 90% of GL were present in the peripheral blood of the two patients, lower percentages of positivity with monoclonal antibodies (HNK-1, OKT10, OKT3, OKM1, OKT8) that have been claimed to react with granular lymphocytes were found (4%-58%). Although HNK-1 monoclonal antibody has been reported to bind to GL in our patients nearly half of the cells displaying GL morphology lack these determinants; this agrees with other observations describing HNK-1-negative GL. In addition, only a few PBMC were activated, as indicated by the reactivity of fresh cells with anti-Tac and OKI1 monoclonal antibodies. These different expressions of surface markers and also different levels of NK in vitro activities may reflect discrete stages of GL maturation.

The main question of whether these expanded populations of GL represent neoplastic proliferations or not is still a matter of discussion. Expanded proliferations of GL have so far been generally included in the heterogeneous group of T cell chronic lymphocytic leukemias (T-CLL) due to their ability to express certain T cell markers. These cases usually showed peripheral and bone marrow lymphocytosis consistent with the diagnosis of CLL, but the number of expanded cells was often low (equal to or below 15 x 10^9/liter, usual minimal criterion for CLL) and their clinical course was usually favorable without any treatment. In these cases, although the homogeneous morphology and immunologic phenotypes indirectly suggest a clonal proliferation, definitive evidence of monoclonality of the expanded cells could not be demonstrated, and therefore it was impossible to confirm the neoplastic nature of the disease. This has prompted some authors to designate these cases with the noncontroversial terms of Tg proliferations or chronic T cell lymphocytosis with neutropenia. We proposed to refer to these patients as having abnormal expansions of LGL, since it is yet to be demonstrated that LGL belong to the T cell lineage, even if they may share some T cell-associated markers.

In the two cases described here, the proliferations of GL have different features. Even if peripheral and bone marrow patterns are compatible with a diagnosis of CLL, and lymphocytosis is sustained by cells with a homogeneous presence of azurophilic granules, we suggest that these patients may have a reactive lymphocytosis. Several lines of evidence support our hypothesis. (A) The heterogeneity of the expanded populations, which is suggested by the heterogeneity of immunologic phenotype and morphology, is in contrast to other previously reported cases of GL proliferations. (B) Both serologic (i.e., EBV titers in patient 1, HBsAg positivity and/or Widal test positivity in patient 2) and histologic (i.e., persistent chronic hepatitis pattern in patient 1, and sinusoidal but not portal infiltrates in patient 2) features suggest that we are dealing with chronically infected patients. (C) Polyclonal hypergammaglobulinemia, often documented in GL expansions (our patients and others), may be interpreted as a consequence of recurrent infections. Further support for this suggestion comes from the finding that an increase of GL, referred to as a leukemoid reaction, has been demonstrated for decades in some patients with chronic infectious diseases. In addition, increased frequency of cells bearing a cytotoxic phenotype and enhanced NK cell activity have been recently reported in a number of infections, notably in chronic hepatitis. The main problem arises from the fact that only in some cases does reactive lymphocytosis express GL morphology and, more importantly, only in occasional patients is the degree of lymphocytosis so high as to simulate a CLL. The explanation of these findings remains speculative at present, but we are probably dealing with a phenomenon similar to the rare but well documented lymphocytosis accompanying solid neoplasia and immunodeficiencies.

Taken together, all these observations lead us to suggest that some GL proliferations already considered as lymphoproliferative disorders might be closely related to the cases reported here. Thus, in agreement with Ault et al., we favor the idea that lymphocytosis in some of these patients could be a secondary, rather than a primary, event. The biologic events leading to GL expansions are unknown, but the role of mediators, such as γ-interferon or interleukin-2, should open new avenues for the comprehension of these diseases.

NOTE

Since this paper was in press, the following monoclonal antibodies against GL have been tested on the
cells from patient 2, with the following results: B73.1,\(^\text{12}\) 68%; VEP13,\(^\text{11}\) 69%; and NK-15 (\textit{J Immunol} 131:1789, 1983) 65%. (Added in proof.)

ACKNOWLEDGMENT

The authors wish to thank Profs. F. Aiuti (Rome, Italy), G. Gasparotto (Padua, Italy), and G. Perona (Verona, Italy) for their helpful criticisms, Dr. A. Fauci (Bethesda, MD), and Dr. T. Uchiyama (Kyoto, Japan) for providing 3A1 and anti-Tac monoclonal antibodies, and Dr. F. Adami for referring patient 2. We also thank I. Caridakis, M. Polisin, and Dr. M. Porcelli for their help in the preparation of the manuscript.

REFERENCES

Clinical features and functional properties of the proliferating cells. Klin Wochenschr 60:133, 1982
36. Cummer CL: Anemia and other blood changes in syphilis. JAMA 91:689, 1928
45. Sala AM, Stein RJ: Carcinoma of the breast with a condition of the blood simulating chronic lymphatic leukemia. Arch Pathol 23:531, 1937
Abnormal expansions of polyclonal large to small size granular lymphocytes: reactive or neoplastic process?

G Semenzato, G Pizzolo, A Ranucci, C Agostini, M Chilosi, I Quinti, G De Sanctis, B Vercelli and F Pandolfi