Complement Alternative Pathway Activation by Chronic Lymphocytic Leukemia Cells: Its Role in Their Hepatosplenic Localization

By F. Praz, G. Karsenty, J. L. Binet, and P. Lesavre

Using affinity-purified $^{125}$I-F(ab')$_2$, anti-human C3, we have investigated the ability of various leukemic cells to activate complement. Lymphocytes from patients with chronic lymphocytic leukemia (CLL) activated the alternative pathway, but cells from patients with other forms of leukemia or normal lymphocytes did not do so. The amount of C3 deposited on the CLL cells was significantly higher in patients with organomegaly (i.e., splenomegaly and/or hepatomegaly). Activation of complement by CLL cells as assessed by C3 deposition on the membrane occurred both in vivo and in vitro and was not related to the N-acetyl-neuraminic acid content of the membrane.

Materials and Methods

Patients

Twenty patients with chronic lymphocytic leukemia (CLL), three with acute lymphoblastic leukemia (ALL), two with hyperleukocytic hairy cell leukemia (HCL), and seven with acute myeloid leukemia (AML) were studied. In all cases, the diagnosis was established on the basis of blood and bone marrow cytologic studies. Clinical and hematologic data of patients with CLL are presented in Table 1. Both the criteria for CLL diagnosis and for staging were done according to the International Workshop on CLL. Diagnosis of ALL and AML was based on a conventional cytochemical study, including periodic acid-Schiff (PAS) and myeloperoxidase staining. The diagnosis of HCL was established on the basis of the typical morphology of the neoplastic cells in the peripheral blood and the presence of acid phosphatase resistance to tartaric acid.

Buffers

VB: isotonic veronal-buffered saline, pH 7.2; VB': VB containing $1.5 \times 10^{-3}$ M CaCl$_2$ and $5 \times 10^{-4}$ M MgCl$_2$; VBE: VB containing $10^{-2}$ M EDTA; VB-MgEGTA: VB containing $2 \times 10^{-3}$ M MgCl$_2$ and $8 \times 10^{-3}$ M EGTA.

Cells

Leukemic and normal cells were obtained from heparinized peripheral blood. Mononuclear cells were separated by a sodium metrizoate/Ficoll density gradient centrifugation (density, 1.077) (Lymphoprep, Nyegaard, Oslo, Norway). Viability was assessed by trypan blue dye exclusion and was at least 95%.

Membrane N-Acetyl-Neuraminic Acid (NANA) Content

Cell suspensions were treated with neuraminidase (0.2 U/10$^6$ cells) for 2 hr at 37°C. The neuraminidase-resistant NANA was measured by the technique of Warren.

Sera

Serum from the patients and a normal healthy donor, referred to as autologous and heterologous sera, respectively, were both used as the complement (C) source. Aliquots of the sera were stored at $-70°C$ and thawed for individual use. Heat-inactivated sera ($\Delta$NHS) were obtained by a 30-min heating at 56°C. MgEGTA-NHS contained $8 \times 10^{-3}$ M EGTA and $2 \times 10^{-3}$ M Mg$^{2+}$. All sera were used diluted 1:5. $\Delta$NHS, NHS, and MgEGTA-NHS were diluted in VBE, VB', and VB-MgEGTA, respectively.

Complement Assays

The hemolytic classical pathway (CP) was evaluated by determination of CH50. AP was assessed by lysis of rabbit erythrocytes and expressed as AH50 units. Immunochemical determinations of components C3 and C4 were measured by the nephelometer method with specific antisera (Hyland Inc., Costa Mesta, CA). Factor B concentra-
The background level was determined after serum, in which the Ca²⁺-dependent CP activity is abolished. The role of the AP by itself was assessed in MgEGTA serum, in which the Ca²⁺-dependent CP activity is abolished. The background level was determined after incubation in a heat-inactivated serum containing EDTA. Because complement activation could be due to the presence of autoantibodies or immune complexes in the leukemic patients’ sera, control experiments were performed in heterologous sera obtained from a normal healthy donor. All the results are expressed as the number of ¹²⁵I-F(ab')₂ anti-C3 molecules per cell, mean value ± SEM. Figure 1 shows that the CLL cells activate the AP with probably no contribution from the CP. Additionally, incubation in autologous normal (A-NHS) or MgEGTA (A-MgEGTA-NHS) serum led to similar C3 deposition, equivalent to 4,450 ± 1,564 and 4,490 ± 794, respectively. Furthermore, CLL cell membrane C3 levels in heterologous sera (NHS) and MgEGTA (MgEGTA-NHS) sera (4,889 ± 1,564 and 4,290 ± 1,011, respectively) did not differ significantly from those in autologous sera, indicating that C activation did not involve antilymphocyte autoantibodies or absorbed immune complexes, possibly occurring in leukemic patients. Further support for this is illustrated by the fact that CLL cells from patients with associated manifestations of autoimmunity (e.g., positive Coomb’s test) known to have raised circulating immune complexes activated the AP to the same extent. Cells harvested after an overnight culture and extensive washings led to the same results. For CLL patients, C3 deposition was also assessed for bound and free antibody, respectively.

Statistical Analysis

A Student’s t test was used to compare mean numbers of C3 deposition in different groups and experimental conditions. The correlation coefficient was calculated with linear regression analysis, using the method of least squares.

RESULTS

Complement Activation by Leukemic Cells

The patients were divided into two groups: the CLL group (Table 1) and the acute leukemia (AL) group, including patients with HCL, ALL, and AML. The contribution of both the classical pathway (CP) and alternative pathway (AP) was determined after incubation of the cells in normal human serum (NHS). The role of the AP by itself was assessed in MgEGTA serum, in which the Ca²⁺-dependent CP activity is abolished. The background level was determined after incubation in a heat-inactivated serum containing EDTA. Because complement activation could be due to the presence of autoantibodies or immune complexes in the leukemic patients’ sera, control experiments were performed in heterologous sera obtained from a normal healthy donor. All the results are expressed as the number of ¹²⁵I-F(ab')₂ anti-C3 molecules per cell, mean value ± SEM. Figure 1 shows that the CLL cells activate the AP with probably no contribution from the CP. Additionally, incubation in autologous normal (A-NHS) or MgEGTA (A-MgEGTA-NHS) serum led to similar C3 deposition, equivalent to 4,450 ± 1,564 and 4,490 ± 794, respectively. Furthermore, CLL cell membrane C3 levels in heterologous sera (NHS) and MgEGTA (MgEGTA-NHS) sera (4,889 ± 1,564 and 4,290 ± 1,011, respectively) did not differ significantly from those in autologous sera, indicating that C activation did not involve antilymphocyte autoantibodies or absorbed immune complexes, possibly occurring in leukemic patients. Further support for this is illustrated by the fact that CLL cells from patients with associated manifestations of autoimmunity (e.g., positive Coomb’s test) known to have raised circulating immune complexes activated the AP to the same extent. Cells harvested after an overnight culture and extensive washings led to the same results. For CLL patients, C3 deposition was also assessed for bound and free antibody, respectively.

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Against the involvement of a cell-surface-associated protease and direct C3 cleavage. On the other hand, neither the AL cells nor the normal lymphocytes activated the complement system. Incubation of the cells from the 12 AL patients in autologous normal, MgEGTA, and heat-inactivated sera led to C3 deposition equivalent to $1,217 \pm 169$, $1,133 \pm 101$, and $733 \pm 69$, respectively. These values were not significantly different from those obtained with cells from 10 normal subjects tested under the same conditions ($1,020 \pm 84$, $1,010 \pm 123$, $600 \pm 45$, respectively), but they were significantly lower ($p < 0.01$) compared to those obtained with the CLL cells. Moreover, membrane C3 levels determined just after drawing, with no further incubation, were significantly higher ($p < 0.01$) for the CLL cells than for either the AL or normal cells suggesting that complement was activated in vivo.

The level of activation of AP by cells of patients with splenic and/or hepatic enlargement was strikingly greater than that by cells of those without organomegaly. After incubation in autologous MgEGTA serum, cells from the 9 patients with organomegaly led to a C3 deposition equivalent to $6,520 \pm 1,448$ 125I-F(ab')2 anti-C3 molecules per cell, significantly higher ($p < 0.02$) than the C3 deposition equivalent to $2,827 \pm 437$ anti-C3 molecules per cell observed with the 11 patients with organomegaly (Fig. 2).

**Absence of Correlation Between Membrane N-acetyl-neuraminic acid (NANA) Residues and AP Activation**

The membrane NANA content of the lymphocytes isolated from 17 patients with CLL was determined after treatment of the cells with neuraminidase. Membrane NANA content ranged from 10.2 to $23.7 \times 10^{-17}$ mole/cell ($15.4 \pm 3.7$) and did not correlate with the AP activating capacity of the cells (correlation coefficient $r = 0.37$, $p > 0.10$) (data not shown).

**Complement Profile in Leukemic Patients**

Both the CP and AP hemolytic activities and C3 and C4 immunochemical levels were found not to be significantly different from normal values. Factor B levels were significantly higher in both the CLL sera ($p < 0.001$) and the AL sera ($p < 0.01$) (data not shown), indicating that, although the CLL cells activate the AP, this activation did not lead to a detectable in vivo AP protein consumption.

**DISCUSSION**

The present report demonstrates that CLL cells activate the alternative complement pathway in vitro. Moreover, a small but significant number of C3 molecules was detectable on CLL cells just after drawing.
with no further incubation in serum, demonstrating that low-grade C3 deposition had already occurred in vivo. In contrast, C3 deposition did not occur in vivo, or in vitro, with any of the other types of leukemic cells or with normal lymphocytes. Complement activation by CLL cells proceeded through the AP with no detectable contribution from the CP, as C3 deposition was identical in the presence or absence of MgEGTA and was abolished in a factor D-depleted serum. Furthermore, the role of antilymphocyte antibodies or absorbed immune complexes, present in CLL patients’ sera, was very unlikely, as C3 deposition was not significantly different in both patient and normal serum. Although apparently contradictory to previous studies designed to detect substantial complement activation by various leukemic cells, including CLL cells, this investigation, using a method able to detect small amounts of C3 deposition by low-grade C activation, clearly demonstrates that CLL cells (but not the acute leukemic cells) activated the human AP. The specificity of the detection system was previously checked by inhibition experiments using free purified C3. In fact, the actual number of C3 molecules per cell, assessed by this method, was 8–10 times greater, compared to the number of F(ab’)_2 anti-C3 molecules per cell. The respective affinity of the antibody for bound and free C3, and the antibody–antigen molar ratio, can both be different for bound and free C3, thus accounting for the observed discrepancy. Then, as this method did not provide a relevant conversion factor to express C3 binding in actual number of C3 molecules per cell, results are expressed as mean number of F(ab’)_2 anti-C3 molecules per cell. It has previously been shown that, in the case of human B cell lymphoma, AP activation is associated with Epstein-Barr virus transformation of the cells. This mechanism cannot account for the C activation in our system, because the CLL cells do not express the Epstein-Barr virus nuclear antigen.

It has been previously suggested that the presence of the C3-C3b receptor was associated with AP activation. However, it is unlikely that the presence of C3b receptor accounts exclusively for the AP activation by CLL cells, as those cells were shown to bear uniformly low C3b receptor density or even no C3b receptor. Moreover, the HCL cells, which constantly expressed a much higher density of C3b receptor than CLL cells, did not activate the C system.

Finally, we could exclude any role of membrane N-acetyl-neuraminic acid residues, a known modulator of the AP activation, because the AP activation extent did not correlate with neuraminidase-susceptible NANA content of CLL cells, which was analogous to that of normal lymphocytes. Therefore, activation by CLL cells, as in the case of the human lymphoblastoid T cell lines, appears to be dependent on undefined properties of the membrane structures and could be either a CLL-specific anomaly or a normal structure expressed during B cell ontogeny, which presents itself during the particular differentiation of CLL cells. In this regard, compared to CLL cells, cells isolated from one patient with prolymphocytic leukemia (PLL) activated the alternative pathway to the same extent (data not shown). The phenotype of both PLL and CLL cells is similar and does not appear to have a normal lymphoid counterpart in the human bone marrow and blood and may represent an early B cell memory. As such, one may speculate that the AP activation by CLL cells could be relevant to the known role of C3 in B cell memory generation.

The role of such a low-grade AP activation by CLL cells, in the expression of the disease, is most likely not cell lysis, as the low surface C3b density cannot lead to C5-convertase and, in turn, the C5b-9 membrane attack on those cells. In addition, we observed that CLL cell viability was not impaired after serum incubation. Interestingly, enough, the cells from CLL patients with organomegaly led to a significantly higher AP activation than those from patients without organomegaly. These observations suggest that the C3 deposited on the CLL cells could interact with both the hepatic and splenic macrophages, thus allowing their hepatosplenic localization.

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