P2X<sub>7</sub> receptor expression in evolutive and indolent forms of chronic B lymphocytic leukemia

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Human leukocytes express a receptor for extracellular nucleotides, named P2X<sub>R</sub>, that in lymphocytes can either mediate cell death or proliferation, depending on the level of activation. The authors have investigated P2X<sub>R</sub> expression and function in 21 patients affected by B-cell chronic lymphocytic leukemia, with an evolutive and with an indolent variant of the disease. Resting cytoplasmic Ca<sup>2+</sup> concentration was significantly higher in lymphocytes from patients with the evolutive compared with indolent variant. Furthermore, in the former, P2X<sub>R</sub> stimulation triggered a Ca<sup>2+</sup> influx significantly larger. Higher Ca<sup>2+</sup> influx correlated with an increased P2X<sub>R</sub> expression in the lymphocytes from patients with the evolutive form. Finally, incubation in the presence of extracellular adenosine triphosphate decreased spontaneous proliferation of lymphocytes from patients affected with the evolutive variant but had no effects on lymphocytes from patients with the indolent form. These results suggest that expression and function of P2X<sub>R</sub> may correlate with the severity of B-cell chronic lymphocytic leukemia. (Blood. 2002;99:706-708)

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**Introduction**

B-cell chronic lymphocytic leukemia (B-CLL) is one of the most common hematopoietic tumors.1-2 Patients with B-CLL experience heterogeneous clinical courses: some survive for more than a decade with minimal therapy, while others die within a few years despite aggressive treatment. Therefore, the identification of novel markers that may serve as prognostic indicators might be of great help. B-CLL cells, in striking contrast with normal B lymphocytes, have a high level of expression of the nucleotide P2X<sub>7</sub> receptor (P2X<sub>R</sub>).3-5 This ligand (adenosine triphosphate [ATP])-gated plasma membrane channel is present in immune cells,6 neurons, fibroblasts, epithelia, and smooth muscle cells.7 Its physiologic function is basically unknown. However, there is little doubt that protracted stimulation causes cell death either by apoptosis or necrosis, depending on the cell type and the experimental conditions.8-10 Peripheral T lymphocytes and monocytes express P2X<sub>R</sub>11,12 even if in some patients this receptor may be nonfunctional.13 We have recently observed that transfection of P2X<sub>R</sub> into human P2X<sub>7</sub>-null lymphoblastoid cells renders these cells capable of growth in serum-depleted culture medium and that proliferation relies on the continuous release of ATP (4-fold larger in the P2X<sub>R</sub>-transfectants compared with mock-transfected cells).14 In the present report we show that P2X<sub>R</sub> is differentially expressed in patients with B-CLL with evolutive B-CLL courses versus indolent and with the evolutive variant but had no effects on lymphocytes from patients with the indolent form. These results suggest that expression and function of P2X<sub>R</sub> may correlate with the severity of B-cell chronic lymphocytic leukemia. (Blood. 2002:99:706-708)

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**Study design**

**Patients**

Twenty-one patients with typical CLL, according to morphologic and immunologic criteria,15 seen at the Section of Hematology, University of Ferrara, during the study period were included in this analysis. All patients were CD5<sup>+</sup>/CD23<sup>+</sup><sup>-</sup>, with weak expression of CD22 and surface immunoglobulins. The patients did not receive cytotoxic therapy for at least 2 months before sampling. All patients had a follow-up of at least 3 years with hematologic workups at 1- to 6-month intervals: the patients here referred to as affected by an “indolent” disease were those who never required cytotoxic therapy, with stable blood counts and no disease progression, whereas those patients with therapy-demanding disease due to a shorter than 12-month lymphocyte doubling time, or to disease progression through the Rai staging system, were classified as “evolutive.” The karyotype was abnormal in 3 of 8 patients (12p<sup>-</sup>, 14q<sup>-</sup> -21 in 1 patient each) in the former group and in 6 of 13 patients in the latter group (11q22-23 abnormality in 3 patients and 13q, 18p<sup>-</sup>, and t(1;6)(q25;p24) in 1 patient each). Approval was obtained from the Institutional Review Board for these studies. Informed consent was provided according to the Declaration of Helsinki.

**Cells**

Peripheral lymphocytes were isolated by Ficoll gradient and resuspended in RPMI medium at a concentration of 10<sup>6</sup>/mL prior to experiments.

**Measurement of the cytoplasmic free Ca<sup>2+</sup> concentration**

Measurements were performed in a thermostat-controlled and magnetically stirred Perkin-Elmer fluorimeter (Beaconsfield, United Kingdom) with the
were separated on 7.5% sodium dodecyl sulfate–polyacrylamide gel. The
ase, and 0.2 benzamidine, 1 mM phenylmethylsulfonyl fluoride, 0.2 m
For [3H]thymidine incorporation, cells were resuspended in RPMI supple-
CA) and expressed as integrated density value.

Statistics
Statistical significance was assessed by the Student t test.

Results and discussion
It was previously reported by Wiley and Dubyak3 that ATP
increases cation permeability of lymphocytes from patients af-
fected by B-CLL but not from healthy patients. We have reevalu-
eted their findings in 2 groups of patients—1 with an evolutive
and 1 with an indolent course of the disease. Exemplificative traces of
resting and ATP-stimulated levels of intracellular Ca2+ ([Ca2+]i)
are shown in Figure 1. Lymphocytes from evolutive B-CLL
showed an elevated resting [Ca2+]i, and a higher ATP-stimulated
peak compared with the patient with the indolent variant. We
screened 13 evolutive B-CLLs and 8 indolent B-CLLs, finding a
mean resting [Ca2+]i, of 88 nm ± 27 and 52 nm ± 23 (P < .01),
respectively. The stimulated [Ca2+]i increase above resting level
was 100 nm ± 30 and 32 nm ± 16 (P < .001) in evolutive B-CLL
and indolent B-CLL, respectively. There was a good correlation

Figure 1. ATP-triggered lymphocyte [Ca2+]i increase. ATP triggers a higher
[Ca2+]i increase in lymphocytes from evolutive (A) compared with indolent (B) B-CLL.
Lymphocytes were isolated as described in “Study design” and resuspended in Ca2+-supplemented or Ca2+-free solution at a concentration of 2 × 10^6/mL. The Ca2+-free solution also contained 500 μM EGTA, ATP and ionomycin (Iono) were 1 mM and 1 μM, respectively.

Reverse-transcriptase–polymerase chain reaction
Total cytoplasmic RNA was extracted as described by Chomczynski and
Sacchi,16 reverse transcribed, and amplified with P2X7-specific primers.14 After hybridization with a digoxigenin-labeled P2X7-specific internal oligoprobe, P2X7 complementary DNA was visualized by chemilumines-
cent detection after incubation with a dilution of antidigoxigenin Fab
fragments conjugated to alkaline phosphatase. As a control for the mRNA
content of the samples, β-actin was used. The β-actin amplification
products are shown as ethidium bromide–stained agarose gel
electrophoresis bands.

Western blotting
Cells were lysed in lysis buffer containing 300 mM sucrose, 1 mM K2HPO4,
1 mM MgSO4, 5.5 mM glucose, 20 mM HEPES (pH 7.4), 1 mM
benzamidine, 1 mM phenylmethylsulfonyl fluoride, 0.2 μg deoxyribonucle-
ase, and 0.2 μg ribonuclease by repeated freeze/thawing (3 cycles). Proteins
were separated on 7.5% sodium dodecyl sulfate–polyacrylamide gel. The rabbit polyclonal anti-P2X7-receptor serum was kindly provided by Dr Gary
Buell (Serono Pharmaceutical Research Institute, Geneva, Switzerland).
The primary antibody was used at a dilution of 1:100 in triethanolamine-
buffered saline buffer (10 mM Tris-HCl, 150 mM NaCl, pH 8.0). The secondary antibody was a goat antirabbit antibody conjugated to alkaline
phosphatase. Intensity of the bands was measured with a Multimage Light
Cabinet Alpha Image 1220 densitometer (Alpha Innotech, San Leandro,
CA) and expressed as integrated density value.

Proliferation
For [3H]thymidine incorporation, cells were resuspended in RPMI supple-
mented with 10% fetal calf serum and plated overnight at a concentration of
10^5/mL in the presence of radiolabeled thymidine (1 μCi [3.7 × 10^6 Bq] per
well). In parallel, cells were also counted with a phase contrast
light microscope.
between an elevated basal \([\text{Ca}^{2+}]_i\), and a higher ATP-stimulated rise \((r = 0.63)\). We confirmed, as previously shown by Wiley and Dubyak,\(^7\) that B-CLL cells do not express functional P2Y receptors and that the whole \([\text{Ca}^{2+}]_i\) increase is due to influx across the plasma membrane following the opening of an ATP-gated ion channel (Figure 1). Because previous studies showed that the main ATP-activated channel of lymphoblastoid cells is P2X7-R,\(^5,17,18\) we investigated the expression of this receptor by reverse-transcriptase–polymerase chain reaction (RT-PCR) in peripheral lymphocytes from all the patients recruited in the 2 groups. Figure 2A reports P2X-R expression in 2 evolutive B-CLL and 2 indolent B-CLL patients representative of the respective groups; human macrophages are shown for comparison. Control β-actin amplification fragments are shown in Figure 2B. Lymphocytes from patients with evolutive B-CLL express P2X-R to a level higher than the patients affected by the indolent variant, and comparable to that of macrophages, a cell type well known for its high expression of this receptor subtype. Patients with a higher ATP-stimulated \([\text{Ca}^{2+}]_i\), showed a higher P2X-R expression (Figure 2A). Figure 2C shows a Western blot of peripheral lymphocytes from the same patients to confirm higher expression of the P2X-R protein in evolutive compared with indolent B-CLL. In our screening of all the 21 patients recruited in this study, we found a good positive correlation \((r = 0.924, P < 0.01)\) between increases in \([\text{Ca}^{2+}]_i\), stimulated by ATP and P2X-R protein expression measured by densitometry of Western blots. We and others have demonstrated in the past that expression of P2X-R confers susceptibility to ATP-mediated cytotoxicity.\(^9,10\) Thus, we selected 8 patients—4 B-CLL indolent (low P2X-R) and 4 B-CLL evolutive (high P2X-R)—and tested their sensitivity to ATP. In the experiment reported in Figure 2D, we show that extracellular ATP inhibits in vitro proliferation of peripheral lymphocytes isolated from patients affected by the evolutive but not the indolent form of the disease. Data on [\text{H}]thymidine incorporation were corroborated by cell number count (not shown).

Receptors for extracellular nucleotides have recently become a focus of interest in immunology and hematology due to their high level of expression in blood cells and the vast potential of therapeutic applications afforded by their modulation.\(^5\) It was originally put forward that P2X-R might participate in shedding of CD23 and CD62L or killing by apoptosis.\(^4,20,21\) We have previously observed that transfection of P2X-R, the most widely diffused P2X receptor subtype in immune cells, confers a substantial proliferative advantage when transfected into human B lymphoblastoid cells that lack it constitutively.\(^14\) The P2X-R transfectants become able to grow in the absence of serum, show an elevated resting \([\text{Ca}^{2+}]_i\), respond to ATP, and secrete large amounts of this nucleotide into the culture supernatant. Thus, we speculated that P2X-R expression might also confer an advantage in vivo and anticipated that patients with the worse prognosis (ie, evolutive B-CLL) might be characterized by a B-cell population with a higher P2X-R expression. Such a cell population might be less affected than normal B lymphocytes, or B-CLLs that express low levels of P2X-R (ie, indolent B-CLL cells), by antimitobolites or might recover more quickly after chemotherapy. It is of interest that while P2X-R expression confers to evolutive B-CLL cells a proliferation advantage, it also makes these cells more susceptible to the cytotoxic effect of high concentrations of extracellular ATP.

It may appear paradoxical that stimulation of a single receptor may bring about such utterly different effects, but this might be explained by the different pattern of intracellular signals generated by a tonic, low-level activation of the P2X-R, such as that presumably responsible for growth stimulation, as opposed to the massive receptor activation that is known to trigger cytotoxicity. In any case, this bifunctional capacity points to P2X-R as a novel and useful target for antitumor therapy.

References

2. Damle RN, Wasi T, Fais F, et al. Ig V gene muta-
3. Wiley JS, Dubyak GR. Extracellular adenosine