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Does the P2X1del variant lacking 17 amino acids in its extracellular domain represent a relevant functional ion channel in platelets?

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variant Ph translocation and this may account in part for the paucity of deletions.

Second, it is possible that a deletion is more likely to accompany a translocation with an *M-bcr* breakpoint. Since *M-bcr* breakpoints occur in the vast majority of patients with CML but in only a minority of patients with Ph-positive ALL, this could account for the rarity of deletions in the latter disease. Although the numbers are small our data are consistent with this idea since deletions were observed in only one of the 54 patients with an *m-bcr* breakpoint compared with 25 of 212 CML patients with a classical Ph translocation¹¹ ($P = .036$).

Third, the rarity of deletions in Ph-positive ALL may reflect features of the target cell in which the translocation occurs. CML results from transformation of a multipotent stem cell,³ whereas ALL more often results from transformation of a committed B-cell progenitor.¹⁴ Lymphoid cells undergo antigen receptor rearrangements that require accurate joining of double stranded DNA breaks¹⁵ and may therefore employ mechanisms that minimize the concomitant occurrence of large deletions.

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To the editor:

Does the P_{2X1del} variant lacking 17 amino acids in its extracellular domain represent a relevant functional ion channel in platelets?

In a recent issue of *Blood*, Greco et al¹ reported on the expression of a novel structurally altered P_{2X1} receptor in platelets and in megakaryocytic cell lines. This P_{2X1} variant lacks 17 amino acids in its extracellular domain due to a deletion within exon 6 of the P_{2X1} gene (GenBank accession no. 17481172). The authors showed that, after heterologous expression in the 1321N1 astrocytoma cell line, P_{2X1del} subunits constitute a channel preferentially activated by adenosine diphosphate (ADP). In reverse transcriptase-polymerase chain reaction (RT-PCR) analyses, they described this variant as the major P_{2X1} mRNA of platelets, thus claiming that P_{2X1del} may play an important role as an ADP-activated ion channel in these cells. These conclusions are in contradiction with other studies^{2,3} that show that the functional platelet P_{2X1} receptor is an adenosine triphosphate (ATP)-gated ion channel that is unresponsive to high-performance liquid chromatography-purified ADP. Indeed, the activation of the P_{2X1} receptor by ATP or by its stable analogs, α,β -methylene ATP and L- β,γ -methylene ATP, produces a rapid, quickly desensitized Ca^{2+} influx⁴ that is responsible for reversible platelet shape change,^{3,5} and that also plays a pivotal role during platelet aggregation induced by collagen.³ These platelet responses

to ATP were found to be antagonized by ADP similarly to the inward current produced by ATP in *Xenopus* oocytes expressing wild-type P_{2X1} receptors (P_{2X1wt}).³ In addition, platelet receptors for ADP have been well characterized and are identified as 2 P_{2Y} receptors: P_{2Y1} and P_{2Y12} (reviewed in Gachet⁶). Both receptors are required for normal platelet responses to ADP, a conclusion recently corroborated in $P_{2Y1}-/-$ and $P_{2Y12}-/-$ knock-out mice.^{7,8} Thus, the existence in platelets of an ADP-activated variant of the P_{2X1} ion channel as the major platelet P_{2X} representative, as hypothesized by Greco et al,¹ can be questioned.

In this letter, we present data that argue against the possibility for a role of P_{2X1del} in platelet function. First, RT-PCR analyses of independent platelet RNA samples followed by sequencing of the PCR products showed major abundance of the P_{2X1wt} mRNA (Figure 1A, lane 2; Figure 1B), whereas the platelet P_{2X1del} mRNA appeared as a minor product. In contrast to the findings of Greco et al,¹ we found comparable relative amounts of the P_{2X1del} mRNA in platelets and in the Dami megakaryocytic cell line (Figure 1A, lane 3; Figure 1B). Second, in order to further demonstrate the presence of P_{2X1del} transcripts in platelets the authors designed 2 different

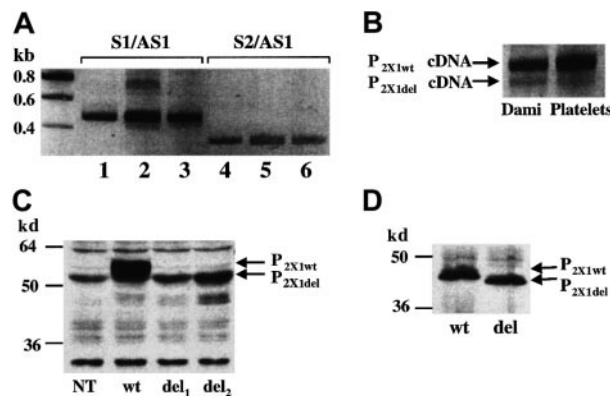


Figure 1. Analyses of P_{2X1del} mRNA and protein. (A) RT-PCR of platelet (lanes 2 and 5) and Dami cell (lanes 3 and 6) RNA; the primer sets S1/AS1 and S2/AS1 are described by Greco et al.¹ In lanes 1 and 4, the pcDNA3- P_{2X1wt} plasmid was used as a PCR template. (B) Enlarged view of lanes 2 and 3. For these experiments, platelets were isolated from freshly drawn blood of at least 10 unrelated healthy volunteers. (C) Western blots of P_{2X1del} and P_{2X1wt} in transfected 1321N1 total cell extracts. The pcDNA3- P_{2X1del} vector was transfected in 2 independent experiments (del₁ and del₂) in parallel with the pcDNA3- P_{2X1wt} vector (wt). A nontransfected cell extract is also shown (NT). (D) Western blots of P_{2X1del} (del) and P_{2X1wt} (wt) proteins synthesized in a *in vitro* T7-coupled transcription/translation rabbit reticulocyte system. The rabbit polyclonal anti-human P_{2X1} antibody used in these experiments was previously described.⁹ Bands corresponding to P_{2X1del} and P_{2X1wt} PCR products and proteins are indicated. Molecular weight ladder is shown on the left.

sets of primers, S1/AS1 to amplify both P_{2X1wt} and P_{2X1del} cDNAs and S2/AS1 to amplify only P_{2X1del} cDNAs. However, this strategy does not enable a quantitative assessment of P_{2X1del} levels. Indeed, when the pcDNA3- P_{2X1wt} plasmid was used as a PCR template, we showed that the primer set S2/AS1 annealed to the P_{2X1wt} cDNA leading to artificial amplification of the 51 base pair deleted cDNA-encoding P_{2X1del} (Figure 1A, lane 4, as confirmed by sequencing). This phenomenon evidently also can occur during RT-PCR analyses of platelet and Dami cell RNA samples containing both P_{2X1wt} and P_{2X1del} mRNA (Figure 1A, lane 5 and 6, respectively). Third, Western blotting experiments performed after transient transfection of 1321N1 cells with a pcDNA3 vector containing either the P_{2X1del} cDNA (del) or the P_{2X1wt} cDNA (wt) revealed only low amounts of P_{2X1del} proteins at the expected size in comparison to the P_{2X1wt} protein levels (Figure 1C). To ensure that the antibody used in this detection would recognize the 2 proteins

with equal sensitivity the P_{2X1del} (del) and P_{2X1wt} (wt) proteins were synthesized in an *in vitro* T7-coupled transcription/translation rabbit reticulocyte system. Western blotting analyses revealed identical amounts of the 2 *in vitro*-translated (nonglycosylated) proteins (Figure 1D). These data thus suggest that the P_{2X1del} protein is not properly produced or is mainly unstable in the transfected 1321N1 cells.

Taken together, our data indicate that the P_{2X1del} variant is unlikely to be a major protein in platelets. Moreover, the fact that Greco et al present this variant as a potential ADP-activated channel is not consistent with all the previous molecular and functional studies of platelet P_2 receptors.⁶ The quantitative and functional relevance of the platelet P_{2X1del} variant should therefore be reconsidered.^{3,4}

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Response:

Functional adenosine diphosphate-activated P_{2X1del} receptor

Oury et al have confirmed our recent identification of a P_{2X1del} variant of the P_{2X1wt} receptor RNA in platelets and megakaryocytic DAMI cells.¹ The complex array of nucleotide receptors they describe in different cell types suggests that questions of identity and function may not be fully resolved. What we have done, no more and no less, is to show that transfection of nonresponsive 1321 cells with the P_{2X1del} -variant cDNA results in the expression in these cells of a selective homomeric receptor sensitive to adenosine diphosphate (ADP).¹

Unfortunately, Oury et al have not provided adequate information for an evaluation of their polymerase chain reaction (PCR) results. We have previously shown that stringent annealing temperatures (60°C) are needed to minimize nonspecific binding due to the high degree of homology of the S2 and AS1 PCR primers.¹ (Fig 1 legend) There are similar difficulties in evaluating their

protein blots because their antibody appears to recognize multiple proteins in nontransfected cells.¹ (Fig 1C, lane 1, "NT") In addition, Oury et al do not take into account the cross-reactivity of available antibodies for the P_{2X1wt} and P_{2X1del} receptors. We do not feel that their conclusion that "the P_{2X1del} protein is not properly produced or is mainly unstable in the transfected 1321N1 cells" can be deduced from these results.

Furthermore, contrary to the comments of Oury et al we have made no claims that "the P_{2X1del} variant is a major protein in platelets." In fact, data from the literature² would suggest that the P_{2X} receptor would be of low abundance in platelets. However, our Ca^{2+} influx studies clearly show that the P_{2X1del} receptor is an ADP-activated channel, not a *potential* ADP-activated channel. Consideration of these problems, taken together with the extensive data reported in our original paper,¹ shows that the question posed

by Oury et al as the title of their communication must be answered in the affirmative.

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To the editor:

Role of surface IgM and IgD on survival of the cells from B-cell chronic lymphocytic leukemia

We read with great interest the paper by Bernal et al¹ on the ability of anti-human IgM antibodies to prevent spontaneous apoptosis of B-cell chronic lymphocytic leukemia (B-CLL) cells in vitro. These findings suggest that receptor engagement by certain antigens, perhaps autoantigens that are constantly available in vivo, may contribute to the survival of the neoplastic cells.

The data, although interesting, are in contrast with previous findings of other groups including our own that showed that exposure to anti- μ antibodies causes rapid apoptosis of certain B-CLL cells.²⁻⁴ More recently we have also demonstrated that when an anti-Ig- δ chain antibody is used, prolonged cell survival instead of apoptosis is observed.⁵ From these findings it appears that the specificity of the antibodies to surface Ig plays a crucial role in the outcome of the experiments. Unfortunately, in the experiments by Bernal et al¹ very little attention is paid to the specificity of the reagent used. If the anti-Ig reagent (a polyclonal goat anti-IgM antibody) that they used also reacted with light chains then the cells could have been stimulated via surface IgM and IgD. In these conditions, the prevailing physiological pathway would be survival or apoptosis depending on which of the 2 signals is more potent.

Differences of about 15% in the Annexin-V staining are barely significant with the presently used methods, according to the experience accumulated by several groups including our own. Thus, 3 B-CLL groups can be distinguished in the work of Bernal et al¹ (Table 1): those that do not respond at all to anti-IgM treatment (cases 31, 86, 89); those that barely respond (cases 47, 69, 72, 96, 104, 106, 108, 111, 114, 121); and those who definitely respond (cases 58, 78). The conclusions of the authors, as mentioned in the title of their article, are based primarily upon the latter 2 cases.

Bernal et al state that for our previous work we selected for cases expressing CD38. These cases might represent a special subset of B-CLL. CD38⁺ B-CLL cells represent 30% to 60% of all the B-CLL (depending upon the different cohorts studied).⁶ Even before CD38 became a fashionable marker, it was already known that the cells from only about half of the B-CLL cases responded to anti-Ig stimulation in vitro as assessed in Ca⁺⁺ mobilization or tyrosine kinase phosphorylation assays.^{7,8} We just pointed out that a correlation existed between CD38 expression and viability of the signal transduction pathway. Based upon these considerations the question arises of how many of the cases utilized by Bernal et al had a functional IgM/IgD-dependent signal transduction pathway initiated by surface Ig cross-linking. This is not trivial, considering the general implications of the paper brought about by their title.

A final comment concerns the observation reported in the paper that certain genes potentially related to antiapoptotic activity are

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up-regulated following exposure of B-CLL cells to anti-IgM antibodies. This up-regulation (which is not very dramatic as apparent from the figures shown by Bernal et al) should not inevitably be taken as evidence for the fact that anti-IgM stimulation causes activation of an antiapoptotic program. In studies that we have not published, we have compared the capacity of anti- μ chain or anti- δ chain stimulation to promote the synthesis of a variety of molecules (M.M. and S.Z., unpublished data, May 2001). There is virtually comparable up-regulation of the same molecules studied by Bernal et al following exposure to anti- δ or anti- μ reagents. However, the 2 types of stimuli had a remarkably different effect on caspase activation and consequently on apoptosis.

In conclusion, the interesting hypothesis proposed by Bernal et al does not appear to be sustained by the available experimental data. It is possible that B-CLL cells are exposed to a variety of apoptotic and antiapoptotic signals in vivo and that the survival of a large fraction of them depends upon the fact that the antiapoptotic signals are prevailing.

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