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To the editor:

Conventional Western blotting techniques will not reliably quantify p210$^{BCR-ABL1}$ levels in CML mononuclear cells

We read with interest the paper by Copland et al,[1] in which they used quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) and Western blotting to demonstrate that the transcript and protein levels of p210$^{BCR-ABL1}$ are elevated in primitive chronic myeloid leukemia (CML) progenitors relative to more mature cells. The work adds substance to the growing body of evidence for increased p210$^{BCR-ABL1}$ activity in the putative CML stem cell, which also provides a partial rationale for the failure of treatments such as imatinib to achieve complete eradication of disease in vivo.

We note that the RT-PCR–based quantitative data and patterns of CrkL phosphorylation that Copland et al[1] report support the hypothesis that p210$^{BCR-ABL1}$ activity is enhanced in more primitive cell populations. However, their Western blotting method uses a conventional lysis buffer to lyse cells in the cold, which is standard procedure. It has been known since 1987[2] that such lysis of CML mononuclear cells (MNCs) releases a degradative activity that very rapidly and selectively destroys p210$^{BCR-ABL1}$ and c-ABL but does not degrade other proteins.[3] This activity has been reported to be primarily restricted to the mature cell compartments,[2-4] hence its influence is commensurately greater in MNC lysates. It is entirely predictable therefore that Copland et al[1] could not recover a p210$^{BCR-ABL1}$ signal or a 210-kDa phosphotyrosine signal from a CML MNC blot and that the signal recovered is greater from CD34$^+$CD38$^-$ cells than CD34$^+$ cells. CD34$^+$ cell fractions typically contain up to 5% contaminating mature cells that would elicit some signal degradation, while double-sorted CD34$^+$CD38$^-$ populations would be expected to contain fewer contaminating cells.

Furthermore, while Copland et al[2] could not recover any p210$^{BCR-ABL1}$ signal from CML MNCs, Guo et al[5] showed that use of an extremely toxic nerve agent and a boiling lysis medium permitted routine p210$^{BCR-ABL1}$ recovery from CML MNCs. We have recently published data showing that the degradative activity is probably an acid-dependent hydrolase and can be neutralized by a high-pH lysis regimen to allow accurate determination of p210$^{BCR-ABL1}$ protein levels. This has the further advantage of permitting coimmunoprecipitation studies, and so, for the first time, investigation of p210$^{BCR-ABL1}$-protein complexes in primary cells from CML patients is now possible.[3] These complexes play an important role in CML pathogenesis, and the amount of p210$^{BCR-ABL1}$ present will be a significant contributory factor. The degradation of p210$^{BCR-ABL1}$ by MNC components of primary cell lysates is therefore a critical variable. Future Western blotting experiments should take account of this inhibitory activity.

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Response:

Conventional Western blotting techniques will not reliably quantify p210 BCR-ABL

We welcome the opportunity to respond to the comments of Patel et al. We were aware of the selective rapid degradation of $p210^{BCR-ABL}$ and c-ABL after lysis of total mononuclear cells (MNCs), which has been reported previously[1] and makes assessment of $p210^{BCR-ABL}$ and c-ABL protein levels in mature cell compartments difficult. In order to overcome this, we used multiple techniques to assess BCR-ABL expression in chronic myeloid leukemia (CML) cells.

First, we measured BCR-ABL transcripts using real-time quantitative reverse transcriptase–polymerase chain reaction (RT-PCR). This showed that, compared with total MNCs, BCR-ABL transcripts were significantly increased in both $CD34^+$ and $CD34^+CD38^-$ populations ($P = .016$ and $P = .031$, respectively). We then developed a novel flow cytometry assay to measure CrKL phosphorylation (P-CrKL), as a marker of BCR-ABL activity in CML cells.[2] This confirmed that P-CrKL was significantly increased in $CD34^+CD38^-$ populations compared with total $CD34^+$ populations. The P-CrKL assay was then validated by Western blotting using a method previously described[3] and comparing MNCs, $CD34^+$ cells, and $CD34^+CD38^-$ cells. Once again the levels were lowest in MNCs—we are not aware that the degradative activity should affect P-CrKL. This Western blotting technique has also been used for the detection of BCR-ABL, c-ABL, and P-Tyrosine (P-Tyr) in BCR-ABL–transduced $CD34^+$ cord blood samples cultured for up to 12 days (Ravi Bhatia, City of Hope National Medical Center, Duarte, CA; personal communication, October 15, 2006). By 12 days, these cultures contain differentiated cells equivalent to an MNC preparation, and we therefore felt confident using this technique for assessment of BCR-ABL and P-Tyr in our experiments. In addition, another group has also successfully developed a flow cytometry assay for measuring total P-Tyr in MNCs and $CD34^+$ CML cells to predict response to imatinib treatment.[4,5]

Based on the combination of techniques used, we do believe that BCR-ABL levels are very significantly lower in MNCs than in $CD34^+$ and $CD34^+CD38^-$ populations. However, we would disagree with the statement that BCR-ABL levels would be expected to be lower in the $CD34^+$ compared with $CD34^+CD38^-$ population due to degradative activity. First, all the $CD34^+$ selected CML samples we used were more than 95% $CD34^+$ after magnetic-activated cell sorting using the CLINIMACS system (Miltenyi Biotec, Bisley, United Kingdom). This was confirmed by flow cytometry after cell sorting, and in some cases the samples were more than 98% purified for $CD34^+$ cells. Therefore, we believe that the degradative activity from contaminating mature myeloid cells in these samples would be negligible. Further, in the report by Maxwell et al,[1] they found that enriching blast cells by Ficoll-Hypaque gradient centrifugation in blast crisis CML samples was sufficient to detect $p210^{BCR-ABL}$ kinase activity.

We would be concerned by the results of Patel et al,[6] who failed to detect BCR-ABL protein expression in $CD34^+$ CML cells. This has never been an issue in our experience. While we find their description of a novel method to overcome the degradative activity of total MNCs using high pH to inhibit an, as yet, unidentified acid-dependent hydrolase interesting, because of the increased degradative activity in total MNCs, we would wish to see it validated in total MNCs as well as $CD34^+$ cells.

We would like to thank Patel et al for highlighting the importance of the degradative activity of total MNCs on BCR-ABL, c-ABL, and P-Tyr levels and will keep this in mind for future Western blotting experiments.

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Conflict-of-interest disclosure: The authors declare no competing financial interests.

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To the editor:

Update on factor V Leiden association with venous thromboembolism in the LITE Study

In a previous article,[1] we reported population-based findings from the Longitudinal Investigation of Thromboembolism Etiology (LITE) on factor V Leiden and risk of venous thromboembolism (VTE). We used a nested case-control design (301 new VTE cases and 630 controls through 1998) from the prospective Atherosclerosis Risk in Communities (ARIC) Study and Cardiovascular Health Study (CHS). The odds ratio, overall, for risk of VTE for carriers of factor V Leiden was 3.67 (95% CI, 2.20-6.12).[1]

We recently extended LITE to additional cases and controls through 2002. In the process, we discovered and corrected an error in the original selection of controls for ARIC that had caused us to oversample participants who had died into the control group for our earlier report. Compared with our published report, the updated sample of 502 cases and 1021 controls yielded an almost identical odds ratio for factor V Leiden of 3.46 (95% CI, 2.20-5.43). Genotype distributions and other odds ratios for factor V Leiden

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