lower end of the 7° axis scatter. Thus, atypical CD4000 platelet plots are not always seen in cryoglobulinemia, although other interferences are usually detected with high efficiency. For comparison, whereas the Technicon H*3 appeared to provide a more immediate visual indication of sample cryoglobulins, large platelet count overestimates were still obtained in most cases. Examination of graphic plots is not used in all laboratories, and its efficiency may be limited when workload is high. Without it, operators depend on the presence of other hematologic abnormalities, instrument flags, or clinical information. Because patients with cryoglobulinemia, both at clinical presentation and during treatment, almost always show abnormal erythrocyte or leukocyte results, morphologic review is likely.

When spurious platelet counts are suggested by graphic or morphologic review, a systematic approach to subsequent investigation can be initiated. Initially, warming samples (>30 min at 37 °C) before instrument analysis generally resolves cryoglobulin interference and enables platelet counting by standard optical or impedance methods. When interference persists after rewarming, other causes should be considered.

When doubt remains regarding the true platelet count, the conventional alternative is manual hemacytometry, but its imprecision is unacceptably high when the platelet count is <20 × 10^9/L. The CD4000 CD61 immunoplatelet count, however, has high precision in thrombocytopenia (4) and, moreover, is not influenced by the presence of cellular or plasma interferences (Fig. 1D). Despite the cost of the test (approximately US $16 in Germany), immunoplatelet counting should be considered in situations such as platelet transfusions and therapeutic interventions with bleeding risk.

Few reports have described platelet count interference by cryoglobulins, although awareness of the potential analytical problems is high. Because the presence of thrombocytopenia was masked in two of the four cases described here, we believe that cryoglobulin interference is an important concern for the clinical laboratory.

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References

Quantitative Reverse Transcription-PCR Comparison of Tumor Cell Enrichment Methods, András Ladányi,1* Richie Soong,2 Karim Tabiti,2 Bela Molnár,1 and Zsolt Tulassey1 (1 Semmelweis University, Second Department of Medicine, Szentkirályi u. 46, 1088 Budapest, Hungary; 2 Roche Diagnostics GmbH, Nonnenwald 2, 82377 Penzberg, Germany; * author for correspondence: fax 36-1-2660816, e-mail ladus@bel2.sote.hu)

The feasibility of detecting disseminated tumor cells through reverse transcription-PCR (RT-PCR) detection of tissue-specific genetic markers in extra-tumor compartments is currently unclear because of conflicting findings on assay sensitivity and specificity between studies [for reviews, see Refs. (1–3)]. One potential source of these assay discrepancies includes the different sample preparations and analysis methods used between laboratories (4). Many studies have used nonenriched (NE) whole blood, although the high population of background cells within these preparations is considered to reduce RT-PCR sensitivity and specificity (5). To improve sensitivity and specificity, some investigators have used density-gradient enrichment (DGE) that is directed toward separating tumor cells in a mononuclear cell fraction from peripheral blood according to cell density (5, 6). Others have tested immunomagnetic separation (IMS) to isolate tumor cells with antibody-coated magnetic beads directed against epithelial cells (7, 8). The lack of standardized procedures and the paucity of quantitative analysis and of studies examining these methods in parallel have made the extent of the influence of sample-preparation methods difficult to determine.

To assess the effect of sample preparation on the RT-PCR-based detection and quantification of disseminated tumor cells, we studied healthy donor blood samples with added colorectal cells. We used a newly developed, quantitative RT-PCR assay for the putative gastrointestinal epithelia-specific marker cytokeratin 20 (CK20) (9–12) to evaluate the sensitivity, specificity, and quantitative PCR effects of DGE, two IMS techniques, and a NE preparation.

For each series, 80 mL of peripheral blood were drawn from the antecubital vein of healthy volunteers with a Vacuette into heparinized tubes (Greiner). To avoid epithelial cells from skin puncture, the first 5 mL were always discarded. We added, per mL, 1000, 100, 10, 1, and 0 HT29 colorectal carcinoma cells to triplicate 5-mL blood aliquots. The 1000 and 100 cells/mL samples were prepared by dilution and the 10 and 1 cells/mL samples by micromanipulation. Each series was processed by either DGE of peripheral blood mononuclear cells with the Ficoll-Histopaque®-1077 (Sigma), IMS with Epithelial Enrich® Ber-EF4-coated microsize beads (Dynal), IMS with a CellSearch Epithelial Cell Enrichment Kit® and anti-EpCAM-coated nanosize beads (Immunocon), or without enrichment. The dilution series were repeated three times for each method with blood from different healthy donors. This gave 9 preparations (triplicates, 3 donors) of each of the 5-cell concentrations and 45 determinations for
each of the 4-sample preparations, although 1 sample (0 cell/mL) of a DGE series was lost. The replicates served to compensate for the sample-to-sample variation of tumor cell loss during the procedure and also the general sampling problem arising from the problematic detection of low copy numbers. This sampling problem could be related to the probability of obtaining a target rather than being able to detect it (13). At the end of each enrichment protocol, samples were resuspended in 300 μL of PBS, and total RNA was isolated with the High Pure RNA Isolation Kit (Roche Diagnostics). Total RNA for the NE series was isolated directly after cell lysis in Stabilization Buffer for Blood/Bone Marrow (Roche Diagnostics). In all cases, the manufacturer’s instructions were rigorously followed.

We performed RT-PCR analysis of RNA samples with the commercially available CK20 Quantification Kit for the real-time LightCycler PCR Instrument (Roche Diagnostics). From the input of a single RNA aliquot, this method provides a relative quantification of CK20 concentrations in a sample that is comparable between PCR runs. The quantities are expressed as the ratio of CK20 to the housekeeping gene porphorynbilinogen deaminase (PBGD) divided by the CK20-to-PBGD ratio in a calibrator sample. Measurement of PBGD is used as a control for RNA loading and integrity, whereas the calibrator provides an interrun normalizer. RT-PCR analysis was performed according to the manufacturer’s instructions with one exception: the usual reagent calibrator was substituted with 10 ng/μL HT29 colorectal cell-line RNA, thereby setting the ratio of CK20 to PBGD in HT29 cells to 10^4. Therefore, samples with a relative ratio of 100 have a CK20-to-PBGD ratio 10^4-fold less than the calibrator. For each enriched sample, 10 μL of RNA, equivalent to 500 μL of whole blood, was analyzed. In the NE series, the 10 μL of analyzed RNA corresponded to only 25 μL of whole blood because of the reduced loading capacity of the procedure.

To examine the impact of sample preparation on CK20 results, we assessed their effects on CK20 sensitivity, specificity, and PCR quantification. The results of this study are summarized in Fig. 1. All methods detected 1000 and 100 cells/mL in 100% of cases, but the detection of 10 and 1 cell/mL samples was inconsistent for all preparation methods. Furthermore, for both IMS techniques, this inconsistency also extended to PBGD detection in the samples with low cell numbers. These results suggest that, rather than sample preparation, the statisti-
cal chance of capturing low cell and/or copy numbers may be a more significant determinant of assay sensitivity. Whether these problems are causes of tumor cell loss during preparation or inconsistent target-copy capture was not examined in this study, and further studies using replicates of the same RNA sample will help to determine this.

Although the detection frequencies of 1–10 cells/mL were greater in the enriched than NE series, the inconsistent detection at these low concentrations and the inequality of loading volumes make it difficult to make direct comparisons. Nevertheless, from the results of this study, it appears for the detection of low cell numbers, analysis of triplicate preparations are required to approach a 100% probability of detection.

In samples without added tumor cells, the CK20 signal was seen with the Immunicon IMS (one in six, 17%) and NE (two in nine, 22%) series, whereas it was undetectable from DGE (zero in eight, 0%) and Dynal IMS (zero in four, 0%) preparations. The improved CK20 specificity of DGE over NE complies with the finding of Jung et al. (5), who found that DGE was essential for the removal of the CK20-expressing granulocyte fraction in unenriched blood. The result of specific CK20 detection after DGE agrees with many CK20 studies (14–16), although this has not been a unanimous finding (17). The ability of IMS to improve CK20 specificity has not been reported previously. These results suggest that Dynal IMS but not Immunicon IMS also provides improved specificity, although caution should be observed when making this conclusion because of the single-case nature of the disparity and the inconsistency of detection at this low concentration. Nevertheless, differences in procedure, bead size, and the epitopes targeted between the two methods could potentially influence the number and type of cells entrapped by the two methods, thus influencing specificity. Further studies performing these IMS procedures on healthy-donor blood samples to characterize CK20-expressing cells will help to clarify this disparity.

To assess the effects of sample preparation on PCR quantification, we restricted our analysis to the 100 and 100 cells/mL samples to avoid complications arising from poor quantitative reproducibility at low copy numbers, (18). Analysis of these samples demonstrated two distinct quantitative results. For DGE and NE samples, the relative CK20 ratio decreased with decreasing cell number, suggesting that results from these preparations may be useful for indicating tumor cell load. In contrast, the relative CK20 ratio for the IMS preparations remained constant at ~10^6 for the same series. The relative ratio of 10^6 in the analyzed IMS preparations suggests that the RNA contained in the samples had the same CK20-to-PBGD ratio as the calibrator. Because the added cells targeted by IMS were HT29 cells and the calibrator in this study was HT29 cell RNA, these results suggest that relative quantification of IMS preparations may be useful for providing an indication of tumor cell identity. Closer inspection of the individual CK20 and PBGD quantities helped to provide an explanation for these two quantitative patterns. Whereas CK20 quantities decreased in proportion to cell number for all preparations, PBGD quantities remained constant for DGE and NE and decreased in proportion with CK20 and cell number for the IMS preparations (results not shown). This result is consistent with detection of PBGD from background nonepithelial cells in DGE and NE preparations and only the isolated epithelial cells in IMS preparations.

In conclusion, these results suggest that sample preparation may play a role in determining assay specificity and relative PCR quantification concentrations. Assay sensitivity at low cell numbers, however, appears to be primarily determined by the probability of tumor cell and/or target gene capture. The inconsistency of detection at these cell numbers and lack of replicates used by most studies may have made a significant contribution to the controversial results published to date. Careful selection of sample preparation methods providing improved specificity and rigorous replicate and quantitative analyses in future studies may provide a better understanding of the significance of RT-PCR-based micrometastasis detection.

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References

Plasma copper is reportedly increased in pregnancy, infections, and inflammation and decreased in corticosteroids (2). Among dozens of chelators, DDC is the one that binds Cu\(^{2+}\) in the presence of ceruloplasmin (7). We confirmed that the present method could be used for plasma containing bilirubin, ascorbic acid, or anticoagulants such as EDTA, oxalic acid, succinic acid, and heparin, although these substances made colorimetric assay of copper difficult (4,9).

ESR measurements were performed on a JEOL JES-FE2XG ESR spectrometer. For comparisons with ESR, copper was also measured by AAS using a Shimadzu AA-6200 flame atomic absorption instrument. A centrifuge with maximum 6000g and a vortex-type mixer were also used. Polypropylene tubes (0.5 mL) were obtained from Eppendorf, pipettes and 0.1–10μL pipette tips were from Gilson, and 20-μL quartz hematocrit capillaries [60 mm long \(\times\) 0.8 mm (o.d.)] were from Drummond Scientific. We could not use glass or plastic hematocrit capillaries because of the strong ESR signals produced by impurities. To seal the capillaries, we used a vinyl plastic putty (CRITOSEAL; Oxford Labware). All chemicals were of atomic absorption grade or biochemical grade from Wako Pure Chemical Ltd. The ultra-pure water (specific resistance, 18 megaohms \(\cdot\) cm) was used. All glassware and plastics were soaked in concentrated HNO\(_3\) or 0.3 mol/L HNO\(_3\), respectively, overnight and rinsed >10 times with ultra-pure water.

On the basis of these findings and using materials and instruments mentioned above, we used the following procedure to prepare plasma and tissue samples for quantification of copper: (a) 5 μL of plasma (or 5 μL of copper calibration solution) was placed in a small tube that could tolerate acid at pH 0 as well as centrifugation at 6000g; (b) 0.75 μL of 1 mol/L HNO\(_3\) was added to the tube and mixed for 10 s in a vortex-type mixer; (c) 0.75 μL of an aqueous solution containing 100 g/L DDC was added and mixed for 10 s (DDC solution stored at 4 °C in the dark is stable for 4 weeks); (d) 6 μL of 1-butanol was added and mixed for 10 s; (e) 3 mg of Na\(_2\)SO\(_4\) was added and mixed for 30 s; (f) the tube was centrifuged at 6000g for 2 min; (g) 5 μL of the top (1-butanol) layer was removed and placed