had no significant effect on the separation or retention times. Lowering the pH of the mobile phase to 5.0 and 4.5 improved the separation between cysteinylglycine and glutathione (Fig. 1B). No interfering peaks were observed in plasma or serum samples for any of the thiols measured. Plasma samples with no internal standard added showed no cysteamine peak.

Calibration curves for homocysteine were linear up to 200 μmol/L for samples prepared in PBS ($r^2 = 0.997$) or in plasma ($r^2 = 0.999$). The limit of detection for homocysteine was 0.16 μmol/L.

The mean recoveries (± SD) of t-homocysteine added to plasma at five different concentrations (3.13–50 μmol/L free thiol), determined on 10 days, were 98.7 ± 2.5% and 96.7 ± 4.7%, calculated with internal and external calibration, respectively. For cysteine, recoveries were 100.6 ± 1.5% and 98.7 ± 3.5%, calculated with internal and external calibration, respectively.

Two plasma specimens containing high total homocysteine (tHcy) concentrations (28.5 and 360 μmol/L) were diluted with PBS 0- to 8-fold. The ratios of the observed/expected values were between 1.0 and 1.1.

The mean intraassay CVs for 20 plasma samples processed in five replicates on 1 day ranged from 1.1% to 1.8% for tHcy and cysteine (tCys). The mean interassay CV of the same 20 plasma samples processed in one replicate on 5 days was 5.6% and 2.4% for tHcy and tCys, respectively. Analyzed over 20 days, the three in-house plasma quality-control (QC) pools showed a variation for tHcy of 6.7% (low pool, 6.5 μmol/L), 5.0% (medium pool, 12.4 μmol/L), and 4.4% (high pool, 29.9 μmol/L) for internal calibration in plasma. The day-to-day variation was higher when internal calibration was performed in PBS or external calibration was performed in plasma. External calibration in PBS produced significantly increased tHcy values for the low and medium QC pools. The slope of the daily calibration curve demonstrated less variation with internal calibration (4.3% vs 8.5% with external calibration).

The correlation between tHcy concentrations for 38 plasma samples covering tHcy concentrations within and greater than the health-related reference range calculated with PBS calibration and with plasma calibration was very good: internal calibration ($r^2 = 1.0000$; slope = 0.9942; intercept = −0.0063), external calibration ($r^2 = 1.0000$; slope = 0.9838; intercept = −1.3309). However, with external calibration the intercept was significantly higher, which especially affected quantification of low homocysteine concentrations.

We performed a direct comparison of the reducing efficiency of TCEP, the newer reducing agent, and tributyl phosphine (TBP), the older reductant (Table 1). Although the relative fluorescence intensities were lower if TBP was used as the reducing agent (approximately two-thirds of the TCEP value), this difference was not apparent in the calculated concentrations of tHcy because of the calibration. For internal calibration in plasma, tHcy concentrations were indistinguishable between TCEP and TBP. Internal calibration in PBS or external calibration in plasma gave the same results if TCEP was used as the reducing agent. The use of TBP led to measured tHcy concentrations up to 20% different from the values obtained with TCEP. Finally, external calibration in PBS led to significantly increased values for TCEP compared with the other three calculation types. For TBP, we found significantly increased values for the low QC pool and significantly decreased values for the high QC pool. The slopes obtained with internal calibration were not significantly different for the two reducing agents: 0.034 (TCEP) and 0.035 (TBP) for calibration in plasma, and 0.032 (TCEP) and 0.031 (TBP) for calibration in PBS.

We studied tHcy and tCys plasma concentrations in 70 healthy subjects (27 men and 43 women; mean age, 43.8 ± 10.6 and 40.7 ± 9.0 years, respectively). The mean tHcy and tCys values were 9.1 ± 1.8 and 298 ± 29 μmol/L, respectively, for men, and 7.8 ± 1.7 and 280 ± 32 μmol/L, respectively, for women. For both thiols, men had significantly higher plasma concentrations than women ($P = 0.0112$ and $P = 0.0287$ for tHcy and tCys, respectively).

In conclusion, the protocol described is a robust, user-friendly, rapid assay, suitable for clinical and pediatric settings. The use of cysteamine as the internal standard significantly improves the precision of this method and overcomes the matrix effect of plasma.

References
p185 is frequently investigated by immunohistochemical techniques. The extracellular domain is shed from tumors in vitro (3) and is detectable in serum by immunoassay (4); however, the considerable overlap between patient and control values renders its estimation of little value in individuals. However, many glycoproteins exhibit carbohydrate changes in cancer (5). Thus a test combining the concentration with changes in glycosylation might be more specific than either alone. We therefore investigated potential glycosylation changes in p185 in the serum of breast cancer patients and controls collected sequentially from a patient series immediately before surgery, using the convenient Lectin ELISA format (6). The protein was specifically captured immunohistochemically and quantified with one of two biotin-labeled lectins binding particular sugars on the protein: wheat germ agglutinin (WGA), with affinity for sialic acid and N-acetylglucosamine, and Concanavalin A (Con A), which binds preferentially to mannose. In preliminary experiments, we investigated these assays in lysates of SKBR3 breast cancer cells, which express p185, and normal lymphocytes. Monoclonal antibody raised against a synthetic peptide sequence of p185 extracellular domain (OM-11-954) was obtained from Cambridge Research Biochemicals. In immunoblotting studies, the antibody detects an ~170-kDa protein in SKBR3 cell membranes blocked by preincubation with synthetic peptide. In immunocytochemical studies using frozen and paraffin-embedded material, staining was blocked by preincubation with the synthetic peptide. The antibody does not recognize the epidermal growth factor receptor. Human breast carcinoma cells, SKBR3, were a gift from Professor B.R. Westley, Pathology Department, University of Newcastle, Newcastle upon Tyne, United Kingdom. The tetramethylbenzidine peroxidase substrate (K-blue) was from Bionostics; all other reagents were from Sigma. Absorbances were measured with a Titertek multiwell spectrophotometer, with an upper limit of detection 3.5.

SKBR3 cells lysates were used as calibrators and were arbitrarily designated such that p185 values were expressed as arbitrary units/L equivalent to protein concentration in mg/L. Competition experiments in serum with synthetic peptide had demonstrated specificity for this sequence in the Lectin ELISA. Microtiter wells (Nunc Maxisorp) were coated with the monoclonal antibody (100 μL) at 0.5 mg/L in 0.1 mol/L carbonate buffer, pH 9.6, and incubated overnight at 4 °C. The solution was then decanted, and the wells were blocked with 10 g/L bovine serum albumin (BSA) solution in deionized water for 1 h at ambient temperature. The wells were washed five times with a solution of 1 mL/L Tween-20 in deionized water, and finally the plates were slapped dry over absorbent paper.

For each assay, 100 μL of lysate calibrator in a solution of 150 mL/L human serum in 0.1 mol/L phosphate buffer, pH 7.4, containing 10 g/L BSA and 1 mL/L Tween-20, or 15 μL of serum sample in 85 μL of the same buffer was added to wells and incubated overnight at 4 °C. The wells were then washed seven times with wash solution containing 2 mL/L Tween-20 and slapped dry. One hundred microliters of biotinylated lectin (2 mg/L) was added and incubated overnight at 4 °C in 0.1 mol/L Tris-HCl buffer, pH 7.6, containing 10 g/L BSA, 1 mmol/L CaCl₂, 1 mmol/L MnCl₂, 1 mmol/L MgCl₂, and 1 mL/L Tween-20. The wells were then washed five times and slapped dry. Streptavidin-HRP conjugate (100 μL/well) in Tris buffer (without divalent cations) and 1 mL/L Tween-20 was added and incubated for 2 h at room temperature. The well contents were then decanted, and the wells were washed five times and slapped dry. K-blue substrate (100 μL) was added to the wells, and the color
was developed for 20 min at room temperature, after which 80 μL of 2 mol/L H₂SO₄ was added and the absorbance was read.

Thirty-five serum samples from breast cancer patients and 14 from women with benign breast growths were examined. The status of the patients and classification of the cancers according to the criteria of Bloom and Richardson (7) were not disclosed before assay. Seven patients (mean age, 61 ± 16.5 years; range, 43–81 years) had cancers classified as grade I; 16 patients (mean age 63 ± 12.8 years; range, 46–80 years) had grade II cancers; and 12 patients (mean age, 64 ± 13.4; range 43–93 years) had grade III cancers. Eight of the cancer patients were <50 years of age. The controls were younger, consisting of patients exhibiting fibrosis and adenosis with a mean age of 36 ± 7.5 (range, 26–45 years).

Dose–response curves for lysate calibrators in diluted serum using WGA and Con A labels indicated relatively high nonspecific binding, which is encountered in lectin assays because lectin binds directly to the IgG coating and serum proteins that absorb to the plastic wells during sample incubation. Little response was observed with WGA in lysates from nondiseased lymphocytes. In contrast, with Con A there was little difference in the response observed between lysates from SKBR3 cells and nondiseased cells.

In the WGA assay (Fig. 1A) results for 11 of the 14 controls were <0.2 units/L, the outliers being 0.4, 1.4, and 1.6 units/L. The mean concentration of these 11 samples was 0.18 ± 0.07 units/L. The 35 cancer samples (and 3 of the controls) had values >0.34 units/L. Thus, all, samples reading <0.34 units/L were from patients without breast cancer.

The results were analyzed using the Wilcoxon rank-sum test, which showed that the results for the controls were significantly lower than the results for the grade I (P <0.05), grade II (P <0.01), and grade III (P <0.01) samples. The differences between grades I and II (P <0.05) and between grades I and III (P <0.01) were also significant.

With Con A (Fig. 1B), there were no significant differences between the controls and any of the cancer groups. The three outliers in the controls were not the same subjects as the WGA outliers. Among the cancer patients, the highest values were in grades II and III samples.

Although the controls were younger, the WGA values for the eight patients less than 50 years of age (0.35–0.95 units/L; ages, 43–49 years) did not overlap with the controls except for the outliers. Furthermore, there was no age correlation of p185 values in the cancer groups; rather, differences were correlated with tumor grade.

Increasing appreciation of the variation of glycosylation in disease has led to interest in tests using lectins as reagents because of their property of binding specific glycans. These results with WGA point to a marked improvement over solely immunochemical detection (4).

In preliminary studies, a response was obtained with WGA in lysates from cancer cells but not from nondiseased lymphocytes. Con A, however, responded to both. We therefore reasoned that because p185 is detectable in serum by immunoassay, application of WGA lectin (but not Con A) might lead to the distinction of the protein in sera from healthy individuals from that of patients.

The results bore this out. With WGA, there was better distinction between sera from breast cancer patients and controls than reported previously. Cancer was excluded in all patients with values <0.3 units/L. The three control outliers made it impossible to conclude that high values represented cancer; nonetheless, 35 of 38 samples with values >0.3 units/L were positive. Any deficiency attributable to high assay background, therefore, did not diminish its ability to provide valuable data.

Although the control women were younger than the patients with breast cancer and galactosylation of some proteins may be related to age, we are unaware of any literature reports suggesting major changes attributable to sialic acid or N-acetylgalactosamine in adult life.

The results for the assay with Con A were similar to those for other immunoassays (4) and were consistent with the lysate studies showing no distinction between the protein from nondiseased lymphocytes and breast cancer cells.

The possible reasons for the control outliers are of interest. No precautions were taken against the presence of heterophilic antibodies that bind to mouse IgG (8) in this preliminary investigation. Because the three Con A outliers were from different patients than the WGA outliers, this possibility would seem to be excluded. The controls may have included patients with other conditions relating to glycosylation changes, such as alcoholism and rheumatoid arthritis (5,9); changes in p185 in such conditions appear to be unlikely, however. Unsuspected cancer of other tissues could not be discounted.

These differences in glycosylation are consistent with reports of altered sialic acid and N-acetylgalactosamine content and branching in other proteins in breast cancer (10,11). This approach, which combines estimation of a protein increased in a proportion of breast cancer patients with a lectin that reveals glycosylation changes, would be anticipated to improve discrimination between cancer and benign growths. The highest WGA values were in grade II cancers, suggesting that N-acetylgalactosamine and sialic acid changes are most significant at this stage. With WGA, differences relate to both qualitative and quantitative changes in the protein; high values would not be revealed unless the appropriate glycan residues were also present.

Additional work is indicated to reduce nonspecific binding, and other lectins might reveal different glycosylation changes. A larger study would include investigation into potential prognostic value. Older controls need to be investigated, although increased p185 values in the youngest cancer cases and lack of age correlation in the cancer groups suggest age mismatch is not the explanation for the differences. On the basis of these preliminary results, WGA might be capable of excluding the diagnosis of cancer. There is no reason to suppose that in other conditions with glycosylation changes p185 itself would be increased; hence the importance of the immunoreac-
tion in regard to disease specificity. However, p185 has been detected in cancers of different organs (4, 12, 13), and diagnostic specificity for breast cancer is unlikely. Application of lectin assays to cancer-associated proteins could be an important contribution to distinguishing breast cancer from benign breast lumps. Thus, in this small sample of subjects, a cutoff point was observed below which all patients were negative; 92% of the patients above this value were positive for cancer.

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References


