sified increased marker concentrations associated with leptomeningeal carcinomatosis as FP test results. When investigating the accuracy of the markers to identify and exclude leptomeningeal carcinomatosis, we classified increased marker concentrations associated with parenchymal brain metastases as FP results. Neither TPS nor TPA was able to discriminate breast cancer metastases involving the meninges from those confined to the brain parenchyma (Table 1). With regard to any CNS metastases, TPS and TPA supplied similar diagnostic information (P >0.1, Fisher’s test), and each marker identified ~80% of patients with CNS metastases.

We found that there was no diagnostic gain when we combined TPS and TPA because none of the patients had increased concentrations of TPS when they had TPA concentrations within reference values, or vice versa; therefore, only one of these markers should be measured. Given the high positive predictive value, the confidence intervals, and the low costs of a cytokeratin test, our data support the view that cytokeratin measurements may be of use as part of a diagnostic protocol for patients suspected of CNS metastases. Thus, breast cancer patients with increased cytokeratin concentrations associated with healthy CNS scan results and the absence of tumor cells in the CSF may have to be evaluated in terms of initiating or continuing further systemic treatment to avoid unnecessary toxicity associated with an ineffective chemotherapy. On the basis of the presented results, we find the data sufficiently encouraging to further investigate the use of cytokeratins as part of a diagnostic strategy in patients suspected of CNS metastases from breast cancer.

Table 1. Diagnostic accuracy of cytokeratin measurements in CSF obtained from breast cancer patients suspected of CNS metastases.

<table>
<thead>
<tr>
<th></th>
<th>Any CNS metastases</th>
<th>Parenchymal brain metastases</th>
<th>Leptomeningeal carcinomatosis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sensitivity, %</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>TPS</td>
<td>85 (68–95)</td>
<td>88 (64–99)</td>
<td>81 (54–96)</td>
</tr>
<tr>
<td>TPA</td>
<td>74 (52–90)</td>
<td>69 (39–91)</td>
<td>80 (44–97)</td>
</tr>
<tr>
<td>TPS + TPA</td>
<td>82 (60–95)</td>
<td>85 (55–98)</td>
<td>78 (40–97)</td>
</tr>
<tr>
<td><strong>Specificity, %</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPS</td>
<td>100b (87–100)</td>
<td>70 (54–83)</td>
<td>66 (50–79)</td>
</tr>
<tr>
<td>TPA</td>
<td>100b (90–100)</td>
<td>83 (62–89)</td>
<td>82 (68–91)</td>
</tr>
<tr>
<td>TPS + TPA</td>
<td>100b (87–100)</td>
<td>81 (64–92)</td>
<td>73 (56–85)</td>
</tr>
<tr>
<td><strong>Positive predictive value, %</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPS</td>
<td>100b (88–100)</td>
<td>54 (34–72)</td>
<td>46 (28–66)</td>
</tr>
<tr>
<td>TPA</td>
<td>100b (80–100)</td>
<td>53 (28–77)</td>
<td>47 (23–72)</td>
</tr>
<tr>
<td>TPS + TPA</td>
<td>100b (81–100)</td>
<td>61 (36–83)</td>
<td>39 (17–64)</td>
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<tr>
<td><strong>Negative predictive value, %</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>TPS</td>
<td>84 (67–95)</td>
<td>94 (79–99)</td>
<td>91 (75–98)</td>
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<tr>
<td>TPA</td>
<td>86 (71–95)</td>
<td>90 (77–97)</td>
<td>95 (84–99)</td>
</tr>
<tr>
<td>TPS + TPA</td>
<td>87 (70–96)</td>
<td>94 (79–99)</td>
<td>94 (79–99)</td>
</tr>
</tbody>
</table>

* Value (95% confidence interval).

References


Adaptation of an Enzyme Immunoassay to Assess Urinary Cotinine in Nonsmokers Exposed to Tobacco Smoke, Denis Roche,1,2 Françoise Callais,1,2 Patrice Reun-Goat,1 and Isabelle Momas1 (1 Hygiene and Public Health Laboratory, Pharmacy Faculty, 75006 Paris, France; 2 Biochemical Laboratory, Georges Pompidou Hospital, 75015 Paris, France; * address correspondence to this author at: Faculté de Pharmacie, 4 Avenue de l’Observatoire, 75006 Paris, France; fax 33-1-4325-3876, e-mail Isabelle.Momas@pharmacie.univ-paris5.fr) Nicotine and its metabolites (1), expired carbon monoxide, and thiocyanates (2) are the most widely used smoking biomarkers. Among these biomarkers, urinary cotinine has been one of the most representative and specific for tobacco smoke exposure (3–5) with regard to active or passive smoking. The methods most frequently used for cotinine quantification are gas chromatography (6) and HPLC (7), coupled or not with mass spectroscopy (8, 9). These methods, however, are difficult to use in large-scale epidemiological studies because they require specialized laboratories. In 1973, Langone et al. (10) proposed the assessment of cotinine by RIA, but RIAs also require specialized laboratories. This last technique was then extended to ELISA (11) and fluorescence polarization immunoassay (12). Recently, an enzyme immunoassay (EIA) that is easier to perform (13) was developed to measure cotinine concentrations between 100 and 200 μg/L, a range that
exceeds concentrations observed in passive smoking. We thus propose an adaptation and automation of this EIA to assess urinary cotinine concentrations <100 µg/L to detect passive smoking. After analytical validation, this technique was applied to subjects exposed and not exposed to environmental tobacco smoke (ETS).

All chemicals, unless specifically noted, were from Prolabo. The cotinine assay (PROCLAIM; Servibio) is a liquid homogeneous EIA based on the competition of a cotinine-labeled enzyme, glucose-6-phosphate dehydrogenase, and the free cotinine in the urine for a fixed amount of cotinine-specific antibody binding sites. Glucose-6-phosphate dehydrogenase activity was determined spectrophotometrically at 340 nm by measuring its ability to convert NAD$^+$ to NADH. Nicotine metabolites and various organic compounds were tested for cross-reactivity by the manufacturer (13); 3'-hydroxycotinine has a 50% cross-reactivity, but at a high concentration of 250 mg/L, a concentration not found in passive smoking.

Cotinine was measured on a Beckman CX7 automated analyzer, although other automated analyzers (open systems) can presumably be used. The analysis rate was 80 samples per hour. Briefly, 7 µL of urine or calibrator was added to 210 µL of reagent A (antibody/substrate reagent). Seventy microliters of reagent B (enzyme conjugate reagent) was dispensed after a 500-s delay and incubated for 196 s. Spectrophotometric measurement was then performed at 340 nm during a 376-s period (the use of a secondary wavelength at 650 nm is recommended). The change in absorbance per minute was calculated, and a four-parameter log-logit function was used to establish the calibration curve. Each series of measurements needs to be calibrated. The calibration curve (Fig. 1) was constructed from a stock 20 mg/L methanolic calibrator cotinine solution (Sigma), with final working solutions of 5, 15, 30, 60, 75, 150, 300, and 600 mg/L in pH 7 buffer. Data are expressed as the mean ± SD.

To increase the sensitivity of the method, urines were concentrated fivefold using SepPack microcolumns (Waters) according to the following procedure: 1 mL of urine was applied to the preconditioned column and then eluted from the column with 0.2 mL of methanol. The eluate was evaporated under a nitrogen stream at 37 °C and redissolved in 0.2 mL of 67 mmol/L phosphate buffer, pH 7. Average recovery was >90%.

The influence of the matrix was studied by comparing the calibration curve in pH 7 buffer and in a fivefold concentrated pooled urinary sample from five nonsmokers not exposed to ETS. Each calibrator was measured in triplicate. The Pearson correlations were $r = 0.99$ in the range 0–600 µg/L and $r = 0.97$ in the range 0–75 µg/L. The slopes of the calibration curves prepared using pH 7 buffer or concentrated urine, although not strictly identical, were not statistically different (Fig. 1), which showed that calibration in pH 7 buffer can, from an epidemiological point of view, be used in routine practice.

The detection limit (mean + 3 SD from 20 determinations of the zero calibrator, pH 7 buffer) was 1.7 µg/L. The limit of quantification (three times the detection limit) was 5.2 µg/L, which actually represents 1 µg/L because the urine was concentrated fivefold. The within-run imprecision (CV) was, respectively, 20%, 19%, 18%, 13%, and 11% at cotinine concentrations of 5, 15, 30, 60, and 75 µg/L ($n = 20$).

The cotinine EIA was applied to urine samples from 53 Paris schoolchildren: 18 exposed to ETS (their exposure, as evaluated by questionnaire, being >10 cigarettes during 48 h before measurement), and 35 not exposed to ETS. This study was approved by the Human Investigation Committee, and informed consent was obtained from all children and parents. Morning urines (14) representing

Fig. 1. Calibration curves in pH 7 buffer and urine matrix. Cotinine concentrations, 0–600 µg/L (A) and 0–75 µg/L (B). ΔDO, change in absorbance at 340 nm.
the exposure over the previous hours (15) were collected and stored at −20 °C until analysis (14). Creatinine was assessed in urine according to a kinetic method adapted from the Jaffé method (16). Urinary cotinine results, calculated taking into account the fivefold urine concentration, were expressed with reference to creatinine values. Results were compared using the Student t-test.

Urinary cotinine values in children exposed to ETS (14.1 ± 11.9 mg/mol of creatinine) were significantly higher (P < 0.001) than those in nonexposed children (5.1 ± 5.4 mg/mol of creatinine). The proposed cotinine EIA thus enables nonsmokers exposed to ETS to be differentiated from those not exposed. The technique is simple, rapid, can be used in any laboratory having an automated analyzer (open system), and can be applied to large-scale studies.

This method can be recommended in epidemiological studies as a control for smoking, an important confounding factor often poorly described in self-administered questionnaires (17).

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References


IgG Autoantibodies against Tissue Transglutaminase in Relation to Antinuclear Antibodies, Gertjan van der Sluijs Veer* and Istoarn Vermes (Department of Clinical Chemistry, Medisch Spectrum Twente Hospital Group, 7500 KA Enschede, The Netherlands; * address correspondence to this author at: Laboratorium Medisch Spectrum Twente, Postbus 50,000, 7500 KA Enschede, The Netherlands; Fax 31-53-487-3075, e-mail labmst@euronet.nl)

The enzyme transglutaminase (tTg; EC 2.3.2.13) catalyzes, among others, the formation of ε-(γ-glutamyl)-lysine bonds between substrate proteins, leading to cross-linked protein polymers (1). The enzyme is synthesized by a broad spectrum of cell types and is widely distributed in human organs (2). Induction and activation of tTg is part of the apoptotic cascade and plays an effector role in this process (3, 4). The enzyme is present in preapoptotic cells and enables the production of a highly cross-linked protein scaffold in apoptotic cells, joining cytoplasmic and membrane proteins and thus maintaining cellular integrity during the formation of apoptotic bodies. This cross-linking of proteins stabilizes the apoptotic bodies and limits the leakage of intracellular components into the extracellular space (1, 5).

Recently, tissue tTg has been shown to be the key autoantigen of the so-called anti-endomysium antibodies (IgA type), which are diagnostic for celiac disease (6). IgG antibodies against tTg (IgG anti-tTG) can be found in other autoimmune diseases in humans (7) and animals (1). There is ample evidence of the relationship between autoimmune disease and dysbalanced apoptosis, but the mechanisms remain hypothetical (8, 9). It has been suggested that a deficiency of the apoptotic removal of autoreactive immune cells breaks the self-tolerance (10). A second hypothesis is that the clearance of apoptotic bodies is deficient, with the result that their contents come into contact with the immune system, leading to autoimmunity as well (11–13). Accordingly, the demonstration of autoantibodies against tTg may be of great interest in the diagnosis and follow-up of autoimmune diseases and may throw a new light on their etiology. We therefore developed a time-resolved fluoroimmunoassay (TRFIA) to measure IgG anti-tTG.

The TRFIA method was as follows. tTg from guinea pig liver (Sigma) was used as antigen. F(ab′)2 fragments from antibodies raised in rabbits against human IgG (Dako A/S) labeled with Eu3+, using the reagent and procedure from Wallac Oy, served as the tracer. Samples (diluted...