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An enzyme immunoassay for determining epidermal growth factor (EGF) in human serum samples using an ultramicroanalytical system

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ABSTRACT
Human epidermal growth factor is a small peptide consisting of 53 amino acid residues, which stimulates cell proliferation and is associated with several human carcinomas. A simple sandwich-type ultramicroELISA assay (UMELISA), based on the advantages of high affinity reaction between streptavidin and biotin has been developed for the measurement of EGF in human serum samples. Strips coated with a high affinity monoclonal antibody directed against EGF are used as solid phase, to ensure the specificity of the assay. The EGF assay was completed in 18 hr, with a measuring range of 39–2500 pg/mL. The intra- and inter-assay coefficients of variation were 4.4–7.3% and 0–5.1%, respectively, depending on the EGF concentrations evaluated. Percentage recovery ranged from 96–104%. Regression analysis showed a good correlation with the commercially available Human EGF Immunoassay Quantikine® ELISA kit (n = 130, r = 0.92, P < 0.01). The analytical performance characteristics of our UMELISA EGF endorse its use for the quantification of EGF in human serum samples.

KEYWORDS
epidermic growth factor; ultramicroELISA; CIMAVax-EGF®; SUMA technology

Introduction
Human epidermal growth factor (hEGF) is a small peptide consisting of 53 amino acid residues with a molecular weight of about 6.2 kDa. As a mitogen, hEGF stimulates cell proliferation and differentiation by binding to the extracellular domain of the EGF receptor (EGFR) dimer. Overactivation of the hEGF signaling pathway is associated with several human carcinomas. This overactivation may cause uncontrolled cell growth. In a clinical context, hEGF is a popular target for anti-cancer therapies because of its potent growth-stimulating effect on a variety of cancer cells that overexpress EGFR on the cell surface, including non-small cell lung cancer (NSCLC) cells.
NSCLC is one of the most common malignant diseases with a high mortality rate worldwide. Only 30% of patients can be treated surgically. For the majority of patients, traditional treatment options have modest efficacy.\cite{3} In Cuba, this cancer type is also the malignant disease with highest incidence, being the leading cause of cancer mortality in the country.

CIMAvax-EGF\textsuperscript{®} is a therapeutic vaccine recently registered in Cuba for advanced NSCLC.\cite{4-13} It is an active immunotherapy, which prevents binding of endogenous hEGF to its receptor, by inducing anti-EGF antibodies, clearing hEGF from circulation, thereby reducing tumor size or preventing its progression.\cite{5} The vaccine is safe even during long-term use and provides survival advantage for patients with advanced NSCLC. However, not all patients respond to it. For this reason it is crucial, knowing that hEGF concentration in sera of NSCLC patients treated in first line with chemotherapy is a biomarker predictor of response,\cite{14} to identify the subset of patients eligible for treatment, to achieve the individualization of therapy and the improvement of overall survival and clinical management.

The aim of this work was to design, develop, and validate an ultramicroELISA for measuring hEGF in human serum samples suitable, among others, for the stratification of NSCLC patients by their hEGF concentrations, to decide about the treatment or not with CIMAvax-EGF\textsuperscript{®} vaccine.

**Materials and methods**

**Chemicals**

All reagents were of analytical grade and solutions were prepared in distilled water. Human recombinant EGF was obtained from the Center for Genetic Engineering and Biotechnology (Havana, Cuba). Human IL-2, murine IL-2, murine IL-4, interferon-α, and interferon-γ were purchased from MP Biomedicals (USA). Na\textsubscript{2}CO\textsubscript{3}, NaHCO\textsubscript{3}, NaN\textsubscript{3}, Biotin, Streptavidin, Glutaraldehyde, HCl, Tween 20 and 4-methylumbelliferyl phosphate were from Sigma (USA). Na\textsubscript{2}HPO\textsubscript{4}, KCl, KH\textsubscript{2}PO\textsubscript{4}, TRIS, Diethanolamine, ZnCl\textsubscript{2}, and MgCl\textsubscript{2} were from Merck (Germany). BSA and Alkaline phosphatase were from Roche (Germany). Sacarose was purchased from Fluka (USA).

**Anti EGF monoclonal antibodies**

CBEGF-1 and CBEGF-2 monoclonal antibodies were obtained from the Center for Genetic Engineering and Biotechnology (Sanctí Spíritus, Cuba). These antibodies are highly specific for human EGF.\cite{15-18}
**EGF-free human serum**

hEGF-free serum was prepared by immunoaffinity purification. CBEGF-1 was immobilized in a cyanogen bromide activated sepharose column. Serum’s flux rate was 5 mL/hr.

**Equipment and accessories**

The Ultra Micro Analytical System (SUMA, acronym in Spanish) technology was used. The system, manufactured by the Immunoassay Center, Havana, Cuba, includes reagents and instrumentation. The equipment comprises a fully computerized spectrofluorimeter-photometer for automatic reading, quantification, validation, and interpretation of results (PR-621) and a plate washer (MW-2001). The reagents arecased in kits in quantities enough for 288 ultramicrotests (10 µL volumes of samples and reagents).

**Immobilization of CBEGF-1 antibodies**

White opaque 96-well polystyrene ultramicro-ELISA plates (Tecnosuma International SA, Havana) were coated with 17–18 µL/well of CBEGF-1 monoclonal antibody at 6 µg/mL in 0.05 mol/L sodium carbonate/bicarbonate buffer, pH 9.6 containing 3 mmol/L of NaN₃. The plates were placed during 18 hr in a humid chamber at room temperature (20–25ºC) and washed with 25 µL/well of 0.15 mol/L of phosphate-buffered saline solution (PBS) containing 1 mol/L of NaN₃ and 1.1 mmol/L of Tween 20. Buffer was removed and the plates were treated with 18 µL of a solution containing 14.5 μmol/L of BSA, 0.15 mol/L of sacarose, and 0.45 mmol/L of Tween 20, to increase their stability. Finally, the ultramicro-plates were dried and conserved with desiccant in polyvinyl sealed bags at 2–8ºC, where they are stable for at least 12 months under these conditions.

**CBEGF-2 biotin labeling**

CBEGF-2 was biotinylated by Bayer and Wilchek’s method, described in 1996.[19]

**Enzyme conjugate**

The streptavidin-alkaline phosphatase conjugate was prepared by a modification of the glutaraldehyde method, as previously described.[20]

**Standards, controls, and samples**

The calibration curve was prepared using hEGF free serum. Recombinant EGF was used for the preparation of 6 standards with concentrations
between 0–2500 pg EGF/mL of serum. The curve was calibrated against international standard of EGF 91/530 from NIBSC. EGF standards were filtered, lyophilized and stored at −20°C. Under these conditions they are stable for at least 12 months.

Controls with a well-known EGF concentration that embraced a wide range of standard curve activity were used for the assay evaluation. Controls were prepared adding known EGF quantities to EGF free serum. Additionally, 130 serum samples, corresponding to 73 healthy subjects and 57 patients with lung cancer were evaluated in duplicate.

**Ultramicro enzyme-linked immunosorbent assay (UMELISA)**

For the measurement of hEGF concentrations, 5 μL of standards, controls and samples were diluted with 45 μL of the biotinilated CBEGF-2 monoclonal antibody in TRIS 0.05 mol/L with 0.15 mol/L of NaCl, 0.03 mol/L of NaN₃, 0.025 mmol/L of ZnCl₂, 1.989 mmol/L of MgCl₂, 0.45 mmol/L of Tween 20, 0.15 mmol/L of BSA, sheep serum 0.7% (W/V), and mouse serum 0.3% (W/V). 30 μL of each diluted specimen were transferred into the wells of the reaction opaque polyestirene ultramicroplates coated with CBEGF-1 monoclonal antibody. The immunological reaction occurred for 16–18 hr at 37°C in a humid chamber and then, the plates were washed 4 times with 30 μL of 0.37 mol/L of Tris-HCl solution, pH 8 containing 3.76 mol/L of NaCl, 1.1 mmol/L of Tween 20, and 76.9 mmol/L of NaN₃ (30 μL per well). 10 μL of streptavidin- alkaline phosphatase conjugate were added and the plate was incubated at 37°C in a humid chamber for 30 min. Afterwards the plate was washed again as described above. The fluorogenic reaction was performed by adding 10 μL of the substrate solution pH 9.6, containing 5.07 mM of 4-methylumbelliferil phosphate, 0.92 mol/L of diethanolamine-HCl, 0.7 mmol/L of MgCl₂, and 7 mmol/L of NaN₃. The ultramicroplates remained at room temperature in a humid chamber for 30 min. Finally, the fluorescence was measured automatically in the spectrofluorimeter-photometer reader. Reading values in fluorescence units (FU) were directly transferred to the computer after measurement, where automatic validation and interpretation of the results were done, using a software developed specially for UMELISA EGF applicability in the stratification of patients, for eligibility to treatment with CIMAvax-EGF® vaccine. Samples with hEGF concentrations above the settled cut-off value were highlighted as the corresponding patients selected for treatment. The settled cut-off value was previously obtained from retrospective data of hEGF concentrations in sera from NSCLC patients before they were treated with CIMAvax-EGF® in a clinical trial study. [14]
Correlation with a commercial colorimetric assay

Correlation with a commercially available Human EGF Immunoassay Quantikine® ELISA kit was studied using 130 human serum samples. The slope, y-intercept of the line-of-best-fit, and regression statistics for the comparison by our ultramicroELISA results versus those obtained on the same serum samples using the Quantikine® ELISA, were obtained using Excel 2000 software.

Statistical analysis

For all datasets, two-tailed Student t-tests (Microsoft Excel) were employed to determine statistical significance, which was considered present when $P < 0.01$. Pearson correlation and concordance correlation coefficients ($r$ and $\rho$ values) were used to calculate correlation between serum EGF levels using UMELISA and Quantikine® ELISA Kit.

Results

Standard curve

A typical standard curve and a precision profile obtained with the UMELISA EGF are shown in Figure 1. The calibration curve was built automatically by the software, by plotting in a graph the mean fluorescence values for each standard (y axis) and their expected theoretical concentrations (x axis). The software automatically interpolates the reading values of the samples in the graphic, and returns the corresponding concentration values in pg of EGF/mL of serum. The FU is directly proportional to the amount of EGF present in the sample. EGF calibrators were measured twofold on 6 consecutive days. The precision profile for fluorescence standard replicates gave coefficient of variation (CV) values between 6% and 9%.

![Figure 1. Typical standard curve and precision profile obtained with the UMELISA EGF.](image-url)
Limit of detection (LOD) and limit of quantitation (LOQ) were determined according to CLSI Guidelines. The zero standard and 3 spiked serum samples (35, 70, and 140 pg/mL) were analyzed 64 times to estimate LOD and LOQ. Means, SDs, CVs, and the slope were calculated. LOD, defined as the lowest amount of hEGF in a sample that can be detected with a probability of 95%, was 10 pg/mL, and it was calculated by: LOD = limit of blank (LOB) + 3×SDs (where LOB=95 percentile of zero standard measurements; SDs = Pooled SD estimate of measurements on spiked samples).

LOQ, the lowest amount of hEGF in a sample that can be quantitatively determined in our UMELISA with an acceptable precision and a suitable accuracy, was 39 pg/mL. The spiked samples were studied to estimate the lowest concentration in the assay for which the CV is less than 20%, and the measured value is within 20% (80–120%) of true value. The LOQ was calculated by this formula: LOQ = LOB + 10× (SDs/slope).

**Precision and recovery**

Precision and recovery were determined following CLSI Guidelines. For evaluating precision, samples representing three different levels of hEGF concentrations were assayed. The repeatability, standard error of the daily means, and within-laboratory precision were calculated from estimating hEGF in 2 replicates of each sample, for 30 operating days. The intra and inter-assay coefficients of variation ranged between 4.4–7.3% and 0–5.1% respectively, depending on the hEGF concentrations evaluated Table 1.

Table 2 shows the results obtained when controls were evaluated. Analytical recovery was performed using pooled serum obtained from healthy volunteers. Four serum controls were prepared adding known amounts of recombinant EGF. The results were averaged of five replicates. Percentage recoveries ranged between 96–103% with a mean value of 98.7 ± 2.9%.

**Linearity of sample dilutions**

Samples representing 4 different levels of hEGF concentration were diluted up to 16 times and assayed in the UMELISA EGF. The linearities obtained are shown in Figure 2. Corrected concentrations were in the range of ±4.74% of the expected concentrations, obtained for the non-diluted samples.

**Specificity**

Cross reactions to some chemokines that are similar to hEGF were tested in human serum by replacing hEGF with concentrations up to 50 ng/mL of human IL-2, murine IL-2, murine IL-4, interferon-α, interferon-γ, murine GM-CSF supernatant, and murine IFN-α4 supernatant. The latter two products were
supplied by the Center of Molecular Immunology. Specificity was satisfactory due to the fluorescence of such chemokines did not exceed that corresponding to a hEGF concentration of 1.2 pg/mL.

### Table 1. Precision for the UMELISA EGF.

<table>
<thead>
<tr>
<th>Sample EGF concentration (pg/mL)</th>
<th>Repeatability</th>
<th>Within-laboratory precision</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$S_r$ CV (%)</td>
<td>$S_{dd}$ CV (%)</td>
</tr>
<tr>
<td>477.2</td>
<td>20.9</td>
<td>4.4</td>
</tr>
<tr>
<td>947.1</td>
<td>54.7</td>
<td>5.8</td>
</tr>
<tr>
<td>1780.6</td>
<td>130.3</td>
<td>7.3</td>
</tr>
</tbody>
</table>

$S_r$, repeatability standard deviation; $S_{dd}$, between-day standard deviation; $S_T$, within-laboratory standard deviation; CV (%), Coefficient of variation = (SD/Mean) x 100.

### Table 2. Analytical recoveries of EGF measured by UMELISA EGF.

<table>
<thead>
<tr>
<th>Expected (pg/mL)</th>
<th>Serum controls</th>
<th>Determined (pg/mL)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>225</td>
<td>226</td>
<td></td>
<td>100.4</td>
</tr>
<tr>
<td>450</td>
<td>432</td>
<td></td>
<td>96.0</td>
</tr>
<tr>
<td>900</td>
<td>917</td>
<td></td>
<td>101.9</td>
</tr>
<tr>
<td>1800</td>
<td>1735</td>
<td></td>
<td>96.4</td>
</tr>
</tbody>
</table>

### Figure 2. Linearity of sample dilutions.

130 human serum samples were evaluated using our assay and the commercial colorimetric Human EGF Immunoassay Quantikine® ELISA kit. Results of correlation analyses are shown in Figure 3. 56.2% of the samples ($n = 73$) corresponds to supposedly healthy individuals and were taken in the Vedado Blood Bank. 43.8% of the samples ($n = 57$) corresponds to patients with NSCLC at different stages of the disease and were supplied by the Department of Clinical
Trials in the Center of Molecular Immunology. The commercial EGF assay was performed according to the manufacturer’s instructions.

The outcomes of both assays were highly correlated by the Pearson correlation ($r = 0.92$, $P < 0.01$), where the slope, y-intercept and linear correlation coefficient were 1.0274, 0.214, and 0.8514, respectively. Values of Pearson correlation coefficient above 0.7 signify the existence of a high positive correlation and values above 0.9 indicate that there is a very high positive correlation between the variables analyzed.\[24\] The absolute agreement between assays was quantified using the concordance correlation. The concordance correlation coefficient ($\rho_c$) combines precision and accuracy measurements. Values greater than 0.85 indicate the existence of a high correlation between the two methods evaluated.\[25\] The results showed high overall concordance between the UMELISA EGF and Human EGF Immunoassay Quantikine® ELISA assay ($\rho_c = 0.91$).

**Discussion**

Biomarkers are a characteristic that can be objectively measured and evaluated as an indicator of normal biological or pathogenic processes or pharmacological responses to a therapeutic intervention and they are increasingly used in the management of cancer patients\[26\]. There exist prognostic and predictive biomarkers. The latter are defined as markers which can be used to identify subpopulations of patients who are most likely to respond to a given therapy. Predictive biomarkers allow selecting the therapy with the highest likelihood of efficacy to the individual patient.\[27,28\]

Retrospective correlation studies, using data from Phase II and III clinical trials carried out with the CIMAvax-EGF® vaccine in Cuban patients with advanced NSCLC (stages IIIb/IV), have demonstrated the prognostic value of hEGF concentrations (pre-treatment, and during treatment) for the overall survival of patients. Survival advantage occurs mainly in patients having high

![Figure 3. Correlation between EGF serum concentrations determined by Colorimetric Human EGF Immunoassay Quantikine® ELISA kit and UMELISA EGF.](image)
hEGF concentrations before treatment. The data also suggest that hEGF levels may be a predictive biomarker for treatment with CIMAvax-EGF*.\textsuperscript{[11,14,29]} That is why it is very important to determine hEGF levels prior vaccination in NSCLC patients, in order to treat only those that will be benefited from the therapy.

There are in the market several immunoassays for EGF estimation mainly in serum, plasma, and urine samples, based on different principles.\textsuperscript{[30–34]} The majority of them fit to our intended purpose of EGF estimation, but they are expensive, and our health programs are conceived to reach a high coverage. In this scenario, it was mandatory to develop our own assay.

In the 1980s, the development of a technology began in Cuba that allowed the study of a great number of samples with a very low cost. Starting from these initial efforts the Ultra Micro Analytic System (SUMA) and the ultramicroELISA (UMELISA) techniques were developed. UMELISAs combine the high sensitivity of the current microELISA tests with the use of ultra-micro-volumes of samples and reagents, thus reducing costs. The SUMA technology is a flexible platform, suitable for developing health programs based on active screening.\textsuperscript{[35]}

Having this SUMA technology and about 30 years of experience with its use, we developed and validated the UMELISA EGF kit for EGF estimation in human serum samples.

The solid phase sandwich-type UMELISA described in this article exhibits similar characteristics to other commercially available assays, in terms of precision, accuracy, and dynamic range, thus, making it useful for the quantification of EGF in human serum samples. The estimated LOD and LOQ are also in the range of those values for ELISAs with similar principle.

The assay exhibited good within-run and between-run reproducibilities in the concentration range from 477–1781 pg/mL, and the sample’s linearity demonstrates its accuracy.

Finally, the results obtained in the test for agreement with the Quantikine® ELISA Human EGF Immunoassay kit, used in all previous studies with CIMAvax-EGF* vaccine, showed a high correlation, so the kit guarantees the continuity of our estimations and studies with the vaccine.

The kit enlarges the product portfolio that SUMA technology offers to the Cuban Health System, making more affordable the treatment and follow-up of our oncology patients. The UMELISA EGF is called to be the companion diagnostic kit for the Cuban CIMAvax-EGF* vaccine for the measurement of EGF in human serum samples from oncology patients, where hEGF levels may be quite variable in a broad range of values.

The detection and quantification limits obtained are more than adequate for the purpose of stratification of NSCLC patients, before treatment with the vaccine. However, cut-off values for any other epithelial cancer, for which EGF concentrations might also be considered a predictor biomarker for CIMAvax- EGF*, will need to be established in the future.
Just now there is an ongoing clinical trial with CIMAvax-EGF® for the validation of prognostic and predictive values of EGF in NSCLC patients, and also a translational study in progress, to evaluate the possible biomarker value of this molecule in other epithelial cancers different to NSCLC.

In summary, the UMELISA EGF as part of SUMA technology is a simple, precise and accurate assay that can be used to quantify EGF in sera from normal subjects, and also in patients with different pathologies that displace the levels of this molecule from the normal values. This is the case of NSCLC and some neurological disorders such as depression, autism, schizophrenia and Alzheimer disease.

The kit allows the processing of many samples at low cost and guarantees efficiency and reliable results.

Acknowledgment

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