Immunoradiometric Assay for $\alpha\gamma$ and $\gamma\gamma$-Enolase (Neuron-Specific Enolase), with Use of Monoclonal Antibodies and Magnetizable Polymer Particles

Elisabeth Paas and Kjetil Nustad

Monoclonal antibodies were raised against neuron-specific enolase, $\gamma\gamma$-enolase, and used in an immunoradiometric assay (IRMA), with mono-disperse magnetizable particles as the solid assay phase. The assay's sensitivity was 0.4 $\mu$g/L and the interassay coefficient of variation was <5% in the working range from 0.4 to 170 $\mu$g/L. Compared with our radioimmunoassay based on polyclonal antibodies, the incubation time is shorter, and precision and sensitivity are improved. The IRMA also improved detection of neuron-specific enolase in sera from patients with lung cancer without a concomitant change in measured enolase in the reference population. The better sensitivity of the IRMA results from its ability to measure $\alpha\gamma$ and $\gamma\gamma$-enolase with equal response. Ninety percent of the small-cell lung carcinoma patients (36 of 40) had increased values before treatment, compared with 7% of non-small-cell lung carcinoma patients (8 of 114).

Additional Keyphrases: lung cancer • tumor markers

The glycolytic enzyme enolase (2-phospho-D-glycerate hydro-lyase, EC 4.2.1.11) exists as several dimeric isoenzymes ($\alpha\alpha$, $\alpha\beta$, $\alpha\gamma$, $\beta\beta$, and $\gamma\gamma$) composed of three distinct subunits, $\alpha$, $\beta$, and $\gamma$ (1). Three isoenzymes are found in human brain: $\alpha\alpha$, $\alpha\beta$, and $\gamma\gamma$ (2). The $\alpha\gamma$- and $\gamma\gamma$-enolase isoenzymes are known as neuron-specific enolase (NSE) because they initially were found in neurons and neuroendocrine cells (3, 4). They also exist in other cells such as blood cells (5, 6), rendering the name neuron-specific enolase rather misleading.

NSE is a valuable tumor marker for cancers of neuroendocrine origin (7), especially for small-cell lung cancer (SCLC) (8–11) and neuroblastoma (12). Hitherto NSE has been measured in serum by radioimmunoassays based on polyclonal antibodies (9, 13–17). Because of its value as a tumor marker, we have generated monoclonal antibodies against human NSE. The new immunoradiometric assay (IRMA) improves the analytical performance of the assay, as well as its value in the clinical management of cancer patients. This is demonstrated in a comparison with a radioimmunoassay (RIA). Both assays measure $\gamma\gamma$-enolase both as $\alpha\gamma$- and as $\gamma\gamma$-enolase. The reason for the improved performance is the increased sensitivity of this IRMA for $\alpha\gamma$-enolase.

Materials and Methods

Purification and radiolabeling of NSE. NSE was purified from human brain as described previously (18). The three isoenzymes $\alpha\alpha$, $\alpha\gamma$, and $\gamma\gamma$-enolase were separated by anion-exchange chromatography. Only the $\gamma\gamma$-isoenzyme was purified to homogeneity, with a specific activity of 91 U/mg. The enzyme was iodinated by the Bolton and Hunter method (19) and stored in an equilibrium solution of ethylene glycol and assay buffer (0.05 mol/L Tris HCl (pH 7.5) containing 1 g of bovine serum albumin and 0.2 g of sodium azide per liter).

Preparation of monoclonal antibodies. Female Balb/c mice (Charles River U.K. Ltd., Kent, England), six to 12 weeks old, were immunized by intraperitoneal injection of 15 $\mu$g of purified $\gamma\gamma$-enolase in Freund's complete adjuvant. A booster dose of 25 $\mu$g was given similarly five months later. The fusion was performed two months later, after four days of final intraperitoneal booster injections of NSE in phosphate-buffered isotonic saline (0.01 mol/L sodium phosphate buffer, pH 7.0, containing 0.15 mol of sodium chloride per liter) as described by Stæhlí et al. (20). The final injections consisted of 30 $\mu$g on the first day and 225 $\mu$g on the subsequent days. The spleen cells were fused with NS0 mouse myeloma cells (21) in a 4:1 ratio of cell numbers, in the presence of polyethylene glycol M, 4000 (Merck, Darmstadt, F.R.G.). The cells, in Dulbecco's Modified Eagle's Medium (GIBCO Ltd., Paisley, Scotland), supplemented with hypoxanthine, aminopterine, thymidine, and a 150 mL/L solution of fetal calf serum (Bio-Rad Labs., Richmond, CA) were seeded into 96-well tissue culture plates (Nunc, Copenhagen, Denmark), containing 10$^4$ mouse peritoneal macrophages per well.

Screening for anti-NSE antibodies. Affinity-purified, species-specific sheep antibodies to mouse immunoglobulins (SAM), 1 $\mu$g per well in 100 $\mu$L of phosphate-buffered isotonic saline, were coated onto 96-well polystyrene chloride microtiter plates (Dynatech Lab., Inc., Alexandria, VA) for at least 24 h at 4 °C. Excess SAM was removed shortly before use. The plates were washed three times with water (purified by reverse osmosis) before we added 20 $\mu$L of hybridoma supernate by use of a transplate cartridge (Costar, Cambridge, MA) and 100 $\mu$L of assay buffer and then incubated for 1 h at room temperature. After three washings of the plates, we added 100 $\mu$L of $^{125}$I-labeled NSE in assay buffer, followed by another incubation for 1 h. The plates were washed three times before the wells were cut, and their radioactivity was counted in a gamma counter. Supernates from antibody-producing hybridomas were re-assayed by displacement analysis, i.e., serially diluted and incubated with $^{125}$I-labeled NSE with and without 10 ng of non-labeled NSE. We separated antibody-bound radioactivity from free radioactivity by

Central Laboratory, The Norwegian Radium Hospital, Montebello, N-0310 Oslo 3, Norway.

1 Nonstandard abbreviations: NSE, neuron-specific enolase; IRMA, immunoradiometric assay; SAM, sheep anti-mouse antibodies; SCLC, small-cell lung cancer; and Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol.

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using SAM bound to acrylate polymer particles (CA-031-A; DynoParticles, Lillesbiocm, Norway) (22). The most promising hybridomas were subcloned twice by limiting dilutions in the presence of 10^4 mouse macrophages per well or in medium containing 100 mL of human epithelial cell supernate per liter.

Production and purification of monclonal antibodies. Hybridoma cells were injected into mice that had previously been primed with pristane (2,6,10,14-tetramethylpentadecane; Aldrich-Chemie, Heidenheim, F.R.G.). Ascites fluid was frozen at -40 °C immediately after the cells were separated from it. Before purifying the antibodies on Protein A-Sepharose (Pharmacia, Uppsala, Sweden) essentially as described by Ey et al. (23), we treated the ascites fluid with "Frigen" (1.1,2-trichlorotrifluoroethane; 113 TR-T; Hoechst, Frankfurt am Main, F.R.G.) to remove lipid. The purified monoclonal antibodies were sterile-filtered (Acrodisc; Gelman Sciences Inc., Ann Arbor, MI) and stored at 4 °C in sodium phosphate/citrate buffer (0.1 mol/L each, pH 7) containing 0.1 mol of NaCl per liter.

Coupling of antibody to magnetizable particles. A new magnetizable polymer particle with primary hydroxyl groups was used as the solid phase. Its properties and details of how the antibodies were immobilized will be presented elsewhere. Twenty milligrams of antibody was coupled to 1 g of particles activated with p-toluene sulfonyl chloride ("Dyna-beads M280"; Dynal AS, Oslo, Norway). The activated particles were washed once in water before use. The coupling was performed in borate buffer (0.1 mol/L, pH 9.5) with use of 50 mg of the particles per milliliter. The antibody–particle solution was rotated at 37 °C for 20 h. Further rotation for 2 h in ethanolamine (0.1 mol/L, pH 7.0) containing 1 mL of Tween 20 surfactant per liter was used to block the remaining activated tosyl groups. The particles were then washed by rotation in Tris hydrochloride buffer (0.1 mol/L, pH 7.0) containing 1 g of bovine serum albumin and 1 g of Tween 20 per liter. Finally, the particles were washed and stored in assay buffer. Coupling efficiency was determined by measuring unbound protein in the supernate after the initial coating of the particles.

"Fast protein liquid chromatography." Samples were diluted in the equilibration buffer (0.02 mol/L Bistris buffer, pH 6.3) and applied to a 0.5 × 5 cm "Mono Q" column (Pharmacia system; Pharmacía, Uppsala, Sweden). The column was eluted with a KC1 gradient from 0 to 0.5 mol/L in the same buffer.

Enzyme activity assay. Enolase activity was measured spectrophotometrically at 340 nm by coupling the reaction with pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.27), resulting in oxidation of NADH (24).

IRMA. Standards, 0 to 170 μg/L, were prepared by adding purified γγ-enolase to a pooled specimen of blood-bank sera with undetectable concentrations of NSE as measured by the IRMA. Antibody E17 was iodinated by the modified lodo-gen (1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril) method (25). Solid-phase antibody E21 (1.4 μg on 0.4-mg particles) and 125I-labeled E17 (10 ng, corresponding to about 100 000 counts/min) in 100 μL of assay buffer supplemented with, per liter, 10 g of bovine serum albumin and 10 mL of normal mouse serum were incubated with 50 μL of standard or sample. The mixture was agitated for 2 h at room temperature, then separated on magnetic racks (Amersham, England). Excess 125I-labeled antibody was removed by washing twice with 0.5 mL of phosphate-buffered isotonic saline supplemented with 1 mL of Tween 20 per liter. Bound radioactivity was counted (LKB/Wallac 1277 Gammamaster; Wallac OY, Turku, Finland), and standard curve and sample values were calculated by use of the LKB/Wallac 1224 Riacalc program.

Radioimmunoassay. NSE was radioimmunoassayed with use of sheep antiserum against γγ-enolase and 125I-labeled purified enzyme (16). The standards were the same as those used in the immunoradiometric assay.

Patients and reference population. All lung-cancer patients in this study attended our hospital. The patients were staged as having limited disease when only one hemithorax and mediastinal lymph nodes were affected or as extensive disease when it had spread outside this region. The staging procedure included physical examination, chest roentgenogram, bronchoscopy, and computed tomographic scans of bone, liver, and brain.

The reference population consisted of 83 blood donors and 100 patients with non–carcinomatous diseases.

Serum was collected and stored at -40 °C until analysis. Serum samples with substances binding murine antibodies were picked up by an interference assay described by Boscato and Stuart (26).

Results

Monoclonal antibody production. One fusion between NSO myeloma cells and spleen cells from one immunized mouse gave 27 clearly positive wells by the initial screening. We re-assayed the 10 most interesting clones by displacement analysis to choose suitable antibodies for an IRMA. Three hybridoma cell lines performed particularly well: E17, E19, and E21. These were subcloned twice, expanded, and injected into mice for ascites production.

Establishment of optimal assay conditions for the IRMA. Practically no response was obtained when the same antibody was used on the solid phase and as labeled antibody. All six possible pair combinations of the antibodies E17, E19, and E21 were evaluated in two-site IRMAs, as soluble or solid-phase antibody, respectively. All combinations gave similar standard curves when purified γγ-enolase was used as the standard. However, pathological sera from SCLC patients showed a marked difference in NSE values obtained with the various assays (see below). The most relevant clinical information was obtained by an assay that recognized αγ and γγ-enolase equally well. The isoenzyme fractions as well as pooled patients’ sera were used for this optimization. Maximal signals from αγ-enolase were obtained with E21 as the solid-phase antibody and E17 as the iodinated antibody, when antibodies and samples were added simultaneously. Equilibrium with αγ-enolase was obtained after a 2-h incubation, whereas γγ-enolase gave maximal binding of radioactivity after 1 h. Other antibody combinations, including two-step assays or delayed addition of solid-phase antibody, required longer incubation for results to be comparable.

Figure 1 shows a typical standard curve and precision profile. The assay has a sensitivity (0 + 2 SD) of 0.4 μg/L and an interassay CV <5% in the working range from 0.4 to 170 μg/L. The assay response reached a maximum at 4000 μg/L and remained at this plateau, even at a concentration of 10 000 μg/L. When αγ- and γγ-enolase were serially diluted, both response curves were linear and almost identical (not shown).

Non-specific binding increased from 0.2% to 0.8% after the labeled E17 antibody was stored for six weeks. Sera
those obtained with our RIA in most of the sera (Figure 2). We studied this discrepancy by analyzing the three isoenzymes αα-, αγ-, and γγ-enolase, using samples of equal enzyme activity. The three fractions were analyzed by the two immunological assays (Table 1). αα-Enolase was not detected by either of the two assays, whereas αγ-enolase gave a signal that was fivefold higher in the IRMA than in the RIA. The two assays measured γγ-enolase with similar response. The αγ-enolase and γγ-enolase were detected with equal response in the IRMA.

Distribution of αγ- and γγ-enolase isoenzyme in patients’ sera. Sera from two patients with increased NSE values that varied considerably with respect to the IRMA/RIA ratio were fractionated on a Mono Q ion-exchange column, and the NSE in each fraction was measured by IRMA (Figure 3). The position of the three isoenzymes was determined in separate chromatographies. Both patients’ sera contained immunoreactive αγ- and γγ-enolase but in very different proportions. The highest αγ-enolase/γγ-enolase ratio was seen in the sample having the highest ratio between NSE values as measured by IRMA and RIA.

NSE-IRMA values in patients with lung cancer. The optimized IRMA was used on a larger number of sera from patients with SCLC and non-SCLC (Figure 4). All samples were collected before treatment. If we used a reference limit of 10 μg/L, about 7% of all untreated non-SCLC patients had increased values, whereas 90% of the untreated SCLC patients had increased values. All patients with extensive disease had increased values, whereas values for 80% of the patients with limited disease were increased at the time they were diagnosed.

Tissue sections from one NSE-positive patient with non-SCLC (NSE 157 μg/L) were examined immunohistochemically. Morphologically, the tumor cells appeared as squamous cells, although somewhat atypical. All cells stained positively with antibodies against NSE (not shown).

Discussion

Assay of NSE in serum is of value in monitoring patients with SCLC and may sometimes be helpful in the diagnostic process. Increased concentrations of both αγ-enolase and γγ-enolase in serum correlate with disease, so measurement of both enzymes simultaneously improves the sensitivity. In the present study we have obtained monoclonal antibodies to human γγ-enolase and applied them in a two-site immunoradiometric assay for NSE. There are few

Table 1. αα-, αγ-, and γγ-Enolase as Measured by IRMA and RIA

<table>
<thead>
<tr>
<th>Fraction</th>
<th>RIA</th>
<th>IRMA</th>
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<tr>
<td>αα-Enolase</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>αγ-Enolase</td>
<td>25</td>
<td>138</td>
</tr>
<tr>
<td>γγ-Enolase</td>
<td>92</td>
<td>129</td>
</tr>
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The measurements were performed on αα-, αγ-, and γγ-enolase fractions with equal enzyme activity. The isoenzymes were obtained from ion-exchange chromatography of human brain homogenate.

Fig. 3. Distribution of αγ- and γγ-enolase in sera from two patients. Serum fractionated on a Mono Q ion-exchange column equilibrated with 0.02 mol/L Bistris buffer and eluted with a KCl gradient (—) from 0 to 0.6 mol/L. In the same buffer, NSE was measured by IRMA in each fraction (C—C). Positions of αα-, αγ-, and γγ-enolase are indicated (—). Values for NSE in serum of the two patients measured by RIA and IRMA were (left) 130 and 284 μg/L and (right) 32 and 150 μg/L, respectively.
reports on monoclonal antibodies to this tumor marker (27, 28). Previous immunoradiometric assays have only applied monoclonal antibodies in combination with polyclonal antibodies to bovine NSE (29). The scarcity of reports may be ascribed to difficulties in raising antibodies to NSE, as we also experienced when raising polyclonal antibodies (18). Our efforts to obtain monoclonal antibodies against NSE did not succeed until we used the immunization procedure reported by Stähli et al. (20). This group recommended a long interval between initial immunization and the final booster doses and the use of large amounts of antigen during the last four days before fusion.

A sensitive two-site IRMA could be obtained with any combination of two of the antibodies E17, E19, and E21. However, there were considerable differences in the NSE values when pathological sera were analyzed by the various assays. The $\alpha$- and $\gamma$-enolase preparations as well as patients' sera were used to choose a suitable antibody combination and the optimal test conditions. The ability to measure $\alpha$-enolase was essential for the choice of antibody combination and incubation time. The resulting IRMA (Figure 1) was more rapid, precise, and sensitive than our RIA. The $\alpha$-enolase gave about the same response in the assay as did $\gamma$-enolase when fractions with equal enzyme activity were measured (Table 1). Serial dilutions of the two isoenzymes gave the same responses in the IRMA, with almost identical curves and a common origin, indicating that one $^{125}$I-labeled E17 molecule is bound per molecule of $\alpha$-enolase and $\gamma$-enolase. The lower detection of $\alpha$-enolase with the RIA reflects the limited ability of $\alpha$-enolase to compete with $^{125}$I-labeled $\gamma$-enolase for the binding to the polyclonal antibodies in this assay.

The presence of both $\alpha$-enolase and $\gamma$-enolase in sera from patients with SCLC has been observed earlier by electrophoretic techniques (30). The variable ratio between the NSE values obtained by the IRMA and the RIA in different sera (Figure 2) could be explained by different proportions of the two isoenzymes in the patients' samples. This was also illustrated by ion-exchange chromatography of sera from two SCLC patients (Figure 3).

$\gamma$-Enolase is a sensitive marker for SCLC. With 10 µg/L as the reference limit, 90% of all untreated patients had increased concentrations of NSE in their serum. The comparable figure with our RIA was 70% (19). Most alternative assays seem to be less sensitive (10). The specificity is also satisfactory because only 7% (eight patients) of the non-SCLC patients had values exceeding 10 µg/L. Some of these patients might have neuroendocrine differentiation in their tumor. It has been suggested that these patients should be treated as SCLC patients rather than according to a non-SCLC regimen (31). This means that the $\gamma$-enolase-specific assay may be helpful when optimal treatment is being selected for lung-cancer patients.

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References