Radioimmunometric Assay for a Monoclonal Antibody-Defined Tumor Marker, CA 19-9

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We describe a solid-phase radioimmunometric sandwich assay for a new tumor marker defined by a monoclonal antibody (19-9). This antibody reacts with a carbohydrate antigenic determinant (CA 19-9) found at low concentrations in sera from healthy individuals but frequently increased in sera from patients with adenocarcinomas. The assay is sensitive and simple to perform. It requires duplicate 100-μL samples and may be performed in 6 h. The concentration of CA 19-9 in samples is determined by reference to a standard curve, which is essentially linear from 0 to 120 arbitrary units/mL. The average CV is approximately 10% in the range of 5.8 to 120 units/mL. The minimum detectable dose is 1.4 units/mL and analytical recovery of CA 19-9 is 97.6 to 100.6%. The average concentration of CA 19-9 in sera from 1020 healthy individuals was 8.4 (SD 7.4) units/mL; only 0.6% of such sera had concentrations >37 units/mL. The assay has high specificity (98.5%), even among patients with benign diseases, and has high sensitivity (up to 79%) for patients with gastrointestinal adenocarcinomas, especially those of the pancreas.

Additional Keyphrases: pancreatic adenocarcinoma cutoff value

Tumor markers; which may be oncofetal or tissue-associated antigens, hormones, or enzymes, have been described for a variety of malignancies. Antisera against such markers have had limited diagnostic value, primarily because of unacceptably high false-positive rates, especially among patients with benign diseases. Nevertheless, assays for tumor markers such as carcinoembryonic antigen (CEA), alpha-fetoprotein, and choriongonadotropin, are useful as noninvasive tests in monitoring cancer patients to detect tumor recurrences, or to assess therapy (1).

Monoclonal antibody technology (2) has given new hope for developing improved methods to define and quantify tumor markers. Monoclonal antibodies with reported specificity for several major classes of tumors have been developed. One such monoclonal antibody, 1116 NS 19-9, prepared by Koprowski et al. (3), was shown to react with a sialylated lacto-N-fucopentaose II (4). In early studies with a competition radioimmunoassay, this antibody was highly sensitive in identifying patients with gastrointestinal adenocarcinomas and highly specific for identifying normal individuals (5,6).

We have developed a "forward sandwich"-type radioimmunometric assay in which the 19-9 antibody is used as both the solid-phase antibody and the iodinated antibody in the tracer solution. The concentration of the 19-9 carbohydrate antigenic determinant (CA 19-9) in serum is quantified by reference to a standard curve. In this report, we show that the CA 19-9 RIA is simple, sensitive, and reproducible; that low concentrations of this antigen are present in serum of healthy individuals; and that concentrations of the antigen are frequently increased in serum of patients with adenocarcinomas, especially pancreatic adenocarcinomas.

Materials and Methods

Preparation of immunoreagents. Murine hybridoma 1116 NS 19-9 (3) was obtained from Dr. Z. Steplewski (Wistar Institute, Philadelphia, PA 19104). Hybridoma cells were cloned, grown in tissue culture, and used to prepare asitic fluid in BALB/c mice primed with Pristane (Aldrich Chemical Co., Milwaukee, WI 53201) (7). Immunoglobulin G (IgG) was purified from ascites by affinity chromatography on protein A-Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ 08854) with elution at pH 4.0 (8). Purity of the isolated IgG was demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, isoelectric focusing, and immunoelectrophoresis (data not shown).

Purified antibody was radioiodinated with ¹²⁵I-labeled N-hydroxysuccinimide ester of p-hydroxyiodophenylpropionic acid (Amersham Corp., Arlington Heights, IL 60005) (9), by the manufacturer's recommended procedure. Specific activity of the ¹²⁵I-labeled 19-9 antibody ranged from 7 to 13 Ci/g. Polystyrene beads (1/4-in. diameter; Precision Plastic Ball Co., Chicago, IL 60641) were coated with the purified antibody (10) and dried.

Assay standards were prepared from partially purified antigen preparations (Klug et al., ms. in preparation) that had been isolated from supernates of a cultured human colorectal adenocarcinoma-derived cell line SW1116 (11) and then diluted into pooled human serum. Because pooled human serum contains small amounts of CA 19-9 activity, "blank" standards were prepared by adding to pooled human serum 100 μg of 19-9 antibody per milliliter, by depleting the CA 19-9 by affinity chromatography, or by substituting gamma-globulin-free horse serum for pooled human serum.

Quantities of CA 19-9 are expressed in units based on comparison with frozen primary reference standards. The CA 19-9 unit is an arbitrary activity corresponding to approximately 0.8 ng of purified antigenic material (Klug et al., ms. in preparation).

Assay conditions. Experimental samples were assayed in duplicate with a "forward sandwich" radioimmunometric assay. We mixed in a reaction tray 100 μL of sera, standards, or positive controls with 100 μL of pH 3.0 buffer [per liter, 5 g of bovine serum albumin (Armour Pharmaceutical, So. Plainfield, NJ 07080), 50 mmol of sodium citrate, 1 mmol of EDTA]. One antibody-coated bead was added to each reaction well and samples were incubated at 37 °C for 3 h. Beads were then washed three times with de-ionized water, then 130 000 dpm of ¹²⁵I-labeled 19-9 antibody in 200 μL of 50 mmol/L sodium citrate buffer, pH 4.5, containing 5 g of bovine serum albumin per liter, was added. After incubating the samples for 3 h at room temperature (18–22 °C), we washed the beads three times and counted their...
radioactivity with a gamma counter. Experimental results were converted into units of CA 19-9 per milliliter by comparison with a standard curve.

Samples. Patients' samples were obtained from the National Cancer Institute, the Mayo Clinic, the Memorial Sloan-Kettering Institute, and the Cleveland Clinic. Clinical data were compiled from reports provided by the institutes. Patients for whom adequate clinical data were not currently available were excluded from the study. Serum samples from 1020 unselected healthy blood bank donors were obtained from Dr. J. Menitove, Southeastern Blood Center of Milwaukee, WI; all of these donors qualified as blood donors, and were negative when tested for hepatitis B surface antigen. Data concerning age and sex were provided by Dr. Menitove and are shown later in Figure 4.

Results

The RIA results for CA 19-9 are essentially linear from 0 to 120 units/mL (Figure 1). To determine the between-assay precision (CV) and the minimum detectable concentration, we assayed nine controls in 20 separate experiments. As shown in Table 1, the CV for positive controls ranging from 5.8 to 120 units/mL averaged less than 10%, indicating good precision over the entire range of the standard curve. In addition, the minimum detectable concentration (defined as the amount of antigen corresponding to the mean cpm of the zero antigen standard plus 2 SD of the mean (12)) was 1.4 units/mL.

Analytical recovery of CA 19-9 from serum of a colorectal cancer patient with a high CA 19-9 concentration or from

![Table 1. Between-Assay (n = 20) Reproducibility of CA 19-9 RIA](image)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>120.3</td>
<td>13.3</td>
<td>11.1</td>
</tr>
<tr>
<td>2</td>
<td>84.8</td>
<td>7.1</td>
<td>8.4</td>
</tr>
<tr>
<td>3</td>
<td>61.3</td>
<td>5.3</td>
<td>8.6</td>
</tr>
<tr>
<td>4</td>
<td>58.2</td>
<td>6.1</td>
<td>10.5</td>
</tr>
<tr>
<td>5</td>
<td>40.9</td>
<td>3.3</td>
<td>8.1</td>
</tr>
<tr>
<td>6</td>
<td>31.2</td>
<td>3.0</td>
<td>9.6</td>
</tr>
<tr>
<td>7</td>
<td>16.3</td>
<td>1.6</td>
<td>8.8</td>
</tr>
<tr>
<td>8*</td>
<td>5.8</td>
<td>0.6</td>
<td>10.3</td>
</tr>
<tr>
<td>9b</td>
<td>0</td>
<td>0.7</td>
<td></td>
</tr>
</tbody>
</table>

*Pooled human serum.

**Pooled serum plus 100 μg of 19-9 antibody per milliliter.

![Table 2. Analytical Recovery of CA 19-9](image)

<table>
<thead>
<tr>
<th>Serum diln. from cancer patient</th>
<th>Observed</th>
<th>Corrected*</th>
<th>Recovered*</th>
<th>% CA 19-9 recovered*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:2</td>
<td>116.0</td>
<td>112.9</td>
<td>225.8</td>
<td>99.0</td>
</tr>
<tr>
<td>1:3</td>
<td>80.3</td>
<td>76.3</td>
<td>228.6</td>
<td>100.3</td>
</tr>
<tr>
<td>1:4</td>
<td>61.5</td>
<td>56.9</td>
<td>227.6</td>
<td>99.8</td>
</tr>
<tr>
<td>1:6</td>
<td>44.5</td>
<td>39.3</td>
<td>235.8</td>
<td>103.4</td>
</tr>
<tr>
<td>Mean (and SD)</td>
<td>229.5</td>
<td>(4.4)</td>
<td>(1.9)</td>
<td></td>
</tr>
<tr>
<td>Partially purified from cell culture</td>
<td>76.2</td>
<td>70.0</td>
<td>2100</td>
<td>100</td>
</tr>
<tr>
<td>1:30</td>
<td>61.1</td>
<td>54.9</td>
<td>2195</td>
<td>104.6</td>
</tr>
<tr>
<td>1:40</td>
<td>36.9</td>
<td>30.7</td>
<td>1842</td>
<td>87.7</td>
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<tr>
<td>Mean (and SD)</td>
<td>2048</td>
<td>(184)</td>
<td>(8.8)</td>
<td></td>
</tr>
</tbody>
</table>

*Observed CA 19-9 concentrations minus endogenous CA 19-9 activity in pooled human serum diluent (6.2 units/mL).

*Corrected CA 19-9 concentration times the dilution factor.

*[(CA 19-9 recovered (units/mL)/expected recovered (units/mL)) × 100.

SW1116 cell-culture supernates was determined by diluting samples with pooled human serum to give concentrations less than 120 units/mL and then assaying with the CA 19-9 RIA. Values were corrected for endogenous CA 19-9 in the pooled human serum diluent and then for dilution. The recoveries of CA 19-9 from serum of the colorectal cancer patient and from the cell-culture supernates were within 10% of the expected values, indicating excellent assay recovery of antigen after dilution with normal human sera (Table 2).

To evaluate the stability of CA 19-9 assay standards, we prepared four dilutions of partially purified CA 19-9 in pooled human serum and incubated them at temperatures up to 45 °C for as long as 78 days. Results were compared with those for frozen assay standards. As shown in Figure 2, the reactivity of samples incubated at 4, 37, and 45 °C remained within 15% of reference values for most points. Gelling of serum after storage at 45 °C for approximately four weeks caused spuriously high values in some samples.

The distribution of CA 19-9 values in sera from 1020 normal blood bank donors is shown in Figure 3. CA 19-9 was detectable in most sera. The mean concentration of CA 19-9 was 8.4 (SD 7.4) units/mL and only six individuals had scores >37 units/mL (range 0–107 units/mL). As indicated in Figure 4, the mean concentration of CA 19-9 among men did not vary significantly with the age of the donor (p > 0.05, Student's t-test). The mean concentration of CA 19-9 in sera of women donors did vary with age. Values were highest in the 20- to 29-year-old group, and lowest between 60 and 69 years of age. Values for women were significantly
higher than for men in all age groups \( (p < 0.05) \) except the 60- to 69-year-old group.

Sera from patients with benign diseases or advanced cancers were also assayed. Among cancer patients, CA 19-9 concentrations of \( >37 \) units/mL \( (\text{range } 0-192 \text{,}000 \text{ units/mL}) \) were observed among 79\% of those with pancreatic adenocarcinomas, and 46\% of patients with gastric adenocarcinomas (Table 3). Further, 46\% of patients with advanced colorectal adenocarcinomas and 8\% of patients with localized colorectal adenocarcinomas (Dukes A and B) had increased CA 19-9 values. Among 21 colorectal cancer patients who had had successful tumor resection and who had no clinical evidence of disease, none had a CA 19-9 concentration exceeding 37 units/mL. Moreover, less than 2\% of patients with a wide range of benign diseases, including pancreatitis, inflammatory intestinal disease, polyps, and other gastrointestinal diseases had CA 19-9 concentrations \( >37 \) units/mL.

**Discussion**

This radioimmunometric assay for the quantification of a carbohydrate antigenic determinant defined by monoclonal antibody, 19-9, is performed in duplicate with 100 \( \mu \)L of sample for each replicate and does not require pretreatment of samples. A “forward-sandwich” format with two incubations of 3 h each is used, so that an assay may be completed easily during a single work day.
The CA 19-9 RIA is sensitive and reproducible. Standard curves are essentially linear over a wide range. The minimum detectable dose, 1.4 units/mL, is well below the mean concentration (8.4 units/mL) found in sera from normal blood-bank donors.

Excellent recoveries of CA 19-9 were observed both for serum from a colorectal cancer patient and for partially purified antigen from cell culture supernates. When observed assay values were corrected for endogenous antigen concentration and for dilution factors, recovered values were very consistent. This consistency over a wide assay range allows accurate estimation of CA 19-9 concentrations in highly positive sera by simple dilution analysis.

Assay standards were prepared from partially purified antigen isolated from cell-culture supernatant fluids and diluted into pooled human serum. The standards are stable indefinitely when frozen, and for at least 10 weeks at temperatures up to 45 °C. The amount of CA 19-9 in unknown serum samples can be expressed by comparison with these standards.

In this study we have briefly summarized the use of the CA 19-9 RIA for analysis of clinical specimens from 1020 blood donors, 314 patients with cancer, and 325 patients with benign diseases. A full description of the latter groups is beyond the scope of this paper and will be the subject of separate reports. On the basis of these data, however, we have chosen a cutoff value of 37 units/mL to discriminate between this group of cancer patients and either normal individuals or patients with benign diseases. This value is very conservative, and was chosen to minimize false positives and to optimize the predictive value and efficiency of the assay (13). Lowering the cutoff value will increase the sensitivity but will also increase the number of false positives. For example, using a cutoff of 25 units/mL increases the sensitivity for pancreatic adenocarcinoma patients slightly (to 86.1%), but decreases the specificity to approximately 95% for normal individuals and for patients with benign disease. (Because of the selection of study groups, it is not appropriate to calculate predictive values.) The sensitivity in this group of patients with colorectal cancer is lower than in other studies involving the 19-9 monoclonal antibody (5, 6). The reasons for these differences are not known but could represent patient status, changes in assay configuration, or other factors. Additional preliminary data indicate that the concentration of CA 19-9 may be influenced by several factors, especially tumor burden and location.

The CA 19-9 RIA appears to be useful in the diagnosis of pancreatic adenocarcinoma. Among patients with pancreatic cancer, 79% had scores >37 units/mL and most had scores in the range of 400–192 000 units/mL. The benign disease group, which included 21 patients with pancreatitis, 56 patients with inflammatory diseases of the gastrointestinal tract, 27 patients with polyps, and 219 with other gastrointestinal diseases, had only five scores >37 units/mL. The high specificity among these latter patients and the high sensitivity among patients with pancreatic adenocarcinoma suggest that the CA 19-9 RIA may be valuable as a diagnostic adjunct for pancreatic cancer.

More extensive studies of CA 19-9 concentrations in sera of patients with cancer and nonmalignant diseases are underway at several major cancer hospitals. In these studies we will evaluate the influence of tumor burden and location, the effect of therapy, and several cutoff values. In addition, we will evaluate the possibility of using a panel of tests for other tumor markers to increase the predictive value and efficiency of these assays.

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References


