Carbocyanine Dye Metachromasia of Sialidase-sensitive Polyanions in Sera from Normal and Tumor-bearing Mice

R. J. Woodman

Cancer Chemotherapy Department, Microbiological Associates, Inc., Bethesda, Maryland 20016

SUMMARY

Motivated by the theory that tumor cells might shed anionic macromolecules from their surfaces into the blood, a microassay for polyanions based on the spectral shift of carbocyanine dye was adapted to the analysis of serum from healthy and tumor-bearing mice, incubated with and without sialidase. Carbocyanine dye-binding polyanion, compared with sodium pectin as a standard, was 131 μg/ml serum from healthy C57BL X DBA/2 F1 (hereafter called BD2F1) mice, 270 μg/ml in L1210 (ascites) leukemia, and 277 μg/ml in mice given i.p. injections of Lewis lung tumor. Non-tumor-bearing, traumatized controls, given i.p. injections of CCl4 to simulate the gross pathology of ascitic tumor growth and s.c. injections of Sephadex gel to simulate the trauma of solid tumor growth, gave carbocyanine dye-binding polyanion values of 173 and 166 μg/ml, respectively. The difference in carbocyanine dye-binding reactivity of serum with and without sialidase incubation gave the sialopolyanion fraction, values of which ranged from 29 to 35 μg/ml serum for healthy or traumatized mice and 135 and 169 μg/ml in mice bearing L1210 leukemia and Lewis lung tumor, respectively.

Sialopolyanion values, measured as a function of time after i.v. implantation of 10⁶ leukemic cells or 10⁶ Lewis lung tumor cells (implanted i.m.), showed a significant elevation in 2 days, whereas it was 8 days before the white blood cell count in leukemic mice increased, and more than 5 days before the Lewis lung tumor became palpable.

Differences between sialopolyanion values of serum from mice bearing a variety of tumors and the corresponding healthy host were similar to those seen for healthy BD2F1 mice and the hosts bearing the L1210 leukemia or Lewis lung tumor.

In support of the theory that tumor cells shed anionic material into the blood in vivo, it was found that the undiluted ascites plasma, from which Krebs tumor cells had been deposited by centrifugation, contained 27% more carbocyanine dye-binding polyanion and 42% more sialopolyanion than did plasma from which Krebs cells had been settled by gravity.

INTRODUCTION

The development of diagnostic methods for the early detection of cancer is of great importance, because prompt initiation of surgical, radiation, or drug treatment can extend survival or even result in cures. Progress has been made in the search for a clinical diagnostic cancer test by analysis of body fluids, such as the polyamine content of urine (21), elevated serum seromucoid (27, 28), and the immunoassay of blood for carcinoembryonic antigens (25) and a-fetoprotein (1, 2). The goal of a single test that is specifically diagnostic for a wide range of cancers at an early stage of disease, however, remains largely unfulfilled and continued research into new diagnostic techniques seems warranted.

Emmelot (8) has recently reviewed the properties of normal and tumor cell surfaces, and one of the important conclusions drawn by this author is that tumor cells release proteolytic enzymes that attack material covering the cell membrane (4, 20, 23). Since artificial proteolytic digestion of both normal and virally transformed cells releases sialic acid containing glycopolymers (5), any unique property of tumor cell surfaces for autolysis in vivo might produce diagnostically useful increases in serum sialopolyanion. The theory motivating this study, namely, that shedding of material from tumor cell surfaces might cause an increase in serum sialopolyanion values, was further supported by the electron micrographic studies of Martinez-Palomo et al. (16). Their electron micrographs of normal and virally transformed cells fixed in situ and stained with the cationic, electron-opaque dye, ruthenium red, showed a much thicker layer of anionic material covering the transformed cell surfaces that appeared to be “streaming” into the ambient culture fluid, although more recent studies have suggested that the anionic material may be serum components precipitated from the culture medium (24).

This evidence prompted the search for a tumor-diagnostic serum assay for anionic macromolecules, the electronegativity of which would be diminished by incubation with sialidase. Attainment of this goal was facilitated by a microassay for polyanions developed by Edstrom (7). The principle of this method, which has been modified for serum in this study, depends on the color change, from magenta to blue, of a carbocyanine dye when bound to closely spaced negative charges on any anionic macromolecule.

1 Supported by Contract NO1-CM-33728 with Division of Cancer Treatment, National Cancer Institute, NIH, Department of Health, Education and Welfare, Bethesda, Md. 20014. Presented in part at the 64th Annual Meeting of the American Association for Cancer Research, April 11, 1973.

2 To whom reprint requests are to be addressed, Cancer Chemotherapy Department, Microbiological Associates, Inc., 5221 River Road, Bethesda, Md. 20016.

Received April 2, 1974; accepted July 23, 1974.
Materials and Methods

Animals and Tumors

Lymphoid leukemia L1210 was implanted i.p. and Lewis lung carcinoma was implanted i.m. into C57BL X DBA/2 F1 (hereafter called BD2F1) male mice according to the protocols described by Geran et al. (9). In one experiment, 10⁶ ascitic L1210 leukemic cells in 0.1 ml sterile 0.15 M NaCl were injected into a tail vein. For other tumor systems, tumor fragments were implanted s.c. into the right flank. For the Krebs-2 and Ehrlich ascites tumors, 0.1 ml of undiluted donor ascites fluid was injected i.p. into each of the test mice. The Madison 109 tumor was carried s.c. and then converted to a brei for i.m. injection into test mice, using 2 × 10⁶ cells in 0.1 ml 0.15 M NaCl per mouse. The AKR mouse leukemia was diagnosed in 6- to 10-month-old hosts by spleen palpation, and gross evidence of disease was confirmed at autopsy. Healthy AKR mice, 6 to 10 months old, were autopsied after bleeding to confirm absence of gross signs of leukemia.

Treatment of Mice to Simulate Trauma of i.p. or s.c. Tumor Growth

Peritoneal Trauma. To simulate the enlarged and pale liver seen in mice approximately 7 days after i.p. injection of L1210 leukemic cells, normal BD2F1 mice were given i.p. injections of 50 μl of carbon tetrachloride and bled 48 hr later. At autopsy the liver appeared enlarged and pale, with a granular pattern on the surface of the organ.

s.c. Trauma. To simulate the tissue trauma of a tumor growing s.c., 0.5 ml of G-50 Sephadex (Pharmacia Laboratories, Inc., Piscataway, N. J.), swelled in 0.15 M NaCl, was injected s.c. into normal BD2F1 mice in the right flank, where it remained as a palpable mass, approximately 6 mm in diameter, until the animals were bled 14 days later. Histological examination of sections stained in hematoxylin and eosin revealed unstained Sephadex enclosed by a capsule of fibroblastic cells. The periphery of the Sephadex:gel mass was infiltrated by lymphocyte-like cells.

Preparation of Serum

Isolation of Mouse Sera. Approximately 0.6 ml of whole blood was taken from the orbital sinus, clotted, and analyzed directly or frozen on Dry Ice and stored at −10°C for several days prior to analysis.

Preparation of Gravity-settled and Centrifuged Ascites Fluid. C3H/He mice were killed 14 days after i.p. injection of 10⁵ Krebs carcinoma cells, and the ascitic fluid (about 12.0 ml/mouse) was aspirated into a tube containing 0.1 ml of 5% sodium citrate to prevent cellular aggregation. The ascites fluid from each mouse was distributed between two 12-mm diameter tubes and stood for 1 hr at 4°C. One of the tubes was then centrifuged at 125 × g for 5 min at 4°C and the sedimented cells and ascitic plasma were mixed by gentle agitation. This process was repeated 2 more times, and then 0.2-ml aliquots of the clear supernatant were removed for CPA and sialopolyanion assay. From the other tube, a clear zone extending about 5 mm down from the meniscus was carefully aspirated and the clear fluid (gravity-settled ascitic plasma) was centrifuged at 125 × g for 5 min to remove traces of debris and erythrocytes. Trypan blue exclusion counts of the tumor cells were made immediately upon their removal from the mouse and after sedimentation by gravity or centrifugation. Ascitic plasma was then processed and assayed for CPA and sialopolyanion as described for serum.

Preparation of Serum Samples for Carboxycyanine Dye Assay. To 0.2 ml of thawed or fresh serum was added 0.8 ml of 0.1 M acetate buffer (pH 5.0) containing 1 mM CaCl₂. To 0.5 ml of this solution was added 0.1 ml of acetate buffer. To the other 0.5 ml, 0.1 ml of acetate buffer, containing 12 units of sialidase (Vibrio cholerae, Grand Island Biological Co., Grand Island, N. Y.) was added, followed by incubation at 37°C for 45 min. Sodium bicarbonate was then added to adjust the pH to 6.5, followed by the addition of 0.1 ml of a water solution containing Pronase (5 mg/ml; Calbiochem, Los Angeles, Calif.) and thimerosal (5 μg/ml; Sigma Chemical Co., St. Louis, Mo.). The samples were incubated at 37°C for approximately 18 hr and then transferred to 6-mm (inflated diameter) dialysis tubing together with about 0.1 ml water used to rinse out the sample tube. Five to 6 samples were contained in each length of dialysis tubing, each sample being separated from its neighbor by knotting the tubing to form a series of turgid sacs. The samples were dialyzed against 200 volumes of 1 mM NaCl at 4°C, changing the water every hr for 3 hr. After removal from the dialysis sacs, the volume of each sample was made to 1.0 ml with water, and 0.4-ml aliquots were mixed with 2.6 ml of carboxycyanine dye solution for the assay described below.

Carboxycyanine Dye Assay

The purpose of this assay was to compare the carboxycyanine dye metachromasia of 1 aliquot of serum, that had been incubated alone, with the dye-binding polyanions in an aliquot incubated with sialidase, the difference between these 2 values being sialopolyanion.

Dye solution was prepared at room temperature immediately before use in the following manner: to 10 ml of dioxyane:water (1:1, v/v) were added 5.6 mg of carboxycya nine dye [1-ethyl-2- [3-[10-ethyl naphthol (1,2d)thiazolin-2-ylidene]-2-methylpropenyl]naphtho(1,2d)thiazolium bromide, from Eastman #2718 (Eastman Kodak Co., Rochester, N.Y.)]. Solution of the dye was facilitated by grinding in a tissue homogenizer. The stock solution of dye was then mixed with 85 ml of water, 0.1 ml of 1 M acetic acid, and 5.0 ml of 0.01 M ascorbic acid in a light-proof, plastic bottle and used within 1 hr.

Two dye blanks were prepared by mixing 0.4 ml water with 2.6 ml of the dye solution and balanced at zero absorbance in a dual-beam spectrophotometer at 560 nm. A narrow slit width (0.1 to 0.2 mm) was used to minimize bleaching of the dye, especially of the reference blank which remained in the light path. Cuvets were siliconized to reduce cumulative binding to their optical faces of free dye and dye-stained material from samples. A standard polyanion curve was prepared by mixing graded amounts of sodium polygalacturonate (1 to 10 μg) in 0.4 ml water with 2.6 ml dye solution, and absorbance was measured at 560 nm against the dye blank. Processed and diluted serum samples, 0.4 ml, were...
Carbocyanine Dye Assay of Mouse Serum

similarly measured, absorbance values were read off the standard curve, and the results were expressed as µg pectin equivalent per ml whole serum. The absorption spectra for pectin and normal mouse serum after Pronase digestion versus the dye blank are shown in Chart 1. The pH of the dye solution, after addition of serum sample (0.4 ml), was 3.7 and gave an absorption maximum of 560 nm with the lot of dye used throughout this study. Absorbance values of dye:serum mixtures were sensitive to changes in pH or the addition of cations, which precluded the use of buffering agents. Recent evaluation of this method (30) has shown that temperature fluctuations, slight differences in dye lots, and binding of the dye to glass surfaces are additional variables that need to be controlled.

Sialic Acid Assay

Serum samples were prepared and assayed in the following manner.

Direct Analysis. Serum (0.1 ml) was diluted with 0.9 ml of 0.1 M acetate buffer (pH 5.0), 0.1 ml 1 N H₂SO₄ was added, and then the mixture was heated at 80° for 1 hr. Aliquots of 0.2 ml from each tube were then assayed in triplicate for sialic acid by the thiobarbituric acid method (26).

PCA Residue. Serum (0.1 ml) was diluted with acetate buffer as described above and chilled, and 0.1 ml 70% PCA was added. After standing at 4° for 15 min, the residue was washed twice with 1.0 ml 7% PCA and then suspended in 1.0 ml of 0.1 N H₂SO₄. The sample was then heated as described above and sialic acid analysis was performed on 0.2-ml aliquots of the supernatant.

Dialized Serum. Serum was diluted in acetate buffer as described above, incubated with or without sialidase as described for the preparation of serum samples, and then dialyzed against water for 3 hr. The sample was then acidified with 0.1 ml of 1 N H₂SO₄, heated at 80° for 1 hr, and assayed for sialic acid.

Pronase-digested and Dialyzed Serum. Serum (0.1 ml) was diluted in acetate buffer as described above, incubated with or without sialidase, and then adjusted to pH 6.5 by addition of sodium bicarbonate. Pronase and thimerosal were then added as described previously and the sample was incubated at 37° for 18 hr. After Pronase digestion, the sample was dialyzed and assayed for sialic acid as described above.

RESULTS

Evaluation of Glycoprotein Recovery from Serum and Optimal Conditions of Sialidase Incubation of Serum Prior to Carbocyanine Dye Assay

Glycoprotein Recovery from Mouse Serum

Based on the hypothesis that most of the CPA measured in serum might be due to glycoprotein sialic acid, an experiment was carried out to determine how easily bovine submaxillary mucin, a glycoprotein that is metachromatic for carbocyanine dye, could be recovered from mouse serum. The results, as presented in Chart 2, show that the CPA component of graded amounts of bovine mucin (Worthington Biochemical Corp., Freehold, N. J.), can be recovered from a constant volume of mouse serum. Pronase digestion and dialysis of mucin did not significantly reduce its CPA content compared with mucin mixed directly with dye solution. At 500 µg of mucin per ml, however, absorbance was reduced approximately 25% by sialidase compared with mucin incubated without this enzyme.

Investigation of Optimal Conditions of Sialidase Incubation of Sera

Optimal pH. Diluted sera from normal mice and mice bearing Lewis lung tumor were incubated for 1 hr at 37° in 0.1 M acetate buffers ranging from pH 4.5 to 6.5 in 0.5 pH increments. Maximum depression of CPA in normal sera, after incubation with sialidase, occurred at pH 5.5 compared with pH 5.0 for sera from mice bearing Lewis lung tumor (Chart 3). On the basis of these data all sialidase incubations were routinely conducted at pH 5.0.

Optimal Concentration of Sialidase. The concentration of sialidase has a differential effect on the amount of SPA measured in normal versus tumorous mouse serum (Chart 4). Incubation of diluted serum with sialidase (10 or 20 units/ml) gave the greatest decrease in absorbance values for tumorous serum versus normal serum. In subsequent experiments, 20
Chart 2. Carbocyanine dye absorbance of mucin in the presence and absence of mouse serum determined after Pronase digestion and dialysis. © dye solution and mucin mixed without prior Pronase digestion or dialysis. Amounts of mucin and serum contained in 3.0 ml dye solution after appropriate processing of the sample.

Chart 3. Effect of pH on sialidase reduction of carbocyanine dye metachromasia of mouse sera. Serum (0.1 ml) was diluted in acetate buffer (0.1 M, 0.4 ml) at pH values shown and incubated with 20 units sialidase per ml for 45 min at 37°. Units of sialidase per ml incubation mixture were therefore routinely used.

Time Course of Polyanion Decrease in Various Sera Incubated with Sialidase. The percentage of decrease in CPA levels of sera from tumor-bearing mice was most rapid during the 1st 20 min of incubation with sialidase; thereafter, the decrease was slower (Chart 5 A and B). Sera from normal mice or mice given injections of carbon tetrachloride showed a rate of decrease that was again slightly greater during the 1st 20 min of incubation. From these data it was concluded that 45 min of incubation with sialidase would be optimal for comparative measurement of sialopolyanion in sera from normal and tumor-bearing mice.

Studies on the Effect of Trauma or Malignant Tumor Growth on CPA and SPA Values of Mouse Serum

CPA and Sialopolyanion Values for Sera from Normal, Traumatized, and Tumor-bearing Mice. The CPA value in normal serum (130 µg pectin equivalent per ml) was approximately 30% higher in sera from mice given injections of carbon tetrachloride or Sephadex and more than 100% higher in sera from mice bearing advanced L1210 leukemia or Lewis lung carcinoma (Chart 6). Incubation of sera from normal or traumatized mice with sialidase yielded sialopolyanion values of 29 to 36 µg pectin equivalent per ml. By contrast, sialidase incubation had a much greater effect on sera from tumor-bearing mice, giving sialopolyanion values of 135 and 169 µg/ml for L1210 leukemia and Lewis lung carcinoma, respectively.

CPA values of the sera from mice bearing leukemia and Lewis lung tumor after sialidase incubation were higher by only 33 µg pectin equivalent per ml and 6 µg pectin equivalent per ml, respectively, than the values from sialidase-incubated normal sera (120 µg pectin equivalent per ml). This suggests that most of the increase in serum CPA levels resulting from tumor implantation was due to the sialopolyanion fraction.

CPA and Sialopolyanion Values for Sera from Mice Bearing a Variety of Murine Tumors. The CPA and sialopolyanion values of sera from several strains of healthy mice and the same strains bearing a variety of solid or ascitic tumors are shown in Table 1. In all cases, sialopolyanion values were at least 3-fold higher in tumor-bearing mice than in their normal counterparts.
Chart 5. Percentage of reduction of carbocyanine dye-binding polyanion in various mouse sera after incubation with sialidase as a function of time. Serum (0.1 ml) diluted with 0.4 ml acetate buffer (0.1 M, pH 5.0) and incubated for times shown with 20 units of sialidase per ml at 37°.

Chart 6. Distribution of sialidase-resistant and sialidase-sensitive CPA in healthy, traumatized, and tumor-bearing mice. LEUK, leukemia; CA, cancer.

Table 1

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Tumor form and site</th>
<th>Host strain</th>
<th>CPAa (μg/ml serum)</th>
<th>Sialopolyaniona (μg/ml serum)</th>
<th>% sialopolyanion of CPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pigmented B16 melanomaa</td>
<td>Fragment s.c.</td>
<td>C57BL/6</td>
<td>257</td>
<td>147</td>
<td>57.0</td>
</tr>
<tr>
<td>Nonpigmented S91 melanoma</td>
<td>Fragment s.c.</td>
<td>DBA/2</td>
<td>445</td>
<td>207</td>
<td>46.5</td>
</tr>
<tr>
<td>Carcinoma 755</td>
<td>Fragment s.c.</td>
<td>C57BL/6</td>
<td>230</td>
<td>118</td>
<td>51.1</td>
</tr>
<tr>
<td>P1798Y leukemia</td>
<td>Fragment s.c.</td>
<td>BALB/c</td>
<td>385</td>
<td>192</td>
<td>49.8</td>
</tr>
<tr>
<td>Madison 109 lung carcinomaa</td>
<td>Brei i.m.</td>
<td>BALB/c</td>
<td>447</td>
<td>187</td>
<td>41.8</td>
</tr>
<tr>
<td>AKR spontaneous leukemia</td>
<td>Systemic</td>
<td>AKR</td>
<td>245</td>
<td>138</td>
<td>56.2</td>
</tr>
<tr>
<td>Krebs-2 leukemia</td>
<td>Ascites i.p.</td>
<td>C2H/He</td>
<td>252</td>
<td>165</td>
<td>65.8</td>
</tr>
<tr>
<td>Ehrlich ascites carcinoma</td>
<td>Ascites i.p.</td>
<td>BALB/c</td>
<td>374</td>
<td>248</td>
<td>66.3</td>
</tr>
<tr>
<td>Non-tumor-bearing host</td>
<td>C57BL/6</td>
<td></td>
<td>144</td>
<td>26</td>
<td>19.4</td>
</tr>
<tr>
<td>DBA/2</td>
<td></td>
<td></td>
<td>250</td>
<td>56</td>
<td>25.9</td>
</tr>
<tr>
<td>BALB/c</td>
<td></td>
<td></td>
<td>315</td>
<td>25</td>
<td>8.0</td>
</tr>
<tr>
<td>AKR (healthy)</td>
<td></td>
<td></td>
<td>212</td>
<td>34</td>
<td>15.8</td>
</tr>
<tr>
<td>C3H/He</td>
<td></td>
<td></td>
<td>179</td>
<td>20</td>
<td>11.0</td>
</tr>
</tbody>
</table>

\( ^a \) Sera diluted 5-fold in 0.1 M acetate buffer, pH 5.0, and incubated with or without sialidase (20 units/ml) for 45 min at 37°.
Chart 7. Time course of increase in sialopolyanion and total white blood cell counts of BD2F mice after i.v. injection of L1210 leukemic cells. Each point is the mean value for groups of 3 mice; blood from each mouse being assayed in duplicate for both serum sialopolyanion and white cell count. Sera incubated for 45 min under conditions described in Chart 4 for sialopolyanion assay. Left ordinate, sialopolyanion; right ordinate, white blood cell count. CPA, on Day 0, 122 μg pectin equivalent per ml; on Day 8, 237 μg pectin equivalent per ml serum. •, sialopolyanion, μg pectin equivalent per ml serum; •, total white cell count x 10^9.

total white blood cell count, however, did not increase sharply until Day 8, when there was an estimated population of 10^8 L1210 leukemic cells in the host. These data also indicate that the increase in CPA from Day 0 to Day 8 is almost totally due to the sialopolyanion fraction, since the values of CPA after sialidase incubation have only increased from 112 to 126 μg/ml during 9 days of tumor growth.

Sialopolyanion was noticeably higher 2 days after i.m. injection of 10^6 Lewis lung carcinoma cells (Chart 8). The tumor was palpable in only 2 of 8 mice by Day 5, at which point the sialopolyanion fraction had almost doubled from the value on Day 2. Not until Day 9 did all the mice have tumors greater than 10 mm in diameter.

Effect of Settlement by Gravity or Centrifugation on CPA and Sialopolyanion Values of Krebs-2 Ascites Plasma

The polyanion content of plasma from gravity-settled ascites fluid was 245 μg CPA per ml compared with 310 μg CPA per ml of plasma from ascites fluid that had been centrifuged and mixed 3 times (Table 2). The gravity-settled plasma contained 99 μg sialopolyanion per ml compared with 140 μg sialopolyanion per ml for centrifuged plasma, a difference of 41 μg sialopolyanion per ml. While this increase in sialopolyanion was not as great as the increment in CPA (65 μg/ml), these data show that the proportion of sialopolyanion in the polyanion, released from Krebs ascites tumor cells during centrifugation, was higher than the proportion of sialopolyanion in polyanion already in the ascites plasma.

Since there was no detectable increase in trypan blue permeability of tumor cells after centrifugation at 4°C, it was improbable that CPA released into ascites plasma by this treatment was due to membrane damage, but more probably due to shearing of CPA from the cell surface.

Sialic Acid Content of Sera from Normal, Traumatized, and Tumor-bearing Mice

In view of the differential effect of sialidase on CPA values of sera from normal or traumatized mice versus sera from tumor-bearing mice, it was considered of interest to determine whether measurement of total (acid-labile) serum sialic acid would show a similar differential effect.

The amount of sialic acid released by direct dilute acid hydrolysis was lowest in serum from healthy mice (467 μg/ml), slightly higher in sera from traumatized mice, and highest in sera from L1210 leukemic (612 μg/ml) or Lewis lung tumor-bearing mice (673 μg/ml) (Table 3). At least 70% of this sialic acid could be recovered from the PCA residue, showing that serum sialoglycoproteins were coprecipitated with other serum proteins. Dialysis of serum prior to mild acid hydrolysis did not produce a consistent decrease in sialic acid content of the 5 categories of serum. Incubation of the sera with sialidase prior to dialysis removed 44 to 58.5% of the sialic acid in a pattern that was unrelated to either trauma or tumor growth. Pronase digestion before dialysis resulted in a 5 to 12% decrease in sialic acid, but again incubation of sera with sialidase prior to these steps resulted in a 45.5 to 54% decrease in sialic acid in a pattern that was unrelated to the pathological condition of the mouse. These data, therefore, show that the total sialic acid content of serum, whether derived from tumor-free or tumor-bearing mice, was similarly sensitive to sialidase. By contrast, arrayed sialic acid, as identified by the carbocyanine dye assay, was more sensitive to sialidase in serum from tumor-bearing mice than in serum from tumor-free mice.

DISCUSSION

The main purpose of this study has been to describe a new
Carbocyanine Dye Assay of Mouse Serum

Table 2

<table>
<thead>
<tr>
<th>Conditions of cell sedimentation&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CPA&lt;sup&gt;b&lt;/sup&gt; (µg/ml ascites plasma)</th>
<th>Sialopolyanion&lt;sup&gt;b&lt;/sup&gt; (µg/ml ascites plasma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gravity-settled</td>
<td>245 ± 10&lt;sup&gt;c&lt;/sup&gt;</td>
<td>99 ± 9</td>
</tr>
<tr>
<td>Centrifuged 3 times (125 x g, 5 min)</td>
<td>310 ± 15</td>
<td>140 ± 16</td>
</tr>
</tbody>
</table>

<sup>a</sup> Trypan blue-stained cells, <3%, both before and after centrifugation.<br>
<sup>b</sup> 0.1 ml ascites plasma diluted 5-fold in 0.1 M acetate buffer, pH 5.0, and incubated for 45 min at 37° with or without 20 units sialidase per ml.<br>
<sup>c</sup> Mean ± S.E. Ascites fluid from 6 mice processed as described in "Materials and Methods."

Table 3

<table>
<thead>
<tr>
<th>Source of serum</th>
<th>Hydrolyzed direct (µg&lt;sup&gt;a&lt;/sup&gt;/ml serum)</th>
<th>Residue of 7% PCA (µg/µl serum)</th>
<th>Serum dialyzed then hydrolyzed</th>
<th>Serum Pronase-digested, dialyzed, then hydrolyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal mice</td>
<td>467</td>
<td>335</td>
<td>405</td>
<td>335</td>
</tr>
<tr>
<td>Mice given i.p. injections of carbon tetrachloride</td>
<td>498</td>
<td>438</td>
<td>432</td>
<td>413</td>
</tr>
<tr>
<td>Mice given s.c. injections of Sephadex</td>
<td>516</td>
<td>405</td>
<td>542</td>
<td>500</td>
</tr>
<tr>
<td>Mice bearing L1210 leukemia (injected i.p.)</td>
<td>612</td>
<td>458</td>
<td>620</td>
<td>567</td>
</tr>
<tr>
<td>Mice bearing Lewis lung carcinoma (injected i.m.)</td>
<td>673</td>
<td>643</td>
<td>605</td>
<td>565</td>
</tr>
</tbody>
</table>

<sup>a</sup> All values are sialic acid in µg/ml serum. % reduction by sialidase, proportion removed by sialidase prior to the acid hydrolysis step.

analytical method and its application to the discrimination between sera from healthy or traumatized mice and sera from mice bearing malignant tumors. The use of traumatized controls was considered especially important because previous clinical studies by Winzler (27, 28) have shown that both malignant and inflammatory disease cause elevation of certain protein-bound carbohydrates soluble in 0.6 M PCA. Macbeth and Bekesi (12, 13) have also demonstrated the elevation of protein-bound sialic acid and carbohydrates precipitated by 0.6 M PCA from plasma or serum from patients with nonmalignant and malignant disease. These clinical studies showed that, while such elevated values were, on an average, higher in malignant disease than in nonmalignant disease, the overlap of the distribution curves of these values precluded the use of this type of blood analysis for cancer diagnosis. The results shown in Table 3 support the findings of Macbeth and Bekesi (12, 13) that both inflammatory pathology and malignant disease produce an increase in protein-bound serum sialic acid. However, the unique effect of sialidase on sialopolyanion of serum from tumor-bearing mice, as measured by the carbocyanine dye method, was not observed when total sialic acid was measured by the thiobarbituric acid assay.

That sialic acid-containing glycoprotein is the most likely blood component identified by the carbocyanine dye test is supported by the fact that nonproteinaceous polyanions, such as hyaluronic acid, are present in amounts less than 10 µg/ml serum from BD2F1 mice used in this study. Although the evidence suggests that most of the sialopolyanion is derived from glycoprotein, the possibility cannot be excluded that some of the sialopolyanion is derived from glycolipids, such as gangliosides. Several studies have shown that the amount of sialoglycolipid in surface membranes of virally transformed cells is lower than the amount of untransformed parent cells (18). This might be due to reduced synthesis of certain glycolipids subsequent to transformation (6, 22), but the possibility remains that viral transformation alters the synthesis of some sialoglycolipids in such a way that they are released into the ambient fluid.

Although much of the serum carbocyanine dye metachromasia may result from arrayed anions other than sialic acid, the sialic acid contributing to the metachromasia may be only a small proportion of the total sialic acid present in serum. This can be interpreted from the data presented in Table 3, which shows that there is a total of 467 µg acid-labile sialic acid per ml serum from normal BD2F1 mice as measured by
the thioarbituric acid method. This amount of sialic acid incorporation in a sialglycoprotein would give a much greater amount of metachromatic polyanion than the amount actually recorded by the carbocyanine dye assay for normal mouse serum (131 μg CPA/per ml).

The sialopolyanion fraction is apparently composed of glycoprotein molecules containing sialic acid accessible to sialidase. This would mean that the sialic acid is terminal to the oligosaccharide chain and not substituted at the C-4 hydroxyl group (10). Furthermore, either a small proportion of such sialic acid is tightly arrayed in such a way as to provide the observed degree of metachromasia as all of the sialic acid is involved in a weak array pattern to collectively produce this same degree of metachromasia.

As far as discriminating between sera from normal or traumatized mice and tumor-bearing mice is concerned, the tumorous sera contain a higher level of the sialidase-sensitive, arrayed, sialic acid (sialopolyanion) than do the nontumorous sera. There is also a tendency for the CPA content of sera from tumor-bearing mice to be higher than that from the non-tumor-bearing host (Table 1), but this is also true of traumatized BD2F1, mice (Chart 6). The correlation between elevated CPA of serum from tumor-bearing or traumatized mice versus normal mice parallels the correlation of elevated serum sialoglycoprotein with inflammatory or malignant disease observed by Macbeth and Bekesi (12, 13). The assay, using carbocyanine dye with sialidase, identifies a more narrowly defined group of blood components than sialoglycoprotein, in that it is specific for those substances that contain sialic acid that is both cleaved by sialidase and “arrayed” (metachromatic).

While the experiments of this study do not provide any direct biochemical mechanisms to explain the observed elevation of sialopolyanion values in serum from tumor-bearing mice, certain hypotheses can be offered. One possibility could be that normal plasma contains higher amounts of sialopolyanion than does normal serum, but in cancerous hosts the blood-clotting process is defective, allowing more sialopolyanion to remain in the separated serum. This situation is difficult to verify experimentally because the conditions required to prepare plasma from whole blood involve either the addition of polyanion (e.g., heparin) or removal of Ca2+ by citrate or Versene (thereby inhibiting the activation of sialidase). However, this theory is largely negated by the evidence of elevated glycoprotein of both plasma and serum in cancer patients (12, 13, 19). A more probable mechanism to explain the observed differences in sialopolyanion of normal and tumorous sera would seem to involve differences in the relative amounts of glycoprotein containing arrayed sialic acid that was terminal to the oligosaccharide chains and unsubstituted at position C-4 (10).

The source of elevated serum or plasma glycoprotein of tumor-bearing rats was originally believed to be the liver (14, 17), but evidence now exists that suggests that tumor tissue itself could be the source (15). Similarly, it would appear that the source of elevated CPA, and in turn sialopolyanion, in tumor-bearing mice is also tumor tissue. This theory is supported by the results shown in Table 2 which suggest that the metachromatic polyanions that are released from the surface of ascitic Krebs tumor cells by centrifugation are the same as those that may be shed in vivo to cause an elevation in serum sialopolyanion (Table 1). If this interpretation is correct, tumor cells would be enclosed in an anionic microenvironment. This concept is supported by the observation of enhanced incorporation of polycation-complexed 5-iodo-2′-deoxyuridine into DNA of tumor cells, but unchanged incorporation into DNA of normal dividing cells when compared with uncomplexed 5-iodo-2′-deoxyuridine (29).

The concept of sialic acid-containing glycoproteins being released from the surface of malignant tumor cells in vivo is also supported by the work of Kim and Tunis (11). These investigators found that a glycoprotein fraction of plasma from mice bearing a metastasizing tumor contained much more sialic acid, bound hexose, and carcinoembryonic antigenic activity than did plasma from mice bearing a nonmetastasizing tumor. Electron microscopy of ruthenium red-stained surfaces of nonmetastasizing tumor cells revealed that the anionic surface coat was much thicker than on the surfaces of similarly prepared metastasizing tumor cells. These results again suggest a shedding of seromucoid from the surfaces of malignant tumor cells into the blood in vivo.

The major finding of this study, namely, the ability of the carbocyanine dye assay to discriminate between sera from normal mice and sera from tumor-bearing mice, can be regarded first for its potential application to the clinical diagnosis of primary tumor growth or its resurgence after various forms of therapy. It is evident from the data shown in Table 1 that the method was diagnostic for a variety of solid and ascitic murine carcinomas and leukemias and gave no “false positive” results for non-tumor-bearing hosts.

Preliminary studies on sera from healthy volunteers, patients with nonmalignant disease, and cancer patients have so far yielded differences in CPA and sialopolyanions values similar to those observed in sera from normal, traumatized, or tumor-bearing mice. Further studies on human serum samples have involved the testing of minor modifications to the method to render it less time consuming and to improve reproducibility of results, which can be adversely affected by several variables. Such modifications have included precipitation by acetone rather than dialysis to recover Pronase-resistant material, heat inactivation of sialidase prior to Pronase digestion, and more extensive control of pH and ionic strength of the sample when mixed with carbocyanine dye. The results of these studies will be reported later.

ACKNOWLEDGMENTS

Human sera from patients with malignant and nonmalignant disease were kindly provided by Dr. Aaron Primack and Dr. Lawrence Broder of the Veterans Administration Hospital, Washington, D. C.

REFERENCES

3. Bissell, M. J., Rubin, H., and Hatie, C. Leakage as the Source of
Carbocyanine Dye Assay of Mouse Serum


Carbocyanine Dye Metachromasia of Sialidase-sensitive Polyanions in Sera from Normal and Tumor-bearing Mice

R. J. Woodman

Cancer Res 1974;34:2897-2905.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/34/11/2897

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.