Sialosylated Lewisx in the Sera of Cancer Patients Detected by a Cell-binding Inhibition Assay

Masaki Hirota, Kiyoyasu Fukushima, Paul I. Terasaki,2 Glenn Y. Terashita, Masaaki Kawahara, David Chia, Naofumi Suyama, and Hiroyuki Togashi

Department of Surgery, UCLA School of Medicine, University of California, Los Angeles, California 90024

ABSTRACT

A new cell-binding inhibition assay to detect tumor-associated antigens in sera was developed. This assay determined that sialosylated Lewisx, as detected by the CSLEX1 monoclonal antibody, is present in the sera of 95% of patients with advanced lung adenocarcinomas. Sera with inhibition titers of 1:16 or higher yielded the following percentages of positive inhibition: lung cancers, 43.9%; stomach cancer, 26.0%; colon cancer, 44.4%; gall bladder and bile duct cancers, 47.8%; pancreas cancer, 37.5%; breast cancer, 26.7%; cancers of the hematopoietic system, 2.9%; benign diseases, 0.9% (332 sera); and normal healthy donors, 0.7% (280 sera).

Within the lung cancer group, 95% of the sera from 21 advanced (Stages III and IV) nontreated adenocarcinoma patients gave positive results with high inhibition titers, whereas only 27% of sera from treated advanced adenocarcinoma patients yielded positive results. The sensitivity of the cell-binding inhibition assay is similar to those of the solid-phase radioimmunoassay and reverse passive-hemagglutination assays. Reproducibility tests yielded an r value of 0.90.

These results suggest that this simple cell-binding inhibition assay could be applied with monoclonal antibodies, such as CSLEX1, to monitor cancer.

INTRODUCTION

Recently, Koprowski et al. (14), Bast et al. (2), and Morgan et al. (18) reported the application of monoclonal antibodies 19-9, OC125, and 205/165, respectively, to detect tumor-associated antigens in the sera of cancer patients. The antigenic determinant of the 19-9 monoclonal antibody, for which a colorectal cancer cell line was used as the immunizing agent, has been identified as sialosylated lacto-N-fucopentaose II (15, 16). The monoclonal immunoglobulin OC125 was raised against the epithelial ovarian carcinoma cell line OVC443. The antigenic determinant defined by this antibody is associated with a high-molecular-weight glycoprotein expressed in the coelomic epithelium during embryonic development (1).

These 2 monoclonal antibodies have been used with radioimmunoassays to demonstrate elevated levels of their tumor-associated antigens in the sera of cancer patients (10, 12). In addition, Morgan et al. (18) used an amplified ELISA3 to detect elevated levels of a M, = 100,000 glycoprotein containing an N-asparagine-linked carbohydrate antigen in sera of patients with malignant melanoma. These studies suggest the potential use of such techniques to diagnose and monitor cancer.

We recently produced a monoclonal antibody, CSLEX1, that recognizes sialosylated Lex (7). In this report, we describe a new inhibition assay to detect antigens in sera using CSLEX1.

MATERIALS AND METHODS

Monoclonal Antibodies. The various monoclonal antibodies in this study were produced according to a modified form of the procedure described by Köhler and Milstein(13). Sixty mouse monoclonal antibodies were produced with various cancer tissues and cell lines as the immunizing agents.

Monoclonal antibody CSLEX1 was produced by immunization with a primary gastric adenocarcinoma and reacted with a sialosylated form of Le^ (sialosyl a_2→3 Le^) (7). This determinant differed from those of the previously reported sialosylated-antigen-reactive antibodies IB9 (9), FH6 (6), and 19-9 (16), which were directed against the following tumor-associated antigens: a sialosyl a_2→6 galactosyl residue; sialosyl a_2→3 difucosylganglioside; and sialosyl a_2→3 Le^, respectively.

Human Sera. Nagasaki University Hospital (Nagasaki, Japan) and National Kinki Chuo Hospital (Osaka, Japan) kindly provided sera from 594 patients with malignant diseases, 332 patients with benign diseases, and 280 healthy persons. Sera were stored at -80 °C until used. Patients were categorized into Stages I to IV according to the International Union Against Cancer classification. Except for the breast cancer sera, most of the sera were from Stages III and IV cancer patients.

Microcytotoxicity Test. The complement-dependent microcytotoxicity test was performed according to standard microtechniques (21). Briefly, 1 μl of antibody was incubated with cells for 0.5 h, followed by a 1-h incubation with rabbit complement of 25 °C. The percentages of dead cells were counted by eosin dye exclusion.

Indirect Immunofluorescence Test. The indirect immunofluorescence test was done by incubating cells with 50 μl of appropriately diluted antibodies at room temperature for 30 min. After washing 3 times with 0.1% sodium azide in PBS, the cells were incubated in 50 μl of fluorescein isothiocyanate-conjugated goat anti-mouse IgM for another 30 min at 4 °C, followed by 3 washings. Cells were examined with a fluorescence microscope.

Reverse Passive-Hemagglutination Test. The reverse passive-hemagglutination test was carried out in U-shaped wells containing 50 μl of 2-fold dilutions of sera and 50 μl of 1% ox RBC sensitized with CSLEX1. Chronic chloride was used in the coupling of antibody to RBC (19). The reaction was read following 2 h incubation at room temperature.

Solid-Phase Radioimmunoassay Assay. The solid-phase radioimmunoassay was performed as described previously (5). Briefly, 50 μl of CSLEX1 antibody (50 to 100 μg/ml) was added to ELISA

1 This work was supported by a grant from the Green Cross Corporation, Osaka, Japan.
2 To whom requests for reprints should be addressed, at the UCLA Tissue Typing Laboratory, 15-30 Rehabilitation Center, 1000 Veteran Ave., Los Angeles, CA 90024.
Received 8/27/84; revised 12/18/84; accepted 1/9/85.

CANCER RESEARCH VOL. 45 APRIL 1985

1901
DETECTION OF SIALOSYLATED LEWIS

wells (Immulon I; Dynatech Laboratories, Inc.), incubated overnight at 4 °C, and blocked by 1% bovine serum albumin for 1 h at 37 °C. Then, 50 µL of sera were added to the wells and incubated for 2 h at 37 °C. The wells were then washed and 125I-labeled CSLEX1 antibody (~50,000 cpm) was added. After a 2-h incubation, the wells were thoroughly washed again. The radiolabeled antibody bound to the wells was counted with a γ-counter. Samples were considered positive when the value of cpm of sample per average cpm of normals was greater than the mean + 2 SD of the normal sample.

Cell-binding and CBI Assays. Terasaki microtest tray wells were coated with antibody by incubating overnight at 4 °C. After the trays were washed, approximately 5 µL of 0.5% FCS in PBS was added to each well. The promyelocytic leukemia line HL60 was treated with 4% formaldehyde at 4 °C overnight for use as the target. Two µL of formaldehyde-fixed HL60 cells (at a concentration of 6 to 8 x 10⁶/mL) was added and incubated for 10 min at room temperature to allow binding to the antibody coating. The trays were then tilted for 30 min at a 60° angle. During this time period, unbound cells fell to one side of the well leaving a clear area in the center. The trays were then laid flat for reading with an inverted light microscope. The results were scored according to the percentage of the well surface covered by bound cells as follows: 0 to 10% coverage, score 1; 10 to 30%, 2; 30 to 60%, 4; 60 to 80%, 6; and 80 to 100%, 8. Reactivity was considered positive if the score was greater than or equal to 6. For the CBI assay, trays were coated with CSLEX1 diluted 1:1000 as described above. After the trays were washed, 2-fold serial dilutions of serum in PBS were added and incubated for 2 h at room temperature. The trays were washed, and HL60 targets were added as described previously. The results were expressed by the titer of serum that inhibited the cell binding, as indicated by a score of 1 or 2. Cancer and benign disease sera, arranged randomly within each panel, were read blind. Results were then compared to the results of the normal control panels.

ELISA. For the micro-ELISA testing, 60-well Terasaki trays were coated overnight at 4 °C with various concentrations of CSLEX1. After washing with 0.5% FCS in PBS, 5 µL of peroxidase-labeled goat anti-mouse IgM (KPL Laboratories) was allowed to react for 1 h at 37 °C. After washing 5 times, 5 µL of o-phenylenediamine was added and incubated at room temperature for 15 min. The reaction was stopped with 2.5 M sulfuric acid. The optical density was measured at 492 nm with a Dynatech TR200 reader.

RESULTS

Comparison of Antibody-coating Detection by ELISA and Cell-binding Assays. ELISA and cell-binding assays were performed on identically coated trays to compare the sensitivity of the 2 assays in the detection of coated antibody. The amount of coated antibody necessary for a positive result in the cell binding assay with CSLEX1 and HL60 target was very close to that of the limit of antibody detection by ELISA. A FCS concentration of 0.5% was selected as the optimal diluting medium for minimizing the nonspecific stickiness of the cells while maintaining high levels of antibody coating, as determined by ELISA.

Comparison of Cell-binding, Cytotoxicity, and Immunofluorescence Tests. The ascites forms of 60 monoclonal antibodies raised against various cancers were diluted 1:1000 for the cell-binding, cytotoxicity, and immunofluorescence tests. Live HL60 cells were used for the cytotoxicity and immunofluorescence tests. Formaldehyde-treated HL60 cells were used in the cell-binding test.

The same reactions by these 3 methods; of these 58, 13 were positive and 45 were negative. Two antibodies reacted positively by the cytotoxicity and immunofluorescence tests but were negative by the cell-binding assay. However, reactivities of these 2 antibodies to HL60 by the immunofluorescence test were weak; only 60% of the target cells were stained to a low degree at the 1:1000 dilution.

Reproducibility of the CBI Assay. The reproducibility of the CBI assay was checked by testing 120 randomly selected cancer sera on different days. The r value for this test was 0.90 (Chart 1).

Table 1
Comparison of cell-binding, cytotoxicity, and immunofluorescence test results with 60 monoclonal antibodies versus HL60 target

<table>
<thead>
<tr>
<th>Assay pattern</th>
<th>Cell binding</th>
<th>Cytotoxicity</th>
<th>Immunofluorescence</th>
<th>No. of monoclonal antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>13</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>2</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>45</td>
</tr>
</tbody>
</table>

r values: cell binding vs. cytotoxicity = 0.91
cell binding vs. immunofluorescence = 0.91

Table 2
Comparison of CBI, sandwich, and reverse passive-hemagglutination assay results for 60 breast cancer and 36 normal sera

<table>
<thead>
<tr>
<th>Assay pattern</th>
<th>CBI</th>
<th>Sandwich</th>
<th>Reverse passive hemagglutination</th>
<th>No. of sera</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast cancer sera</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>12</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>2</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>2</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>3</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>38</td>
</tr>
<tr>
<td>Normal sera</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>35</td>
</tr>
</tbody>
</table>

r values: CBI vs. reverse passive hemagglutination = 0.74
CBI vs. sandwich = 0.76
Reverse passive hemagglutination vs. sandwich = 0.72

CANCER RESEARCH VOL. 45 APRIL 1985

1902
TABLE 2. The CBI, radioimmunosandwich, and reverse passive-Hemagglutination Assays. Sixty breast cancer sera were positive, and 38 were negative by the radioimmunosandwich method, r values were calculated by $\chi^2$ between pairs of methods and ranged from 0.72 to 0.76.

Comparison of CBI, Radioimmunosandwich, and Reverse Passive Hemagglutination Assays. Sixty breast cancer sera (11 Stage I, 18 Stage II, 23 Stage III samples) and 36 normal sera were tested using these 3 assays. These panels were read blind. The resulting reaction patterns are shown in Table 2. The CBI, radioimmunosandwich, and reverse passive-hemagglutination methods yielded positive results in 26.7, 28.3, and 25.0% of the breast cancer sera, respectively. Twelve of the 60 breast cancer sera were positive, and 38 were negative by all 3 assays. There was a lack of consensus for 10 of the sera. Only one serum from the normal group showed a weak positive reaction by the radioimmunosandwich method. $r$ values were calculated by $\chi^2$ between pairs of methods and ranged from 0.72 to 0.76.

Sialosylated Le\textsuperscript{a} Titors among Patients and Controls. Table 3 summarizes the CBI assay results of sera from 524 cancer patients, 70 hematopoietic malignant disease patients, 332 benign disease patients, and 280 healthy blood donor controls. CSLEX1 was used in all serum testing.

Samples that showed inhibition titers of 1:16 and above were considered positive in this assay; 278 of 280 (99.3%) normal control sera showed titers of 1:16 or less.

Twenty-nine (58%) of the 50 positive sera from patients with lung adenocarcinomas yielded relatively high inhibition titers of 1:128 or higher. In contrast, only 4 of 22 (18%) positive sera from patients with squamous cell carcinomas yielded titers of 1:128 or higher.

The frequency of sialosylated Le\textsuperscript{a} detection in the various subgroups of solid tumor patients' sera ranged from 28.0 to 47.8%. In contrast, only 2.9% of 70 sera from patients with hematopoietic cancers (leukemia, malignant lymphoma, and myeloma) showed positive inhibition.

Relationship between Clinical Stage of Lung Cancer and CBI Titer. The correlation between the clinical stage of lung cancer and CBI titer was as follows in the CBI assay of 201 lung cancer sera, summarized according to stage (Chart 2): Stage I, 31.3%; Stage II, 18.2%; Stage III, 40.0%; and Stage IV, 51.5%. Although the positive percentages observed in the advanced stages (III and IV) were higher (46.7 versus 25.9%) than those observed in the "early" stages (I and II), statistically significant differences between the titers of the 4 stages were not observed (Kruskal-Wallis test), possibly due to the limited number of samples in the early stages.

Effect of Clinical Treatment of Patients on CBI Test Results. The lung cancer patients were divided into 2 categories, treated and nontreated. Treatments included chemotherapy, radiation, and/or surgery. Patients showing evidence of tumor loading who had not been treated in the 6 months prior to bleeding but who may have had previous histories of therapy were classified as
nontreated for this study. In advanced (Stages III and IV) adenocarcinomas, 20 of 21 (95%) nontreated patients' sera showed relatively high inhibition titers, whereas only 13 of 48 (27%) sera from treated patients in this group gave positive test results (Chart 3). This observed difference is significant (P < 0.001, Wilcoxon paired rank sum test).

In the squamous cell and other types of lung carcinomas, significant differences between sera of treated and nontreated patient groups were not observed. Despite their classification as advanced stage samples, 4 of 9 sera from nontreated squamous cell carcinoma patients were negative. However, it is encouraging that 3 cases (2 squamous and 1 small cell carcinoma) of the early stage samples could be detected.

**DISCUSSION**

We have described here the detection by a new CBI assay of a tumor-associated antigen, sialosylated Le\(^a\), in the sera of cancer patients. The assay uses the well-known fact that cells will bind to solid-phase surfaces coated with antibody (4). Among the unique features that have been introduced is the use of formalin-fixed cells as targets. This permits standardization of the assay since the target is fixed and does not vary according to culture conditions. In addition, the treated target cells can be stored at 4 °C for over 1 month. By using microtest wells, it is possible to test large numbers of sera very simply using minute quantities of reagents. Perhaps most importantly, tilting the tray at a 60° angle provides a very simple method of applying a constant force to the cells on the plastic plate. With a semiautomated stage mover attached to an inverted phase-contrast microscope, it is possible to read and record reactions in less than 1 s/well. Although the readings are subjective, optical readers could eventually be designed.

In comparison with the cytotoxicity and immunofluorescence assays, essentially the same degree of sensitivity was noted for the cell-binding assay. In addition, the reactivity of sera from cancer patients to the monoclonal antibody CSLEX1 was compared for the CBI, radioimmunosandwich, and reverse passive-hemagglutination assays. Comparable results with r values of 0.72 to 0.76 were obtained among these 3 tests. However, it should be noted that the CBI assay is simpler than other assays such as the sandwich and reverse passive-hemagglutination assays described here. The CBI assay does not require radioactive labeling, sensitization of RBC, or application of secondary antibodies. Also, being an inhibition assay, CBI may be more useful for detecting antigens present in monovalent forms, whereas the sandwich and reverse passive-hemagglutination assays require polyvalent antigens. With respect to reproducibility, 120 randomly selected cancer sera yielded an r value of 0.90.

We have shown previously that CSLEX1 detects a sialosylated Le\(^a\) epitope in cancer tissue (7). In 2 other studies, this epitope was found to be present in the serum of cancer patients by the sandwich and reverse passive-hemagglutination assays (5, 11). Here we have shown that sialosylated Le\(^a\) in the sera of cancer patients can also be detected by the new CBI assay. The 3 assays detect the antigen in essentially the same sera. It was further noted that the presence of the hapten in breast cancer sera was stage dependent in that 7% of the early-stage patients were positive, whereas 45% of those in Stages III and IV yielded positive test results. Also, the titers of inhibition for the nontreated group of lung cancer patients were significantly higher than those of the treated group, with as many as 95% of the sera giving a positive result. However, since information regarding the patients' tumor burden was not available, temporal studies of individual patients are needed to determine the precise relationship between serum levels of sialosylated Le\(^a\) and the response of the patient to therapy. Nevertheless, from our preliminary results, it appears that the sialosylated Le\(^a\) hapten may be useful to monitor and evaluate therapy for various cancers.

Although the sialosylated Le\(^a\) hapten is present in normal tissue, it is more abundant in tumor tissue (7) and is currently associated with cancer in sera. Only 0.7% of 280 normal control sera and 0.9% of 332 sera from patients with benign diseases were positive, whereas 37% of 524 sera from carcinoma patients were positive. This marked association with cancer is similar to that noted for the sialosylated Le\(^a\) hapten detected by monoclonal antibody 19-9 (20). Elevated levels of sialosyltransferase activity in cancer tissue (3) as well as in the sera of cancer patients (8) have been reported.

CSLEX1 reacted with particularly high percentages of sera from patients with adenocarcinomas of the lung, gall bladder or bile duct, and colon. It is possible that the sialosylated Le\(^a\) hapten is secreted by adenocarcinomas into the circulation as a mucin, as in the case for sialylated Le\(^a\) in colon carcinomas (17). CSLEX1 also detected sialosylated Le\(^a\) in other non-mucin-secreting types of cancer, with the antigen possibly being shed from the cancer cell membrane as a glycoprotein or a glycolipid.

The ability of CSLEX1 to distinguish groups of cancer sera from those of normal and benign disease patient sera by the CBI assay is quite striking. The assay is very simple, does not require any special instruments, can be performed within 4 h, and is as sensitive as the radioimmunosandwich and reverse passive-hemagglutination assays with good reproducibility. The CSLEX1 antibody along with the CBI assay has potential for applications in the mass screening and/or monitoring of cancer patients.

**REFERENCES**

DETECTION OF SIALOSYLATED LEWIS*


Sialosylated Lewis^X in the Sera of Cancer Patients Detected by a Cell-binding Inhibition Assay

Masaki Hirota, Kiyoyasu Fukushima, Paul I. Terasaki, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/45/4/1901

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.