Sandwich ELISA Using a Mouse/Human Chimeric CSLEX-1 Antibody

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BACKGROUND: An assay using a mouse antisyalyl Lewis X (sLeX) antibody (CSLEX-1) is used clinically for screening and monitoring patients with breast cancer in Japan. However, the IgM isoform of CSLEX-1 is not preferred for the assay because the bulkiness of IgM generally causes poor accessibility to the antigen. To solve this problem, we developed an antisyLeX mouse/human chimeric IgG antibody, CH-CSLEX-1, using transgenic silkworms. The performance of a homologous sandwich ELISA of CH-CSLEX1 was then evaluated.

METHODS: To generate CH-CSLEX-1, we used a GAL4/UAS binary gene expression system in transgenic silkworms. The reactivities of CSLEX-1 and CH-CSLEX-1 were determined in a Biacore analysis. To confirm antigen specificity, 3 antigens [sLeX, sLeA, and Lewis Y (LeY)] were used.

RESULTS: CH-CSLEX-1 formed correctly as an IgG class of immunoglobulin molecule with an isoelectric point close to the predicted value. The best combination for capturing and probing in a sandwich ELISA was determined as a homologous combination of CH-CSLEX-1. The CH-CSLEX-1 assay specifically detected sLeX, but not sLeA and LeY. A correlation analysis with 107 human samples showed good concordance between the conventional CSLEX-1 assay (homologous sandwich ELISA using CSLEX-1) and the CH-CSLEX-1 assay ($r = 0.98$). Moreover, the CH-CSLEX-1 assay was not affected by either human antimouse IgG antibodies (HAMA IgG) or HAMA IgM.

CONCLUSIONS: The mouse/human chimeric antibody CH-CSLEX-1 allowed the establishment of a highly specific sandwich ELISA for sLeX that was not affected by HAMA.

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The sialyl Lewis X (sLeX)4 and sLeA antigens were identified and recognized as tumor markers in the early 1980s via a series of investigations using monoclonal antibodies against cancer tissues (1, 2). Various types of antisLeX antibodies exist that recognize unique sLeX antigens: FH6 and antibody (CSLEX-1) against sLeX as a carbohydrate antigen, and NCCST439 against sLeX presenting on the mucin protein (3–6). To quantify the serum concentration of such carbohydrate antigens, a homologous sandwich ELISA was constructed in which the same clone of monoclonal antibody was employed for both capturing and probing, because the antigen presents in serum as multiple epitopes on core protein(s). Currently, NCCST439 and CSLEX-1 homologous sandwich ELISAs are used in clinical practice in Japan to monitor patients with advanced breast cancer (7, 8).

Several lines of evidence have shown the association between sLeX and the prognosis of patients with breast cancer, colorectal cancer, and non–small-cell lung carcinoma (9–12). Interestingly, cimetidine, which is largely used for the treatment of heartburn and peptic ulcers, has been shown to dramatically improve survival in patients with colorectal cancer exhibiting tumor cells that expressed high concentrations of sLeX and sLeA (13), suggesting that cimetidine blocks the adhesion of tumor cells to the endothelium and prevents metastasis via the suppression of the expression of E-selectin, which binds to sLeX and sLeA, on the surface of vascular endothelial cells (14). A similar observation has been reported in lung cancer (15).

Because CSLEX-1 is an IgM and its bulkiness is thought to cause low accessibility to the antigen, we postulated that the analytical performance of the CSLEX-1 assay would be improved by changing the isoform to an IgG type. sLeX is thought to be a T-cell–independent antigen and to elicit exclusively an IgM-class antibody response in the mouse. Therefore, the only way to establish an antibody using an IgG isotype against the CSLEX-1 antigen is via the biotechnological cassetting of
the binding site of CSLEX-1 into a recombinant IgG expression system. We chose the human IgG expression system to add the human IgG constant region to the CSLEX-1 binding site, thus expecting to eliminate human antimouse antibody (HAMA) reactions causing a pseudopositive diagnosis of the HAMA-positive samples. The ELISA systems used in clinical practice are generally applied by taking measures to prevent a HAMA reaction, such as adding neutralizing antigen(s) to HAMA into the reaction buffer. However, proving complete prevention of this reaction for all samples is not possible. Therefore, chimerization to the constant region of human IgG could be a fundamental resolution for this problem.

We employed an expression technology in transgenic silkworms (16, 17) for the production of a chimeric mouse/human anti-CSLEX-1 IgG1 antibody (CH-CSLEX-1), because the technology has been proven to be practical for the industrial production of recombinant materials, and the methodology from rearing silkworms to the extraction and purification of materials has been approved as a production system for reagents of clinical diagnosis by the Japanese Ministry of Health, Labor, and Welfare. Here, we report the binding characteristics of CH-CSLEX-1, and compare the performance of a homologous sandwich ELISA for CH-CSLEX-1 with that of a conventional sandwich CSLEX-1 ELISA.

Materials and Methods

PLASMID CONSTRUCTION

The sericin1 promoter and its 3’-untranslated regions (3’-UTRs) were amplified from the genomic DNA of B. mori using the primers (TCTAGATTGGCGGTTCGAGTCGGGAGTCTGCACTGATGTGGTATGGCTGACCTAGTTAGCACTCGCC) and (GGCCAAGGCCCATCTTACAGTCTTACGGC), and sequences were confirmed. DNA templates for CH-CSLEX-1 and human IgG1 in SOE-PCR were synthesized (GenScript).

A new expression vector was constructed using the amplified CH-CSLEX-1 L chain, the 3’-UTR of SV40, the UAS promoter, and the CSLEX-1H chain via PCR with 8 primers: CH-CSLEX-1Lf (GGGACTAGTTTGGAGGCTGAAAAGAACAGTGGCTGCCCTCACTCGAGCTGATGCTTCTATTCCAT), CH-CSLEX-1Lr (GATGAACACTGACGGGGCACGCAATGCTGACCTAGTTAGCACTCGCC) and sequences were confirmed. DNA templates for CSLEX-1 and human IgG1 in SOE-PCR were synthesized (GenScript).

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PURIFICATION OF CH-CSLEX-1
The silk glands of transgenic silkworms were pulled from their bodies and homogenized in extraction buffer (20 mmol/L phosphate, pH 7.0) and centrifuged at 70000 g for 30 min. A saturated solution of ammonium sulfate was added to the supernatant up to 10% volume. The supernatant was centrifuged at 70000 g for 30 min and filtered (pore size, 0.45 μm). The solution was injected into a Protein A column (GE Healthcare) and separated using elution buffer (100 mmol/L glycine buffer, pH 2.7). The fractions were neutralized using alkaline buffer (1 mol/L Tris, pH 9.0) and pooled. The buffer of the fraction was changed to PBS (10 mmol/L phosphate, 150 mmol/L NaCl, and 0.05% azide) and filtered (pore size, 0.45 μm). The concentration of CH-CSLEX-1 was calculated using a BCA analysis kit (Thermo Fisher Scientific).

SDS-PAGE AND ISOELECTRIC FOCUSING
CH-CSLEX-1 and CSLEX-1 solutions were mixed with SDS-PAGE buffer (500 mmol/L Tris, pH 6.8, containing 20 g of SDS, 10 mL of glycerol, and 200 mg of bromophenol blue per liter) and analyzed by 5%–20% gradient SDS-PAGE with (reducing) or without (nonreducing) 100 mmol/L dithiothreitol. Isoelectric focusing was performed using IEF (isoelectric focusing)–PAGE pH3–10 (TEFCO).

HORSE RADISH PEROXIDASE– OR ALKALINE PHOSPHATASE– CONJUGATED ANTIBODIES
Horseradish peroxidase (HRP)- or alkaline phosphatase (ALP)-conjugated CSLEX-1 and CH-CSLEX-1 were prepared using peroxidase and alkaline phosphatase labeling kits (DOJINDO), respectively.

EVALUATION OF REACTIVITY WITH ANTIGEN-BOUND PLATE
For examination of reactivity in a wide pH range, the following procedure was employed. Antigens derived from colo205 cells were diluted with 50 mmol/L carbonate–bicarbonate buffer, pH 9.6, and applied to the polystyrene plates. The plates were incubated for 1 h at 25 °C, aspirated, and coated with 10 mmol/L phosphate-buffered saline, pH 7.4, containing 10 g of casein per liter at 4 °C overnight. HRP-conjugated CH-CSLEX-1 and CSLEX-1 were diluted in reaction buffer (26.1 mmol/L Britton–Robinson buffer, containing 10 g of Triton X-405 and 20 g of normal mouse serum/L), pH 2.0–9.0, and were incubated on the antigen-bound plate for 2 h at 37 °C. The plates were washed 3 times using wash buffer (10 mmol/L PBS, pH 7.4, containing 0.5 g of Tween-20 per liter). The plates were incubated with 3, 3′, 5, 5′-tetramethylbenzidine for 10 min at 37 °C and were analyzed using Model 680 Microplate Reader Accessories (Bio-Rad Laboratories). The reaction curves obtained using the plates without the antigens were subtracted from the reaction curves obtained using the plates with the antigens. The data were standardized based on the highest values.

For examination of the reactivity in the presence of NaCl, ALP-conjugated antibodies diluted in the reaction buffer at pH 6.0 with NaCl (0–150 mmol/L) were applied to the antigen-bound plate as described above. At a substrate reaction step, Lumi-Phos® 530 was applied and incubated at 37 °C for 10 min and analyzed using an Ascent Microplate Fluorometer (Thermo Fisher Scientific).

For competitive binding assay, 2 kinds of antigen-bound plate were prepared: sLeX-acetyl phenylenediamine (APD)-human serum albumin (HSA) and antigens derived from colo205 cells. At a reaction step, ALP-conjugated CSLEX-1 or CH-CSLEX-1 diluted in reaction buffer was applied with nonlabeled CSLEX-1 or CH-CSLEX-1 (0–40960 ng/well).

REACTION KINETICS OF CH-CSLEX-1 AND CSLEX-1
A kinetics analysis was performed using a Biacore T-200 apparatus (GE Healthcare). sLeX-APD-HSA (IsoSep AB) was diluted in 10 mmol/L HEPES buffer, pH 7.4, containing 150 mmol/L NaCl, 3 mmol/L EDTA, and 0.5 g of surfactant P20 per liter, whereas CH-CSLEX-1 and CSLEX-1 were diluted in 20 mmol/L citrate buffer, pH 6.0, containing 50 mmol/L NaCl and 2 g of Triton X-100 per liter, respectively. The sLeX solution was flowed over a Series S Sensor Chip CM5 (certified grade) and the reaction between sLeX-APD-HSA and the sensor was confirmed. The solution that did not contain sLeX-APD-HSA was flowed over another sensor chip (reference sensor chip). The solutions of the 2 antibodies were flowed over the sensor chips for 120 or 80 s and the solutions without antibodies were flowed for 600 s at a flow rate of 30 μL/min. The reaction curves obtained using the reference sensor chips were subtracted from the reaction curves obtained using the sensor chips with sLeX-APD-HSA. The data were analyzed via kinetics analysis using the Biacore T-200 Evaluation Software, version 1.0, and Kon, Koff, and Kd (Koff/Kon) were calculated.

SANDWICH ELISA
CSLEX-1 or CH-CSLEX-1 was diluted with 50 mmol/L carbonate–bicarbonate buffer, pH 9.6, and applied to the polystyrene plates (0.5 μg/well). The plates were incubated for 1 h at 25 °C, aspirated, and coated with 10 mmol/L PBS, pH 7.4, containing 10 g of casein per liter at 4 °C overnight. Samples diluted in reaction buffer were applied to the CH-CSLEX-1 plates. Both plates were washed 3 times using wash buffer. ALP-conjugated CSLEX-1 or CH-CSLEX-1 diluted in reaction buffer was incubated at 37 °C for 2 h. Then, the plates was washed 3 times using wash buffer. Lumi-Phos® 530 was applied and incubated at 37 °C for 10 min and analyzed using an Ascent Microplate Fluorometer (Thermo Fisher Scientific).
applied to the plates, which were incubated at 37 °C for 10 min and analyzed using an Ascent Microplate Fluorometer.

For examination of NaCl tolerability at the first reaction, the antigens from colo205 cells were diluted in reaction buffer with 0–600 mmol/L NaCl and applied at a sample reaction step.

For evaluating HAMA effects, 2 human samples confirmed to be harboring HAMA activity were obtained as follows. One showed a pseudopositive diagnosis in a CSLEX-1 assay and the cause was confirmed to be HAMA IgM, a human antimouse IgM, in our laboratory. The other was a sample with HAMA IgG, not IgM, purchased from Access Biologicals LLC (Vista). The serum was mixed with and without the HAMA-blocking reagent THBR2 (Institute of Immunology) and applied as the test samples in experiments.

For evaluation of antigen specificity, HSA-conjugated glycans, sLeX-APD-HSA, sLeA-APD-HSA (Glyco Tech), LeY- aminophenylethyl (APE)-HSA (Glyco Tech), or HSA (1, 2, and 4 μg/well) was applied to the CSLEX-1 or CH-CSLEX-1 plates as a sample. The intensities of HSA-conjugated glycans and HSA were standardized by dividing by the intensities obtained without the glycans and HSA.

ANALYSIS OF CSLEX CONCENTRATIONS IN SERA OF SAMPLES
Sera from 107 samples were analyzed in a homologous CH-CSLEX-1 sandwich ELISA and a commercially available CSLEX-1 assay kit (NITTOBO MEDICAL Co., Ltd.) as a homologous CSLEX-1 sandwich ELISA. The sera were obtained from the following sources: 50 healthy donors (BioreclamationIVT), 37 patients with breast cancer (Access Biologicals LLC), and 20 HAMA-positive patients (ProMedDx). The patients with breast cancer had stage III disease and had been analyzed for CA15-3 values using Immulite (Siemens). The analyses were similarly performed using the method described above. The second reaction of ALP-conjugated CH-CSLEX-1 was replaced by HRP-conjugated CH-CSLEX-1. Before the analyses, we examined whether the serum samples harbored HAMA activity; 20 among 107 samples showed HAMA activity.

STATISTICAL ANALYSIS
The Pearson correlation coefficient between the CSLEX-1 and CH-CSLEX-1 assays was determined using MedCalc Software (version16.2.1). P-value and 95% CI for the correlation coefficient were similarly determined.

Results
CONSTRUCTION OF A CHIMERIC CSLEX-1 EXPRESSION SYSTEM
To construct a production system of CH-CSLEX-1, we generated a new transgenic silkworm strain (Fig. 1). First, the genes that encode the CH-CSLEX-1 L and H chains
were amplified and inserted into an expression vector (Fig. 2A). The expression vector was injected into the eggs of silkworms, and a transgenic silkworm strain was selected with a color marker using KMO (19, 20). To confirm the production of the CH-CSLEX-1 antibody, we extracted antibodies from the silkworm between the different generations (#1–3) and purified them using a Protein A column. An SDS-PAGE analysis performed using the fractions revealed 2 bands of about 25 kDa and 50 kDa under reducing conditions and a single band of about 150 kDa under nonreducing conditions (Fig. 2B). Electro focusing showed that the isoelectric point of CH-CSLEX-1 was between pH 8.0 and 8.3 (Fig. 2C), which was in agreement with the theoretical value of pH 8.02 (calculated using the ExPASy ProtParam tool). These results clearly demonstrated that the newly generated silkworm strain was able to produce CH-CSLEX-1.

CHARACTERISTICS OF THE CH-CSLEX-1 ANTIBODY

To characterize this CH-CSLEX-1 antibody, the pH dependency of the reaction was examined using a tumor cell extract as the antigen (Fig. 3A). In this analysis, we used an antigen-bound polystyrene plate and HRP-conjugated CSLEX-1 and CH-CSLEX-1. The

![Fig. 2. Construction of the CH-CSLEX-1 generation system using transgenic silkworms.](image)

![Fig. 3. Characteristics of the CH-CSLEX-1 antibody.](image)
optimal pH was observed to be identical for both CSLEX-1 and CH-CSLEX-1 (pH 5.0–6.0), suggesting an absence of substantial alterations of reactivity by the chimerization, although the isoelectric points were markedly different (Fig. 2C). Subsequently, we determined the association velocity (Kon) and the dissociation velocity (Koff) of CH-CSLEX-1 and CSLEX-1 using a Biacore analysis with sLeX-conjugated HSA (13 sLeX per mole of HSA). The analysis showed that the Kon of CH-CSLEX-1 was analogous to that of the original CSLEX-1 (CH-CSLEX-1, 7.5 \times 10^5; CSLEX-1, 5.3 \times 10^5), but its Koff was much faster than that of CSLEX-1 (CH-CSLEX-1, 9.8 \times 10^{−3}; CSLEX-1, 2.8 \times 10^{−3}). Consequently, the calculated Kd was substantially different between CSLEX-1 and CH-CSLEX-1 (CSLEX-1, 5.2 \times 10^{−10}; CH-CSLEX-1, 1.3 \times 10^{−8}; Table 1). In an experiment of NaCl tolerability, the binding of CH-CSLEX-1 was more highly susceptible to NaCl concentration than that of CSLEX-1 (Fig. 3B).

Next, we examined whether CH-CSLEX-1 bound exactly to the same epitope of the CSLEX-1 antigen. For that purpose, competition assays were performed using sLeX-APD-HSA and a crude extract of colo205 cancer cells as an antigen, independently. The binding of CH-CSLEX-1 was completely inhibited by the addition of CSLEX-1 at a molar ratio of 5:1 in both experiments. However, CSLEX-1 binding was hardly inhibited by CH-CSLEX-1 (Fig. 3C).

**ESTABLISHMENT OF A CH-CSLEX-1 ASSAY**

To establish a sandwich ELISA for the quantification of the sLeX antigen in the serum, the best combination for capturing and probing was first determined. As expected, the homologous combination of CH-CSLEX-1 (a CH-CSLEX-1 assay) showed the best analytical sensitivity at 0 mol/L NaCl compared with the other 3 combinations, CSLEX-1/CSLEX-1, CSLEX-1/CH-CSLEX-1, and CH-CSLEX-1/CSLEX-1 (Fig. 4A). Although the bind-

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<th>Koff, s⁻¹</th>
<th>Kd, mol/L</th>
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<tr>
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<td>CSLEX-1</td>
<td>Human IgG₁</td>
<td>7.5 \times 10^5</td>
<td>9.8 \times 10^{−3}</td>
<td>1.3 \times 10^{−8}</td>
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Table 1. Kinetics analysis of CSLEX-1 and CH-CSLEX-1.

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**Fig. 4.** Establishment of a CH-CSLEX-1 assay.

(A), Evaluation of solid-phase CSLEX-1 or CH-CSLEX-1 on ELISA plates. First reactions were performed with antigens from Colo205 cells at various concentrations of NaCl. Second reactions were performed with ALP-conjugated CSLEX-1 or CH-CSLEX-1. (B), Specific reactivity of CSLEX-1 and CH-CSLEX-1, as assessed using HSA-conjugated synthetic glycans: sLeX, sLeA, and LeY. Control used only HSA. The structures of carbohydrates are shown at the right of the graph.
ing of CH-CSLEX-1 was affected by NaCl, even at a low concentration (37.5 mmol/L) in the direct binding assay (Fig. 3B), the δ intensity (subtraction of the background signal with no antigen) was tolerated up to 150 mmol/L of NaCl in the CH-CSLEX-1 assay (Fig. 4A). Therefore, we used 150 mmol/L NaCl in the reaction buffer for antigen reaction step (1st reaction buffer), because this is normal condition for preventing nonspecific binding in ELISAs. The finalized conditions of buffers in the CH-CSLEX-1 assay are as follows, 1st reaction buffer; 100 mmol/L citrate buffer at pH6.0 with 150mM NaCl, containing 10 g of Triton X-405, second reaction buffer; 25mM maronic acid buffer, pH6.0, containing 10 g of Triton X-405, and wash buffer; 10 mmol/L phosphate-buffer at pH 7.4 with 150mM NaCl, containing 0.5 g/L of Tween-20.

To confirm the antigen specificity of the CH-CSLEX-1 assay, 3 types of synthetic glycans, i.e., sLeX-APD-HSA, sLeA-APD-HSA, and LeY-APE-HSA, were measured. The CH-CSLEX-1 assay showed a high specificity to sLeX-APD-HSA, similar to that of the conventional CSLEX-1 assay (Fig. 4B).

Finally, we examined whether the CH-CSLEX-1 assay was able to escape the effects of HAMA. For this purpose, 2 types of HAMA samples were obtained: one harboring antimouse IgM activity and the other harboring antimouse IgG activity. The results clearly showed that the CH-CSLEX-1 assay was not affected by either of the HAMA types (see Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol62/issue11).

CORRELATION BETWEEN THE CSLEX-1 AND CH-CSLEX-1 ASSAYS
The correlation between the values of the CH-CSLEX-1 assay and those of the conventional CSLEX-1 assay was validated using 107 samples containing 37 breast cancer samples. The slope of the correlation curve was 0.89 and the correlation coefficient was 0.98 (Fig. 5).

Discussion
In this report, we used the unique bioengineering technology of silkworms and established a chimeric antibody (CH-CSLEX-1) with a human IgG constant region and the original binding site of the CSLEX-1 mouse IgM monoclonal antibody. The transgenic silkworm stably generated CH-CSLEX-1 over 7 generations (data not shown). CH-CSLEX-1 retained the binding site of CSLEX-1 in a competition assay (Fig. 3C). The results of a Biacore analysis showed that Kon was analogous between CH-CSLEX-1 and CSLEX-1, strongly suggesting that CH-CSLEX-1 also retains the affinity per 1 valent against the antigen. A drastic difference in reactivity was observed between the 2 antibodies, as corroborated by the following 3 results. The first was that the Koff of CH-CSLEX-1 was much faster than that of CSLEX-1 (Table 1). The second was that the binding of CH-CSLEX-1 was more susceptible to NaCl concentration than that of CSLEX-1 (Fig. 3B). The third was that the CSLEX-1 binding was hardly inhibited by CH-CSLEX-1 (Fig. 3C). These 3 differences could be explained by the 5-times lower valency of CH-CSLEX-1 as an IgG subclass (bivalent) compared with CSLEX-1 as an IgM subclass (10 valents), thus causing a lower CH-CSLEX-1 avidity compared with CSLEX-1. Better tolerability of CSLEX-1 than CH-CSLEX-1 in alkaline conditions (Fig. 3A) could also explain the difference in avidity. Our observation was in agreement with the previous 2 reports of chimeric antibodies with analogous specificity, albeit with different reactivity, vs those of the original antibody, because of immunoglobulin subclass (21, 22).

Despite the fact that the avidity of CH-CSLEX-1 was substantially lower than that of CSLEX-1, the analytical sensitivity of the CH-CSLEX-1 assay (a homologous sandwich ELISA of CH-CSLEX-1) showed the best sensitivity compared with the other sandwich assays, including the conventional CSLEX-1 assay. This could be explained by the accessibility of the probing antibody (the second antibody) after the reaction of the capturing antibody (the first antibody), as the accessibility of probing antibodies to antigens with multiepitopes, such as sLeX antigens in the serum, is strictly restricted by the distance/space from the epitope occupied by the capturing antibody. Therefore, a smaller IgG vs IgM harbors a strong advantage for the establishment of the homologs sandwich ELISA for measuring multiepitope antigens.
The correlation analysis of 107 samples showed that the values of the CH-CSLEX-1 assay were about 10% less than the values of the CSLEX-1 assay (slope, 0.89), although the correlation coefficient between the assays was good ($r = 0.98$, $P$-value $<0.0001$, 95% CI $0.97–0.98$). This may have been caused by the better accessibility of CH-CSLEX-1 compared with CSLEX-1 to a calibrator prepared from a culture supernatant from a human pancreatic cancer cell line, CEU, obtained from UCLA. The density of epitopes on the core molecule should be different between the antigens in the sera of cancer patients and the culture supernatant of a cancer cell line, leading to different accessibility between IgG and IgM. The density should also vary among patients and cancer species. This also strongly indicates the need for better accessibility to achieve an accurate quantification of sLeX in the serum of cancer patients.

In summary, we demonstrated that the mouse/human chimeric antibody allowed the establishment of a sandwich ELISA for sLeX without HAMA effects. The improved clinical relevancy of the CH-CSLEX-1 assay should be confirmed by measuring a statistically sufficient number of samples from various types of cancer.

**References**