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Original article

An ELISA method for detection of human antibodies to an immunotoxin

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Abstract

Introduction: The use of biological molecules, such as immunotoxins, as pharmaceuticals is limited by the presence and development of human antibodies to these agents. This immune response can cause significant inflammatory-related toxicities and can interfere with the efficacy of the biological agent. Therefore, a clinically applicable method to detect these human antibodies is needed for screening patients prior to enrollment and for monitoring patients during treatment. The SS1(dsFv)-PE38 immunotoxin currently in clinical trials is a hybrid molecule targeted against mesothelin-expressing cancer cells via the Fv portion of a murine antibody linked to the *Pseudomonas* exotoxin (PE), which can inhibit protein synthesis leading to cell death. The objective of this study was to determine if an enzyme-linked immunosorbent assay (ELISA)-based method could be used to detect human anti-SS1(dsFv)-PE38 antibodies in patient serum. **Methods:** Human antibodies to the immunotoxin in serially diluted serum specimens were captured on immunotoxin-coated ELISA plates, and detected using a secondary goat antihuman antibody linked to biotin in combination with horseradish peroxidase linked to avidin D (HRP–Avidin). The color was developed with tetramethyl benzidine (TMB). Curves of optical density (OD_{630}) versus dilution for 44 serum specimens were compared with positive and negative control serum specimens to classify the serum as positive or negative for anti-immunotoxin antibodies. **Results:** Ten out of the 40 patients screened were positive for anti-immunotoxin antibodies. Repeated testing of seven samples produced the same results in two independent experiments. The first two patients treated with the immunotoxin developed anti-immunotoxin antibodies during treatment. The results were in perfect concordance with a tissue culture-based neutralization assay performed by an independent laboratory. **Discussion:** An ELISA-based strategy using an immunotoxin to capture human anti-immunotoxin antibodies provides a consistently accurate technology for screening and monitoring patient serum specimens in clinical trials.
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Keywords: Antibodies; ELISA; Exotoxin; Immunotoxin; Mesothelin; Methods; *Pseudomonas*; Serum

1. Introduction

Current approaches to treat cancer with cytotoxic therapies that differentially affect cancer cells to greater extents than normal cells have met with limited success and cause significant toxicities that decrease quality of life. Novel approaches to develop strategies with improved therapeutic ratios (efficacy/toxicity) target biological molecules to proteins that are differentially expressed in cancer cells in comparison to normal cells. Immunotoxins are hybrid proteins targeted to cancer cells via monoclonal antibody domains that recognize antigens known to be overexpressed on cancer cells in comparison to normal cells (Kreitman, 2001). The antibodies' domains are fused to truncated bacterial toxins that can kill the cell after the immunotoxin is internalized. A major problem with immunotoxin therapy is that humans generally develop an antibody response to the immunotoxin, or may already have antibodies to the bacterial toxin. Major dose-limiting toxicities of immunotoxin therapies are liver damage and other inflammatory toxicities caused by the human immune response to the immunotoxin (Onda et al., 2000). Also, the presence of antibodies in treated patients is likely to interfere with the efficacy of the immunotoxin. Therefore, only patients who do not already have antibodies to the biological agent should be eligible for the clinical trials, and patients treated with the biological agent

Abbreviations: ADP, adenosine diphosphate; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; HRP–Avidin, horseradish peroxidase linked to avidin D; MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2*H*-tetrazolium inner salt); PBS, phosphate-buffered saline; PBST, PBS buffer containing 0.05% Tween 20; PE, *Pseudomonas* exotoxin; TMB, tetramethyl benzidine.

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need to be carefully monitored for the development of these antibodies. This necessitates the development of clinically applicable techniques to detect human antibodies to the immunotoxin in patient serum.

Immunotoxins targeted against mesothelin are currently in clinical trials. Mesothelin is a glycosylphosphatidylinositol-linked cell-surface glycoprotein that has been found to be expressed in malignant mesothelioma and in cancers of the ovary, cervix, lung, head and neck, esophagus, and pancreas at higher levels than in normal tissue (Argani et al., 2001; Chang & Pastan, 1996; Chang, Pastan, & Willingham, 1992; Hassan et al., 2000). SS1(dsFv)-PE38 is an immunotoxin targeted to mesothelin via the Fv portion of a murine anti-mesothelin antibody, which is fused to a mutant form of the *Pseudomonas* exotoxin (PE) (Onda et al., 2001). PE kills mammalian cells by causing irreversible ADP-ribosylation and inactivation of elongation factor 2, which results in cessation of protein synthesis (Hwang, Figzgerald, Adhya, & Pastan, 1987). The PE protein consists of three functional domains responsible for cell binding, translocation and ADP-ribosylation (Hwang et al., 1987). The mutant form of PE used in this immunotoxin contains the translocation and ADP-ribosylating domains, but not the cell-binding domain of the PE protein. The nonspecific toxicity of the SS1(dsFv)-PE38 immunotoxin was decreased by lowering the isoelectric point of the Fv portion of the molecule (Onda et al., 2001). The SS1(dsFv)-PE38 immunotoxin was found to be taken up into mesothelin-positive xenograft tumors at higher levels than present in serum of treated animals (Hassan et al., 1999) and was found to induce apoptosis in organotypic cultures prepared from primary ovarian and cervical cancers (Hassan et al., 2002). Two options to measure human antibodies to the immunotoxin are currently available. One of these options is a tissue culture assay to measure the ability of patient serum to neutralize the cytotoxicity of the immunotoxin against the mesothelin-expressing A431 K5 cell line (Hassan et al., 2000). This neutralization assay is cumbersome and would be difficult to utilize for evaluation of large numbers of patients screened and enrolled in clinical trials planned for SS1(dsFv)-PE38. Alternatively, one of several enzyme-linked immunosorbent assay (ELISA) methods to detect human antimurine antibodies that have been developed could be utilized (Kricka, 1999), but these would not detect human anti-PE antibodies. The objective of this study was to determine if an ELISA could be developed to detect human antibodies to SS1(dsFv)-PE38.

2. Methods

2.1. Patient serum

The Institutional Review Board (IRB) at the University of Oklahoma Health Sciences Center approved this study. Ten milliliters of blood was drawn from patients volunteering for this study into red top Vacutainer tubes and allowed to clot for 30 min to 1 h at room temperature for screening studies, or allowed to clot overnight at 4 °C for treatment monitoring studies. The blood was then centrifuged at $1000 \times g$ for 10 min. The serum layer was removed with a transfer pipette and aliquoted into prelabeled screw cap cryogenic vials, which were immediately stored in an ultracold freezer at − 70 °C.

2.2. Chemicals

SS1-PE38 was provided by NeoPharm (Bannockburn, IL). An antihuman IgG linked to biotin and horseradish peroxidase linked to avidin D (HRP–Avidin) were obtained from Vector Laboratories (Burlingame, CA). A biotinylated goat antihuman secondary antibody and tetramethyl benzidine (TMB) were obtained from Zymed Laboratories (South San Francisco, CA). Phosphate-buffered saline (PBS), bovine serum albumin (BSA), and Tween 20 were obtained from Sigma (St. Louis, MO).

2.3. ELISA

Ninety-six-well Costar ELISA plates were coated with 2 μg/ml SS1(dsFv)-PE38 in 10 mM PBS buffer (pH 7.4) and incubated at room temperature for 2 h. The plates were washed four times in PBST (PBS buffer containing 0.05% Tween 20), and then blocked within PBS buffer containing 3% BSA at room temperature for 2 h, followed by four washes in PBST. These plates were stored overnight at − 70 °C. Serum from patients and negative control serum were diluted 1/25, 1/50, 1/100, 1/200, and 1/400, or 1/100, 1/200, 1/400, 1/800, and 1/1600 in PBST and incubated in the coated ELISA plates at room temperature for 1 h. The plates were washed four times in PBST, and then 4 μg of goat antihuman IgG linked to biotin was incubated in the wells for 40 min at room temperature. Plates were washed four times in PBST, followed by incubation with a 1:1000 dilution of HRP–Avidin for 30 min at room temperature. After washing four times in PBST, the color was developed with TMB for exactly 5 min and the optical density (OD) was read at 630 nm. The assays conducted during the development phase were performed in triplicate and the assays performed to test the patient samples were performed in duplicate. The average OD_{630} of duplicate or triplicate wells was plotted against the dilution factor for each test specimen on the same graphs with positive and negative serum specimens.

2.4. Neutralization assay

The serum samples were also evaluated for anti-immunotoxin antibodies using the neutralization tissue culture assay in the laboratory of Dr. Robert J. Kreitman. In the neutralization assay, the ability of serum samples to inhibit cell death induced by the immunotoxin in the mesothelin-expressing A431 K5 cell line was measured. This cytotoxicity assay was performed as previously described, except that the endpoint of cell survival assay using MTS (3-(4,5-

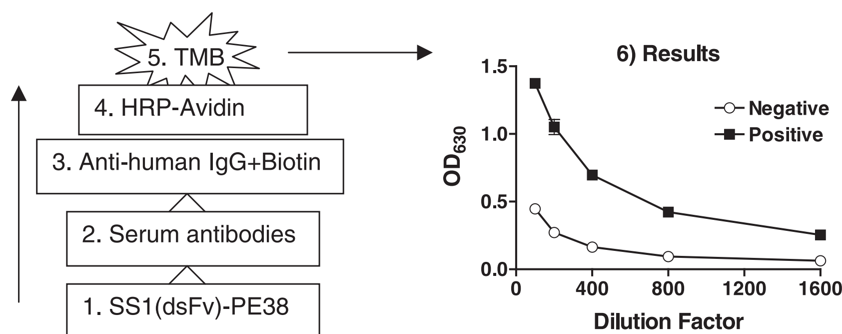

Fig. 1. ELISA to detect human antibodies to SS1(dsFv)-PE38 immunotoxin. (1) The wells of the ELISA plate are coated with the SS1(dsFv)-PE38 immunotoxin. (2) Dilutions of control serum and patient serum are incubated in the wells. (3) Bound human anti-immunotoxin antibodies are detected with a biotinylated antibody conjugated with biotin. (4) Bound biotin is detected with HRP–Avidin. (5) TMB is added and metabolized by bound HRP. The color intensity is determined spectrophotometrically. (6) The optical density (OD_{630}) is plotted against the dilution factor for controls and patients.

dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2*H*-tetrazolium inner salt) was used instead of the endpoint of protein synthesis inhibition measured by ^{3}H leucine incorporation (Hassan et al., 2000).

3. Results

3.1. Development and optimization of assay conditions

A method to quantify human antibodies to the SS1(dsFv)-PE38 immunotoxin could not be developed due to the ethical reason that human antibodies would need to be made and used as standards. Therefore, the assay was limited to determining positivity and negativity based on comparison with positive serum collected from a volunteer who was found to have antibodies to the immunotoxin (most likely from a *Pseudomonas* infection) and negative serum isolated from an immunotoxin-naive volunteer. Sufficient quantities of these serums were obtained for assay development and evaluation of patients for the Phase I clinical trial.

The ELISA strategy designed in this study is illustrated in Fig. 1. In order to capture both human antimouse and anti-PE

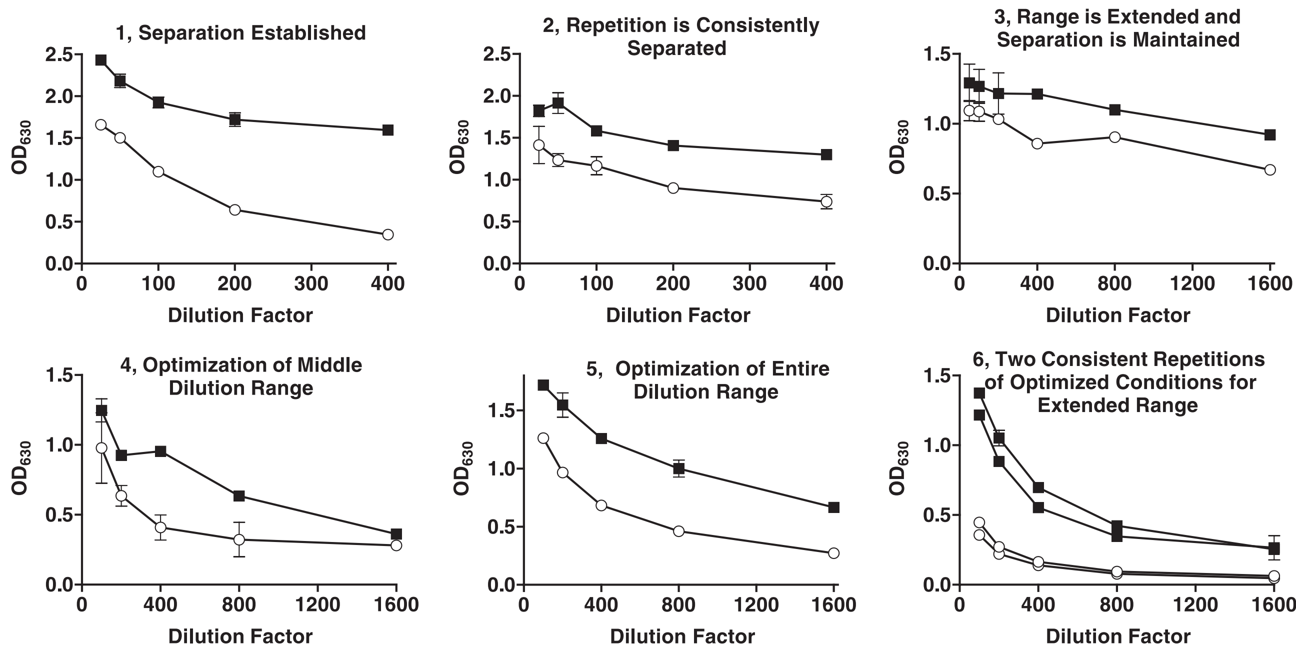

Fig. 2. Optimization of ELISA to detect human antibodies to SS1(dsFv)-PE38 immunotoxin. Graphs 1 to 6 depict the chronological step-wise optimization of the ELISA after initial conditions for separation of positive (■) and negative (○) curves were established. Each graph represents a separate experiment. Panels 1 and 2 demonstrate that experimental conditions that consistently resulted in clear separation of the positive and negative curves were obtained. Panel 3 demonstrates that these conditions effectively allowed separation of the positive and negative curves over an extended dilution range. Panel 4 demonstrates that increased separation of the middle dilution range was achieved by increasing the percentage of BSA in a blocking buffer. Panel 5 demonstrates that the entire range was further optimized by decreasing the incubation times. Panel 6 demonstrates that the optimized conditions were repeated in two additional assays with consistent results.

antibodies in the serum samples, the immunotoxin was coated onto the bottom of the ELISA plate wells. Captured human antibodies were detected by incubating the wells with an antihuman IgG antibody linked to biotin, followed by incubation with HRP–Avidin and followed by incubation with TMB for color development. Repeated washing steps

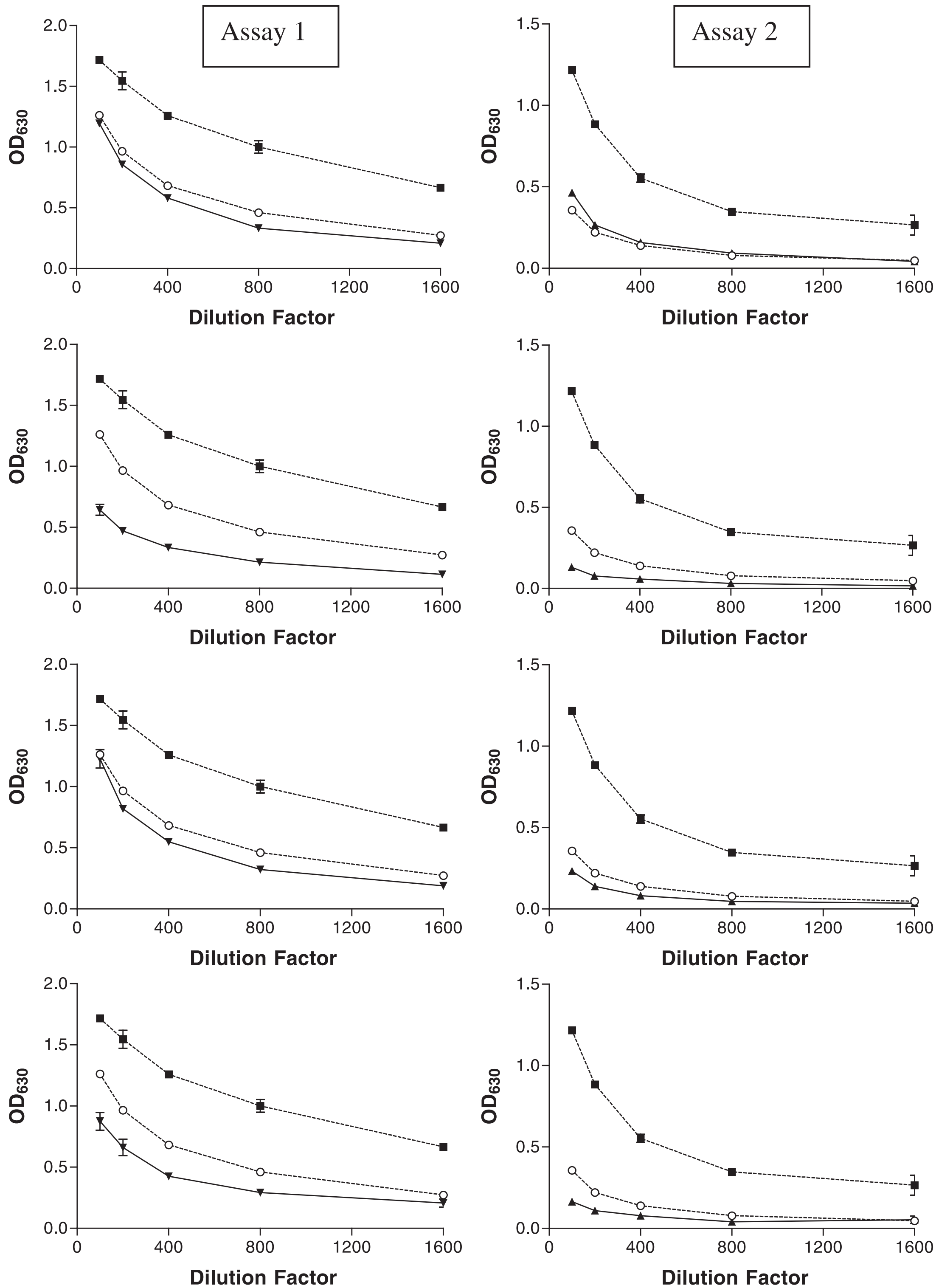

Fig. 3. Two independent ELISAs demonstrate consistent classification of serum from the same patients as negative. The positive (■) and negative (○) controls are represented by dashed lines, while the patient serum specimens (▲) are represented by solid lines. Each row represents an individual patient and each column represents an independent experiment.

were performed in between each of the incubation steps. The color development was measured by reading the OD_{630} after a consistent reaction time was completed. The OD_{630} for a series of dilutions of the positive and negative control serums was plotted against the dilution factors. Individual parameters of this assay were optimized by altering one condition at a time in a series of experiments comparing the positive and negative control serum samples over a series of dilutions (Fig. 2). Once conditions that consistently resulted in clear separation of the curves for the positive and negative control serum specimens were obtained (Fig. 2, panels 1 and 2), the range of dilutions was extended (Fig. 2, panel 3). Increased separation of the middle dilution range was achieved by increasing the percentage of BSA from 1% to 3% in the blocking buffer that was used before adding the serum (Fig. 2, panel 4). The entire range was further optimized (Fig. 2, panel 5) by decreasing the incubation times as follows. Incubation of the immunotoxin on the wells was changed from overnight at 4 °C to 2 h at room temperature. The serum samples were incubated at room temperature for 1 h, instead of overnight at 4 °C. The incubation time for the secondary antibody was reduced from 1 h to 40 min, and the incubation time for the HRP–Avidin was reduced from 1 h to 30 min. These conditions reduced the overall time of the assay and were repeated in two additional assays with consistent results (Fig. 2, panel 6).

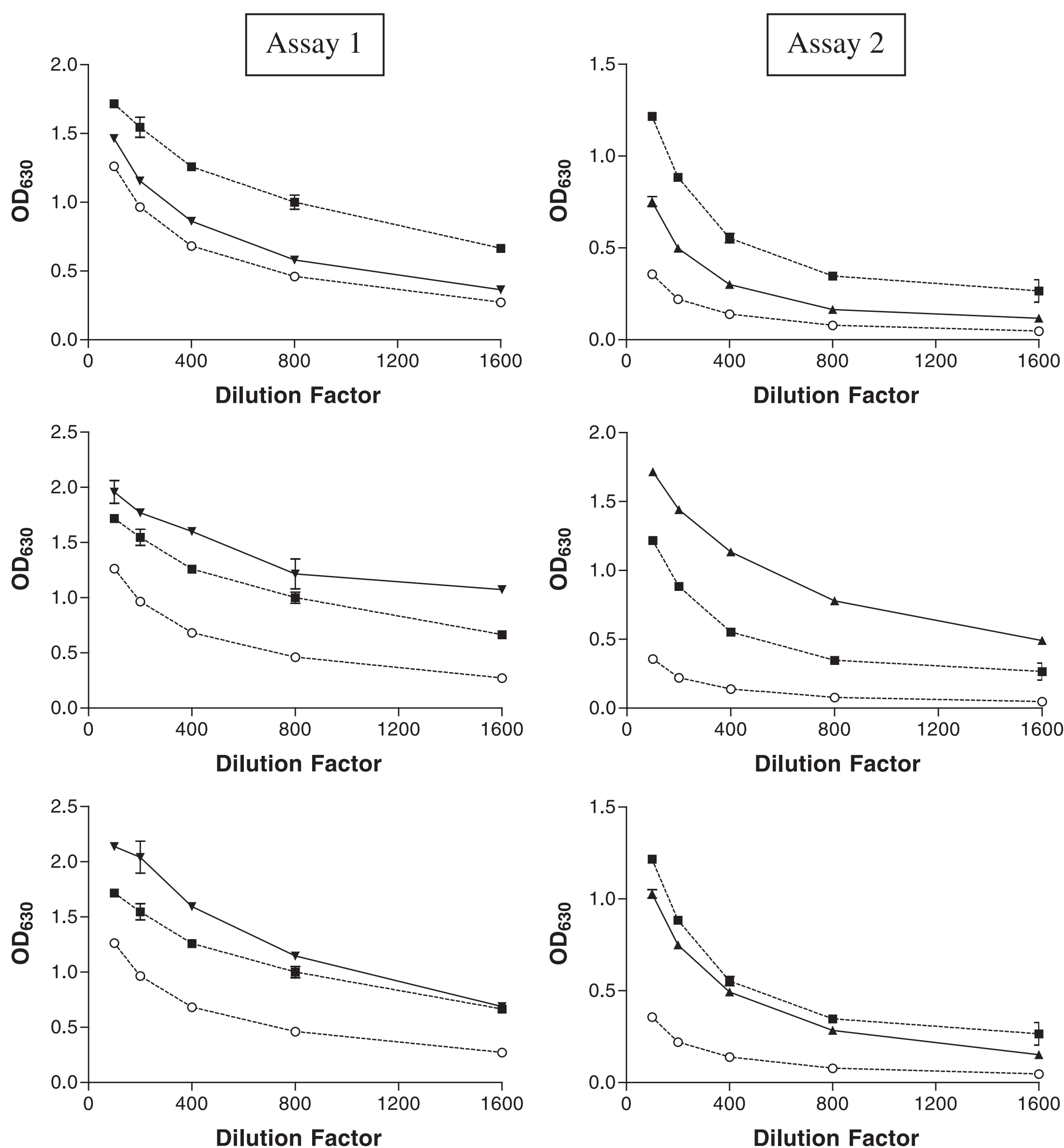

Fig. 4. Two independent ELISAs demonstrate consistent classification of serum from the same patients as positive. The positive (■) and negative (○) controls are represented by dashed lines, while the patient serum specimens (▲) are represented by solid lines. Each row represents an individual patient and each column represents an independent experiment.

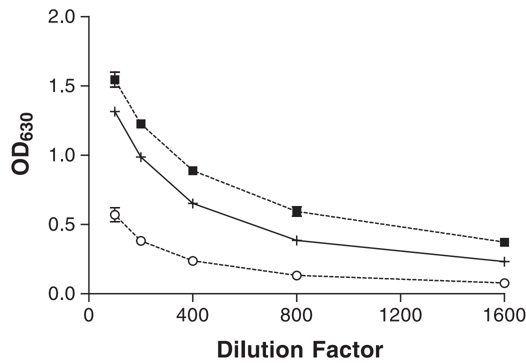

Fig. 5. Negative serum spiked with positive serum exhibits predictable ELISA results. The 100% positive serum (■) and 100% negative serum (○) samples are represented by dashed lines, while a 50:50 mixture of these positive and negative serum samples (+) is represented by a solid line.

3.2. Establishment and validation of criteria for classification of patient samples

Forty patient serum specimens were screened for human antibodies to the SS1(dsFv)-PE38 immunotoxin and classified as either positive or negative based on the following criteria.

3.2.1. Positive classification

Serum was classified as positive if the test serum curve crossed the positive control curve or was above the positive control curve. Serum was also classified as positive if the test serum curve did not cross the positive control curve, but a single point of the test serum curve was greater than a single point of the positive control curve within two 2-fold dilutions.

3.2.2. Negative classification

Serum was classified as negative if the test serum curve was below the positive control curve and did not cross this positive control curve. If the serum curve reached the same OD value as the positive control curve but at greater than two 2-fold dilutions, it was considered negative.

3.2.3. Validation

To evaluate the accuracy of this ELISA, seven of the serum specimens were evaluated in two independent assays. Four of these specimens were classified as negative in each of the two assays (Fig. 3). Three of these specimens were classified as positive in each of the two assays (Fig. 4). A total of 40 specimens were screened and 10 of them were classified as positive. These results were compared to classification of the same samples performed using the neutralization assay in an independent laboratory. The neutralization assay measures the ability of patient serum samples to inhibit the cell-killing activity of the immunotoxin. This inhibition is indicative of the presence of anti-immunotoxin antibodies in the patient serum that can bind to the immunotoxin and antagonize its ability to kill mammalian cells. There was 100% concordance between the two assays for the serum samples screened prior to enrollment on the trial and for the sequential serum samples taken from two treated patients as described in the next section.

Further validation of the ELISA was performed by evaluating the predictability of the results of negative serum that was spiked with positive serum. The negative and positive control serum samples were mixed in a 50:50 ratio and evaluated with the ELISA in comparison to 100% negative and 100% positive serum control samples. As expected, the 50:50 mixture exhibited a curve that was located in between the positive and negative curves at all levels of dilution (Fig. 5).

3.3. Development of immune response to the SS1(dsFv)-PE38 immunotoxin

Serum was collected from the first two enrolled patients during and after treatment and was evaluated for development of antibodies to the SS1(dsFv)-PE38 immunotoxin. These patients had been classified as negative prior to being enrolled in the trial. Their initial serum specimens taken prior to treatment were compared with serum collected during and after treatment. The ELISA demonstrated that both patients

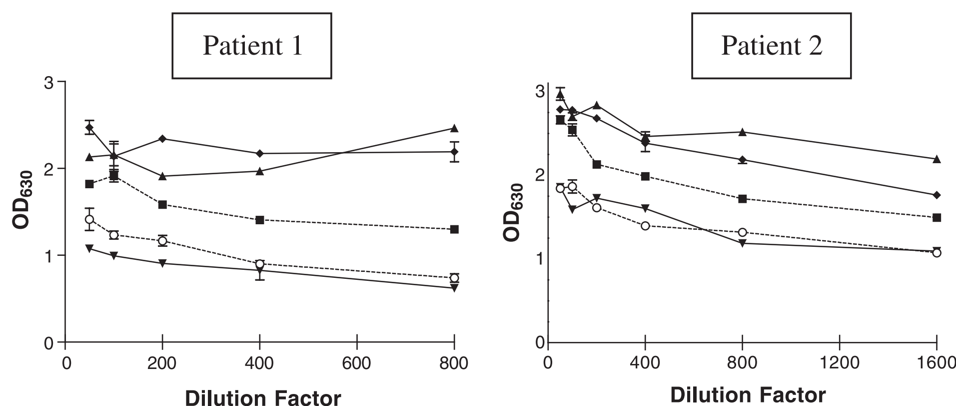

Fig. 6. ELISA detection of the development of an immune response in treated patients. The positive (■) and negative (○) controls are represented by dashed lines, while the patient serum specimens before treatment (▼), during treatment (▲), and after treatment (◆) are represented by solid lines.

developed antibodies to the immunotoxin during the treatment (Fig. 6). This expected result further validates the SS1(dsFv)-PE38 ELISA.

4. Discussion

In the present study, experimental parameters for an ELISA were developed to detect human antibodies to the SS1(dsFv)-PE38 immunotoxin. The lack of an ethical mechanism to obtain human antibodies precluded the ability to quantify the human antibodies in patient serum. The availability of positive and negative serum samples, however, allowed the classification of serum samples as positive or negative, based on dose–response curves of serial dilutions of the test samples in comparison to the positive and negative controls. The accuracy of the results of the ELISA was validated by (1) consistent results between two independent assays of seven patient specimens, (2) perfect concordance of the results with the neutralization assay performed in an independent laboratory, and (3) the ability of the assay to detect the expected immune response in treated patients.

The ELISA offers several benefits over the tissue culture-based neutralization assay. The ELISA plates can be prepared the day before the assay is run, and the actual assay can be performed within less than one 8-h time period. Also, the ELISA does not require the maintenance and use of a tissue culture cell line as in the neutralization assay (Hassan et al., 2000), which can cause significant variations in the assay due to natural variability inherent in biological systems.

Use of the immunotoxin to capture the antibody offers benefit over other ELISAs developed to detect human anti-animal antibodies (Kricka, 1999), because it may also capture antibodies to the PE portion of the immunotoxin. The orientation of the immunotoxin when it is adhered to the ELISA plate could affect whether the murine antibody or the PE portion of the hybrid molecule is available to capture the serum antibodies. If the orientation of the immunotoxin on the ELISA plate is random, then it would be expected that both types of anti-immunotoxin antibodies would be detected in the ELISA. Although further studies would need to be performed to determine if this ELISA detects both antimurine and anti-PE antibodies, the perfect concordance of this assay with the neutralization assay indicates that human antibodies that are developed in response to treatment with the SS1(dsFv)-PE38 immunotoxin are being detected regardless of the specific antigen on the immunotoxin being recognized. The detection of the development of antibodies in the first two treated patients further validates this ELISA technique.

In conclusion, an ELISA-based strategy using an immunotoxin to capture human anti-immunotoxin antibodies provides a consistently accurate technology for screening and monitoring patient serum specimens in clinical trials.

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References

Argani, P., Iacobuzio-Donahue, C., Ryu, B., Rosty, C., Goggins, M., Wilentz, R. E., Murugesan, S. R., Leach, S. D., Jaffee, E., Yeo, C. J., Cameron, J. L., Kern, S. E., & Hruban, R. H. (2001). Mesothelin is overexpressed in the vast majority of ductal adenocarcinomas of the pancreas: identification of a new pancreatic cancer marker by serial analysis of gene expression (SAGE). *Clinical Cancer Research, 7*, 3862–3868.

Chang, K., & Pastan, I. (1996). Molecular cloning of mesothelin, a differentiation antigen present on mesothelium, mesotheliomas, and ovarian cancers. *Proceedings of the National Academy of Sciences of the United States of America*, *93*, 136–140.

Chang, K., Pastan, I., & Willingham, M. C. (1992). Frequent expression of the tumor antigen CAK1 in squamous-cell carcinoma. *International Journal of Cancer*, *51*, 548–554.

Hassan, R., Lerner, M. R., Benbrook, D. M., Lightfoot, S. A., Brackett, D. J., Wang, Q. -C., & Pastan, I. (2002). Anti-tumor activity of SS(dsFv)PE38 and SS1(dsFv)PE38, recombinant anti-mesothelin immunotoxins against human gynecologic cancers grown in organotypic culture in vitro. *Clinical Cancer Research*, *8*, 3520–3526.

Hassan, R., Viner, J. L., Wang, Q. C., Margulies, I., Kreitman, R. J., & Pastan, I. (2000). Anti-tumor activity of K1-LysPE38QQR, an immunotoxin targeting mesothelin, a cell-surface antigen overexpressed in ovarian cancer and malignant mesothelioma. *Journal of Immunotherapy*, *23*, 473–479.

Hassan, R., Wu, C., Brechbiel, M. W., Margulies, I., Kreitman, R. J., & Pastan, I. (1999). 111Indium-labeled monoclonal antibody K1: biodistribution study in nude mice bearing a human carcinoma xenograft expressing mesothelin. *International Journal of Cancer*, *80*, 559–563.

Hwang, J., Figzgerald, D. J., Adhya, S., & Pastan, I. (1987). Functional domains of *Pseudomonas* exotoxin identified by deletion analysis of the gene expressed in *E. coli*. *Cell*, *48*, 129–136.

Kreitman, R. J. (2001). Toxin-labeled monoclonal antibodies. *Current Pharmaceutical Biotechnology*, *2*, 313–325.

Kricka, L. J. (1999). Human anti-animal antibody interferences in immunological assays. *Clinical Chemistry*, *45*, 942–956.

Onda, M., Nagata, S., Tsutsumi, Y., Vincent, J. J., Wang, Q., Kreitman, R. J., & Lee, B. I. P. (2001). Lowering the isoelectric point of the Fv portion of recombinant immunotoxins leads to decreased nonspecific animal toxicity without affecting antitumor activity. *Cancer Research*, *61*, 5070–5077.

Onda, M., Willingham, M., Wang, Q., Kreitman, R. J., Tsutsumi, Y., Nagata, S., & Pasta, I. (2000). Inhibition of TNF-α produced by Kupffer cells protects against the nonspecific liver toxicity of immunotoxin anti-Tac(Fv)-PE38, LMB-2. *Journal of Immunology*, *165*, 7150–7156.