

New multiplexed flow cytometric assay to measure anti-peptide antibody: a novel tool for monitoring immune responses to peptides used for immunization

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Peptide-based vaccination has a great potential for the prevention and treatment of various diseases. There is, however, no appropriate monitoring system to measure immune responses to vaccinated peptides, which hampers the development of therapeutically effective vaccine regimens to various diseases. In this study a new multiplexed flow cytometric assay using the Luminex™ system to monitor humoral immune responses to vaccinated peptides is described. Although the sensitivity is mostly equal to that of the traditional enzyme-linked immunosorbent assay (ELISA), the new assay has several advantages over ELISA in that it minimizes the amount of sera needed, running costs and working periods, and thus will be a novel tool for monitoring immune responses to vaccinated peptides.

Key words: Antibody; Luminex™ system; peptide vaccination; screening

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INTRODUCTION

Recent advances in immunology and molecular biology have resulted in the identification of a number of antigens and epitopic peptides involved in tumor immunity, infections, allergies and autoimmune diseases [1–4]. Some of these peptides involved in tumor immunity and autoimmune diseases have been used for clinical

trials of peptide-based therapy, but these initial clinical trials are associated with rare clinical responses as well as with dim levels of immune responses to vaccinated peptides [5–8]. We hypothesized that this failure could partly be due to the lack of an appropriate monitoring system to measure immune responses to vaccinated peptides. We previously reported a new high-throughput fluorometric microvolume

assay technology system to measure cellular immune responses to peptides [9–11], and recently we reported that humoral response, but not cellular responses, correlated well with overall survival of cancer patients under peptide vaccination [12]. However, humoral responses to peptides have been measured using a conventional enzyme-linked immunosorbent assay (ELISA), which requires a number of plates, large amounts of sera, an excessive quantity of reagents and a long working time. Subsequently, development of a new assay system is needed. Multiplexed flow cytometric assay technology has been growing rapidly during the past few years [13–17], allowing us rapidly to measure multiple targets in the wells of 96-well microplates. This assay uses microspheres as the solid support for conventional immunosorbent assay. The antibody (Ab) or antigen of each immunosorbent assay is coupled to one of individual microsphere bead sets. During data acquisition, different bead sets can be pooled together for the assay. We utilized this technology and in this study report for the first time on a new multiplexed flow cytometric assay that can rapidly and accurately measure immune responses to vaccinated peptides using a minimum amount of sera and with cost-saving.

MATERIALS AND METHODS

Materials

The peptides used in this study have the ability to induce cytotoxic T lymphocyte (CTL) reacting to tumor cells [10], and thus are currently being used in clinical trials as cancer vaccines by our group [18, 19] (Table I). The peptides were purchased from Multiple Peptide Systems (San Diego, CA,) under the conditions of Good Manufacturing Practice. Color-coded beads, Luminex 100TM, and the Luminex XYPTM platform were obtained from Luminex Corp. (Austin, TX). The 96-well filter plates (MABVN1250) and vacuum manifold apparatus (MAVM 09601) were obtained from Millipore Corp. (Bedford, MA). Biotinylated goat anti-human IgG (gamma chain-specific) (BA-3080) was purchased from Vector Laboratories Inc. (Burlingame, CA). Streptavidin-PE (S-866) was purchased from Molecular Probes (Eugene, OR). Disuccinimidyl suberate (DSS)

TABLE I. Amino acid sequences of peptides used in this study.

Peptides	Sequences
SART-1 _{690–698}	EYRGFTQDF
SART-2 _{93–101}	DYSARWNEI
SART-2 _{899–907}	SYTRLFLIL
SART-3 _{109–118}	VYDYNCHVDL
SART-3 _{315–323}	AYIDFEMKI
Lck _{208–216}	HYTNASDGL
Lck _{488–497}	DYLRSVLEDF
Lck _{486–494}	TFDYLRSVL
ART1 _{170–178}	EYCLKFTKL
ART4 _{13–20}	AFLRHAAL

These peptides are now under clinical trials as cancer vaccines for cancer patients in Kurume and Hokkaido University Hospitals.

(21555) and 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) (22980) were obtained from PIERCE (Rockford, IL), and a 96-well Nunc covalink flat plate (478042) was obtained from Nalge Nunc Int. (Pittsburgh, PA). Rabbit anti-human IgG (gamma chain-specific) and horseradish peroxidase-dextran polymer conjugated anti-rabbit Ig was purchased from DAKO (Glostrup, Denmark). Tetramethyl benzidine substrate solution was obtained from KPL (Guildford, UK). Sera from cancer patients attending to the phase-I clinical trials of peptide vaccines at Kurume and Hokkaido University Hospitals were used for this study. All patients were required to comprehend and sign an informed consent form before entry into the clinical trials. To purify the anti-SART3_{109–118} Ab, IgG fraction of the post-vaccination (18th) serum of a colon cancer patient who received 3 mg SART3_{109–118} peptide vaccination 18 times every 2 weeks was partially purified by protein G affinity column chromatography. The IgG fraction with reactivity to each of the five peptides (SART2_{93–102}, ART1_{170–178} Lck_{208–216}, Lck_{486–495}, Lck_{488–496}) was also partially purified by protein G affinity column chromatography from the post-vaccination serum of a cancer patient who repeatedly received the corresponding peptide. We used the singular fraction but not plural fractions for each of the six positive controls.

Coupling of peptides to color-coded beads

The peptides were coupled to color-coded beads according to the modified instructions of

the manufacturer. In brief, 100 μ L color-coded beads were washed with 0.1 M MES buffer, pH 4.5, followed by mixing with 100 μ L peptide (1 mg/mL in 0.1 M MES buffer, pH 4.5). The peptide-loaded beads were then incubated with EDC (1 mg/mL) at room temperature for 30 min in darkness, and the beads were washed with Tween-PBS. The beads were treated with 2-aminoethanol for 10 min at room temperature in darkness followed by washing twice, and were then re-suspended with 1 mL of 0.05% NaN₃ in Tween-PBS.

Assay to measure anti-peptide antibody (Ab)

Sera were incubated with 25 μ L of the peptide-coupled color-coded beads for 2 h at room temperature on a plate shaker (set to 300 rpm) in a 96-well filter plate. After incubation, the plate was washed by vacuum manifold apparatus and incubated with 100 μ L biotinylated goat anti-human IgG (gamma chain-specific) for 1 h at room temperature on a plate shaker (set to 300 rpm). The plate was then washed followed by the addition of 100 μ L streptavidin-PE into wells, and was incubated for 30 min at room temperature on a plate shaker (set to 300 rpm). Optimal concentrations of anti-IgG or Streptavidin-PE were described in the preliminary studies (data not shown) and these were used for the assays. The bound beads were washed three times followed by the addition of 100 μ L Tween-PBS into each well, and the plate was placed for 3 min on a plate shaker (set to 300 rpm). Fifty microL of the sample was detected using the LuminexTM system. Each sample was analyzed in the duplicate determinates with a fluorescence intensity of more than 100 beads which gated to the simple bead group to assure the reliability, precision and accuracy of the assay. The method for the standard ELISA has been described in a previous report [19]. Briefly, the peptide (20 μ g/mL)-immobilized plates were blocked with Block Ace (Yukijirushi, Hokkaido, Japan) and washed with Tween-PBS; 100 μ L of the serum sample was added to the plate. After 2 h incubation at 37°C, the plate was washed and incubated further for 2 h at 37°C with rabbit anti-human IgG (gamma chain-specific). The plate was then washed, followed by the addition of 100 μ L horseradish peroxidase-dextran polymer conjugated anti-rabbit Ig, and was

incubated at room temperature for 40 min. After washing, 100 μ L tetramethyl benzidine substrate solution was added, and the reaction was stopped by the addition of 1 M phosphoric acid. The optical density was measured at 450–620 nm.

RESULTS

Preparation of the positive control Ab

To prepare the positive control Ab for the assay, we used protein G column chromatography to obtain the partially purified IgG fraction reacting to the SART3_{109–118}, ART1_{170–178}, and SART2_{93–101} peptides from post-vaccination sera of cancer patients. The reactivity of each of the purified fractions was then measured by the new assay using the LuminexTM system. All peptide-coated beads comprised a uniform and distinctive proportion of color-code. The fluorescence intensity of this fraction increased in a dose-dependent manner with the linear dynamic range (Fig. 1A). Peptide reactivity of this fraction was also confirmed with ELISA (Fig. 1B). The cross-reactivity was examined by absorption tests (Fig. 1C). As expected, each of the purified fractions had no cross-reactivity to any of the irrelevant peptides tested. We also obtained the partially purified IgG fraction reacting to the Lck_{208–216}, Lck_{486–495}, and Lck_{488–496} peptides from the post-vaccination sera of cancer patients. The fluorescence intensity of each of these fractions increased in a dose-dependent manner within the linear dynamic range (Fig. 1D). All the results given in Figures 1A–D are the means $\pm$ standard deviations (SD) of the triplicate determinants. Based on these results, these fractions were used as the positive controls each time the assay was conducted in the following experiments.

Comparison of the new assay with the conventional ELISA

To test whether this new assay could accurately quantify Ab levels reacting to vaccinated peptides during the kinetic study, pre- and post-vaccination sera from cancer patients were provided for their reactivity to peptides using both the conventional ELISA

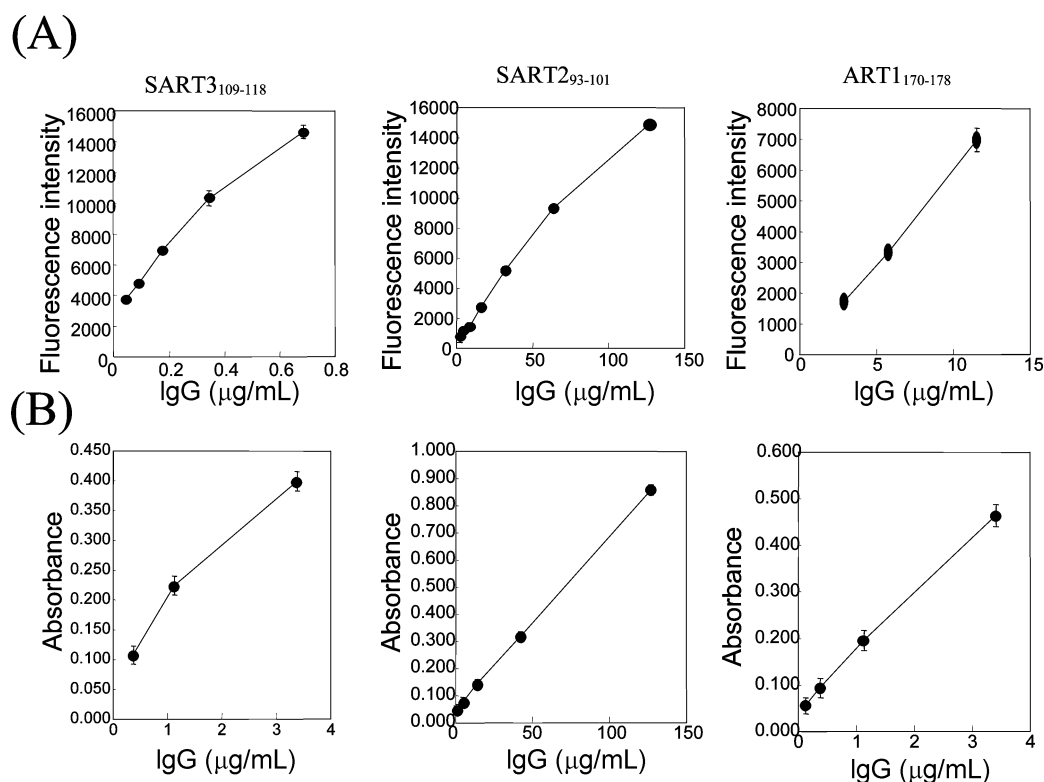


FIG. 1.

and the new assays in a wide range of dilutions at seven different points. The representative results of four cases (Patients 1–4) are shown in Figure 2A. Both of the assays could detect the Ab reaction to the vaccinated peptides in a dose-dependent manner with the linear dynamic range. Furthermore, the standard curves allowed us readily to estimate the Ab reaction to the vaccinated peptides when each of the fluorescence intensities was converted to that of the standard Ab. We also examined sera from the three cases (Patients 5–7) with similar results, and the data of new assay are presented in Figure 2B.

We then investigated whether the new assay could detect the increment of vaccination-induced humoral responses in the post-vaccination sera from the patients who received the peptide vaccination. Representative results of the eight cases are shown in Figure 3. Increased humoral responses to at least one of the vaccinated peptides were observed in the post-vaccination sera from 7 of 8 patients tested

with different patterns of reactivity among the patients. For example, the significant level of anti-Lck_{208–216} Ab became detectable in the post-vaccination (9th) serum from a cancer patient (Patient 8) who was vaccinated with the Lck_{208–216}, Lck_{486–494}, and Lck_{488–497} peptides. The significant levels of IgG reactive to all three vaccinated peptides became detectable in the post-vaccination serum (9th) of Patient 9 who received the Lck_{208–216}, Lck_{486–495} and SART3_{109–118} peptides. In the remaining patient (Patient 10), the vaccinated peptide (Lck_{488–496}) failed to increase the humoral response. Similar results were obtained in most cases when the standard ELISA was used for the detection (data not shown).

Lastly, we assumed the advantages of this new assay as a laboratory marker for monitoring immune responses to vaccinated peptides from different practical points of views (Fig. 4). One of the most critical points of the assay could be saving in amounts of sera since the source is derived from blood and thus is always limited.

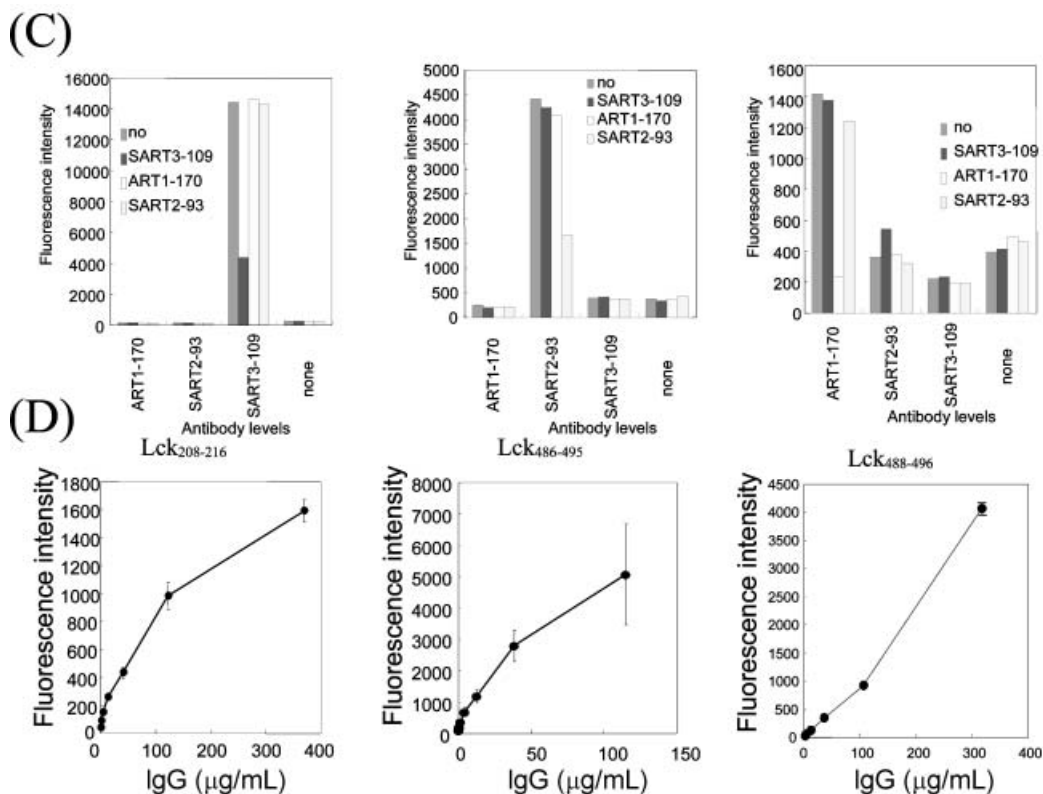


FIG. 1. Preparation of the control Ab. A. Reactivity of the partially purified anti-SART3₁₀₉₋₁₁₈, and anti-ART1₁₇₀₋₁₇₈, anti-SART2₉₃₋₁₀₁ Abs was measured by the new assay. Each point represents the mean $\pm$ SD of triplicate assays. B. Peptide reactivity of the positive controls was also measured by ELISA. Each point represents the mean $\pm$ SD of triplicate assays. C. The peptide-specificity of this fraction was confirmed by an absorption test. This fraction was incubated with either the SART3₁₀₉₋₁₁₈ or SART2₉₃₋₁₀₁ or ART1₁₇₀₋₁₇₈ or with no peptide, and the supernatants were assayed for their reactivity to each peptide by the new assay. Each bar represents the mean $\pm$ SD of triplicate assays. D. Reactivity of the partially purified anti-Lck208-213, anti-Lck486-495 and Lck488-496 was also measured by the new assay. Each point represents the mean $\pm$ SD of triplicate assays.

With the new assay each peptide can be distinguished on the surface of color-coded beads that are identified by each color-code; even if the modality of the peptide increases up to as much as 100-fold, it could be measurable in a single well. Therefore, the new assay can measure many kinds of anti-peptide Ab using only 1.5 μ L of serum samples even if the numbers of peptide measurements are at a maximum of 100, whereas the ELISA needs 900 μ L for measurement of 100 peptides (Fig. 4A). Running costs and working time are also critical for a laboratory marker. The new assay allows measurement of as many as 96 kinds of sera at the same time, within a short time, since it uses each well of a 96-well

microplate, whereas the ELISA requires many wells and plates, many expensive reagents in large quantities, and also needs a considerable amount of working time for measurements of 96 sera. Subsequently, the new assay also has great advantages regarding running costs and work periods. For example, the cost of the new assay is estimated at approximately one-tenth of the ELISA for measurement of 100 different peptides (Fig. 4B). The occupation period using the new assay is estimated to be as short as 1/240 of that of the ELISA for measurement of 100 peptides in sera of 1000 patients (Fig. 4C). These advantages become greater when the number of patients increases. In addition, the new assay has an advantage

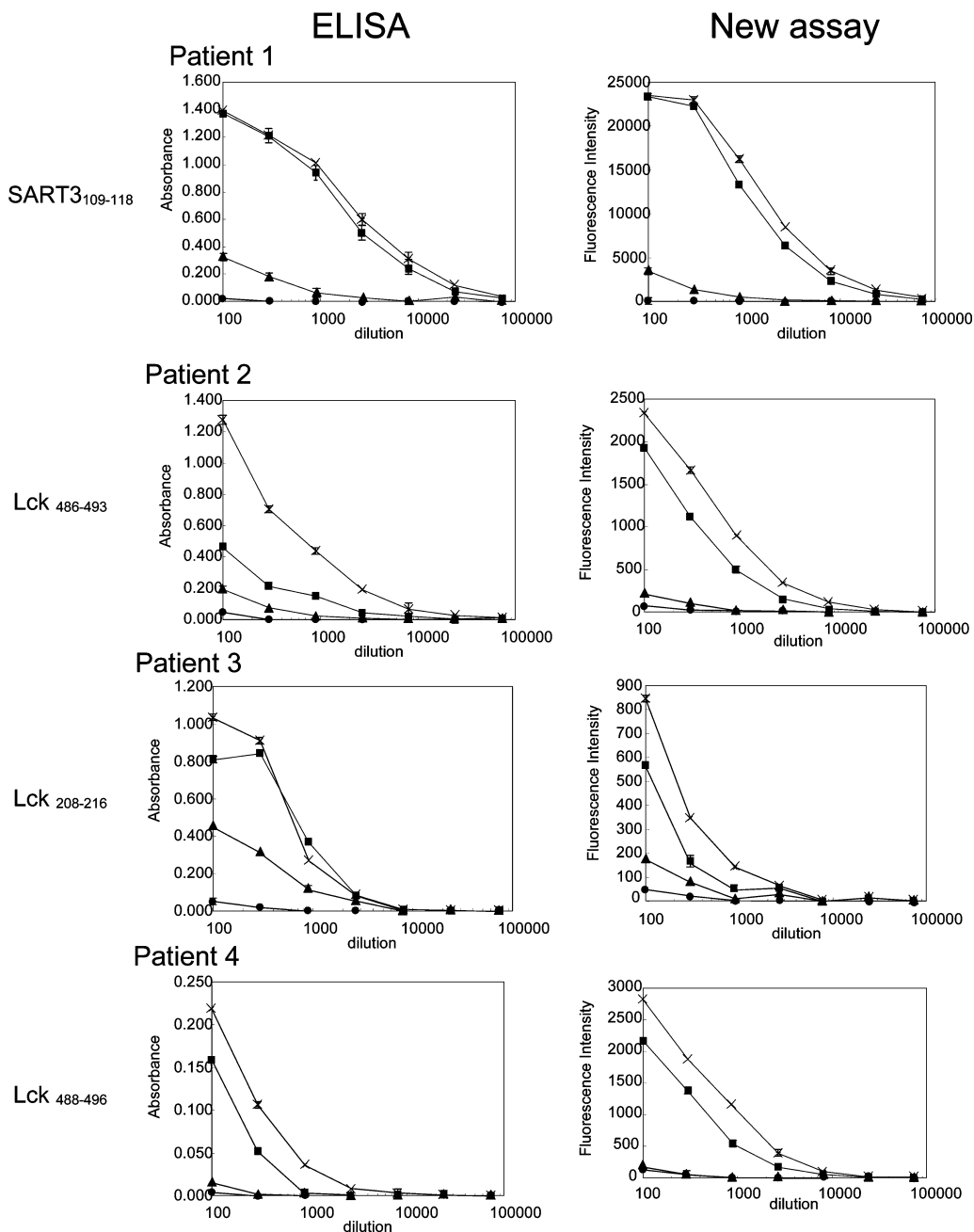


FIG. 2.

from an environmental point of view since it needs less laboratory space and produces smaller amounts of biohazard wastes (sera, infectious samples, chemical reagents and plastic plates) as compared to those with ELISA.

DISCUSSION

CTL precursor frequency analysis, ELISA, the ELISPOT assay and cytotoxicity assays are generally used as laboratory markers to measure cellular immune responses to vaccinated

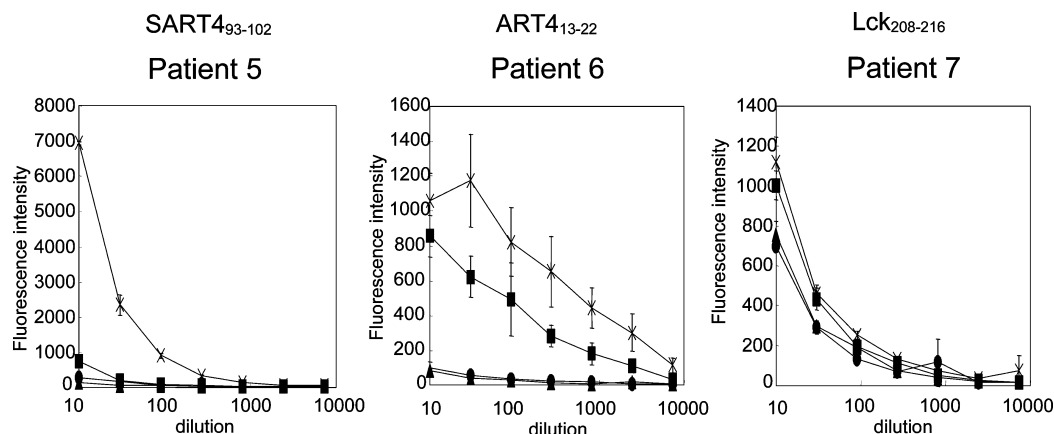


FIG. 2. Comparison of the two assays. The serum samples were diluted ($\times 100$ to $\times 72\,900$) in Block Ace containing 0.05% Tween-20. These diluted sera from pre-vaccination ($\bullet$) and post-vaccination (3rd: $\blacktriangle$; 6th: $\blacksquare$; and 9th: $\times$) of cancer patients were measured for their reactivity to the SART₃₁₀₉₋₁₁₈, Lck₄₈₆₋₄₉₄, Lck₂₀₈₋₂₁₆, Lck₄₈₈₋₄₉₆, SART₂₉₃₋₁₀₂ and ART₄₁₃₋₂₂ peptides by both ELISA and the new assay. Results of the two assays are presented in (A), while those of the new assay only are given in (B), to save space. Each bar represents the mean $\pm$ SD of triplicate assays.

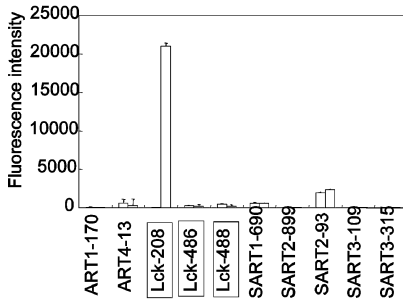
peptides [4–8, 18, 19]. Although these assays are well-established monitoring systems, none of them are highly reproducible mainly because the CTL precursors in the sample are usually very low (probability is 1/1000 to 1/100 000), and the frequency of CTL precursors in the sample must be between 1/3000 and 1/30 000 cells for detection by these assays [4–10]. Lower reproducibility of these cellular assays is also due to *in vitro* biases, including the condition of the cryopreservation of cells, the medium for *in vitro* culture, culture conditions and the numbers and viability of cells at the time of harvesting. In contrast, measurement of humoral immune responses will be relatively reproducible since Ab molecules are generally stable and abundant in serum samples. We recently reported that most vaccinated peptides elicited humoral responses in cancer patients [18, 19]. More importantly, we recently reported that humoral response, but not cellular responses, correlated well with overall survival of the patients under peptide vaccination [12]. This new observation allowed us to establish a new assay for the measurement of anti-peptide Ab as a tool for monitoring immune responses to vaccinated peptides.

We tried to prepare the six different, partially purified, anti-peptide Abs as the positive control in order to 1) normalize unknown patients' sample data each time

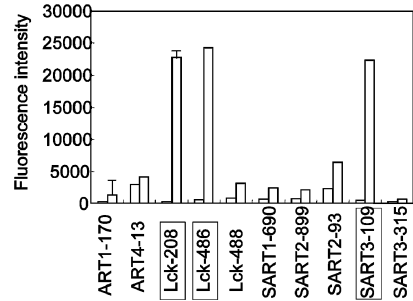
of the assay, 2) gauge assay reproducibility over time, and 3) indicate the quality of the peptide-coupled microspheres over time and after recoupling. For example, anti-SART₃₁₀₉₋₁₁₈ at a concentration of approx. 0.37 $\mu\text{g/mL}$ gives a fluorescence intensity of 10 000, whereas it takes approx. 60 $\mu\text{g/mL}$ anti-SART₂₉₃₋₁₀₁ to reach the same fluorescence intensity. It is considered that the peptide concentration or method used for coupling is not optimal for each peptide, or the populations of the individual antibodies bound to peptide are different from each other, although this circumstance cannot provide actual Ab levels bound to the other peptide if the binding affinity to the other peptide is different. Nevertheless, counting of relative anti-peptide Ab may facilitate a quantitative analysis of the results for kinetic studies of immune responses to vaccinated peptides, which is one of the major problems when CTL assays are used for monitoring [4–10, 18, 19].

Development of a humanized monoclonal Ab specific to each peptide is needed to solve the problems mentioned above. Subsequently, we have developed a mouse monoclonal Ab as the first step towards preparation of a humanized monoclonal antibody to the SART₃₁₀₉₋₁₁₈ peptide. As expected, however, our preliminary data showed that this mouse monoclonal Ab showed a different pattern of fluorescence

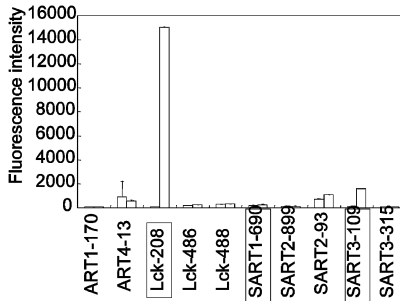
Patient 8



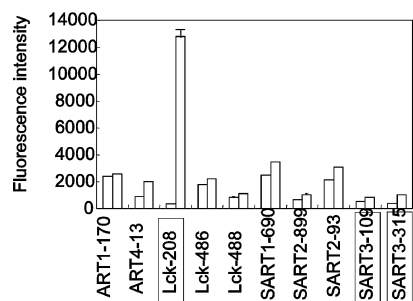
Patient 12



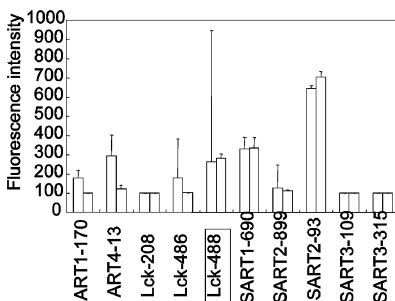
Patient 9



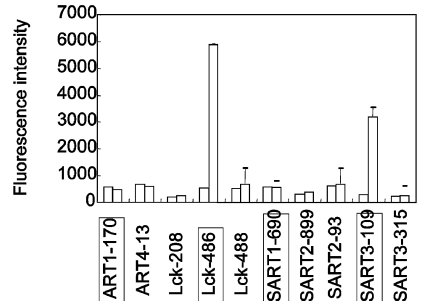
Patient 13



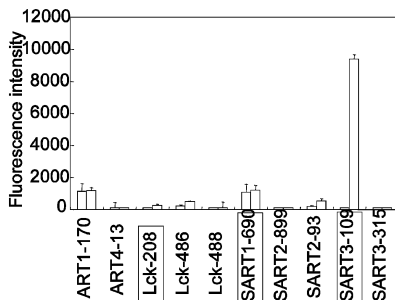
Patient 10



Patient 14



Patient 11



Patient 15

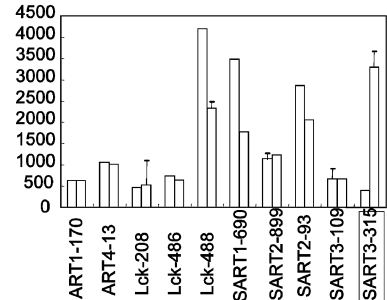


FIG. 3. Measurement of the vaccination-induced humoral responses. Both the pre- and post-vaccination sera (100-fold dilution in Block Ace containing 0.05% Tween-20) were measured by the new assay for their reactivity to the vaccinated peptides (boxed) as well as unvaccinated peptides (unboxed). Representative results of the 8 cases are shown. Each bar represents the mean $\pm$ SD of triplicate assays.

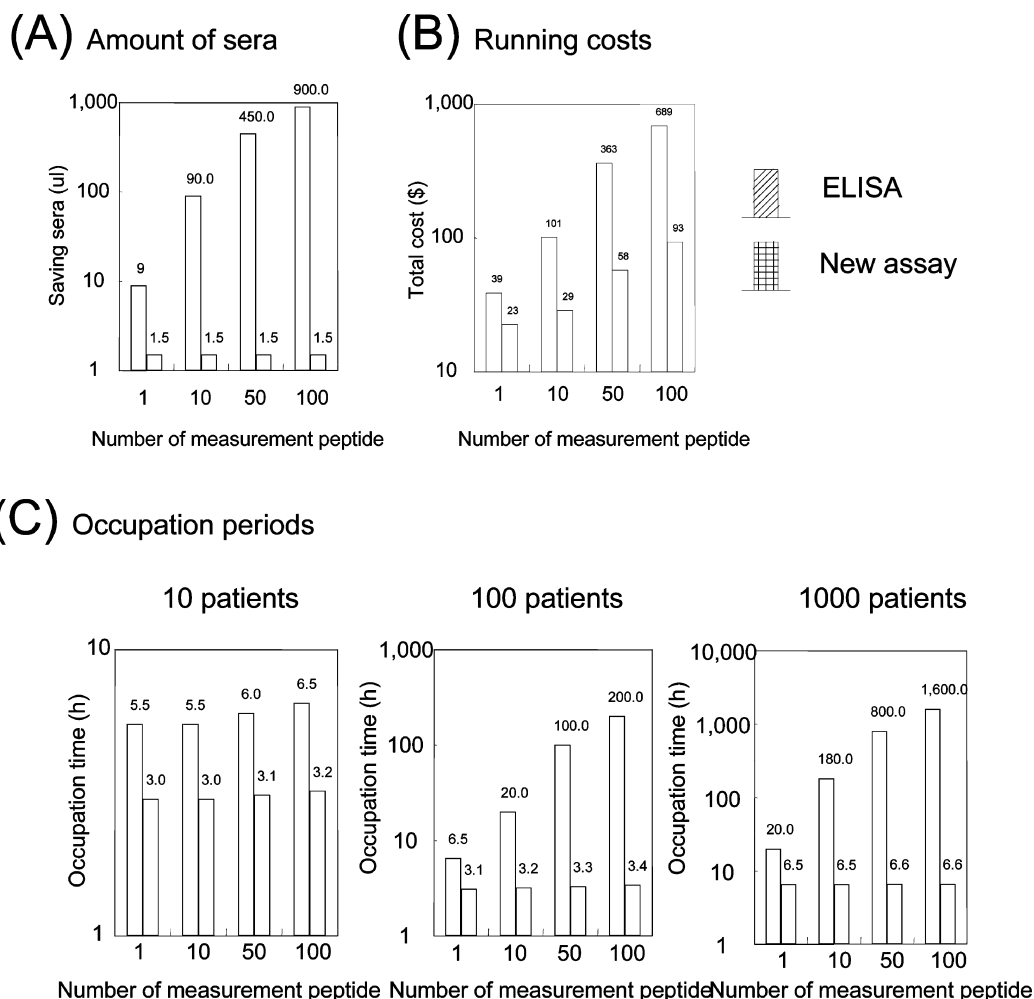


FIG. 4. Feasibility of the two assays as monitoring systems. A. Amounts of sera required for assay. Amounts of sera required for four different settings (1-, 10-, 50- and 100 measurement peptides) in ELISA and the new assay are shown. B. Running costs. The estimated running costs of these assays for one patient are shown. C. Occupation periods. The estimated occupation periods to measure sera from 10-, 100- and 1000 patients at four different settings are shown.

intensity as compared to that of the partially purified anti-SART3_{109–118} from human sera (data not shown). These results suggest that the currently described characteristics of the new assay may well be highly antiserum specific. Since peptide-binding efficiency to beads is different in each peptide, the limit of sensitivity should determine the individual for each peptide.

We compared the results of the new assay with those of the ELISA in order to test whether the new assay could accurately quantify Ab against peptide in the post-vaccination

sera of cancer patients. The results demonstrated that both the assays detected anti-peptide Ab, producing mostly identical results. Furthermore, the new assay was as sensitive as the ELISA for detection of dim levels of humoral responses.

We previously presented evidence of the existence of IgG reactive to CTL epitope peptides in the pre-vaccination sera of both cancer patients and healthy donors [19–21]. Some of these IgG Abs were either deficient or unbalanced in patients with atopic disease [22].

These results were confirmed in this study since the modest levels of peptide antibody were detectable in pre-vaccination sera of certain patients (Fig. 3). These levels, however, were not changed in sera of post-vaccination in most cases if the peptide was not vaccinated.

Finally, we investigated the feasibility of these assays as laboratory markers for monitoring immune responses to vaccinated peptides from different practical points of views. All the results indicate that the new assay can be useful in monitoring immune responses to vaccinated peptides in clinical studies of peptide-based immunotherapy against various diseases. This new assay will facilitate the development of therapeutically effective vaccine regimens for cancer, infectious diseases and autoimmune diseases.

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