INTRODUCTION

Recombinant human miniantibodies, also known as single chain variable fragments (scFv), are extensively used as in biotechnology and therapeutic applications. The most widespread method of generating such miniantibodies is phage-display technology. Miniantibodies generated after three or four rounds of selection on the antigen can specifically recognize the target antigen in a mixture of homologous antigens. For a number of variable antigens, a simple procedure of biopanning on an antigen can be used to generate antibodies with affinity to other proteins of the same family.

The goal of our study was to develop a simple and rapid method for generating scFv miniantibodies on the surface of filamentous bacteriophage M13 that are specific to Staphylococcus enterotoxin type C1 (SEC1).

RESULTS

Rounds of selection of phage miniantibody library. A non-immune human miniantibody (scFv) library with diversity of $6 \times 10^9$ in a phage-display format was used to search for specific antibodies. At the initial stage, specific antibodies within the bacteriophage were detected in 2–3 rounds of selection on enterotoxin C1 adsorbed on the plate surface. As was shown in previous work, the generated miniantibodies interacted with enterotoxin C1 and other Staphylococcus enterotoxins (A, B, D, E, G and I); however, it was desirable to develop a method for generating miniantibodies within a bacteriophage that would be able to bind specifically with only enterotoxin C1. To achieve this goal, a procedure of step-by-step panning was proposed similar to that used for generating from polyspecific to monospecific polyclonal antibodies. The procedure of panning was based on performing rounds of selection against SEC1 from the non-immune human phage library of single-chain miniantibodies. During the first and second rounds of selection, the library in the concentration $2 \times 10^{12}$ and $10^{12}$ phage particles per well was incubated with $1 \, \mu g$ and $0.5 \, \mu g$ SEC1, respectively. Using indirect ELISA of polyclonal phage particles the efficiency of the phage library enrichment at the first and second rounds of selection was compared (Fig. 1). Further the same method was applied to compare the signal of the total phage library of single-chain miniantibodies after the second round of selection using SEC1 with various antigens of the SE family: SEA, SEB, SED, SEE, SEG and SEI with high homology of the three-dimensional structure.

As seen from Figure 2, the library enriched using enterotoxin C1 after the second round of selection interacts with various antigens.
members of the family of Staphylococcus enterotoxins at different intensity levels.

For more effective panning of the library, it was necessary to choose a corresponding concentration of phage antibodies at which it would be possible to remove nonspecific clones and generate clones specific to enterotoxin C1 in one stage. To this end, we compared various concentrations of phage particles enriched as a result of two rounds of selection on the SEC1 library and capable of interacting with enterotoxins (SEA, SEB, SEC1, SED, SEE, SEG and SEI) at 100 ng per well. It was thus determined that at the concentration of 3.125 x 10^{10} phage particles per well (100 μl), non-specifically interacting clones could be successfully removed.

The order of panning (Fig. 3) on a certain toxin was chosen arbitrarily. To perform panning, phage particles were alternately incubated with each of the enterotoxins adsorbed on the immune plate surface for 50 min at 37°C. The choice of the incubation time was explained by the fact that in the previous selections 30 min were sufficient to perform an efficient procedure of interaction of phage antibodies with the antigen adsorbed on the immune plate surface. To increase the specificity of desorption, the bound phage particles were eluted from the well containing SEC1 using trypsin. The E. coli TG1 culture was infected using phage particles. The obtained culture was used for amplification of the selected phage particles. The efficiency of the performed panning was determined with an indirect immune enzyme analysis of phage miniantibodies on antigens against which the panning was done: SEA, SEB, SEC, SED, SEE, SEG and SEI (Fig. 2).

The library obtained after the panning on enterotoxins and elution from enterotoxin C1 was used to infect 1 ml of the E. coli TG1 culture, which was then spread over solid growth medium 2YT to form individual colonies. Eleven (a random choice) colonies were subsequently analyzed for the specificity of interaction with single enterotoxins.

It was found that five of the analyzed eleven clones interacted only with SEC1 and produced no cross-reactions with the other antigens (Fig. 4), while the remaining six clones had no high specificity. The proposed method thus allows us to generate clones of single-chain miniantibodies within a phage particle that are highly specific to Staphylococcus enterotoxin C1.

**Discussion**

Generation of specific antibodies is a fundamental task of analytical immunochemistry. Such antibodies are highly pertinent in technologies for detection and monitoring of the composition of various compounds. To generate monospecific polyclonal antibodies, the method of negative sorption is commonly used when undesired antibodies existing in the mixture are separated by sorption either on conjugated antigen carriers or in solution forming precipitates isolated by centrifugation from specific antibodies remaining in the solution. When generating recombinant antibodies from phage display libraries, it is also possible to isolate individual clones, control their specificity in ELISA and choose clones that interact only with one antigen.

**Figure 1.** Analysis of interaction of phage particles of the total library with SEC1 after the first and second rounds of selection. (A) Interaction of phage particles with SEC1. Triangles show the initial phage miniantibody library. Squares show the miniantibody library after the first round of enrichment on SEC1; rhombi designate the background signal. Phages were deposited as triple dilutions of the concentration 10^{13} pfu/ml; the concentration of toxin SEC1 was 500 ng/well. (B) Interaction of phage particles of the library with SEC1 after the first and second rounds of enrichment on SEC1. Rhombi show the phage library after the first round of enrichment on SEC1; squares show the library after the second round of enrichment on SEC1; triangles designate the background signal. Phages were deposited as triple dilutions of the concentration 10^{13} pfu/ml, the concentration of toxin SEC1 was 250 ng/well.

**Figure 2.** Comparative analysis of interaction of phage particles of the total library on SEC1 prior to (A) and after panning rounds of (B) against a number of toxins of the SE family (1-SEA, 2-SEB, 3-SEC1, 4-SED, 5-SEE, 6-SEG and 7-SEI, respectively). Phages were deposited at the concentration of 10^{12} pfu/ml, the concentration of toxins was 100 ng/well.
However, this approach requires analysis of a vast number of clones, which would be laborious, time-consuming and costly. Using the procedure proposed herein, clones specifically interacting with the target antigen—Staphylococcus enterotoxin C1—can be chosen in less than 24 h from the selection enriched with two rounds on a library antigen. It should be noted that the proposed technology is highly specific. When generating scFv in a phage format from the library of miniantibodies that was used in previous work in reference 5, four rounds of selection on the antigen were performed and polyspecific clones to Staphylococcus enterotoxins were obtained. In our case, the same miniantibody library is used.

The traditional technique of phage selection involves a few (2–4) rounds of panning using one target antigen with subsequent determination of specific properties of phage-scFv with ELISA. The probability of low-affinity and cross-reactive antibody panning is very high in this situation. In our study, we found that five of eleven analyzed clones interacted only with SEC1 and produced no cross-reactions with the other antigens. Our method, which excludes use of special techniques, was thus shown to be efficient for the generation of antibody clones that specifically interact with enterotoxin C1. The results suggest that it can also be used to generate specific antibodies to other antigens from homologous families.

Materials and Methods

Materials. Anti-M13 monoclonal conjugate consisted of horse-radish peroxidase conjugated to mouse anti-M13 monoclonal antibody that reacts specifically with the bacteriophage M13 major coat protein gene of product gene VIII (gp8) (Amersham); 1 M IPTG (Serva, Germany) Staphylococcal enterotoxins were prepared as described previously in reference 8.

RODS of selection of phage miniantibody library. The selection of a phage miniantibody library was made on 96-well ELISA plate (Costar Medium Binding, Sigma, USA). 100 μl antigen SEC1 at concentrations of 10 μg/ml and 5 μg/ml in 0.1 M NaHCO3 buffer, pH 9.6, were incubated in the ELISA plate wells overnight at 4°C for the first and second rounds of selection, respectively. Free binding sites on the plate were immobilized with 2.5% BSA in PBS. 2 x 1012 and 1012 phage particles (100 μg/ml) were deposited on the antigen for the first (overnight at 4°C) and the second (1 h at 37°C) rounds of selection, respectively. After incubation, non-specifically bound bacteriophages were removed by washing the plate wells with PBS-T (10 times) and PBS (once).

A trypsin-sensitive helper phage was used to improve the bio-panning efficiency. This modified helper phage included modified gene III, which encodes a peptide sequence that is cleaved by trypsin between domain D2 and D3. By applying trypsin cleavage following the selection of the phage antibody library, the phage particles not carrying an antibody-pIII fusion lose domain D1 and domain D2 of protein 3, and thus lose their ability to infect bacteria, whereas phage particles carrying an antibody-pIII fusion retain their infectivity due to the absence of the protease-sensitive site in gene III of the fusion. The unspecific phages were deleted by treatment with 100 l trypsin solution (1 mg/ml) in PBS at 37°C for 15 min. 1 ml E. coli TG1 (OD600=0.6) was added to the remaining phages and infected for 1 h at 37°C.

The infected culture was then placed in 5 ml 2YT medium containing ampicillin (100 μg/ml) and 0.4% glucose, grown to the optical density OD600 = 0.4 and 1013 phage particles of trypsin helper bacteriophage M13K07 were added. Incubation was performed for 1 h at 37°C. The infected culture was plated in 15 ml 2YT medium containing ampicillin (100 μg/ml) and 0.5 mM IPTG. The culture was grown overnight at 30°C and 220 rpm. Phage particles were isolated using PEG/NaCl precipitations.

Indirect ELISA of polyclonal phage particles. The antigen at concentrations of 5 μg/ml (for comparison of the interaction of phage particles of the total library prior to the rounds of selection and after the first round of selection on SEC1), 2.5 μg/ml (for comparison of the interaction with SEC1 of phage particles of the total library after the first and second rounds of selection on SEC1) and 1 μg/ml (in the remaining ELISA) was adsorbed on the ELISA plate (Costar Medium Binding, Sigma, USA) overnight at 4°C in carbonate buffer, pH 9.6. To block non-specific binding sites on the plate, BSA-T was used. 100 μl initial crude phage library, the library after the first and second rounds of selection at concentrations of 1013 phage particles per ml (for analysis of the interaction of the phage library with toxin SEC1 prior to enrichment on SEC1, after the first and second rounds of selection on SEC1) and 1012 phage particles (for the remaining ELISA) in 2.5% BSA-T solution were deposited in the ELISA
plate wells at consecutive triple dilutions and incubated for 1 h at 37°C. Non-specifically bound bacteriophages were removed by washing the ELISA plate wells with PBS-T (three times) and PBS (once). Bound phage particles were detected using the conjugate of polyclonal murine antibodies anti-M13 HRP at dilution 1:1,000 in PBS-T (100 μl per well). Ortho-phenylenediamine was used as a chromophore. The optical density was measured at the wavelength 492 nm (TiterTekMultiscan, Finland).

**Panning of phage library.** The phage library was selected as described in Figure 4. To deplete the phage library, 100 μl antigens at the concentration of 1 μg/ml in carbonate buffer, pH 9.6, were incubated overnight at 4°C in the ELISA plate 96 wells (Costar Mediasorb, USA) in the following order: SEA in the first well, SEB in the second, SED in the third, SEE in the fourth, SEG in the fifth, SEI in the sixth and SEC1 in the seventh. The sites of non-specific binding of the ELISA plate were immobilized with BSA-T for 1 h at 37°C. 100 μl of the solution (3.125 phage particles per ml) in 2.5% BSA-T of the total phage library were then deposited in the first well of the ELISA plate after the second round of selection on SEC1, incubated for 50 min and the solution was transferred from the first well to the second one, incubated for 50 min, etc. Non-specifically bound bacteriophages were removed from the seventh well by 9-fold washing with BSA-T and one washing with BSA-T, while the specifically bound bacteriophages in the seventh well were used to infection *E. coli* with previous trypsin treatment. (100 μl trypsin solution (1 mg/ml) in BSA for 15 min at 37°C).

**Disclosure of Potential Conflicts of Interest**
No potential conflicts of interest were disclosed.

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