Fingerprint-like Analysis of "Nanoantibody" Selection by Phage Display Using Two Helper Phage Variants

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ABSTRACT **This paper discusses the selection of mini-antibody (nanoantibody, nanobody® or single domain antibody) sequences of desired specificity by phage display-based method using a generated library of antigen-binding domains of special heavy-chain only antibodies (single-stranded antibodies) of immunized camel. A comprehensive comparison of the efficiency of parallel selection procedures was performed by using the traditional (M13KO7) and modified (with N-terminal deletion in the surface gIII protein) helper phages. These two methods are partly complementary, and by using them in parallel one can significantly improve the selection efficiency. Parallel restriction analysis (fingerprinting) of PCR-amplified cloned sequences coding for mini-antibodies (HMR-analysis) is proposed for identifying individual clones, as a replacement to sequencing (to a certain extent). Using this method, unique data were collected on the selection of mini-antibody variants with the required specificity at various stages of a multi-stage selection procedure. It has been shown that different sequences coding for mini-antibodies are selected in different ways, and that, if this feature is not taken into account, some mini-antibody variants may be lost.**
KEYWORDS **immunisation, phage display, helper phage, recombinant mini-antibody, fingerprinting.**
ABBREVIATIONS **ELISA—enzyme-linked immunosorbent assay, BSA—bovine serum albumin, hp—helper phage, PCR— polymerase chain reaction, HA-tag – antigenic determinant, a fragment of 9 amino acids (YPYDVPDYA) from the hemagglutinin protein of the influenza virus, this epitope can be efficiently detected by commercially available antibodies, HMR-analysis—a method proposed in this paper of parallel restriction analysis (using three restriction enzymes - HinfI, MspIand, and RsaI) of PCR-amplified cloned mini-antibody sequences, TNF (TNF-α)—tumour necrosis factor, a multifunctional anti-inflammatory cytokine, PBS—phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4).**

INTRODUCTION

This work was performed in the framework of projects aimed at developing new methods for passive immunisation for preventive care and immunotherapy of infectious diseases, particularly rabies and anthrax, in humans and animals. In this paper, we put special emphasis on an analysis of sequences encoding specific mini-antibodies at various stages of selection that is based on the phage display technology [1–3].

Starting from the establishing in 2003 of collaboration work with Prof. S. Muyldermans' laboratory at Vrije Universiteit Brussel, Belgium, we have been developing and using a novel technology for generating specific camel single domain mini-antibodies (also called nanoantibodies or nanobodies®) in the Institute of Gene Biology, Moscow, Russia.

Nanoantibodies are antigen recognition molecules that are fragments (variable domains) of unusual antibodies found in nature, along with classic antibodies, only in *Camelidae* family animals and in some cartilaginous fishes such as sharks and ratfish [4, 5]. These "camel-specific" antibodies consist of dimers of a single, short (without the first CH1 domain) immunoglobulin heavy chain and are fully functional in the absence of the immunoglobulin light chain. Only a single variable domain of this antibody is necessary and sufficient in order to specifically recognize an antigen and bind to it. In contrast to the majority of recombinant antibodies, generated single-stranded antibodies usually demonstrate a rather high affinity to a given antigen, because the first stage of their formation takes place in the animal's organism (*Camelidae*), via affinity maturation *in vivo*. Nanoantibodies have several advantages over traditional antibodies and may have great prospective use in the future in research and in new biotechnological devices, as well as in clinical diagnostics and treatment. The advantages of nanoantibodies include their smaller size, new structural features (better penetration into tissues and the ability to recognize epitopes hidden, inaccessible to conventional antibodies), the possibility to be economically and efficiently mass-produced (in bacteria and yeast), good solubility, resistance to sig-

nificant changes in temperature and pH, and simplicity when used for various genetic engineering operations [5, 6]. It is important to note that these camel-variable domains do not cause an evident immune response in primates, and their structure is closely homologous to the variable domains of one subclass of human IgG immunoglobulins (IgG3). It has been shown that these camel mini-antibodies can be "humanized" without significant loss of their specific activity, via few point amino acid substitutions [7]. This indicates a potential for the broad use of mini-antibodies as a passive immunisation treatment to prevent various dangerous infections [5, 7–9].

In our method, the first critical stage of nanoantibody production is inducing specific antibodies in the camel's (or llama) organism by immunisation, and the second critical stage is selecting the clones of the nucleotide sequences of the required nanoantibodies by phage display from the generated library of the entire repertoire of variable domains of special antibodies of the immunised animal. The latter stage is quite nontrivial and has not been studied extensively. In this work, we have carefully studied the selection of the required sequences and compared the techniques using two different helper phages. Recently, we proposed a modified helper phage for a more efficient selection of the antigen recognition domains of the special single-stranded camel antibodies (nanoantibodies) by phage display based on the filamentous phage M13 [10]. The use of a mutant M13KO7 phage called hpΔMBpIII (with N-terminal deletion in the M13 phage gIII protein, which makes the phage unable to infect the bacterial cells but does not stop the formation of the phage particle) as a helper phage can in some cases significantly increase the selection efficiency. This has been demonstrated on a model system in the final (third) selection stage of nanoantibodies binding to the tumour necrosis factor, TNF-α. The nanoantibodies were selected from a library that had been specifically pre-enriched in two traditional selection procedures. In this work, we performed a comprehensive comparison of the selection efficiency using both the traditional and mutant helper phages.

EXPERIMENTAL

Antigens and camel immunisation

A preparation of the Rabies virus and a recombinant protein corresponding to the *Bacillus antracis* lethal factor synthesized in a bacterial expression system were used as antigens for camel immunisation. The anthrax lethal factor was kindly provided by Dr. A. Kolesnikov, Laboratory of Biocatalysis, Institute of Bioorganic Chemistry, RAS. A rabies vaccine based on an inactivated attenuated RB 71/10 strain of Rabies virus, produced at the Pokrov bioplant (Vladimir region), was kindly provided by Prof. B. S. Naroditsky (N.F.Gamaleya Research Institute of Epidemiology and Microbiology). This vaccine was used to make the Rabies virus preparation. Virus particles were separated from the culture medium proteins by ultrafiltration through a membrane that is permeable to particles smaller than 300 kDa (Vivaspin 20 ml Concentrator, VS 2051, Vivascience, USA) but not to much larger virus particles. In parallel, the virus preparation was concentrated (approximately to 10^7 virus particles in 10 ml of standard PBS solution). The obtained preparation was divided into six equal parts and frozen at −70°C for storage (five parts were used for five successive immunisation injections, and the sixth part was used for the selection and analysis of mini-antibodies). Before each immunisation stage, one of the aliquots stored at −70°C was thawed, then combined with 650 μg of the *Bacillus anthracis* lethal factor and mixed with an equal amount of Freund adjuvant. Thus, a five-stage immunisation of a 6-year-old female *Camelus bactrianus* was performed by using the two antigen preparations described above, mixed with equal amounts of Freund adjuvant (complete—only for the first injection, and the other 4 times—with incomplete). The second injection was made 4 weeks after the first, while the other three injections were made in 10-day intervals, one after another. Blood samples were taken 5 days after the last injection.

Construction of a nucleotide sequence library of nanoantibodies

DNA cloning of the entire set of variable domains of specific single-stranded antibodies from the B-lymphocytes of immunised camel peripheral blood was performed in accordance with the standard procedure (RNA isolation, cDNA synthesis, two-stage PCR with specific primers, insert restriction and purification, and insertion into phagemid vector [11–14]). This procedure resulted in a new specific nucleotide sequence library of mini-antibodies in the pHEN4 phagemid vector [11]. This library consisted of two sub-libraries, which were different in the restriction sites used for insertion into the phagemid vector of sequences coding for antigen recognition domains, (i) NcoI + NotI and (ii) PstI + NotI. *Escherichia coli* cells (strain TG1) were transformed by electroporation with recombinant plasmid DNA obtained by ligation of the two insert sub-libraries and two corresponding vector sequences. Standard cloning, bacterial and bacteriophage M13 procedures were performed following the techniques described in ref. [15, 16]. The "Basic library of immunised camel nanoantibodies" satisfied the criteria of 10^7 clones and about 80%

of inserts of the expected size. Nanoantibodies with the required specificity were selected from the library.

Selection of nanoantibodies by phage display

The selection of camel mini-antibodies (nanoantibodies) by phage display, production of nanoantibodies in the bacterial periplasm, and the analysis of the nanoantibodies' ability to recognize a given antigen were performed using the described techniques [11–14], with the following modifications. For selection and ELISA, MICROLON 600 (Greiner Bio-One) high binding polystyrene microtiter plates were used. Five percent Skim milk (Bio-Rad) and 1% BSA (Sigma-Aldrich) in PBS were used as blocking agents. The working concentrations of ampicillin and kanamycin were 100 μg/ml and 70 μg/ml, respectively.

E. coli K12 / TG1 {*supE thi-1 (lac-proAB) (mcrB-hsdSM)5(rK- mK-) [F' traD36 proAB lacIqZ M15]*} (Stratagene) and *E. coli* K12 / WK6 {Δ*(lac-pro), galE, strA, nal; F' lacIq ZΔM15, pro*+} strains were used [17], as well as M13KO7 bacteriophage (New England Biolabs) [18] and pHEN4 phagemid [11]. The *E. coli* strains and phagemid were provided by Prof. S. Muyldermans (Vrije Universiteit Brussel, Belgium). We had previously prepared hpΔMBpIII modified bacteriophage [10] by deleting a region between the MspAI (1634) and BamHI (2221) sites from the M13KO7 genomic DNA. The helper phage corresponding to the mutant bacteriophage was produced by a two-stage sequential transformation of *E. coli* cells (strain TG1). At the first stage, the cells were transformed with pHEN4Δf1ori mutant DNA with a deleted fragment coding for replication initiation of the f1 filamentous phage (between the EcoRI (1650) and Alw44I (2345) sites). This deletion preserves the plasmid's ability to replicate as a two-stranded DNA (by still having the corresponding section for replication initiation), but it is no longer capable of either phagemid single-stranded replication nor of packing to a phage particle. At the second stage, the cells containing pHEN4Δf1ori, and therefore resistant to ampicillin, were transformed with mutant genomic DNA (hpΔMBpIII) of the M13KO7 bacteriophage (replicative two-stranded form) containing the kanamycin resistance gene. Then, the cells were cultured with both antibiotics. One of the cell colonies was used to produce the modified helper phage. The phage was grown overnight in 2xTY medium containing kanamycin and 1 mM of isopropyl-β-*D*-1-thiogalactopyranoside (used for activating the synthesis of the wild-type gIII protein, coded for by pHEN4). Then, the phage was purified via the traditional PEG precipitation technique [15, 16]. The resulting phage is effective in infecting bacterial cells; however, its genomic DNA only codes for the gIII mutant protein. As a result, the daughter phages cannot infect bacteria in the absence of an additional plasmid coding for either the wild-type gIII or gIII with a mini-antibody inserted sequence.

Nanoantibodies binding to the Rabies virus preparation or antrax lethal factor were selected from the same library using two different methods in parallel. The first method was traditional phage display with the M13K07 helper phage in three selection/amplification cycles. The blocking agents used for the cycles were 5% Skim milk (Bio-Rad Laboratories, USA) in PBS in the first cycle, 1% BSA (Amresco, USA) in PBS in the second cycle, and the 5% milk again in the third cycle. On average, ~10^{11} phage particles from the original library were introduced into a well with an immobilized antigen; after incubation and washing, ~10^{6} phage particles were eluted and remained active after the first selection stage, while 10 times more (~10^{7} out of ~10^{11}) amplified phage particles remained active after the second selection stage. As a result of the third stage, ~10^{8} out of loaded ~10^{11} phage particles were selected, which is, similarly, about 10 times more than after the previous stage. This is usually an indication that the selection proceeds well and that specifically binding phage particles proliferate, with mini-antibodies getting exposed on the particle's surface, presumably ensuring its specific binding properties. After the last elution and neutralisation, the phage particle solutions at various dilution levels were used to infect *E. coli* cells (WK6 strain), then separate colonies were prepared on a Petri dish for the final analysis.

In parallel to the traditional procedure, nanoantibodies able to bind to the Rabies virus preparation or anthrax lethal factor were selected from the same library, using the mutant hpΔMBpIII helper phage with N-terminal deletion on the surface gIII protein [10]. The selection procedure was analogous to that described for the traditional helper phage. We should note that, in this case, the number of colonies grown after each selection stage (which corresponds to the number of active phage particles with nanoantibodies) was significantly smaller than in the case when the traditional helper phage was used (100 times less after the first stage and 10 times less after the second stage). Helper phage was less reliable for subsequent infection of bacteria and amplification of eluted phage particles, which sometimes resulted in the loss of selected clones. Infecting the WK6 strain cells with the modified phage after elution is apparently ineffective (and almost impossible); therefore, in the case of the modified helper phage, *E. coli* cells of the TG1 strain were only used for infection with eluted phage particles, after all selection stages, including the final one.

A periplasmic extract containing a mini-antibody with a C-terminal HA tag was used to estimate the specificity and efficiency of the nanoantibody binding to the preparation of the antigen immobilised in the immunological plate with traditional ELISA [13, 14]. Anti-HA-monoclonal antibodies (CHGT-45P-Z, ICL, Inc., USA) conjugated with horseradish peroxidase were used as secondary antibodies to the HA-tag. Horseradish peroxidase activity was determined by using ABTS (2,2'-azino-bis(3-ethylbenzothiasoline-6-sulphonate) as a chromogenic substrate. The absorbance was measured with a plate fluorimeter at 405 nm. There was no antigen in the control wells, but they were also blocked and processed in parallel with the experimental wells containing the antigen. The number of "+" signs in the "ELISA signal" column in Tables 1 and 2 corresponds to the relative ELISA absorbance value (reflects the summarized efficiency of expression/availability and binding of the nanoantibody from the periplasmic extract to the antigen immobilised in the well).

RESULTS AND DISCUSSION

HMR analysis of nanoantibody cloned nucleotide sequences

For interim analysis of the mini-antibody clone samples, PCR-amplification (on a given colony directly, colony PCR) is usually performed. A nucleotide sequence coding for the mini-antibody with small adjacent sections of the pHEN4 vector phagemid is to be amplified. The PCR product is then subjected to restriction endonuclease (restrictase) treatment, usually HinfI. In the sequences coding for the nanoantibodies, there are both relatively conservative regions, which contain conservative HinfI recognition sites, and rather long hypervariable regions (the third region, CDR3, is the longest one). In the hypervariable regions, the number and location of the sites recognized by restrictases vary remarkably, hence the strong variation in the number and size of the split DNA fragments. We have found that in order to identify a given clone (a mini-antibody sequence variant), parallel digestion of the PCR product with three different restrictases (HinfI, MspI, RsaI) yields the most reliable results.

A forward primer, RP (5'-cacacaggaaacagctatgac-3'), and a reverse primer, GIII (5'-ccacagacagccctcatag-3'), were used for PCR amplification of the cloned sequence coding for the nanoantibody with small adjacent segments of the pHEN4 phagemid. PCR was carried out in a volume of 20 μl, then the mixture was distributed into three tubes (6 μl of mixture into each tube), where parallel treatment of the PCR product was done (in 15 μl total volume) with three different restrictases, one in each tube: HinfI (Fermentas, Lithuania), MspI (Fermentas, Lithuania), and RsaI (SibEnzyme, Russia). The three hydrolysates were loaded into adjacent 2.5–3% agarose gel wells for electrophoresis. The "low range" mixture (Fermentas) was used as a DNA fragment marker. The resulting electrophoretogram (three gel lanes with DNA treated with the HinfI, MspI, and RsaI restrictases, respectively, and the fourth lane with marker DNA) is a reliable fingerprint image of a given mini-antibody coding sequence cloned in the pHEN4 phagemid. We called this extended fingerprint analysis technique, which can identify a variant of a cloned mini-antibody sequence, "HMR analysis." We used the HMR analysis to study the selection results (samples of mini-antibody clones).

Figure 1 shows the HMR analysis data of some (presumably the most common) clones of the initial mini-antibody library obtained as a result of immunisation and subsequent cloning. We selected and analysed all 94 clones grown after 10^{-5} dilution of the base library consisting of two sub-libraries. We found 85 fingerprints, with only 9 of them repeating twice in this sample, which meant that the library was very heterogeneous without dominating sequences. The majority of the fingerprints (61) corresponded to the clones in which the sequences coding for the nanoantibody were inserted into the PstI–NotI vector restriction sites. The minority of the fingerprints (33) denoted with the letter "N" corresponded to the clones in which the sequences coding for the nanoantibody were inserted into the NcoI–NotI vector restriction sites. Very similar fingerprints found in both sub-libraries are underlined. *Figure 1* confirms that the parallel use of three restrictases instead of one reveals the differences in many more clones. Thus, if we had used only HinfI, we would not have been able to distinguish the following clone groups: 1) 3, 38, 52, 53, and 66 and 2) 55, 56N, 60N, 62N, 74, and 75N. Analogously, much fewer clones could have been differentiated using only one of the other two restrictases: MspI or RsaI.

This result indicates a rather wide diversity of clones in the original library and the high-resolution capability of the HMR analysis. We should note that when only one restrictase is used (out of the three: HinfI, MspI, and RsaI), the number of different fingerprints is much smaller and, hence, the analysis less reliable; therefore, we decided that it was essential to use the full version of the HMR analysis at all further stages of the mini-antibody clone analysis.

We used the following algorithm for nanoantibody sampling. With the HMR analysis, we studied a series of the most represented clones (those that formed no fewer than 24 colonies at the highest dilution of the library or of the eluted phage particles). All clones with unique fingerprints were used to produce the

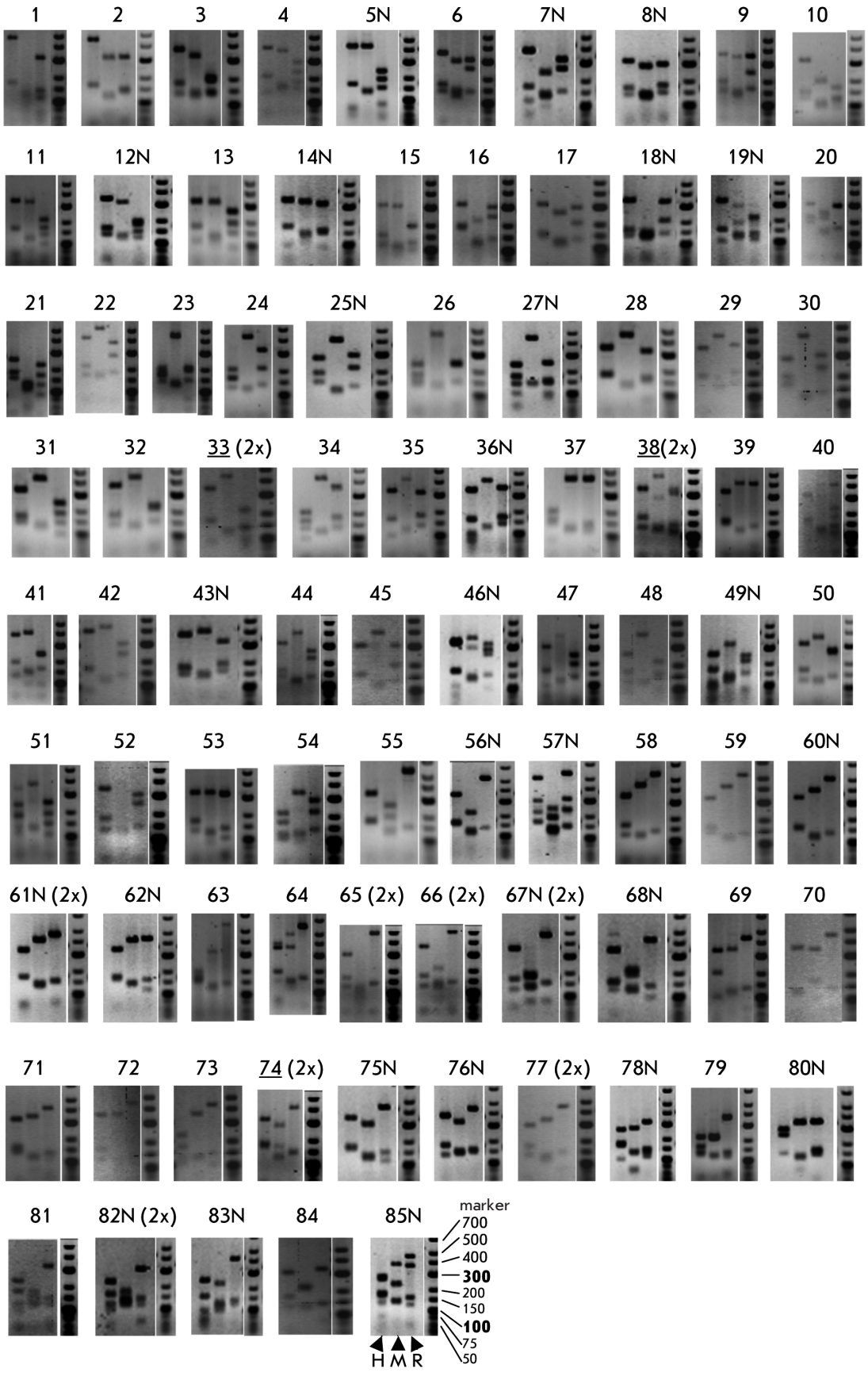

Fig. 1. HMR analysis data (HMR fingerprints) of clones from the original library of cloned miniantibody nucleotide sequences. Ninety-four clones were selected and analysed. Eighty-five different fingerprints were identified with only 9 of them twice as repeats in this set (marked as 2x). The majority of fingerprints correspond to clones with PstI-NotI insertions of adapted nanoantibody sequences. The smaller part of fingerprints (marked by the letter N) corresponds to clones with NcoI-NotI insertions of adapted nanoantibody sequences into the plasmid vector. Fingerprints found in both sub-libraries are underlined. Each HMR fingerprint is an electrophoretogram consisting of three gel lanes with separated DNA fragments obtained after parallel treatment of the PCR product (an amplified nanoantibody sequence) with one of three restrictases, HinfI (H), MspI (M) or RsaI (R), and the forth lane with marker DNA (the sizes of the marker DNA fragments are shown at the bottom of the figure).

Table 1. Relative representation of fingerprint variants of selected nanoantibody clones recognizing the anthrax lethal factor (alf), at various selection stages.

alf clone number	1st stage, normal helper phage	1st stage, mutant helper phage	2nd stage, normal helper phage	2nd stage, mutant helper phage	3rd stage, normal helper phage	3rd stage, mutant helper phage	ELISA signal
1	3/28	0/27	3/30	0/43	**3**/42	**5**/43	++
2	0/28	1/27	**3**/30	0/43	0/42	0/43	+++
3	1/28	0/27	0/30	0/43	**3**/42	**3**/43	++
4	2/28	0/27	0/30	0/43	**1**/42	0/43	+
5	2/28	0/27	0/30	1/43	0/42	**1**/43	+
6	3/28	0/27	12/30	0/43	**12**/42	**13**/43	++
7	0/28	0/27	0/30	0/43	0/42	**1**/43	+++
8	0/28	0/27	0/30	0/43	0/42	**2**/43	++
9	1/28	0/27	0/30	1/43	**1**/42	**1**/43	++
10	1/28	0/27	3/30	0/43	**2**/42	**1**/43	+++
11	0/28	0/27	0/30	0/43	0/42	**1**/43	++
12	0/28	0/27	0/30	0/43	**1**/42	**1**/43	++
13	1/28	0/27	2/30	0/43	**6**/42	**2**/43	++
14	0/28	0/27	2/30	1/43	**2**/42	**2**/43	+++

Note: Here and in Table 2 the clones used for the production and ELISA of corresponding nanoantibodies are in bold font. The number of "+" signs correlated with the relative increase in the ELISA signal (absorbance).

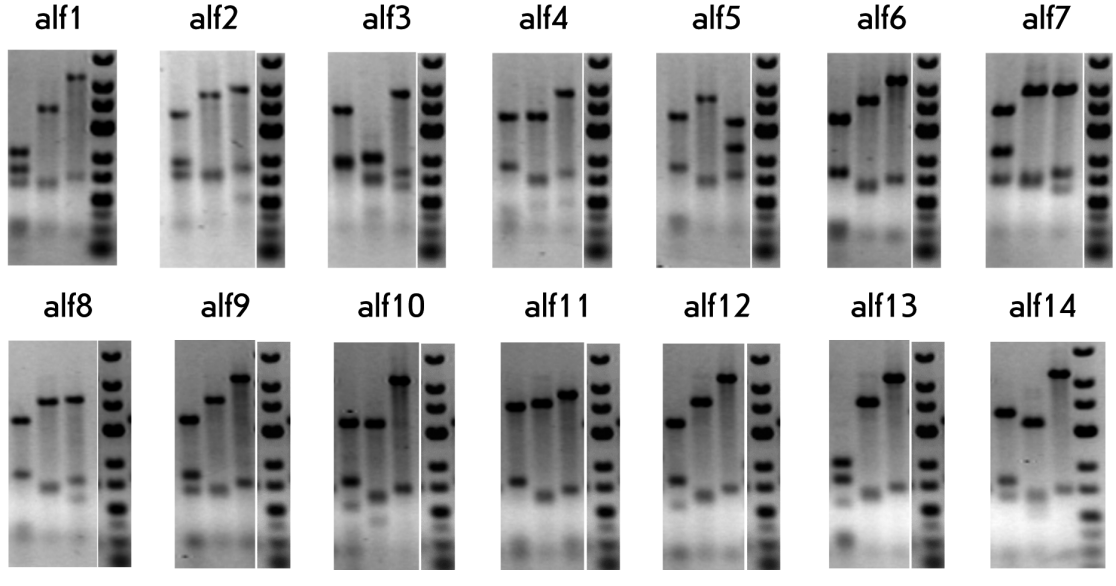

Fig. 2. HMR fingerprints of the selected nanoantibody clones that bind in ELISA to immobilised recombinant anthrax lethal factor. These clones are designated as "alf" with a number.

corresponding nanoantibodies in the host bacteria's periplasm. The periplasmic extracts were then tested with ELISA for the presence of a nanoantibody that binds to a given antigen. The same preparations of the Rabies virus and anthrax lethal factor that were used for camel immunisation were used as antigens for the selection procedures and ELISA. The increase in the absorbance value at 405 nm in the plate wells reflected two processes: the level of expression/availability of the nanoantibody and the strength of its interaction with the immobilized antigen. Information pertaining to the selection of the final nanoantibody clones binding to a given antigen is presented in *Figs. 2* and *3* and in Tables 1 and 2.

It is necessary to exert caution when interpreting the data. It is possible that some clones differing in nucleotide and amino acid sequences will have similar fingerprints, especially when sampled clones are compared to random clones from the original library. Sequencing can, naturally, provide more information. Our experience, however, shows that in most cases of clone identification the HMR analysis can replace

Table 2. Relative representation of fingerprint variants of selected nanoantibody clones recognizing Rabies virus preparation (aRv), at various selection stages.

aRv clone number	2nd stage, normal helper phage	2nd stage, mutant helper phage	3rd stage, normal helper phage	3rd stage, mutant helper phage	ELISA signal
1	0/67	3/45	**1**/30	**2**/58	+
2	7/67	1/45	**5**/30	**2**/58	+
3	13/67	0/45	**8**/30	**11**/58	++
4	0/67	0/45	**2**/30	0/58	+++
5	4/67	1/45	**1**/30	0/58	+
6	0/67	0/45	**1**/30	0/58	+
7	0/67	0/45	0/30	**1**/58	+++
8	**2**/67	0/45	0/30	0/58	+
9	**1**/67	**1**/45	0/30	0/58	+
10	**1**/67	0/45	0/30	0/58	+
11	**1**/67	0/45	0/30	0/58	++
12	**2**/67	0/45	0/30	0/58	+++
13	4/67	0/45	0/30	**2**/58	++
14	0/67	**2**/45	0/30	0/58	++
15	**1**/67	0/45	0/30	0/58	+
16	**1**/67	0/45	0/30	0/58	+++
17	3/67	1/45	**2**/30	**1**/58	++

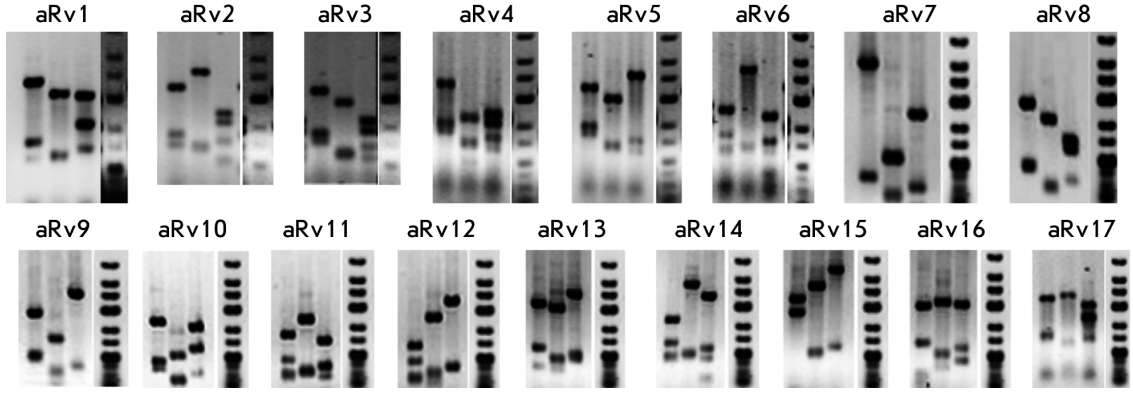

Fig. 3. HMR fingerprints of the selected nanoantibody clones that bind in ELISA to immobilised Rabies virus. These clones are designated as "aRv" with a number.

sequencing; therefore, it makes it a more economical technique.

HMR analysis of nanoantibodies recognizing anthrax lethal factor

Figure 2 shows the HMR fingerprints of 14 variants of the selected nanoantibody samples (alf) that recognize the anthrax lethal factor. There were only 5 out of the 14 for which we could find similar fingerprints among the 85 variants from the original library - the corresponding pairs being alf3—68N, alf6—59, alf8—62N, alf9—58, and alf10—64. Even though these 5 pairs are similar, that doesn't necessarily make them identical. Thus, the alf8 clone fingerprint variant was not found among 28–30 analyzed clones after the first and second selection stages; it appeared only after the third selection stage and only when the mutant helper phage was used. On the other hand, the alf6 clone fingerprint variant may well correspond to one of the most representative variants in the original library, since this variant remains one of the most represented after each selection stage.

We would like to note here a rather unexpected phenomenon related to the modified selection method using the hpΔMBpIII helper phage. When nanoantibodies to each of the two antigens were being selected (Tables 1 and 2), enrichment of specific clone variants was observed during the first two selection stages, which were different from the 85 variants presented in Fig. 1 and those enriched using the traditional helper phage. Unfortunately, the nanoantibodies coded for by those clones did not bind to the corresponding antigens

in ELISA. Those clones were effectively sorted out at later selection stages. We believe that those temporarily selected clones bind to the blocking agent and are then sorted out when the blocking agent is replaced. Apparently, some nanoantibodies, more easily available on the surface of the mutant phage, can either bind to a component of Skim milk (at the first stage) or to BSA (at the second stage). At the third selection stage, when Skim milk was used again, a sharp clearing of the nonspecific background occurred. The nonspecific clones enriched at the first and second stages were disappearing, and the fingerprints of the sought specific clones appeared, many of them identical to the clones selected in the parallel traditional procedure (Table 1, clones alf1, alf3, alf6, alf9, alf10, alf12, alf13, and alf14). Interestingly, similar to our previous work [11], some nanoantibody variants could only be selected with the modified selection procedure (Table 1, clones alf5, alf7, alf8, and alf11). There were also variants that could only be selected with the standard procedure (clone alf2 at the second stage and clone alf4). Interestingly, the nanoantibody clones that produce the strongest ELISA absorbance signal were not necessarily the most represented among the selected clones and could even disappear after the subsequent selection stages (alf2). The most enrichment was observed for the clones that produced medium intensity ELISA absorbance signal.

HMR analysis of nanoantibodies recognizing Rabies virus preparation

The findings described above were reaffirmed during the selection of nanoantibodies (aRv) recognizing the Rabies virus preparation (Fig. 3, Table 2).

In that case, a similar fingerprint could only be found for 5 out of the 17 finally selected clones among the 85 variants in the original library: aRv2—32, aRv3—12N, aRv5—75N, aRv13—76N, and aRv17—46N. The aRv3 clone fingerprint may well be one of the most represented variants in the original library, since this variant remains the most represented after (repeating) each selection stage with the standard helper phage. When the mutant helper phage was used for the selection of this fingerprint variant, it was clear that, as in the case of selection of nanoantibodies recognizing the anthrax lethal factor, the nonspecific background cleared after the third stage with blocking agent replacement. In this case, the parallel use of the traditional and the modified selection methods also ensured a wider variety of selected nanoantibody clones. Thus, in using only the traditional procedure, the fingerprint variants corresponding to the following clones were selected: aRv4, aRv6, aRv8, aRv10, aRv11, aRv12, aRv15, and aRv16. But while using the modified procedure alone, variants aRv7, aRv13, and aRv14 were selected. The aRv13 variant was also selected after the second stage of the traditional procedure, but it disappeared after the third stage.

It is interesting that in this case as well, the nanoantibody clones producing the strongest ELISA absorbance signal were not the most represented among the selected clones. Only a small number of the aRv4, aRv7, aRv12, and aRv16 clones were selected, and by using only one of two selection methods. Some of these clones (aRv12 and aRv16) can be easily lost at the third selection stage.

CONCLUSION

Parallel selection using the traditional helper phage and a modified helper phage with N-terminal deletion in the gIII surface protein is recommended for improving the efficiency of phage display selection of nanoantibodies with a required specificity. The modified procedure should take into account the higher nonspecific background, which is apparently due to the selection of phage particles containing nanoantibodies that bind to the blocking agents or to other nonspecific components. We cannot exclude a possibility of specific binding to the antigen of a special kind of nanoantibody, which is happening only when it is exposed on the phage's surface and which disappears in the case of stand-alone nanoantibody. The sequential use of different blocking agents in combination with the proposed mutant helper phage reduces the nonspecific background significantly after three selection stages. One should be aware that some important nanoantibodies might be lost during additional amplification/selection stages. We recommend analyzing selected clones after the second and third selection stages when performing the traditional procedure, and after the third stage when performing the modified procedure. Good results are also obtained when the modified method is used after the initial stage of the traditional selection procedure. ●

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