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INVESTIGATION / RESEARCH

Antibody response after hepatitis B vaccine boost mapped with peptide-phage display

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ABSTRACT

Recombinant hepatitis B virus vaccines confer protection by eliciting specific antibodies against the hepatitis B surface antigen (HBsAg), known as anti-HBs. However, the performance of rapid anti-HBs diagnostic tests generates concerns regarding consistency. Novel indicators of protection might be developed by monitoring changes in targeted HBsAg-epitope profile after vaccination. In this work, we test the feasibility of our peptide-phage display platform in identifying B-cell epitopes targeted at different time-points after hepatitis B vaccination. We combined this platform with a unique approach for *in silico* analysis of enriched sequences. Serum samples collected from one single patient who had two boosting immunizations against hepatitis B virus were used in two-rounds of selection experiments. Five epitope candidates from HBsAg were identified *in silico*; most of them were previously reported in the literature. Our results suggest that the number of recognized HBsAg epitopes is related to the decrease of anti-HBs over time.

Keywords: peptide-phage display, epitope mapping, hepatitis B virus vaccination

INTRODUCTION

Hepatitis B virus infection (HBV) is considered a major public health problem by the World Health Organization (WHO). Deathly consequences from HBV infection are mainly related to chronic liver disease and primary liver cancer¹. The hepatitis B vaccination at birth has been successfully implemented in 97 countries, decreasing the percentage of children younger than 5-years-old chronically infected with HBV to only 1.3% in 2015². The hepatitis B surface antigen (HBsAg) is the major component of the virion envelope and also the current main antigen used in recombinant hepatitis B vaccines^{2,3}. The vaccine confers protection by eliciting specific antibodies against

HBsAg and developing HBsAg-specific B and T cell-mediated immune memory . The main indicator of serological protection after hepatitis B vaccination is the IgG antibody-titer against HBsAg (anti-HBs). Long-term protection against HBV infection is considered ensured when anti-HBs titer of ≥ 10 mIU/mL is reached 1 or 2 months after completing the vaccination series ². Although the efficacy of hepatitis B vaccination in infants is clear in the long term, it can drop below 60% in elderly patients ⁵. Currently available data suggests that boosting immunizations are not needed in immunocompetent patients that completed the vaccination routine, however this is not yet validated ^{2,5}. Today, hepatitis B boosting immunizations are advised mainly in high-risk groups when anti-HBs titers are lower than 10 IU/L ². Rapid diagnostic tests to detect anti-HBs are available, but their performance is not consistent amongst manufacturers ². Therefore, the improvement of diagnostic tests could be aided by identifying diagnostic and/or clinically relevant HBsAg epitopes.

The antibody response elicited after hepatitis B vaccination has been thoroughly characterized. Several epitopes have been reported. However, out of 72 HBsAg human B-cell positive epitopes reported in the Immune Epitope Database (IEDB; www.iedb.org), 55 epitopes are reported as peptides comprising 10 to 48 amino acid residues (in average, 22 residues). Shorter epitopes were identified mainly by mapping monoclonal antibodies isolated from immunized patients using time-consuming and expensive methods such crystallography, nuclear magnetic resonance (NMR), computational docking and site-directed mutagenesis ⁶.

Peptide-phage display has led to the identification of ligands and serum biomarkers relevant for the treatment or diagnosis of a large variety of diseases such as cancer, viral infections, multiple sclerosis, degenerative joint disorders and traumatic brain injuries ^{6,7}. Although the systematic identification of epitopes targeted by circulating antibodies is currently not feasible, B-cell and T-cell epitope mapping is done using peptide-phage display based methods ^{6,8}. These remarkable efforts have however resulted in a very limited amount of epitopes or mimotopes ⁶. The characterization of larger number of sequences selected from peptide phage display has been improved by combining it with Next Generation Sequencing (NGS) platforms ⁹. However, this also increased the challenges regarding to the search of good binders from a larger pool of non-specific peptides ⁹. The distinct epitope recognition profiles between different patients demand rapid, efficient and cost-effective methods to identify epitopes recognized by circulating antibodies ^{8,10}. Rapid epitope identification directly from patient sera was reported using a combination of peptide-phage display, next-generation sequencing (NGS) and a unique approach for *in silico* analysis ^{10,11}. By using a similar approach, we test its feasibility in identifying B-cell epitopes at different time-points after boosting injections with hepatitis B vaccination directly from one single patient's sera.

Serum samples from an adult, male donor who completed primary hepatitis B vaccination on adulthood were kindly provided by the Ligand Development Unit at Fraunhofer IZI. In 2010, the patient (born in 1971) had non-sufficient anti-HBs titer to ensure protection and therefore a boosting injection was advised. Serum samples were collected after two boosting injections in 2010 and 2016, and also at irregular intervals between immunizations as described below:

Serum ID	Description
S1	First boosting. Serum was collected 42 weeks after vaccination with Enderix-B (GlaxoSmithKline, UK). Sampling date (MM/YY): 11/2010
S1a	2 years after boosting. Sampling date (MM/YY): 12/2012
S1b	4 years after boosting. Sampling date (MM/YY): 02/2014
S1c	5 years after boosting. Insufficient anti-HBsAg titer to guarantee protection. Sampling date (MM/YY): 12/2015
S2	Second boosting. Sample was collected 5 weeks after immunization with Enderix-B. Sampling date (MM/YY): 01/2016

Serum aliquots were stored at -20 °C until use. An aliquot of each serum sample corresponding to 24 µg of total protein was used in two selection rounds following the peptide phage display protocol used by Kern *et al.*, 2018. NGS of bound peptide phage was performed using illumina MiSeq® (San Diego, USA) and the output data was pre-processed and converted into peptide sequences¹⁰. Potential epitope candidates were identified *in silico* according Kern *et al.*, 2018. This approach applies a statistical analysis of peptide sequences obtained after the first and second selection rounds as follows: The sequence pools are analyzed in terms of those motifs similar to HBsAg which are enriched above a theoretically expected value. This analysis based on statistics is feasible due to a relatively even amino acid distribution of the library. Sequences sharing enriched motifs are retrieved from a database corresponding to each serum sample. Next, sequences are aligned for the identification of peptides sharing more extensive similarities the antigen. In other words, this method replaces the search for individually enriched sequences, as it is done in most of the current peptide-phage display protocols, by the analysis of sequences containing enriched 4-mer fragments.

This approach led to the identification of five HBsAg epitope candidates. An example is illustrated by the motifs located in the C-terminus of HBsAg. Increased enrichment values of the C-terminal motifs were observed mainly on the datasets corresponding to the sera S1 and S2, which were collected after boosting immunizations. After the second-selection round, the C-terminal motif WVYI was enriched 3.2- and 2.8-fold following the first and second boosting immunization, respectively. An important enrichment was also observed on the C-terminal motifs from the dataset obtained from serum S1c (i.e. collected 5-years after first boosting). When comparing alignments of peptide sequences containing the enriched motifs retrieved from different datasets (Figure 1), a higher frequency in the datasets obtained from samples S1 and S2 in comparison to the datasets S1c is observed (*frequency* i.e. the ratio of occurring sequences containing the motif to total sequences included in the dataset). This is suggested by a clear increase on the occurrence of sequences in datasets obtained from samples S1 and S2 in comparison to the dataset obtained from S1c. The enrichment of peptides binding to serum antibodies becomes evident when these alignments are compared to alignments containing sequences from the naïve library dataset (i.e. selection without patient serum) (Figure 1- A).

A: Naive library	B: Five years after first boosting	C: After boosting immunizations
HBsAg C-terminus (UniProt accession number P30019) SPFLPLLPFFCLWVYI-----	HBsAg C-terminus (UniProt accession number P30019) SLYSILSPFLPLLPFFCLWVYI-----	HBsAg C-terminus (UniProt accession number P30019) ILSPFLPLLPFFCLWVYI-----
count=2 ---PWSQFSILLWVYIGF----	count=5 -----HGF CWVYI AIARVYC----	> S2 count=8 -----I INCWVY GYERFHSD----
count=3 ---PWS SLVYI QHDQEC-	count=7 -----S IFC WVYEDWPFVYC----	> S2 count=7 -----I HSCWVY QVPHIRC----
count=2 ---TYQST LWVY FFLFD--	count=4 -----Y GC WVYEDIRCEFA----	> S1 count=5 -----I IPCWVY QCQGNVD----
count=2 --- F CWVY QPIQSNVD	count=2 -----L PC WVYCPEDIVHI----	> S2 count=3 -----I WMCWVY CYHAHLA----
count=2 --- F CWVY QEPICARD	count=4 -----S QC WVYKYCVDAF----	> S2 count=5 -----I ARCWVY QEDVGVG----
count=2 HTTCH PCWVY HTD----	count=7 -----A KPC WVYQPYNHVEF----	> S1 count=2 -----H FPCKILVYI ICGD----
count=2 VDNSH YICWVY HFF----	count=2 -----K EA CWVYLSGGHFAI----	> S2 count=2 -----I BACWVY KLYHHNDN----
count=2 -----E THC WVYRSRDFKQY	count=3 -----Q SPC WVYEHIGFLTC----	> S2 count=2 -----I QTCWVY DEIGCID----
count=2 -----N STC WVYRHQHVGYC	count=3 -----P FC WVYQVYVYFY----	> S1 count=2 -----Q FACWVY YACDGF----
count=2 -----Y VC WVYFYISGTRN	count=2 -----R NHC WVYVYFY----	> S2 count=3 -----P IFC WVYAKRDCQPC----
count=2 -----D AHC WVYQPFCEFY	count=3 -----L YR CLWYLSQFVSPA----	> S2 count=9 -----L KFCWVY EERYDYPN----
count=2 -----E QC WVYQGGICAC	count=17 -----D NC WVYRBYICVY----	> S1 count=6 -----N NFC WVYYPYHNFHAF----
count=2 TGRSV WILCLWY NDY----	count=3 -----R NA CLWYFPQHALGF----	> S1 count=5 -----T EW CWVYAVEQSEHA----
count=2 -----N WC WVYVGPYCVVI	count=5 -----Y TV WVYVQGEVNRD----	> S1 count=3 -----E DHSYS DN CWVY RVF----
count=2 -----S AH CLWYQIPDPYED	count=4 -----V F CWVYVQGEVNRD----	> S1 count=5 -----D Q TST SIN CWVYPTI----
count=2 IGM WDFCLWY QLF----	count=3 -----Q F CWVYDCECEAY----	> S1 count=5 -----P DR CWVYRPEAKDNA----
count=2 -----H SE CLWYQEGRVSEN	count=2 -----N F CWVYRAWTSEAA----	> S1 count=9 -----V VS WVYHAYVHHGY----
count=2 -----F LN CLWYRACIEVEN	count=4 -----I BA CWVYKLYHHNDN----	> S1 count=3 -----L VS WVYICYQLHFI----
count=2 PQR CRYA CLWYHCY----	count=2 -----V TR CLWYITEQPQFA----	> S1 count=4 -----H IV WVYAHVYHTVE----
	count=8 -----F KRC WVYSLWVYICF----	> S1 count=4 -----R VV CLQYRH WVY RY----
	count=2 -----G VL SSLWYI GA QFAY----	> S1 count=5 -----Q YS Q WVY I IF SYN----
	count=2 -----L WV I F VLVRIQQVHI	> S1 count=2 -----A I HC D WVYI GV CHKY----
	count=7 -----S GR SW QCLWV I IN	> S1 count=7 -----T V CLWYH HH VE FP VGY----
	count=2 -----E V NCYR Q -A DE L VYI	> S1 count=7 -----D Q W CLWY H Q V Q FL KVI
	count=5 -----F WH CK F SN S R L F VYI	> S1 count=9 -----D Q W CLWY P VE T L GGD----
	count=3 -----D EL S VYI D PE Y L A I Q F	> S1 count=7 -----R YV WVY GV IG IV GYA----
	count=2 -----T H GS H EN P -N DR L F V YI	> S2 count=4 -----E L A CLWV P V CE EH PD----
		> S2 count=3 -----H N C WVY E AD W VH Y Q I----
		> S2 count=3 -----N V A CLWV Q AW E TR SD----
		> S2 count=6 -----S N C WVY H C N I N Y H I A----

Total sequences occurring more than once containing the motif: 39

Frequency: 1.8×10^{-5}

Frequency = Occurring sequences / Total sequences in dataset

Total sequences occurring more than once containing the motif: 113

Frequency: 4.5×10^{-4}

(Only 30 unique sequences are shown)
Total sequences occurring more than once containing the motif: 319

Frequency: 6.7×10^{-4}

Figure 1. Alignment of peptides containing C-terminus motifs (CLWVYI) retrieved from datasets corresponding to: A) the naïve ENTE1 library, B) serum S1c (5-years after first boosting) and C) S1, S2 (after boosting immunizations). Highlighted residues match their counterparts within the primary sequence of the C-terminus of HBsAg (UniProt accession number P30019). The occurrence of each peptide is shown as counts. The frequency of all sequences containing the motifs is calculated as indicated. The highest frequency was observed on those datasets obtained after boosting immunizations.

By applying this approach, five HBsAg epitope candidates were identified *in silico* from data generated from peptide-phage display experiments using patient sera. A unique set of enriched epitopes was identified on datasets corresponding to different time-points (Table 1). Up to four epitopes were enriched after boosting immunizations. The number of identified enriched epitopes decreased over time, reducing up to two epitopes identified 5-years after boosting.

Sample ID	HBsAg epitopes candidates identified <i>in silico</i>				
	48 VCLGQ	104 LPVCPL	135 PSCCC	178 PFVQWF	221 CLWVYI
	52	109	139	183	226
S1		✓	✓	✓	✓
S1a	✓		✓	✓	✓
S1b		✓	✓		
S1c	✓				✓
S2		✓	✓	✓	✓

Table 1. Overview of the epitope candidates identified in this study. A larger number of enriched epitopes were identified after first and second boosting immunization (S1 and S2). Except $_{48}\text{VCLGQ}_{52}$, all motifs were previously reported by other groups as part of larger immunogenic regions.

According to the patient's vaccination records, the vaccination schedule suggested by the manufacturer was completed in 2004 and two boosting immunizations were performed in 2010 and 2016, when his anti-HBs titers were low. This correlated with the number of epitopes identified after boosting immunizations (S1 and S2). The different number of epitope candidates identified in each peptide dataset suggests that different vaccine-specific antibodies are produced over time.

Most of these epitope candidates were reported previously as human B-cell epitopes in the Immune Epitope Database (IEDB; www.iedb.org), supporting the efficacy of our technology for the mapping of circulating antibodies directly from patient sera (Table 2).

Epitope candidate	Reported epitope(s)	Description	References
$_{47}\text{VCLGQ}_{51}$	No human epitope reported		
$_{104}\text{LPVCPL}_{109}$	$_{101}\text{QGMLPV}_{106}\dots$ $_{163}\text{WEW}_{165}$	Discontinuous epitope mapping of four monoclonal antibodies by peptide phage display.	Chen <i>et al.</i> , 1996
	$_{101}\text{QGMLP}_{105}$	Epitope localization of murine monoclonal antibodies by peptide phage display	Reynaud <i>et al.</i> , 2001
	$_{104}\text{LPVCPLLPG-}$ $_{-}\text{TSTTSTGP}_{120}$	Epitope mapping of human monoclonal antibodies with synthetic peptides	Kazuto <i>et al.</i> , 2010
$_{135}\text{PSCCC}_{139}$	Reported in more than 20 studies		
$_{178}\text{PFVQ}_{181}$	$_{178}\text{PFVQWFVGL}$ $_{186}$	Epitope mapping of murine monoclonal antibodies directed against native HBsAg obtained from infected patient sera by peptide scanning analysis.	Paulij <i>et al.</i> , 1999
$_{221}\text{CLWVYI}_{226}$	$_{214}\text{PLLPIF}_{219}$	See above	Reynaud <i>et al.</i> , 2001

Table 2. Summary of the epitopes candidates identified in this study and the reported human B-cell epitopes available on the Immune Epitope Database (IEDB; www.iedb.org).

Several conformational epitopes have been reported within the α -determinant (residues 124-147). This region is highly targeted by anti-HBs and confers protection against all subtypes, since it is shared by all serotypes and genotypes of the virus ³. This region is thus considered the most immunogenic region and an important target for diagnosis and immune prophylaxis ³ (3). However, we could identify only one enriched epitope candidate within the α -determinant: the motif PSCCC. This epitope is particularly interesting, because it was identified in all peptide datasets, except the dataset corresponding to S1c (Table 2). A plausible explanation for the identification of a single epitope candidate in such a well-known immunogenic region is the presence of a number of methionine residues. Since this residue was largely avoided during the ENTE1 library design, enriched motifs containing methionine were not identified in the peptide datasets.

Variations of the specificity of the circulating vaccine-specific antibodies might be related to the identification of additional epitope candidates ($_{104}$ LPVCPL $_{109}$, $_{178}$ PFVQ $_{181}$, $_{221}$ CLWVYI $_{226}$). As shown in Table 2, the epitope candidate $_{47}$ VCLGQ $_{51}$ has not been reported as a human B-cell epitope. Further studies involving a larger amount of patients are nevertheless needed to validate this epitope candidate in humans. On the other hand, the remaining epitope candidates were previously reported as epitopes targeted by human and murine HBsAg-specific monoclonal antibodies.

Five peptides (peptide length between 11-13 residues) containing three HBsAg epitope candidates identified in this study were selected from the generated peptide datasets, synthesized and tested in a modified peptide ELISA-like assay. Although the method has not been fully validated yet, serum IgG binding from S1c and S2 was positive for two out of five peptides. This encourages the viability of our method in identifying peptide ligands specific against HBsAg (Figure 2).

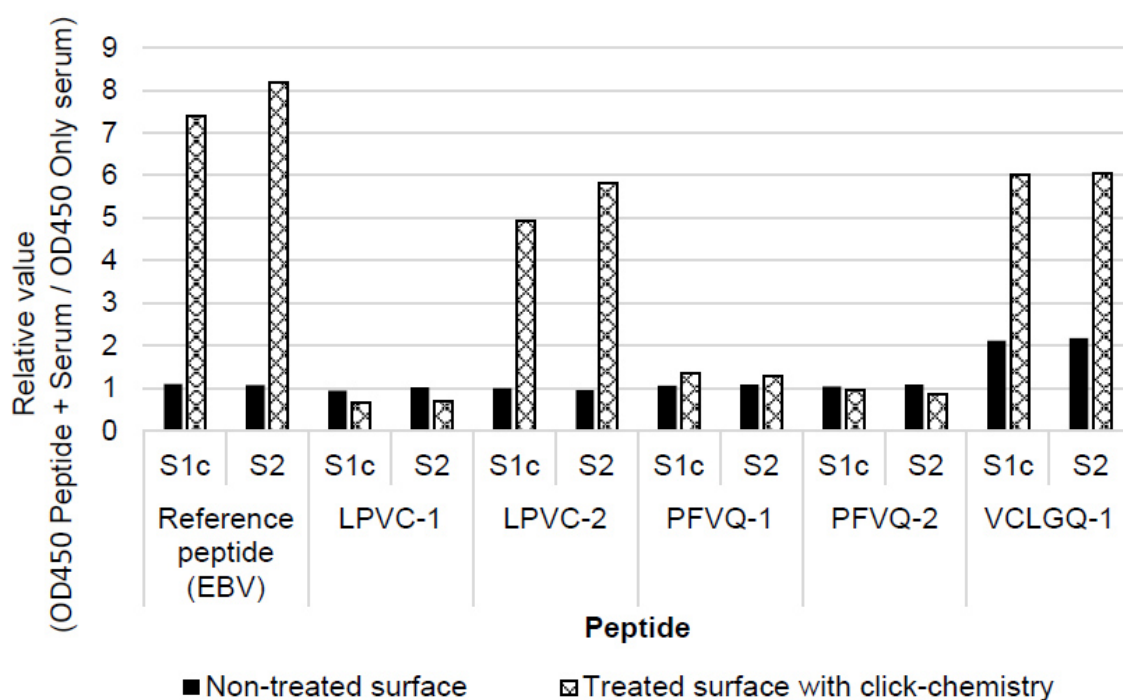


Figure 2. ELISA-like assay of synthetic peptides selected from datasets obtained from peptide-phage display experiments on patient sera. Peptides were coated by triplicates in treated 96-well microplates by click-chemistry. Subsequently, peptides were incubated with patient sera (S1c, S2) and IgG binding was detected with goat anti-human IgG-peroxidase antibody (Sigma-Aldrich). Results were expressed as relative values, i.e. dividing the mean absorbance (OD450) of peptide-serum reactions by the mean absorbance of the reactions performed without peptide at the same wavelength. Peptides LPVC-2 and VCLGQ-1 showed a remarkable IgG binding. An EBNA1-specific peptide (Epstein-Barr-virus nuclear antigen 1) was used as reference.

The increased number of identified epitopes (Table 2) and the larger occurrence of peptides containing an enriched motif in datasets S1 and S2 (Figure 1) might be related with a 10-100 fold increase of HBsAg-specific antibodies that typically follows shortly after re-exposure of the antigen to the memory B-cells⁵. The protection by HBV vaccination is guaranteed when anti-HBs titer > 10 UI/L are reached 1–2 months after immunization². Most identified epitopes were detected in these datasets, suggesting an enhanced response upon boosting immunization in this patient. This was confirmed by satisfactory anti-HBs titer after both boosting injections.

Our results support those studies that suggest a successful prevention against acute viral infection even when anti-HBs titers drop below 10 UI/L¹². Although there is strong evidence supporting the efficacy of HBV vaccine in the prevention of acute and chronic infection up to 15 years after immunization of infants⁵, two boosting injections were advised to the patient evaluated in this study within a period of 12 years. The chronological identification of an epitope candidate within the *a*-determinant correlated to the decreasing anti-HBs titer that ultimately determined the need of boosting immunizations.

CONCLUSION

The profiling of antigenic regions from patient sera by phage display and NGS has been previously reported^{6,8}, nevertheless a very different approach is used in this study for the identification of potential epitopes. Here, the identification of enriched motifs is supported by a robust statistical analysis within the sequenced phagemid pools, taking into consideration the library design and its complexity. Despite the complex diversity of circulating antibodies in patient sera, the *in silico* analysis of antigen's 4-mer motifs from phagemid pools allowed the identification of previously reported HBsAg epitopes in a single patient over time. The diversity of epitopes identified at different time-points supports that changes in the specificity of the immune response occur after immunization within the same subject.

This is the first study where the chronological response to vaccination was evaluated in terms of the identification of B-cell epitopes directly from patient sera. Our results show that the largest number of B-cell epitopes was identified in the datasets corresponding to sera collected after boosting immunizations. Since antibodies specific to other fragments of HBsAg are also produced, the development of more precise immunoassays for the quantification of anti-HBs might be feasible. The potential use of synthetic peptides for the validation of epitopes candidates identified by this approach was preliminary demonstrated, although several improvements in the experimental setup and the selection of peptides from the phagemid pools remain to be done. This technology might improve the rational antigen design and aid the quality control of newly produced vaccines⁸. Finally, further studies in a larger number of patients may contribute to the improvement of the current understanding of the specificity of antibodies elicited after vaccination or other immunotherapies.

REFERENCES

1. World Health Organization. Global hepatitis report 2017; 2017. Available in: <http://www.who.int/iris/handle/10665/255016>.
2. World Health Organization. Hepatitis B vaccines: WHO position paper – July 2017. Weekly Epidemiological Record [Internet]. 2017;92(27):369–92. Available in: <http://www.who.int/iris/handle/10665/255873>.
3. Golsaz-Shirazi F, Mohammadi H, Amiri MM, Khoshnoodi J, Kardar GA, Jeddi-Tehrani M, et al. Localization of immunodominant epitopes within the "a" determinant of hepatitis B surface antigen using monoclonal antibodies. Arch Virol. 2016; 161(10):2765–72. doi: 10.1007/s00705-016-2980-y.
4. Brunskole Hummel I, Zitzmann A, Erl M, Wenzel JJ, Jilg W. Characteristics of immune memory 10-15 years after primary hepatitis B vaccination. Vaccine. 2016;34(5):636–42. doi: 10.1016/j.vaccine.2015.12.033.
5. van Herck K, van Damme P. The immunological basis for immunization series: Module 22: Hepatitis B Immunization, Vaccines and Biologicals. Geneva, Switzerland: World Health Organization; 2012.
6. Wu C-H, Liu I-J, Lu R-M, Wu H-C. Advancement and applications of peptide phage display technology in biomedical science. J Biomed Sci. 2016;23:8. doi: 10.1186/s12929-016-0223-x.
7. Zambrano-Mila MS, Sánchez Blacio KE, Santiago Vispo N. Peptide Phage Display: Molecular Principles and

Biomedical Applications. Ther Innov Regul Sci. 2019. doi: 10.1177/2168479019837624.

8. Domina M, Lanza Cariccio V, Benfatto S, D'Aliberti D, Venza M, Borgogni E, et al. Rapid profiling of the antigen regions recognized by serum antibodies using massively parallel sequencing of antigen-specific libraries. PLoS ONE. 2014; 9(12):e114159. doi: 10.1371/journal.pone.0114159.
9. He B, Jiang L, Duan Y, Chai G, Fang Y, Kang J, et al. Biopanning data bank 2018: hugging next generation phage display. Database (Oxford). 2018;2018. doi: 10.1093/database/bay032.
10. Kern K, Havenith H, Delaroque N, Rautenberger P, Lehmann J, Fischer M, et al. The immunome of soy bean allergy: Comprehensive identification and characterization of epitopes. Clin Exp Allergy. 2019;49(2):239–51. doi: 10.1111/cea.13285.
11. Havenith H, Kern K, Rautenberger P, Spiegel H, Szardenings M, Ueberham E, et al. Combination of two epitope identification techniques enables the rational design of soy allergen Gly m 4 mutants. Biotechnol J. 2017;12(2). doi: 10.1002/biot.201600441.
12. Rosado MM, Scarsella M, Pandolfi E, Cascioli S, Giorda E, Chionne P, et al. Switched memory B cells maintain specific memory independently of serum antibodies: the hepatitis B example. Eur J Immunol. 2011;41(6):1800–8. doi: 10.1002/eji.201041187.

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