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RESEARCH ARTICLE

High Affinity Peptides in Processes of IgG Purification, Chromatographic Column Virus Inactivation/ Elimination and Titer of Anti-Rubella IgG Enrichment

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ABSTRACT

According to "The Proteome Code" concept introduced by J. Biro and our early development of affinity peptide calculation method it was studied the possibility of high affinity peptide chromatographic gels development for IgG_{1-4} separation from the donor plasma. Given the next step of virus inactivation of IgG directly in the chromatographic column, the affinity gel had bind IgG at several spatially spaced points in order to limit the degree of freedom of the protein for retention IgG at high buffer flow rate or elevated buffer temperatures without denaturation. In addition, the possibility of creating highly specific affinity sense-antisense peptides against Rubella virus in order to increase the titer of ^{aR}IgG in plasma or even its isolation in highly purified form was studied. Based on previous experiments, an affinity multi-peptide chromatographic gel with the following properties was developed: the DBC with enough residence time 10 min was around 50-54 mg × mL^{-1} of total 98.0% purity of IgG with natural proportion of the 1-4 subclasses, any other immunoglobulins were not found. The virus inactivation/elimination on this gel directly in chromatographic column showed a highly effective virus elimination (log_{10}>9) for both nonenveloped and lipid enveloped viruses.

Using RV sequence from UniProt_KB and dates from more than 20 literature sources on the virus proteins interaction, affinity peptides were calculated against virus proteins C and $E_{1,2}$. Then these peptides were modified to reach more affinity enhancement and affinity-peptide chromatographic gel was synthetized. By this gel from total mass IgG_{1-4} contained 6644 IU anti-Rubella IgG with specificity 6.64 IU × mg^{-1} were isolated 5382 IU ^{aR}IgG (> 80%) with a specificity of 791 IU × mg^{-1}.

ABBREVIATIONS

DBC: Chromatographic Gel Dynamic Binding Capacity; TDC: Chromatographic Gel Temperature-Dependent Capacity; $aa_{(s)}$: Amino Acid(s); $hIgG_{1,2,3,4}$: Human Immunoglobulin Class G Subclass 1, 2, 3 or 4; RV(s): Rubella virus(es); ^{aR}IgG: Anti-Rubella Immunoglobulin Class G (total); S/D: Solvent/Detergent; TNBP: Three-*N*-Butyl-Phosphate; T°: Temperature; FVD: Decreasing the Factor of Virus Concentration; LEV(s): Lipid Enveloped Virus(es); NLEV(s): Nonenveloped Virus(es); SpA: *Staphilococcal* Protein A; SpG: *Streptococcal* Protein G; PpL: ***Peptostreptococcal*** Protein L; MOG: Myelin Oligodendrocyte Glycoprotein.

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INTRODUCTION

Previously we have shown that the development of chromatographic adsorbents with high Dynamic Binding Capacity (DBC) gave us the opportunity to transform the method of virus-inactivation of proteins in solution into the method of inactivation and elimination of viruses directly in the chromatographic process at an elevated temperature of 30-45˚C [1,2]. The parameter "temperature-dependent capacity of the adsorbent (TDC)" offered by us as a function of DBC shows that if the adsorbent DC is higher, its TDC will be higher, since the temperature-dependent rotations/hesitation of protein in physical essence are quite close to the microturbulence of the chromatographic mobile phase with dissolved protein [2]. In the case of multipoint adsorption of the target protein, it is actually "crucified" on the adsorbent; its degrees of freedom and rotations/hesitation of individual parts of the molecule tend to zero. Therefore, an increase in the temperature of the "protein-adsorbent-buffer" chromatographic system does not cause a noticeable denaturation of the protein, even as labile proteins as FVIII. A high DBC allows keeping the protein in a "crucified" state at elevated temperatures. The development of the above method included as model proteins and polypeptides that differ by molecular weight, simplicity or complexity of the molecule, presence/absence of enzymatic activity: fragment of streptokinase SK^{1-61}, lysozyme, fibrino(geno)lytic enzyme from the *Agkistrodon blomhoffi* venom [1], and also a complex of coagulation factors VIII/von Willebrand (FVIII/VWF) [2]. In all cases, any denaturation or loss of protein activity was avoided, and the FVIII yield in the complete process of its isolation from donor blood plasma was significantly increased.

At the beginning, the most versatile and capacious, as well as fairly inexpensive adsorbents were used - ion-exchange SP and Q Sepharose HP, WorkBeads 40S and 40Q, hydrophobic interaction Source 15RPC [1,2]. It is known that increasing temperature promotes the exposure of new hydrophobic sites on the surface of the protein globule [3-5], in fact, increasing the number of binding sites with the adsorbent. For Source 15RPC it was shown that the DBC for insulin was increased from 57 to 78 mg × ml^{-1} when the temperature rises from 20 to 45˚C and the linear velocity of the buffer remained stable 300 cm × h^{-1} [1]. The same mechanism works with reverse phase chromatography, where the binding of the target protein depends on the exposure of hydrophobic sites on the protein surface under the solvents influence [3,6]. As for affinity adsorbents, high-molecular ligands of a protein nature (staphylococcal protein A - SpA, streptococcal protein G - SpG, peptostreptococcal protein L - PpL), even if they withstand elevated temperatures [7-9], then their DBC significantly decreases [8-10], and the leaching from the matrix is intensified [11,12]. Nonspecific and monoclonal antibodies as chromatographic ligands for the process under temperatures above 30-35˚C are not considered due to their thermal lability [13]. The behavior of short-chain (6-15 amino acids) peptide ligands used in affinity chromatography under such conditions has not yet been adequately studied. However, it would be appropriate to combine in one stage the affinity purification of immunoglobulins and their virus-inactivation at elevated temperatures, which would significantly reduce the process time and cost. Recently, we have shown that the using of Biro "Proteome Code" concept [14] makes it possible to significantly simplify, respectively, accelerate and reduce the cost of calculating the affinity peptide ligand for almost any protein [15]. In addition, we expected that a 10-15 amino acids (aa_s) peptide attached to a neutral adsorbent such as WorkBeads 40/1000 ACT would not be susceptible to denaturation and excessive leaching, bearing in mind that the same ligand had not previously shown appreciable flow for 250 complete chromatographic cycles at 20˚C in previous experiments [15]. In the same development, it was found that the affinity of the calculated peptide can be "ordered" higher or lower, which was measured by the pH level of desorption of 50% of the protein from the sorbent ($pH_{50\%}$). In the case of the peptide affinity adequate $pH_{50\%}$ = 3.65 ÷ 3.80, the interaction of sense-antisense aa_s was determined as average, coinciding with the level of the affinity of Proteins A/G for IgG. The strong level of affinity ($pH_{50\%}$ = 3.40 ÷ 3.47) was characterized by hydrophilic-hydrophobic and also electrostatic interactions between sense-antisense aa_s. In this case, complete desorption of the protein from the sorbent occurred at pH < 2.5 [15], encouraging that a virus inactivation of immunoglobulins directly in the chromatographic column will be possible. In addition, the level of denaturing temperature-dependent rotations/hesitation of the protein can be significantly reduced in the case of simultaneous linking to the adsorbent of two or three ligands that will bind the target protein at different as sites located at the different parts of molecule. About 35 peptide ligands for affinity chromatography of natural, recombinant immunoglobulins and their fragments have already been published in the literature [16-18]. To obtain the top performing affinity peptides rapidly, many screening methods have been developed such as combinatorial chemistry, mRNA display, phage display, computer-based virtual screening technology and other. Our goal in this study was to show a fast, inexpensive way to calculate the high affinity peptide ligands for IgG from human plasma. The above-mentioned Biro concept "Proteome Code" allows the interaction of accessible linear regions of two polypeptides [19]. Moreover, the complete concept insists that it is precisely such an interaction can be predetermined by the genetic code [19]. It can be assumed, for example, that in the event of a virus attack, the mammalian organism reads a certain aa sequence of the viral (most likely capsid) protein, which is responsible for the interacting with the cell receptors. Then immunoglobulin (M, A or G) is synthesized with the inclusion of antisense aa sequence in the variable region of the light/heavy chain. And thus, the "landing place" of the virus on the cell can be blocked by a specific immunoglobulin [20,21]. The literature contains enough

data describing peptide/polypeptides of viral proteins that can interact with cell receptors. Based on these data and our developments [15], it is possible to calculate a hypothetical site of interaction and use it as an affinity ligand for the isolation of a specific immunoglobulin. This last problem is much more interesting than the previous two, but it remains the most speculative. The simplest case for the such study seems to be the specific anti-rubella IgG (^{aR}IgG), since in any vaccinated human body it is contained in the amount of ~40 IU × mL^{-1} until the end of the life [22]. The level of vaccination against rubella in Ukraine is 78%, and among the younger generation (up to 25 years) - more than 82% [23]. Thus, in the total pool of donor blood, the ^{aR}IgG titer can actually range from 5 to 40 IU × mL^{-1}. This level is quite sufficient to assess the affinity of the developed peptides, the possibility of direct column virus inactivation at elevated temperatures, and also for the attempt using the high affinity peptide ligand to increase the ^{aR}IgG titer, separating it from other nonspecific immunoglobulins. Cong, et al. [24] showed that one of the host cell receptors identified to bind the E1 protein, is Myelin Oligodendrocyte Glycoprotein (MOG), a member of the immunoglobulin superfamily mainly expressed in the central nervous system and in other tissues such as spleen, liver and thymus of mice. Due to the restricted expression of MOG in cells of these tissues, the role of other receptors and co-receptors in RV attachment cannot be excluded [24]. For example, not excluding the mechanism of transplacental vertical RV infection or direct fusion of the viral envelope with the endosomal membrane due to a conformational change in the E1 and E2 glycoproteins cannot understand [25]. Given that capsid protein C is the other contender for interaction with the cell receptor [26] all three capsid proteins, C and E1 and E2, would be the ideal choice for a determination of peptide epitopes of RV interaction with cell.

Thus, this study was aimed to solve three problems, namely: 1. The development of 3-4 peptide ligands to spaced sites of the human plasma IgG in order to obtain an affinity adsorbent with high DBC/TDC and multi-point IgG binding to prevent possible denaturation at elevated temperatures of virus inactivation in the column; 2. The determination of the virus inactivation parameters for the IgG directly in the chromatographic column and the levels of inactivation/elimination of model viruses; 3. The development high specific affinity peptide(s) for ^{aR}IgG in order to significantly increase its titer and create an inexpensive pharmaceuticals to prevent rubella infection in pregnant or HIV-infected people.

MATERIALS AND METHODS

All reagents were purchased from Sigma-Aldrich (Green Chemistry LLC, distributor in Mongolia) unless otherwise indicated. Reagents and equipment from other manufacturers are indicated below. Human (donor's) plasma, obtained by plasmapheresis, was bought from several regional blood centers in Ukraine.

Native human IgG_1, IgG_2, IgG_3 and IgG_4 (Cat. No. ab 90283, 90284, 118426 and 183266) proteins >95% purity, recombinant RV proteins E_1, E_2 and C (ab107947, ab43033, ab43034, respectively) and kits for determination human IgG (*h*IgG) total (ab195215), *h*IgG_1 (ab), *h*IgG_2 (ab), *h*IgG_3 (ab 100548, 202402, 201284) were purchased from Abcam office at China. The *h*IgG_4 concentration was calculated in the needed case according to the difference between total IgG and IgG_{1-3} quantities. The ELISA kits for RV IgG determination were purchased from Creative Diagnostics distributor Filgen, Inc., Japan (Cat. No. DEIA011) or from **Cepham Life Sciences, Inc.**, Fulton, MD, USA (Cat. No. 10935).

Total human IgG (*h*IgG) was re-purified [27,28] to 99.4% purity from commercial donor's plasma 10% intra venous immunoglobulin "Bioven" manufactured by pharma plant "Biopharma LCC", Bila Tserkva, Ukraine.

The total protein in the collected samples was determined by Bradford method [29] with Stoscheck modification [30].

The calculation of the high affinity peptides to total hIgG1,2,3,4 and to aRIgG

Peptide calculation was carried out according to the "Algorithm of peptide affinity calculation" and table of "Sense-antisense aa_s for the both 3'-5' and 5'-3' direction of gen reading" published early [15] using aa sequences from RV proteins shown in the table 1.

Array peptides synthesis and SPOT peptide array experiments

The peptide's array library, containing calculated affinity members (~50-100 nmol per spot), was synthesized through short aa spacer as described earlier, using Fmoc chemistry and MultiPep SPOT synthesizer (membranes and equipment from Intavis AG, Germany) [15]. All controls, positive and negative, were same as shown at the previous development [15].

The peptide-membrane after synthesis was subjected to washing [15] and incubation with 0.01 mM model (purified from donor's plasma) IgG or RV proteins solution. Reactive spots were visualized with Typhoon Trio, λ_{Ex} = 295 nm, λ_{Em} = 340 nm, followed after 3 × 2 min washing of unbonded *h*IgG/E_1/E_2/C. Further peptide-membrane was incubated 3 min at 20°C with citric acid-sodium citrate buffer, pH 3.70,... 2.20, that was made by lowering pH in increments of 0.05 pH units. The steps gradient pH was made with Orion Star 9102 pH Automated Titrator (Thermo Fisher Scientific, China). After each washing the fluorescence detection were carried out for affinity determination according $pH_{50\%}$ - the buffer value at which 50% of investigated peptide was desorbed [15].

The apparent K_d

The apparent K_d for binding of each protein-protein, protein-peptide and peptide-peptide was determined with

Table 1: Review of the identified aa or peptides of the immunoglobulins Fc, defined as sites or effectors of the interaction with cell receptors, complement, rheumatoid factor, etc.

Subclass	Region	Fc peptide sequence / amino acid as a possible participant of interaction	Subject of interaction	Literature
IgG_3	Hinge	^{216}ELKTPLGDTTHTCPRCP^{232}, ^{263}EPKSCDTPPPCPRCP^{277}	C_{1q}	[38]
IgG_3	Hinge CH_2	^{234}LLGGPS^{239} ^{328}LPAPIE^{333}	FcγRI	[39]
IgG_1	CH_2	^{239}SVFLFPP^{245},^{274}KFNWYVD^{280}, ^{297}NSTYRVVSVLTVLHQDWL^{314}	RF	[40]
IgG_1	CH_3	^{343}PREPQVYTLPPSRD^{356},^{401}DGSFFLY^{407}, ^{417}WQQGNVF^{423}, ^{425}CSVMHEALHNHY^{436}, ^{439}KSLSLSP^{445}	RF	[41]
IgG_1	Hinge CH_2 CH_3	^{233}ELLGGPS^{239} ^{267}SHEDPE^{272},^{293}EEQYNSTYR^{301}, ^{331}PIEKTISK^{338} ^{430}EALHNHY^{436}	FcγRs	[42]
IgG_1	CH_3	^{384}NG^{385}, ^{424}S, ^{428}M, ^{433}HNHY^{436}, ^{438}Q, ^{440}S	RF	[43]
IgG_3	Hinge	^{216}EPKSCDKTHTCPPCP^{230}	FcγRIIIa, C_{1q}	[44]
IgG_1	CH_2//CH_3	^{243}F, ^{292}R, ^{300}Y, ^{305}V // ^{396}P	FcγRs	[45]
IgG_1	CH_2//CH_3	^{252}MIS^{254} // ^{430}EALHNHY^{436}	$TRIM_{21}$	[46]
IgG_1	CH_2	^{236}G, ^{239}S, ^{332}I	FcγRIIa, FcγRIIIa	[47]
IgG_1	Hinge	^{226}CPPC^{229}	C_{1q}	[48]
IgG_1	CH_2	^{249}D, ^{253}I, ^{255}R, ^{280}D, ^{288}K, ^{310}HQ^{311}, ^{315}D, ^{317}K, ^{333}E,	SpA	[49]
IgG_1	CH_3	^{350}TLPPSRD^{356},^{436}YTQKSLSLS^{444}	RF	[50]
IgG_1	CH_2//CH_3	^{234}LLG^{236},^{239}S,^{243}F, ^{268}H,^{270}D, ^{292}R, ^{298}S, ^{300}Y, ^{305}V, ^{330}A, ^{332}I, ^{334}A // ^{396}P	FcγRIIIa	[51]
IgG_1	CH_2//CH_3	^{253}I // ^{345}E, ^{430}E, ^{433}HN^{434}, ^{439}KS^{440}	C_{1q}	[52]
IgG_1	CH2	^{245}PKPKDTLMISRTPE^{258}	FcγRs, C1q	[53]
IgG_1	CH_1 CH_2 CH_3	^{157}SWNSGALTSGVHTF^{170},^{186}VTVPSSSLGTQT^{197}, ^{243}FPPKPKDTLM^{252},^{334}KTISKAK^{340} ^{341}GQPREPQV^{348}, ^{433}HNHYTQKSLSLS^{444}	FcγRIIIa, FcRn	[54]
IgG_1	CH_2 CH_3	^{252}MISRT^{256} ^{429}HEALHNHY^{436}	$TRIM_{21}$	[55,56]
IgG_1	Hinge CH_2	^{232}PELLGGPSVFL^{242} ^{265}DVSHEDPEV^{273},^{291}PREEQYNST^{299}, ^{323}VSNKALPAPI^{332}	FcγRIIIa	[57]
IgG_1	Hinge	^{233}ELLGGPS^{239}	FcγRI	[58]
IgG_1	CH_2 CH_3	^{228}PCP^{230},^{250}TLMISRTPEVTCVVV^{264}, ^{307}TVLHQDWLN^{315} ^{378}AVEWESNGQPEN^{389},^{428}MHEALHN^{434}	FcRn	[59]
IgG_1- IgG_4	Hinge CH_2 CH_3	^{216}EPKSCDKTHTCPPCPAPELLGG^{237} ^{268}H,^{274}KFNWY^{278},^{300}Y,^{309}L,^{327}ALPAP^{331} ^{355}RDEL^{358},^{384}N,^{392}KTTPPV^{397},^{419}Q,^{422}V,^{435}HY^{436}	FcγRs, C1q	[60]
IgG_1	CH_2//CH_3	^{252}MISRTPE^{258} // ^{435}H	FcRn	[61]
MAbs	CH_2	^{243}FPPKPKDTLM^{252}	FcRn	[62]
IgG_1	Hinge	^{216}EPKSCDKTHT^{225}	FcγRIIIa	[63]
IgG_1	CH_2//CH_3	^{251}LMI^{253},^{310}HQDWL^{314} // ^{382}ESNG^{385},^{431}ALHNHYTQKSLS^{442}	RF (YES8c)	[64]
IgG_1	Hinge CH_2 CH_3	^{226}CPPCPAPELLGGP^{238} ^{262}VVVDVSHEDPEV^{273}, ^{291}PREEQYNST^{299}, ^{323}VSNKALPAPIEKTISKAK^{340} ^{341}GQPREPQVVTLPPSRDELTKN^{361}, ^{384}NGQPENNYKTTPPVL^{398}, ^{436}YTQKSLSLSPGK^{447}	FcγRs	[65]
IgG_1	Hinge CH2	^{233}ELLGGPS^{239} ^{265}DVSHED^{270},^{309}L,^{322}K,^{329}PAPI^{332}	FcγRs	[66]
IgG_1	CH_1 CH_2 CH_3	^{186}VTVPSSSLGTQT^{197} ^{242}LFPPKPKDTL^{251},^{263}VVDVSHEDPEVKF^{275}, ^{278}YVDGVEVHNAKTKPREEQYNST^{299}, ^{19}YKCKVSNKALPAPIE^{333} ^{381}WESNGQPENNYKTTPPVL^{398}	FcγRIIIa	[67]
IgG_1	CH_1	^{131}SSKSTSGGT^{139}	C_{1q}	[68]
IgG_3	CH_2 CH_3	^{250}DTLMISRTPEVTCVV^{256} ^{308}VLHQDWLNGKE^{318}, ^{365}LTCLVKGFYPSDI^{377}	Ficolin	[69]

Note 1: The similar color of the field and letter is shown the identical (short or long) sequence/aa from the different literature sources.
Note 2: The author's aa numbering is coordinated (if necessary) with EU numbering taking into account the kappa light chain of IgG.

standard procedure described by Baja, et al. [31] for intact *h*IgG, and virus proteins E_1, E_2, and C in our modification for our pares of interaction substances at their variable concentration under constant proteins concentration.

Fmoc solid phase peptide synthesis

~0.75 mmol/~500 mg of each 13-mer peptides (8-mer affinity peptide plus 5-mer spacer) which were chosen as applicant of the affinity ligand were synthesized using Fmoc chemistry, and purified by reversed-phase flash chromatography, and confirmed by MALDI-TOF mass spectrometry [32].

Peptide-affinity chromatographic gel synthesis

200 mg of each selected as affinity ligand peptide were dissolved in 25 mL sodium carbonate-bicarbonate buffer, pH 9.4, and the seven solutions were mixed together and pumped with flow 1 mL×min^{-1} through the ECO^{PLUS} Glass Column TAC25/125SLPE0-AB-2 (YMC Europe GmbH, Germany) packed with 20 mL of WorkBeads 40/1000 ACT (Bio-Works, Sweden), during 48 hours at 4 °C. The peptides density on the WorkBeads gel was determined by quantifying the difference between total peptides quantity in start solution and unbound peptides by fluorescence of tryptophan in the spacer measured by Typhoon Trio Variable Mode Imager 6 with Image Master 2D Platinum 6.0 DIGE software (GE Healthcare AB, Sweden). All other manipulations, including blocking unreacted active groups, were done according to instruction of manufacturer [32]. Finally, the column was rinsed with 500 mL of deionized water to remove the blocking agent and, before using, with working buffer for equilibration or, before storage, with 20% ethanol. Monopeptide affinity chromatographic gel was synthesized at the same manner in 5 mL column BabyBio ACT (Bio-Works, Sweden).

Determination of gel Dynamic Binding Capacity (DBC) and Temperature-Dependent Capacity (TDC)

0.5 mL of peptide-affinity gel packed into 0.8 cm diameter, 1.0 cm height column was equilibrated with 10 Vc citric acid-Na_2HPO_4 buffer, pH 7.4, and 1 Vc of 5 mg×mL^{-1}. *h*IgG in equilibration buffer was applied into column with flow 0.002-0.25 mL × min^{-1} (2-25 min residence time). DBC was determined at 10% breakthrough [33]. The column was washed with equilibration buffer, and the elution was performed with citric acid-Na_2HPO_4 buffer, pH 2.6. The washing and elution flow rate was 0.25 mL × min^{-1}.

TDC was determined by the method described before [2]: model *h*IgG was applied on peptide-affinity column in quantity 10% lower than DBC of adsorbent under 20°C. After sampling and washing the column and buffer (pumping into column with 0.5 Vc × min^{-1}) temperature was slowly raised by 5 degree each step and IgG was determined in the eluate.

Total IgG peptides-affinity purification from normal donor's plasma with virus inactivation/elimination directly during chromatographic process

As IgG sample served re-purified IgG to 93.4% purity, obtained after EBA STREAMLINE SP XL (GE Healthcare AB, Sweden) [27,28] captured from low molecular weight protein fraction [34]. Eluate was subjected to membrane filtration to achieve protein concentration 8-9 mg × mL^{-1}.

The concentrated eluate was spiked by model viruses as shown before [2] to achieve protein concentration 7.5 mg × mL^{-1}. The infected *h*IgG sample (85 mL, that given ~70% loading the affinity ligands) was loaded onto ECO^{PLUS} Glass Column (TAC25/125SLPE0-AB-2) with 20 ml peptide-affinity adsorbent. Column was equilibrated with 10 Vc of 20 mM citric acid-Na_2HPO_4 buffer, pH 7.4. The equilibration flow rate was 0.5 Vc × min^{-1}, the application – 0.1 Vc × min^{-1}.

The S/D treatment was beginning after IgG capturing on the affinity column. The experimental scheme and stages of the virus-inactivation/elimination process, developed and presented earlier, did not change when studying the behavior of chromatographically bounded IgG under the temperature-dependent treatment [2]. The exceptions were temperature levels and the usage of one R/D blend only: TnBP/Triton X-100.

The common conditions of S/D treatment were the following:

1. First washing: 30 Vc of equilibration buffer (0.5 Vc × min^{-1}) to complete absence of virus material in eluate.
2. The raising of S/D inactivation buffer gradient: 30 Vc inactivation buffer with flow rate 0.5 Vc × min^{-1} was automatically created by programming linear increasing concentration from 0 to 100% and applied on the column. The final inactivation buffer included 1.0%/2.5% TnBP/Triton X-100.
3. The raising inactivation temperature gradient: on the beginning of inactivation process for inactivation buffer and column the termostabilized gradient was started from 20 to 45°C during 60 min, therefore a temperature gradient grew in parallel to S/D gradient in all cases.

Stages 2 and 3 combined in one process named in the finale table Gr.

4. The second washing after raising gradients – actual treatment process: column was washed by 90 Vc under 45°C with same flow rate 0.5 Vc × min^{-1}; the column temperature was kept at corresponding buffer temperature (Gs).

5. The falling down S/D inactivation buffer and temperature gradients: after the washing the reverse S/D and T° gradients were used to reach the initial conditions for 60 minutes - 30 Vc with same flow rate 0.5 Vc×min^{-1} (**Gf**).

6. The third column washing: when the start T° 20°C was reached simultaneously with falling down gradient finishing, the column was washed by 10 Vc equilibration buffer, flow rate 0.5 Vc × min^{-1}.

The elution of IgG was carried out with 2 Vc of 20 mM citric acid-Na_2HPO_4 buffer, pH 2.6, 0.04 Vc × min^{-1}. All the eluates were collected for concentration/yield and purity of target proteins determination and their impurities and ligands determination by ELISA, and electrophoresis, and HPLC analysis.

The solvent level in the final IgG solution after S/D treatment was measured by GC method with FID, the detergent level - by HPLC with UV-detector (both instruments from E-Chrom Tech, Taiwan) as described previously [1,2].

Column regeneration and sanitization were performed with 2 Vc of 0.1 M glycine-HCl, pH 2.5, and 2 Vc of 0.05 M NaOH with flow rate 0.5 Vc × min^{-1}.

Virus models

In the virus inactivation/elimination experiments we used model viruses from the American Type Culture Collection (ATCC) which have become standard in our developments: Bovine Viral Diarrhea Virus (BVDV), Canine parvovirus (CPV), Bovine Enterovirus Type 3 (BEV), Murine Leukemia Virus Type C (MuLV), Pseudorabies or Suid herpes virus 1 (PRV) and Duck Hepatitis B Virus type 1 (DHBV) [1,2].

Virus titration and quantification

Virus titers were determined using the classical method of Kaerber [35] and Spearman [36] and all calculations were done according to our earlier publications [1,2].

For quantification virus analysis we used quantitative real-time PCR and the data were expressed as log10 of ration between virus nucleic acid detected in the protein sample before and after purification and designated in the results tables as parameter $t_{T°C}$ (example t_{30} = 4.15) [1,2].

^{aR}IgG peptides-affinity separation

^{aR}IgG peptides-affinity separation from total IgG purified from normal donor's plasma was carried on double adaptor column XK 16/20 (GE Healthcare AB, Sweden) with 25 ml synthetized peptide-affinity to RV capsid proteins WorkBeads 40/1000 gel as described in section "Peptide-affinity chromatographic gel synthesis" but for one selected affinity ligand only.

Chromatographic process conditions for ^{aR}IgG separation were the same as in case of total IgG peptide-affinity purification excluding virus inactivation/elimination stage.

Statistical analysis

The statistical processing of results was carried out by the standard methods [37]. A value of $p < 0.05$ was considered statistically significant. Data was presented as a mean ± Standard Errors (SEM) of at least 5 independent experiments unless otherwise indicated.

RESULTS AND DISCUSSION

High affinity peptides calculation to the human IgG different subclasses, their affinity determination

A picky and careful analysis of the literature sources shown that there are several aa sequences in the IgG molecule that are available for interaction with other proteins (SpA, SpG, PpL), various cellular receptors (FcγRs and $TRIM_{21}$), Rheumatoid Factor (RF), C1q complement. Basically, the sites of the above-mentioned interaction are located in the hinge region and CH_2 and CH_3 domains of the IgG molecule. Although some authors have shown that in the light chain there are available linear sequences that interact with cellular receptors. The most interesting information in the context of this investigation is summarized in the table 1.

This table also includes possible interaction sites located in other parts of the IgG molecule, which were important in the light of the study objectives. In addition, IgM and $IgA_{1,2}$ aa sequences were tested for analogous sequences to avoid possible cross-linking of these immunoglobulins during affinity isolation of IgG. The several analogous or similar sites and their parts were detected and discussed below.

The results of studies by a number of authors [38-69] systematized in the table 1 are not exhaustive, but are most relevant for considering possible interaction sites that may be available in the IgG molecule. We have noted that the data on IgG light chain kappa/lambda interaction sites were not considered as these chains present in all immunoglobulin spices and their peptide sequences would guarantee the binding of IgAs, IgD, IgE and IgM by future developed affinity chromatographic gels.

Most studies identify aa sequences or individual aa_s that have a direct or indirect effect on IgG (in this case IgG_1) interaction with cells effectors. All tabulated aa sequence clearly covers the middle (^{150}FPE...SGV^{167}) and lower (^{193}VVT...ICN^{209}) parts of the CH1 domain, top (^{224}EPK...THT^{233}), middle (^{234}CPPCP^{238}) and lower (^{239}APELLGGPS^{247}) parts of the hinge, and smears the whole CH_2 domain (^{249}FLF...TPE^{266}, ^{270}VVV...YVD^{280}, ^{288}KTK...NGK^{317}, ^{319}YKC...KTI^{340}) and two short parts of CH_3 domain. But if we consider the aa_s which are significant for binding only the two shorter essential sequences can be distinguished at the CH_1 domain: ^{158}FPE...SGA^{170} and

^{193}VVT...GTQ^{204}, five at the CH_2 domain: ^{241}FLFPPKPK^{248}, ^{252}MISRTPEV^{259}, ^{266}VSHEDPEV^{273}, ^{294}EQYNSTYR^{301}, ^{331}PIEKTISK^{338}, and two at the CH_3 domain: ^{380}ESNGQPEN^{388} and ^{429}HEAL**HNH**YTQK^{439}.

The latter sequence of the CH_3 domain interacts with the PRYSPRY domain of the homodimeric $TRIM_{21}$ with very high affinity 0.6 nM [70]. Identical (except the IgG_3 Y/F mutation) sequences were found in the IgG_{2-4} molecules with K_d = 37 ÷ 200 nM [71]. Unfortunately, analogues are also present in the domains of IgM and IgA_{1-2}. Although the affinity of IgM and IgA for monomeric $TRIM_{21}$ PRYSPRY is much weaker, 17 and 50 μM, respectively [70,72], which is due to large differences in the core aa compositions (IgM: HEALPNRVTER and IgA: HEALPLAFTQK), the ability to interact remains quite high. Therefore, this sequence ^{429}H-K^{439} of the CH_3 domain was excluded from the following experiments, but was taken into account for the development of an affinity peptide for the total isolation of immunoglobulins A, G_{1-4} and M classes. As for other selected sites, their analogues were not found in IgM/A_{1-2}/D/E.

Anti-sense sequences to selected sites on the IgG1 molecule in 8-aa_s overlapping peptides were by our proposed level of 50% desorption of immunoglobulin from membrane-synthesized peptide ($pH_{50\%}$) [15] and presented in the table 2. Fragments of sense peptides, which according to our analysis of physicochemical properties may represent the interaction sites of the Fc domain (CH_{1-3}) of IgG_1 and Fc receptors ($D_{1,2}$ domains FcγRn, FcγRI/II/III), highlighted in bold.

Incubation of anti-sense peptides synthesized on the membrane with *h*IgG_1 showed varying degrees of peptide-protein interaction in all cases membrane-synthesized. At the previous study it was shown that a nonspecific interaction carries on at $pH_{50\%}$>4.00, specific weak interaction - at $pH_{50\%}$ = 3.75 ÷ 4.00, specific average - at $pH_{50\%}$ = 3.60 ÷ 3.75, specific strong - at $pH_{50\%}$ = 3.40 ÷ 3.60, specific powerful - at $pH_{50\%}$ = 3.20 ÷ 3.40, specific superpower - at $pH_{50\%}$ < 3.20. Specific superpower interaction is, of course, a subjunctive term, but complete protein desorption from the peptide in this case requires lowering pH to 2.0 and below, which always leads to marked denaturation of the protein.

The analysis of the results, presented in the table 2, determined a number of peptides synthesized on the membrane, which forcefully captured IgG_1 during the 5 min incubation: GHWHRTFR (No.1), HWHGRRRD (No.2), GRGLDDPPGRHKEK (No.3), YYRAWGHWGFKH (No.4), GLHFKFTI (No.5), RGYLFWYR (No.6), and PVGLFFIF (No.7). It should be noted that all the affinity peptides calculated by our method closely coincide with the affinity for IgG peptides developed by SPR (surface plasmon resonance) [73], several chemical modifications with mass spectrometric analysis [74], biomimetic design strategy [75], combinatorial phage-display library screening [40,76,77].

The aa_s physic-chemical properties of potential IgG_1

Table 2: The intensity of IgG1 interaction with Fc antisense-peptides shown at the literature sources as a possible interaction sites from CH-1,2,3 domains.

aa_s sequence of IgG_1 domains		IgG_1 interaction with antisense-peptides, synthetized on membrane, determined according $pH_{50\%}$ index			
CH-1	sense	^{155}DYFPE**PVT**^{162}	^{160}**PVTVSWNS**^{167}	^{165}**WNS**GALTS^{172}	^{170}LTSGVHTF^{177}
	antisense	LIKGLG**HW**	**GHWHRTFR**	**TFR**PREWR	EWRPHLWK
		3.70÷3.75	3.45÷3.60	3.65÷3.75	4.10÷4.20
	sense	^{189}SLSSV**VTV**^{196}	^{194}**VTVPSSS**L^{201}	^{199}**SS**LGTQTY^{206}	^{204}QTYICNVN^{211}
	antisense	RDRRH**HWH**	**HWHGRRRD**	**RRD**PWVWI	VWIYTFHF
		3.65÷3.75	3.50÷3.60	3.70÷3.75	0
CH-2	sense	^{222}KV**EPKSCD**^{229}	^{228}**CDKTHTCP**^{235}	^{234}**CPPCPAPE**^{241}	^{240}**PELLGGPS**^{247}
	antisense	FHLGFRTL	TLFWLW**TG**	**TGGTGRGL**	**GLDDPPGR**
		3.80÷3.90	3.70÷3.85	3.40÷3.55	3.30÷3.40
	sense	^{246}**PSVFLFPP**^{253}	^{252}**PPKPK**DTL^{259}	^{258}TL**MISRTP**^{265}	^{264}**TPEV**TCVV^{271}
	antisense	**GRHKEK**GG	GGFGFLWE	WE**YYRAWG**	**WGLH**WTHH
		3.60÷3.75	0	3.50÷3.60	3.75÷3.85
	sense	^{269}CVVVDVSH^{276}	^{274}VSHE**DPEV**^{281}	^{279}**PEVKFNWY**^{286}	^{284}**NWYV**DGVE^{291}
	antisense	THHHLHRL	HRLLL**GLH**	**GLHFKFTI**	**FTI**HLWHL
		0	3.80÷3.95	3.45÷3.55	3.85÷4.00
	sense	^{328}KCKVSNKA^{335}	^{332}NKAL**PAPI**^{340}	^{338}**APIEKTIS**^{345}	^{343}**TISK**AKGQ^{350}
	antisense	FTFHRFFR	FFRDG**RGY**	**RGYLFWYR**	**WYR**FRFWV
		0	3.80÷4.00	3.40÷3.55	3.85÷3.95
CH-3	sense	^{388}EWESNG**QP**^{395}	^{393}G**QPENNYK**^{400}	^{398}**NYKVTT**PP^{405}	^{403}**T**PPVLDSD^{410}
	Antisense	LTLRF**PVG**	**PVGLFFIF**	**FIFHW**CGG	CGGHDLRL
		3.65÷3.75	3.40÷3.50	3.55÷3.60	0

Note 1: Antisense-peptides calculated as reported in [15] against IgG1 sense-peptide from the shown aa sequence.
Note 2: The interaction intensity was measured by the pH level of the 50% of IgG1 desorption - pH50% [15].
Note 3: White cell - unspecified IgG1 adsorption; light yellow cell - weak IgG1 interaction with peptide; light green cell - medium interaction; yellow cell - strong interaction; rose cell - very strong interaction. Red bold letters - possible affinity peptide for IgG purification.

interaction sites and identified peptides carried out the change-points of aa_s according to date of table 3 to improve the effectiveness of the IgG1 capture. In addition, the reduction of up to 8 aa_s long peptides was carried out in order to standardize their synthesis.

From the proposed (Table 3) modified peptides, the best results of $IgG_1 CH_1$ binding showed peptides no.1-7 GRCRRTFR ($pH_{50\%}$ = 3.30, Kd = 32 nM) and no. 2-6 RCRGRRRD ($pH_{50\%}$ = 3.35, Kd = 51 nM), $IgG_1 CH_2$ binding - No. 3-4 DDPPGRRR ($pH_{50\%}$ = 3.20, Kd = 14 nM), no. 4-4 NRAWGLRW ($pH_{50\%}$ = 3.40, Kd = 67 nM), no. 5-6 GLRFRFTI ($pH_{50\%}$ = 3.45, Kd = 72 nM) and no. RGELFWER ($pH_{50\%}$ = 3.40, Kd = 61 nM), $IgG_1 CH_3$ binding - no. 7-6 ALFFMFRW ($pH_{50\%}$ = 3.50, Kd = 82 nM). The most effective peptide was DDPPGRRR, but each of the seven listed can be used for chromatographic purification of IgG/antibodies as an affinity ligand. Their chromatographic properties will be determined in a separate study. So far, we have obtained seven high affinity peptides that interact with the immunoglobulin molecule at seven spaced sites, which, as previously planned, will provide a multipoint interaction of the IgG with chromatographic gel.

Multipoint peptide-affinity chromatographic gel properties, determination DBC and TDC

After peptides "sewing" the following chromatographic gel was obtained: DDPPGRRR- and 7-PEPs-WorkBeads 40/1000 with a total peptide density 21.8 mg × mL^{-1} (~22.3 µmol × mL^{-1}), each peptide was sewed in approximately equal proportion.

It was shown that target chromatographic fraction eluted with citric acid-Na_2HPO_4 buffer, pH 2.6, contained $IgG_{1,2,3,4}$ in proportion 67.3:20.8:8.5:3.4 approximately as at the total IgG re-purified sample for both gels. The IgA and IgM in the eluate were not found. The purity of the total IgG was 98.6% and 98.0, corresponding.

The DBC with enough residence time 10 min for both gels was around 62-65 and 50-54 mg × mL^{-1} of total IgG, corresponding (Figure 1).

The DBC and TDC of a monopeptide affinity gel, as expected, dropped sharply with increasing speed (>200 cm × h^{-1}) or buffer temperature (>30˚C), while the same parameters of a multi-peptide affinity gel practically didn't change at a linear buffer flow rate at 500 cm×h^{-1} or increasing its temperature to 45˚C at a stable speed of 300 cm × h^{-1} (Figure 1). The data obtained indicate that the IgG molecule bounded at several sites on the chromatographic gel is sharply limited in mobility, which in turn preserves its native state.

The other conclusion following from the results of the 7-PEPs-WorkBeads 40/1000 gel TDC determination were following: the direct process of IgG virus inactivation and elimination was possible to carry on under the temperature 45˚C and buffer flow rate maximum 250 cm × h^{-1}. The temperature around 50˚C is already in the zone of the critical retention of immunoglobulin by chromatographic gel.

Direct virus inactivation by S/D treatment during IgG purification process by peptide-affinity chromatography method

S/D treatment of IgG was performed according to scheme shown earlier [1,2]. Our published dates and previous studies on virus inactivation of immunoglobulins dates (in full will be published shortly) have shown that proper virus removal

Table 3: The modification of affinity peptides, capturing IgG1, to improve the capturing effectiveness and standardize peptide/chromatographic gel synthesis.

		The change-point of the aa_s sequence of affinity to IgG_1 peptides			
		Peptide No. 1	**Peptide No. 2**	**Peptide No. 5**	**Peptide No. 6**
		GHWHRTFR	HWHGRRRD	GLHFKFTI	RGYLFWYR
New peptide index	-1	GH**C**HRTFR	**K**WHGRRRD	GL**K**FKFTI	RGYLF**C**YR
	-2	G**K**WHRTFR	**R**WHGRRRD	GLHF**R**FTI	RGY**F**FWYR
	-3	G**R**WHRTFR	H**C**HGRRRD	GL**R**FKFTI	RG**E**LFWYR
	-4	GHW**K**RTFR	HW**R**GRRRD	GLHFKFT**M**	RGYLFW**E**R
	-5	GHW**R**RTFR	**R**W**R**GRRRD	GL**K**FKFTI	RG**E**LFW**E**R
	-6	G**R**W**R**RTFR	**RCR**GRRRD	GL**R**F**R**FTI	RG**E**LF**CE**R
	-7	G**RCR**RTFR	**KCR**GRRRD	GL**R**F**R**FT**M**	RG**EF**F**CE**R

		Peptide No. 3	**Peptide No. 4**	**Peptide No. 7**
		GRGLDDPPGRHKEK	EYYRAWGLHWTH	GLFFIFHW
New peptide index	-1	GRGLDDPP	EYYRAWGL	GLFFIF**K**W
	-2	GLDDPPGR	E**NN**RAWGL	GLFFIF**R**W
	-3	DDPPGRHK	YRAWGLHW	GLFFIF**KC**
	-4	DDPPGR**RR**	**N**RAWGL**R**W	GLFFIF**RC**
	-5	PPGRHKEK	AWGLHWTH	GLFF**M**F**RC**
	-6	PPGR**RE**R	AWGL**R**WT**R**	**A**LFF**M**F**R**W
	-7	PPGR**RRDR**	AWGL**RC**T**R**	**A**LFF**M**F**RC**

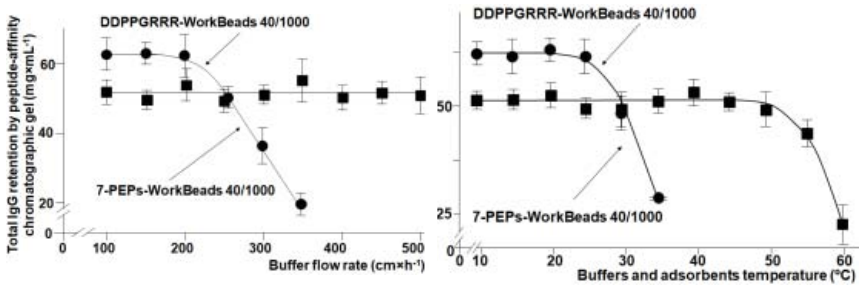

Figure 1 The binding and retention of total IgG (IgG_{1-4}) by mono-(DDPPGRRR-WorkBeads 40/1000) and multi-peptide (7-PEPs-WorkBeads 40/1000) affinity chromatographic gels under rising linear buffer flow rate or buffer and gel temperature: experiments were done with 0.5 ml gels packed into Ø 0.8 × 1.0 mm glass columns.

during chromatographic purification depends on the nature of the Solvent/Detergent (S/D) and their concentration, time and temperature of action on the IgG adsorbed by the chromatographic gel. The most effective virus inactivation/elimination conditions were selected for the presented work, namely: TnBP/Triton X100 concentrations 1.0% and 2.5%, respectively; temperature 45˚C and exposure to high T˚ for 5 hours (linear buffer gradient, which T° increases from 20 to 45˚C during 1 hour; exposure to constant buffer T˚ 45˚C during 3 hours; linear buffer gradient, which T˚ decreases from 45 to 20˚C during 1 hour).

Results of the S/D virus inactivation of IgG directly in a chromatographic column depending on process temperature are presented in table 4.

For further comparison we continue to evaluate the virus inactivation by three or two mentioned methods in the present study. In fact, we received full repetition of previously detected pattern: the method of the virus titer determination gave an adequate result according to target protein infectivity in the case of LEV and NLEV models [1,2]. The LEV titer determination didn't leave the hope to calculate process kinetics since the loss in virus infectivity due to the virus particles destruction and protein denaturation. The virus titration was more acceptable for NLEV infectivity mass balance determination - maximum 18% of the total infectivity was lost against 4-6% determined by RT-PCR. Unfortunately, the titration accuracy was very poor again (variants deviation more than 15-20% in the best cases) and didn't allow definite conclusions.

Determination of viral proteins by IFA approximately simulated the virus titer and nucleic acids measurement but could in no way to confirm the presence or absence of infectivity. Quantification of viral nucleic acids by RT-PCR in target proteins and buffer fractions obtained during inactivation allowed to calculate the process mass balance within 95.4 ± 4.9%. It means that stability of the virus nucleic acid (in most experiments not more than 3-4% of losses, rarely up to 6.5%) allows to calculate the process kinetic to assess what happens to model virus in the process of S/D treatment with small variant's diversity not higher than 8-10%. In contrast, diversity of variants of virus titer of target proteins was more than 25-30%.

On the other hand, the virus titer determination compared with the determination of its nucleic acid in the eluate samples is very indicative in terms of resistance of the virus to the S/D mixture. For example, if the titer of most LEVs at the eluate (phase *Gr*) was almost on the limit of detection, and nucleic acids amount was 2-4 Log, it can mean only one thing: S/D mixture destroyed the virus envelope with loss of virulence and nucleic acids were released into solution. If we found equal amount of virus in the eluate by titer and nucleic acid, it means that LEVs almost were not damaged by S/D, but S/D caused dissociation of virus and IgG and virus was washed out of the column. Thus, the process of virus elimination occurs with CPV^{NLEV} and BEV^{NLEV} (Table 4, stage *Gr*, *Gf*). Thus, it's well known that LEVs destruction by S/D is 3-4 times more effective than that for NLEVs [78] that also supported by our data shown before [1,2] and in the table 4. $BVDV^{LEV}$ conduct itself is a bit asymmetric, namely: partially destroyed and partially undamaged washed out, which confirms its previously determined medium resistance to S/D treatment [79].

In the present investigation we demonstrated again that virus inactivation by S/D treatment of peptide/protein preparation directly in the chromatographic column cooperates two processes, namely: 1) destruction of a virus particles by solvent and/or detergent (mainly for LEVs with minor effect for NLEVs) and 2) dissociation and washing away of virus material which was associated with protein where the outcome that was successful both for LEVs and NLEVs. The IgG yield and purity were not measurably changed when the virus-inactivation/elimination was introduced in the chromatographic purification process.

High affinity peptides to the human ^{aR}IgG calculation and development

The level of vaccination in Ukraine against RV is about 78% and among the younger generation (up to 25 years) - more than 82% [23]. The quantity of the specific anti-

Table 4: Direct virus inactivation by S/D treatment during IgG chromatographic purification process: virus titer, nucleic acid content and protein quantity functional assessment.

S/D treatment stage	Virus determination by:	Decreasing the factor of virus concentration (FVD) as a log_{10} (M ± SEM, (n = 5)					
		BVDV^{LEV}	DHBV^{LEV}	MuLV^{LEV}	PRV^{LEV}	CPV^{NLEV}	BEV^{NLEV}
Gr	titer	1.36 ± 0.32	0.03 ± 0.01	0.07 ± 0.02	0.31 ± 0.08	4.58 ± 0.85	3.71 ± 0.53
	NA	2.38 ± 0.35	3.97 ± 0.38	1.83 ± 0.15	2.21 ± 0.14	4.39 ± 0.34	3.53 ± 0.27
Gs	titer	4.31 ± 0.68	0.02 ± 0.01	0.03 ± 0.01	0.05 ± 0.02	4.65 ± 0.57	5.64 ± 0.71
	NA	7.14 ± 0.71	5.71 ± 0.40	7.45 ± 0.79	7.23 ± 0.58	4.37 ± 0.39	6.07 ± 0.65
Gf	titer	0.03 ± 0.01	bsl	bsl	bsl	0.04 ± 0.1	0.03 ± 0.01
	NA	0.05 ± 0.01	bsl	0.41 ± 0.03	0.13 ± 0.02	0.02 ± 0.01	0.02 ± 0.01
Target IgG elution	titer	0	0	bsl	bsl	0	0
	NA	bsl	0	0.05 ± 0.01	0.12 ± 0.01	0	0
Whole process summed FVD	titer	5.67 ± 0.75	0.05 ± 0.01	0.10 ± 0.02	0.36 ± 0.08	9.27 ± 1.02	9.38 ± 0.89
	NA	9.57 ± 0.79	9.68 ± 0.58	9.74 ± 0.80	9.69 ± 0.60	9.38 ± 0.50	9.62 ± 0.70

Note 1: Gr. Gs. Gf - process stages. more explanation at the section "Materials and Methods - Total IgG peptides-affinity purification…"
Note 2: NA - Nucleic Acid.
Note 3: bsl - value was below the method's sensitivity level.

rubella IgG (^{aR}IgG) was determined in the donor blood near 29 IU×mL^{-1}, that left the possibility of a noticeable purification of specific IgG during the experiment. A total 4 g primary purified IgG (93.4% purity or 3736 mg pure IgG) from 1 L plasma was processing over 5 chromatographic cycles through the ECO^{PLUS} Glass column (TAC25/125SLPE0-AB-2) packed with 20 ml of 7-PEPs-WorkBeads 40/1000 and received 3.734 g of target fraction with amount of IgG_{1-4} - 3660 mg, that means a purity 98,0%. The total anti-RV activity was 24308 IU, thus specific activity was calculated according to pure IgG as 6.64 IU×mg^{-1}.

Of course, we hoped that RV has a sufficiently specific receptor on the cell surface for the primary invasion to calculate on this basis a possible high-affinity peptide against ^{aR}IgG. In addition, a sufficient number of publications offers enough options for the interaction of RV capsid proteins with cells, thus exposing potential sites of interaction [26,80-100]. The capsid protein C is the second contender for interaction with the cell receptor [26]. So, these three capsid proteins would be the ideal choice for a determination of peptide epitopes that interact with cell surface, i.e., capsid proteins. In the table 5 the most important data concerning the determination of possible sites of interaction on the RV capsid proteins were systematized. The table dates shown that at least 10 epitopes of RV proteins E1, E2 and C are available for interaction and several of them are possible and are specific for interaction with the cellular receptor Myelin-Oligodendrocyte Glycoprotein (MOG) [32].

The analysis of the dates at the table 5 gives the chance to concentrate attention at the 4 capsid C sites with confident interaction (no. 1-C - ^{10}EDLQKALET^{18}; no. 2-C - ^{59}PRRRRGNR^{66}; no. 3-C - ^{78}PPPPEERQ^{85}; no. 4-C - ^{98}RAPPQQPQ^{105}), on the 3 protein E_2 sites (no. 5-E_2 - ^{354}VLPGHWLQ^{365}, no. 6-E_2 - ^{543}VLLVPWVL^{550}, no. 7-E_2 - ^{574}QGYNPPAY^{582}) and on the 5 protein E_1 sites (no. 8-E_1 - ^{742}HTETRTVW^{749}, no. 9-E_1 - ^{829}GATPERPR^{836}, no. 10-E_1 - ^{861}VIGSQARK^{868}, no. 11-E_1 - ^{889}IHAHTTSD^{896}, no. 12-E_1 - ^{918}RTLAPPRN^{925}). We do not rule out that other sites listed in the table interact with different cellular effectors, but we choose for experimental study those that are confidently identified by first researchers and confirmed by other.

Against selected peptides we developed anti-sense peptides [15] shown in the table 6 and tested their affinity by incubation with corresponding capsid proteins. Peptides LLDVFRDL (no. 1-1-C), GAAASPFA (no. 2-3-C), HDDHGTHD (no. 6-4-E_2), HYPGVRAF (no. 10-3-E_1) and AWDRGGAF (no. 12-1-E_1) showed effective interaction during incubation on the level $pH_{50\%}$ = 3.45 ÷ 3.65. The following aa_s replacement $L^{1,2}/F^{1,2}$ and D^3/H^3 in peptide no. 1-1-C; A^2/S^2 and P^6/R^6 in peptide 2-3-C; P^3/R^3 in peptide no. 10-3-E_1; W^2D^3/C^2K^3 in peptide 12-1-E_1 raised the level of interaction to a strong one ($pH_{50\%}$ = 3.40 ÷ 3.50) for last peptides: FFHVFRDL, GSAASRFA, HDDHGTHD, HYRGVRAF and ACKRGGAF. K_d for each peptide interaction with corresponding KM capsid protein were 29 ÷ 41 nM.

^{aR}IgG separation from total IgG by peptide-affinity chromatography

As described in section "Material and methods" five peptide affinity gels were synthetized and their chromatographic properties were examined. The peptide density of each gel was 20.3 ÷ 22.6 mg × mL^{-1} (~21.9 ÷ 23.2 µmol × mL^{-1}).

It was shown that target chromatographic fraction was eluted from these chromatographic gels with citric acid-Na_2HPO_4 buffer, pH 2.6, contained from 41 to 81% of total anti-rubella activity. The best result was received with RDHHGTHE-WorkBeads 40/1000 gel 81%), a little worse – RHDHGTHE-gel (78%, the difference was statistically unsignificant compare with gel no. 6-6-E_2, $p > 0.1$) and 5-6-4/2/3/1-, and 5-6/5/4/3-E_2-gels (66-52%, corresponding;

Table 5: Amino acid sequence of RV proteins which are possible the sites of RV interaction with cell membranes or with cell mitochondrial protein.

RV capsid protein	aa sequence of RV proteins recognized by T-cells lines. host proteins. etc.	Literature
C	^{98}RAPPQQ^{103}. ^{120}PELGPPT^{126}. ^{138}RPPLH^{142}. ^{207}RAYN^{210}. ^{218}GVWG^{221}. ^{272}HPWR^{275}	[80]
	^{126}TNPFQAAVARGLRPP^{140}. ^{206}VRAYNQPAGDV^{216}. ^{265}IETRSARHP^{273}	[81]
	^{9}MEDLQKALET^{18}	[26]
	^{255}APLPPHTTERIETRSARHPWRIRFGA^{280}	[83]
	^{14}KALETQSRALRAELAA^{29}	[86]
	^{265}IETRSARHP^{273}	[88]
	^{1}MASTTPITMEDLQKALETQSRALRAELAA^{29}. ^{88}RSQTPAPKPSRAPPQQPQPPRMQT^{111}	[89]
	^{46}STTGDDSGRDSGGPRRRRGNRGRGQRRDWSRAPPPPEERQETRS^{89}	[93]
	^{1}MASTTPITMEDLQKALETQSRALRAELA^{28}	[94]
	^{30}GASQSRRPRPPRQRDSSTTGDDSGRDSGGPRRRRGNRGRG^{69}	[95]
	^{78}PPPP^{81}. ^{100}PPQQPQPP^{107}. ^{174}FTNLGTPPL^{182}. ^{258}PPHT^{261}	[100]
E_2	^{340}HHGGTLRVGQHH^{351}. ^{354}ASDVLPGHWLQG^{365}	[26]
	^{354}ASDVLPGHWLQG^{365}	[82]
	^{434}GFLSGCGPMRLRHGADTRCGRLI^{456}.^{468}TRFGCAMRWGLPP^{480}	[89]
	^{387}MDFWCVEHDRPPPATPTPLTT^{407}	[91]
	*^{574}QGYNPPAYG^{582}	[92]
	^{406}TTAANSTTAAATPATA^{420}. ^{463}AQYPP^{467}. ^{500}PAHP^{503}. ^{542}FVLLVPWVL^{550}	[100]
E_1	^{784}DLVEYIMNHT^{793}. ^{808}HGPDWASPVC^{817}. ^{827}LVGATPERPR^{836}. ^{848}RTAPGPGEVWVT^{859}	[26]
	^{636}PTDVSCEGLGAWVPTAPCARI^{656}. ^{746}RTVWQLSVAGVSCNVTTEHP^{765}. ^{854}GEVWVTPVIGSQARKCGLHI^{873}. ^{889}IHAHTTSDPWHPPGPLGLKF^{908}	[83]
	^{790}MNHTGNQQSRWGLGSPNCHGPDWASPVCQRHS^{821}	[84]
	^{722}SVFALASYVQHPHKTVRVKF^{741}. ^{739}VKFHTETRTVWQLSVAGVSC^{758}. ^{789}IMNHTGNQQSRWGLGSPNCH^{808}. ^{906}LKFKTVRPVALPRTLAPPRN^{925}. ^{940}VEGLAPGGGNCHLTVNGEDL^{959}	[85]
	^{784}DLVEYIMNHT^{793}. ^{808}HGPDWASPVC^{817}. ^{826}RLVGATPERPRLRLVDA^{842}. ^{846}LLRTAPGPGEVWVTPV^{861}	[86]
	^{861}VIGSQARK^{868}. ^{966}KFVTAALLN^{974}. ^{993}RVIDPAA^{999}	[87]
	^{836}RLRLVDADDPLLRTAPGPGEVWVTPVIGSQA^{866}. ^{883}VEMPEWIHAHTTSD^{896}. ^{971}ALLNTPPPYQVSCGGESDRA^{990}.	[89]
	^{795}NQQSRWGLGSPNCHGPDWASPVCQRHS^{821}. ^{836}RLRLVDADDPLLRTAPGPGEVWVTPVIGSQAR^{867}	[90]
	^{739}VKFHTETRTVWQLSVAGVSC^{758}.	[91]
	^{825}SRLVGATPERPRLRLVDADDPLLR^{848}	[26]
	^{801}GLGSPNCHGPDWASP^{815}	[96]
	^{795}NQQSRWGLGSPNCHGPDWASPVCQRHS^{821}	[97]
	^{790}MNHTGNQQSRWGLGSPNCHGPDWASPVCQRHS^{821}	[98] [99]
	^{683}YFNPGGSYY^{691}. ^{693}QYHPTAC^{699}. ^{777}QVPPD^{781}. ^{815}PVCQRHSP^{822}	[100]

Note 1: Hereinafter. our aa numbering corresponds to the EU numbering in the UniProtKB database.
Note 2: *Authors shown peptide PY4 274RGTTPPAYG282. But upon careful consideration the aa$_s$ of Structural polyprotein Rubella virus. Strain RA27/3 vaccine. P19725 from UniProtKB database an analogue of said peptide 574(274)QGYNPPAYG582(282) was determined.
Note 3: **By the same frames and titers color were housed peptides with identical peptide sequences found at different investigations.

the difference was statistically significant compare with gel no. 6-6-E2, $p < 0.001 \div 0.02$). All gels binding capsid proteins on the level more than 50% marked by blue color, less than 50% - by light green color in the table 6. Given that DBC of peptide affinity column was around 50 mg × mL^{-1} on the XK 16/20 column with 25 ml RDHHGTHE-WorkBeads 40/1000 gel was uploaded 20 ml of 5% solution of virus-inactivated/eliminated IgG$_{1-4}$, that means 1000 mg IgG with ~6640 IU of ^{aR}IgG was uploaded. 959.3 mg IgG/1135 IU ^{aR}IgG were removed from column with first washing by equilibration buffer and second washing by equilibration buffer including 200 mM NaCl, pH 7.4. The target fraction eluted by 20 mM citric acid-Na_2HPO_4 buffer, pH 2.6, included 6.8 mg IgG and 5509 IU of ^{aR}IgG. The last 35.9 mg IgG and 127 IU of ^{aR}IgG were removed from column during sanitization process by 0.1 M glycine-HCl, pH 2.5, and 0.05 M NaOH. The table 7 showed the process mass-ballans.

A 10 min of residence time was enough for coupling the ~83% of target activity ^{aR}IgG activity by peptide-affinity

Table 6: The modification of affinity peptides. Capturing RV capsid proteins. to improve the capturing effectiveness and standardize peptide/chromatographic gel synthesis.

		The change-point of the aa_s sequence of peptide affinity to RV capsid proteins			
		Peptide No. 1-C	**Peptide No. 2-C**	**Peptide No. 3-C**	**Peptide No. 4-C**
		EDLQKALE	**PRRRRGNR**	**PPPPEERQ**	**RAPPQQPQ**
New peptide index	-1	LLDVFRDL	GAAAAPFA	GGGGLLAV	ARGGVVGV
	-2	LLEVFRDL	GAASAPFA	GGGGLLSV	SRGGVVGV
	-3	LLDVFREL	GAAASPFA	GWGGLLAV	ARGWVVGV
	-4	LLEVFREL	GAAAAPFS	GWGGLLSV	SRGWVVGV
	-5	LLHVFRHL	GAAAARFS	GWGGFFSV	SRAAVVAV
	-6	FLHVFRHF	AAAAARFS	AWGAFFSV	GRAAVFAF
		Peptide No. 5-E_2	**Peptide No. 6-E_2**	**Peptide No. 7-E_2**	**Peptide No. 8-E_1**
		VLPGHWLQ	**VLLVPWVL**	**QGYNPPAY**	**LCLCACHT**
	-1	HDGPLTDV	HDDHGTHE	VPMFGGRM	LWLCACHT
	-2	QDGRLTDV	RDDHGTHE	VPIFGGRM	LCLWACHT
	-3	HEGPLTDV	HHDHGTHE	VPMFGGRI	LCLCAWHT
	-4	HDWPLTDV	HDHHGTHD	VPIFGGRI	LWLWAWHT
	-5	RDWRLTDV	RHDHGTHE	VHIFGGRI	LWLWAWRT
	-6	QHGPLTDV	RDHHGTHE	VRIFGGRI	LWLCAWRT
		Peptide No. 9-E_1	**Peptide No. 10-E_1**	**Peptide No. 11-E_1**	**Peptide No. 12-E_1**
		GATPERPR	**VIGSQARK**	**IHAHTTSD**	**RTLAPPRN**
	-1	PRWGLAGA	HYPRVRAF	YLRLWCRL	AWDRGGAF
	-2	PRCGLAGA	HYPAVRAF	YLRLCCRL	ACDRGGAF
	-3	PRWGLSGA	HYPGVRAF	YLRLWWRL	SWDRGGAF
	-4	PRWGLAGS	QYPRVRAF	YLRLWCAL	ACDRGGAL
	-5	PRCGLSGS	QYPGVRAF	YLRLCCAL	SCDRGGAL
	-6	RRCALSGS	RYRGVRAF	YFRLCCAF	SCDRAAAL

Note 1: By blue color was marked all peptides that in affinity gels binding capsid proteins on the level more than 50%.
Note 2: By light green color was marked all peptides that in affinity gels binding capsid proteins on the level less than 50%.

Table 7: Mass-balance of aRIgG separation from unspecific IgG1-4 by peptide-affinity chromatography.

Chromatography purification stage	IgG_{1-4} quantity (mg)		^{aR}IgG activity (IU)		Specific ^{aR}IgG activity ($IU \times mg^{-1}$ IgG)	
IgG uploading on column		1000.0 ± 34.7		6644 ± 40		6.64
Fist & second column washing Coupling on column	959.3 ± 57.5		1135 ± 79		1.18	
		40.7 ± 4.9		5509 ± 51		135.36
Elution the target fraction Left on column	6.8 ± 0.8		5382 ± 95		791.47	
		35.9 ± 3.4		127 ± 16		3.54
Column sanitizing Left on column	25.6 ± 3.6		89 ± 14		3.48	
		10.3 ± 1.2		38 ± 5		3.69

gel (the difference between total and coupling activity on the uploading and washing stages). The residence time increasing did not lead to significant changes in the binding of target activity. Note that the main part of this activity was associated with 4% of the IgG only, that suggested the coupling in basic the ^{aR}IgG. 35.9 mg IgG with specific activity 3.54 IU × mg^{-1} IgG. We do not refer to significant losses of ^{aR}IgG. Most likely this is due to the presence in the human immunoglobulin family the IgGs with higher affinity to the synthesized peptide. But its IgGs do not have high ^{aR}IgG activity. The proteins washed at the sanitization stage were the denatured IgGs that quantity around 1% is normal for chromatographic process.

The anti-Rubella IgG separated from the total human

IgG_{1-4} family has a specific activity closed to 800 IU × mg^{-1}. Given that the ^{aR}IgG titer in the donor blood was about 29 IU × mL^{-1}, and in 1 mL of plasma was about 4 mg IgG, it means that specific activity was ~7,5 ÷ 8.0 IU × mg^{-1} and that we achieved an increasing in ^{aR}IgG titer by 100 times.

In a total for the 4 chromatographic cycles during 2 hours 27 mg of immunoglobulin with a total anti-rubella activity 21,500 IU was isolated from 1 L of standard donor plasma. Thus, it can be argued that the main goal of the investigation was achieved - the developing a peptide affinity chromatographic gel with satisfactory properties for the isolation of specific anti-rubella immunoglobulin. We do not rule out that on further work on the described approach we will have not only a more effective adsorbent, but also adsorbents for isolation from standard donor plasma of anti-herpes, anti-measles, anti-polio, anti-diphtheria and other IgG, according to which large-scale vaccinations with long-life immunity can be carried out on these diseases.

The high ^{aR}IgG titer of separated immunoglobulins show that affinity RDHHGTHE peptide and its family (6-1/2/3/4/5/6-E_2) or their closed by physic-chemical properties peptides no. -3-6 from 5-E_2 (shown in the table 6) captures exactly this ^{aR}IgG. Cong, Jiang and Tien back in 2011 found that the role of MOG, expressed in the central nervous system and in other tissues such as spleen, liver and thymus of mice, as a receptor of RV attachment to cell cannot be excluded [24]. Nobody has denied this fact so far, due to which we suggested that MOG could have a similar analogue of the peptide no. 6-6-E_2 for RVs binding on the cell surface. In order to verify, we performed analysis of MOG aa_s sequence represented at UniProt_KB (Q16653 MOG human, Q63345 MOG rat, Q61885 MOG mouse, P55803 MOG bovine, Q9BGS7 MOG monkey macaca, and Q29ZQ1 MOG monkey marmoset). Particular attention was paid to the sites of possible interaction MOG with RV capsid proteins (^{131}DHSYQEEAAMELK^{143} and ^{236}AGQFLEELR^{244}) indicated by the authors early publication [24]. The site ^{196}TFDPHFLRV^{204} [24] was rejected as inadequate to interact with the virus because it was topologically located inside the cell, not even transmembrane. On the base both analyzed sites were determined following peptides at human MOG: ^{130}RDHSYQEE^{137} and ^{232}HRRLAGQF^{239}.

A comparison of the developed RDHHGTHE and the found peptide ^{130}RDHSYQEE^{137} shows that they are identical by 50% «^{1}R^{2}D^{3}H...^{6}E» and other almost does not differ in physic-chemical properties. Thus, at ^{4}H/S replacement that should interact with V at ^{543}VLLVPWVL^{550} of E_2 the histidine looks more acceptable amino acid than serine, due to the fact that it will interact with valine not only by hydrophilicity but also by charge, while serine interacts with valine only due to pronounced hydrophilicity. The charge of serine and valine, which is close to zero, has no special effect on the interaction (HPI_H = -1.7, pI_H = 7.6; HPI_S = -1.1, pI_S=5.7; HPI_V = 2.3, pI_V = 6.0). The same reasoning applies to another pair of amino acids ^{7}H/E, where histidine will also be preferable to glutamine in interaction with valine (HPI_H = -1.7, pI_H = 7.6; HPI_E = -2.6, pI_E = 3.2; HPI_V = 2.3, pI_V = 6.0). At the ^{5}G/Y replacement that should interact with P the glycine due to high hydrophilicity more preferable for interaction (HPI_G = 0.7, pI_G = 6.0; HPI_Y = 0.1, pI_Y = 5.7; HPI_P = -0.3, pI_P=6.5). In the ^{6}T/Q pair, tyrosine is the only one antisense aa for tryptophan [14,15], so it will be preferred in any case (HPI_T = -0.8, pI_T = 5.9; HPI_Q = -2.9, pI_Q = 5.7; HPI_W = 1.5, pI_W = 6.0).

The peptide RDHHGTHE as analog of the MOG ^{232}HRRLAGQF^{239} has a greater potential to interact with another site of RV E_2 – ^{354}VLPGHWLQ^{36} [26,82], namely: a powerful positive charged core with high hydrophilicity (RDHH) against the low negative charge and medium hydrophobicity (VLPG) and three couples of the sense-antisense aa_s (^{2}D/^{2}L, ^{6}T/^{6}W, ^{7}H/^{7}L). The level of interaction is lower than with ^{543}VLLVPWVL^{550} due to the presence of antagonistic by the physical sense aa_s (the same charge and hydrophobicity) at peptides positions ^{3}H/^{3}P and ^{8}E/^{8}Q.

Another developed peptide QHGPLTDV (shown 66% ^{aR}IgG activity coupling as an affinity ligand) corresponds to the possibility of interaction with ^{543}VLLVPWVL^{550}, but showed low efficiency. This may be caused both by the only partial conformational availability of ^{543}VLLVPWVL^{550} and by the weak interaction due to the absence of amino acids with a strong charge and sufficient hydropathy properties.

The same potential RV-receptor core peptides were found at the MOG aa_s sequences of other animals with a short offset of 1-2 aa positions. The presence of T-cell epitopes on E_2 protein of RV with same or closed aa_s sequence [82] and immune epitope with anti-sense sequence on the developed against RV' E_2 protein [100] add confidence that human MOG peptides ^{130}RDHSYQEE^{137} and ^{232}HRRLAGQF^{239} both or one of them are/is the key site(s) of cell receptor and RV interaction. Which one peptide is including into RV-receptor, will be possible to recognize in the next study.

CONCLUSION

With each new study, we are increasingly finding that biochemical process of protein-protein interaction occurs due to hydropathic and electrostatic contact between *linear sequences* built from amino acid partner couples. This emphasizes once again the non-randomness and the logical genetic program (sense and antisense sequences on each of the interaction partner proteins) of recognition, such as receptor recognition by the effector. Conversely, the interaction due to conformational convergence of partner amino acids seems random and very difficult for genetic programming. From the same point of view, it no longer seems speculative and fantastic that the interaction amino acids partner couples (or by J. Biro sense-antisense) are the same genetic code for proteins as the nucleotide pairs for nucleic acids.

Based on the above the definition at the MOG receptor

RV the linear peptides of effective interaction with virus antisense peptides and more than 66–81% extraction of ^{aR}IgG from a mixture of IgG_{1-4} plasma donors suggests that MOG peptides ^{130}RDHSYQEE^{137} or ^{232}HRRLAGQF^{239} may be a key receptor's sequence for RV. On the other hand, we hope that peptides QHGPLTDV/RDHHGTHE, which we developed in this study, could work no less effectively in the living organism as blockers of the virus landing site on the cellular receptor. If the assumptions made in the following experiments are proven, it will pave the way for the development of reliable natural peptide safeguards pharmaceuticals not only against viruses and bacteria.

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Conflict of Interest Statement

Authors declare no conflict of interest. The study was performed at a time when authors were employees of Neutromics Ukraine TOV and working together at the biotechnology pilot plant in Raining (Boroo) Valley, Mongolia.

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by all authors too. The first draft of the manuscript was written by Serhiy P. Havryliuk and Heorgii L. Volkov and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethical Approval

This article does not contain any studies with human participation or animal performed by any of the authors.

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