

APPENDIX

In addition to the authors, the following investigators contributed to the trial: *Data Monitoring and Safety Board*: E. Marth, R. Konior, F. Sonnenburg; *Baxter Clinical Study Team*: K. Birthistle, T. Dvorak, S. Geyer, M. Kraft, M.C. Leitzgeb, F. Maitsch, L. Phillipson, E. Robotka. *Austria: Medical University of Vienna, Vienna General Hospital (Study Center Management)*, Vienna: A. Abraham, M. Bauer, M. Brunner, A. Cornea, C. Drucker, Z. Erdogan, J. Griss, B. Heinisch, F. Kovar, E. Lackner, C. Lambers, O. Langer, I. Leitner, C. Marsik, W. Poeppel, M. Popovic, R. Sauermann, R. Schaberl, G. Sodeck, C. Thallinger, F. Traunmueller, C. Wagner, M. Zeitlinger; *Singapore: Changi General Hospital (Study Center Management)*: S.K. Chua, S. Chuin, R. Fong, A.S. Foo, A.G. Koh, P.K. Lim, S.Y. Yap, L.H. Yew; *National University Hospital (Study Center Management)*: J.W.L. Goh, L.Y. Hsu, C.W.P. Loke, J.Y.C. Ng, E.L. Toh, P. Weatherill, Y.P. Zhou.

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一般的名称		研究報告の公表状況	Identification of trimeric peptides that bind porcine parvovirus from mixtures containing human blood plasma. Heldt, C.L. et al, Biotechnol. Prog., 24, 554-560 (2008).	公表国 カナダ		
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研究報告の概要	<p>本稿では、ヒト血漿溶液から従来の除去法で十分に取り除けなかったパルボウイルス（粒径が小さい非エンベロープウイルス）を除去する新たな手法について記載する。固相法で三アミノ酸残基よりなるペプチドライブラリーを樹脂上に合成し、種々の溶液に添加したブタパルボウイルス（PPV）への選択的な結合能を有するペプチドをスクリーニングした。カラムから溶出した PPV は、その後の感染性検査で感染能を測定した。その結果、16 種のペプチドを単離し、配列決定したところ、塩基性アミノ酸の存在が PPV 結合に非常に重要であることが判明した。PPV をリン酸緩衝生理食塩水（PBS）に混ぜた場合、すべてのペプチドが最初の 9 カラム体積でウイルスを完全に除去した。著者らは、この方法が浄水への適用に有用と示唆している。それに対してウイルスを 7.5% のヒト血漿（蛋白質 5 mg/mL）を含有する溶液に添加した場合には、アミノ酸配列がトリプトファン-アルギニン-トリプトファン（WRW）の場合のみ、最初の 3 カラム体積で検出可能な PPV を除去することができた。しかし、その後血漿はペプチド樹脂へのウイルスの結合を阻害し始めた。本手法は、改良の余地があるものの、短いペプチドを特定のウイルスの除去プロセスに利用できることを示している。</p>					使用上の注意記載状況・ その他参考事項等
	<p>報告企業の意見</p> <p>本研究は、まだ対処されていない非エンベロープウイルスに対する効果的な病原体除去法に対応し得る新規の手法が述べられている。</p>					<p>今後の対応</p> <p>現時点で新たな安全対策上の措置を講じる必要はないと考えるが、今後も利用可能な本手法に関する情報収集に努める。</p>

Identification of Trimeric Peptides That Bind Porcine Parvovirus from Mixtures Containing Human Blood Plasma

Caryn L. Heldt,[†] Patrick V. Gurgel,^{†,‡} Lee-Ann Jaykus,[§] and Ruben G. Carbonell^{*,†}

Department of Chemical and Biomolecular Engineering and Department of Food, Bioprocessing and Nutrition Science, North Carolina State University, Raleigh, North Carolina 27695, and ProMetic Life Sciences, Montreal, Canada

Virus contamination in human therapeutics is of growing concern as more therapeutic products from animal or human sources come into the market. All biopharmaceutical processes are required to have at least two distinct viral clearance steps to remove viruses. Most of these steps work well for enveloped viruses and large viruses, whether enveloped or not. That leaves a class of small non-enveloped viruses, like parvoviruses and hepatitis A, which are not easily removed by these typical steps. In this study, we report the identification of trimeric peptides that bind specifically to porcine parvovirus (PPV) and their potential use to remove this virus from process solutions. All of the trimeric peptides isolated completely removed all detectable PPV from buffer in the first nine column volumes, corresponding to a clearance of 4.5–5.5 log of infectious virus. When the virus was spiked into a more complex matrix consisting of 7.5% human blood plasma, one of the trimers, WRW, was able to remove all detectable PPV in the first three column volumes, after which human blood plasma began to interfere with the binding of the virus to the peptide resin. These trimer resins removed considerably more virus than weak ion exchange resins. The results of this work indicate that small peptide ligand resins have the potential to be used in virus removal processes where removal of contaminating virus is necessary to ensure product safety.

1. Introduction

The removal of viruses, pathogenic microorganisms, and toxins is an important problem in the growing area of human therapeutics. Every year, more therapeutic products are produced from animal, human, or cell culture sources (1), and these sources contain an inherent risk of viral contamination. Therapeutic products from human blood plasma, antibodies, albumin, and factor VIII, just to name a few, could be infected with human immunodeficiency virus (HIV), hepatitis B, B19 virus (formally known as parvovirus B19), SARS coronavirus, or one or more emerging viruses that have yet to be identified (2). Cell cultures are often contaminated with retrovirus particles, belonging to the family of viruses that include HIV (3). Cell culture lines often used in the production of human antibodies may contain viruses such as murine parvovirus (MVM) or cytomegalovirus (4). While this contamination has been greatly reduced since the requirement of strict characterization of cell culture lines and the careful screening of human plasma donors (3), the risk of low levels of contamination still exists.

The FDA requires that any process that uses materials from living sources must have two viral clearance steps to lower the risk of contamination (5). These steps must demonstrate a distinct mechanism of virus clearance and achieve a minimum of 4 log removal, or 99.99%. There are two broad categories for viral clearance, inactivation and removal (6). Inactivation is often performed toward the beginning of the purification of a therapeutic and could involve a lowering of pH or heating of

the product. Both of these processes work well against enveloped viruses, but caution must be taken to not harm the desired protein product. Virus removal is often done at the end of a process and most commonly involves nanofiltration of the final product directly before formulation. Nanofiltration works well for viruses of large size. However, small viruses like parvoviruses are often of approximately the same size as the protein product, making it difficult to separate them by filtration (2). Complete removal of small non-enveloped viruses with 20 nm pore size filters has been accomplished, but there is significant fouling of these small-pored membranes that can lead to reductions in production rates (7, 8). Filtration of parvoviruses has been improved by flocculation of the virus particles through addition of cationic polymers (9) or amino acids (10), which allows the use of larger pore membranes that do not foul as quickly. Virus removal can also be accomplished using functionalized membrane surfaces. Quaternary amine groups have been attached to membrane surfaces to facilitate the removal of viruses through an ion exchange mechanism (11–13). Viral clearance validation may be achieved by conducting spiking experiments on normal process steps used in the purification of a therapeutic (i.e., chromatography columns, precipitation) (14), but care should be taken if a chromatography step is to be used concurrently as a viral clearance step and a protein purification step. If both the virus and the protein bind to the resin, it is possible for viruses to accumulate in the column. Without proper cleaning, the virus may elute from the column in subsequent batches and contaminate the therapeutic product (14).

Affinity adsorption is rarely used to remove viruses from process streams because the most common affinity ligands for viruses are antibodies. Antibodies are expensive to produce, often cannot withstand the harsh conditions required for the

* To whom correspondence should be addressed. Ph: (919) 515-7054. Fax: (919) 515-5831. Email: ruben@ncsu.edu.

[†] Department of Chemical and Biomolecular Engineering.

[‡] ProMetic Life Sciences.

[§] Department of Food, Bioprocessing and Nutrition Science.

cleaning of process equipment, and carry an inherent risk of being contaminated with viruses in their own right (3). However, affinity adsorption has been applied to the reduction of infectious prions from blood (15, 16), and a small peptide ligand has been found to remove staphylococcal enterotoxin B, a small toxin, from *E. coli* lysate (17). Small peptides are more robust than antibodies, and they are also less expensive and can be chemically synthesized, eliminating the risk of virus contamination. Peptides can handle the cycling of production and cleaning much better than antibodies, and by using small peptides, there is no three-dimensional structure that may be destroyed during processing. In this work, several trimeric peptides have been discovered that remove PPV from phosphate buffered saline containing as high as 7.5% human blood plasma. The peptides were found when a synthetic, solid-phase combinatorial library was screened for ligands that bind to porcine parvovirus (PPV). Solid-phase libraries allow screening directly on the chromatographic support that will be used as the separation media and have been successful in the discovery of many affinity peptide ligands (18–21). The discovered peptides can completely remove any detectable PPV from PBS and completely remove any detectable PPV from the first 3 column volumes when 7.5% human plasma is present. This work demonstrates that small peptides may offer a novel and effective method for removing viruses from complex mixtures.

2. Materials and Methods

2.1. Materials. Phosphate buffered saline (PBS) containing 0.01 M phosphate, 0.138 M NaCl, and 0.0027 M KCl, pH 7.4 was purchased from Sigma (St. Louis, MO), and human blood plasma was a donation from the American Red Cross (Rockville, MD). Amino acids, phenol red, sodium carbonate, sodium phosphate, glucose, calcium chloride, sodium chloride, potassium chloride, and magnesium sulfate were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Waltham, MA). Eagles Minimum Essential Media (EMEM) was purchased from Quality Biologicals (Gaithersburg, MD). MEM vitamins, sterile PBS, trypsin, gentamicin, and glutamine, all for cell culture, were purchased from Invitrogen (Carlsbad, CA).

2.2. Virus Propagation and Titration. The porcine parvovirus (PPV) NADL-2 strain was titrated and propagated on porcine kidney (PK-13) cells, which were a gift from the American Red Cross (Rockville, MD). The PK-13 cells were maintained and the PPV propagated as described in Heldt et al. (22) using complete media, which consisted of EMEM supplemented with 2 mM glutamine, 1x gentamicin, and 10% non-heat-inactivated fetal calf serum (Hyclone, Logan, UT). Upon propagation of the virus, the cell culture flasks were frozen at -20°C and thawed at room temperature. The cells were then scraped from the flask, and the solution was clarified by centrifugation at 3000 rpm for 10 min in an IEC Centra CL2 centrifuge (Thermo Electron, Waltham, MA). This solution was then stored at -80°C until further use.

Radioactive PPV was prepared by metabolically incorporating a radiolabel during propagation by addition of ^{35}S methionine and cysteine to the cell culture media. This was done by seeding the cells at 6×10^5 cells per 75 cm² flask. The next day, the flask was infected with 10^3 MTT units of PPV in 1 mL of PBS. An MTT unit was defined as the concentration of virus where 50% of the cells were considered viable, as determined by the metabolic cleavage of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide salt (MTT) as previously described (22). The MTT concentration can be observed optically and quantified by spectrophotometry. The flasks were placed in the incubator

at 5% CO₂, 37 °C, and 100% humidity for 1 h. At this time, 5 mL of complete media was added to the flask, which was returned to the incubator. The next day, the media was removed from the flask and starvation media was added, which contained the same amino acids and essential nutrients as the EMEM, except for methionine. The cells were exposed to 5 mL of this starvation media for 1.5 h, and then EasyTag Protein Labeling mixture (Perkin-Elmer, Waltham, MA) was added to a final concentration of 50 $\mu\text{Ci/mL}$. The cells were frozen at -20°C when approximately 90% cytopathic effect was observed, usually after 4–5 days. Virus purification was done by CsCl gradient centrifugation, as described elsewhere (22), after which solutions were dialyzed against PBS for 3 days at 4 °C, stored at 4 °C, and used within 2 weeks.

All infectivity measurements were made using the MTT assay, which previously has been correlated to a TCID₅₀ (50% tissue culture infectious dose), a common method for the titration of infectious viruses (22).

2.3. Primary Screening of Library. A solid-phase combinatorial trimer library was made by Peptides International (Louisville, KY) using the divide-couple-recombine technique (23) on Toyopearl Amino 650 EC (Tosoh Bioscience, Montgomeryville, PA). The library had an alanine and 2 mini-PEG spacer arms [Toyopearl resin-Ala-(COCH₂-(OCH₂CH₂)₂-NH)₂-X-X-X], where X is any naturally occurring amino acid except cysteine or methionine. The library was swelled in 20% methanol in DI water overnight and then buffer exchanged with PBS three times, with the last buffer exchange being overnight. Ten milligrams of dry library was taken and mixed with 50% human blood plasma in PBS for 1 h. ^{35}S -Labeled PPV was added to the library at 10,000 CPM (about 1×10^{-3} μCi) and allowed to equilibrate for about 1.5 h. The library was then placed in a disposable 10 mL fritted column (Bio-Rad Laboratories, Hercules, CA). The beads were washed with PBS followed by PBS containing an additional 1 M NaCl or KCl until no radioactivity could be detected from the wash. The beads were washed again in PBS to remove excess salt and then put into 20 mL of 1% low melt agarose (Bio-Rad Laboratories, Hercules, CA). This was poured onto a 160 mm \times 180 mm GelBond (BioWhittaker Inc, Walkersville, MD) and allowed to dry for 3 days. Kodak BioMax MR Film (Kodak, Rochester, NY) was placed onto the dried gel for 10 days and developed with a Konica Medical Film Processor (Tokyo, Japan). A proprietary ligand found by the American Red Cross that binds to PPV (positive control) and a negative control of Amino 650M were used as markers to line up the film and the gel for visualization of radioactive beads. Positive beads were excised from the gel, boiled in water for 10 min each, and vortexed and the water changed for a total of three repeats to remove the agarose and the bound PPV from the beads. The beads then were sent to the Texas A&M Protein Laboratory (College Station, TX) for sequencing by Edman degradation.

2.4. Chromatography to Verify Screening Results. Peptide resins were synthesized on Toyopearl Amino 650M resin (Tosoh Bioscience, Montgomeryville, PA) by Peptides International (Louisville, KY) and were packed into disposable PIKSI columns (ProMetic Biosciences Ltd, Cambridge, England) with a total of 0.5 mL of settled resin in PBS per column. A Rainin (Oakland, CA) 8-channel peristaltic pump was used to add a solution of PPV supernatant in either PBS or 7.5% human blood plasma in PBS, at a rate of 0.1 mL/min. Ten 0.5 mL fractions were collected and tested for infectivity using the MTT assay and compared to the titer of the starting material before addition to the column.

2.5. Acetylated Control. The acetylated control was made by the acetylation of Toyopearl Amino 650M resin. About 50 mL of settled resin was added to a sintered glass funnel and allowed to drain. The resin was washed three times with 100 mL of 0.1 M NaOH. The resin was then washed with deionized water until the pH was below 8. The resin was placed into three separate 50 mL conical tubes and 30 mL of 0.5 M sodium acetate was added to each tube followed by end-over-end rotation for 10 min. A 100% excess of acetic anhydride (Riedel-Haen, Germany), which amounted to a total of 755 μ L, was dissolved into 3 mL of acetone, and 1.2 mL of the solution was added to each conical tube. The tubes were mixed for 2 h. The resin was then returned to the sintered glass funnel and washed three times with 100 mL of DI water, four times with 100 mL of 0.5 M NaOH, and finally at least 10 times with 100 mL of DI water, until the pH of the rinse was below 8. The acetylation was confirmed by taking 50 μ L of acetylated resin, 50 μ L of Toyopearl Amino 650M resin, and 50 μ L of DI water and adding two drops of ninhydrin reagent, 2% solution (Sigma, St. Louis, MO). After 1–2 min the resins were observed for color change; the acetylated resin and the DI water remained yellow, whereas the amino resin turned purple.

3. Results and Discussion

3.1. Library Design. Many hexameric peptide ligands have been found that can purify proteins (19, 24, 25) and toxins (17). Each of these peptide ligands were selected from a hexamer library, which contains over 34 million different combinations, when 18 of the 20 naturally occurring amino acids are used for library production. It would take a tremendous amount of effort to screen all of these sequences, and it is not necessary when purification is the intended use of the ligand. In general, a purification ligand is useful if it can bind over 90% of the target protein and is specific enough to produce an eluted protein that is 80–90% pure, but for virus removal, the goal is reduction of $\geq 99.99\%$ of a virus, which is at femtomolar to picomolar concentrations. To improve the possibility of finding a ligand that can accomplish this, a trimeric library was designed and screened. This library contained only 5832 different sequences and could be screened many times over to compare different screening conditions. Further, by screening the entire library, there was a greater probability that one or more strongly binding ligands would be found, which would not necessarily occur with a hexamer library.

A spacer arm of two sets of two ethylene glycol units separated by a peptide bond (26) (designated AEEA-AEEA by Peptides International) was added to the library to increase the chances of finding a peptide ligand that bound to a conserved area on the virus surface. This spacer arm separated the peptide approximately 15 Å from the undisclosed spacer on the Toyopearl resin. It has also been shown that hydrophilic ethylene glycol does not bind proteins and makes a flexible yet inert spacer arm that allows movement of the ligand, improving binding (27–29). A surface map of PPV shows that there are canyons on the surface of the virus that are approximately 15 Å in depth (30), and so the spacer arm was designed to allow the peptide to reach into the depths of the canyons. For most non-enveloped viruses, it is accepted that the conserved amino acid sequences are located in the depth of these canyons because these are often the location of the receptor binding sites.

3.2. Primary Screening. The library beads were originally blocked with 50% human blood plasma before the virus was added. This blocked any of the peptides that had a high affinity for plasma proteins before the addition of PPV to the library.

Table 1. Peptide Sequences Found from Primary Screening

wash	sequence	
1 M NaCl	KNY	AKL
	WRW	KTF
	KKK	VWR
	KGK	RAA
	KYY	KRR
	FVV	
1 M KCl	FRH	KHR
	KAA	RTG
	RQQ	

After incubation with PPV, the beads were washed to remove any nonspecifically bound virus. One screening run was washed in 1 M NaCl and yielded a total of 24 positive beads from about 10,000; another screening run was washed in 1 M KCl and gave a total of 9 positive beads. Only those positive beads that had a large signal to size ratio (i.e., a small bead that gave a large signal), as determined by visual inspection, were chosen for sequencing. The results of the returned sequences are shown in Table 1. To better determine the significance of the different chemical groups, the amino acids were counted and compared to their probability of random occurrence (Table 2). A random occurrence was determined as the number of amino acids in the chemical group divided by the number of different amino acids in the library and then multiplied by the total number of amino acids found from the sequencing. For example, there are five different aliphatic amino acids, so the random number of aliphatic amino acids is calculated by dividing 5 amino acids by the 18 different amino acids used in this study, and the result multiplied by the 48 total amino acids in the 16 trimers found by screening. This gives the random occurrence of aliphatic amino acids of 13.3 indicated in Table 2. If the number of amino acids from a certain chemical group was close to the random occurrence number, then it was suspected that the chemical group was just randomly found and may have little to do with the binding of the virus. However, if the number was much higher than that expected to occur randomly, then that group was considered to be significant in the binding of the virus.

Table 2 shows the importance of basic amino acids in the binding of PPV and, to complement this, the lack of acidic groups associated with ligands found to bind to PPV. The results show that positive charges are important for the binding of virus. This is an expected result because canine parvovirus, a related parvovirus, has an isoelectric point of 5.3 (31), leaving it negatively charged at physiological pH, which corresponded to the conditions used in the screening studies. There was a random distribution of aromatic and aliphatic groups with seven sequences that contained aromatics and seven sequences that contained aliphatics. The sequences were then categorized into the following: those containing an aromatic amino acid, those containing an aliphatic amino acid, and those containing neither. Since all but one sequence contained a basic group, all sequences chosen for further screening contained a basic amino acid. From these categories, five sequences were chosen for additional screening using column chromatography: WRW and KYY, which contain aromatics; RAA, which contains an aliphatic; and KHR, which contains a histidine. Also, KKK and KRR were combined to form KRK, which contains basic residues.

3.3. Column Chromatography. The resins were packed into disposable columns and tested for breakthrough of PPV in the eluent using infectivity as the enumeration method. First, cell culture supernatant containing PPV was diluted with PBS to a final titer of about 6–7 log (MTT/mL) (approximately a 1:100

Table 2. Chemical Characterization of Sequences

amino acid type	random ^a	actual ^b
aliphatic	13.3	11
cyclic imino	2.7	0
imidazole	2.7	2
basic	5.3	21
aromatic	8.0	9
hydroxy	5.3	2
amide	5.3	3
acidic	5.3	0

^a The number of amino acids expected from a random distribution of amino acids in 16 trimeric peptides. ^b The number of each type of amino acid that was present in the 16 trimeric peptides.

to 1:10 dilution) and was filtered through a 0.22 μ m filter. Virus spiking studies should not be carried out at a dilution more concentrated than 1:10, as the virus solution, which contains contaminants from the cell culture from which it was created, may start to interfere with the virus clearance mechanism (3). However, the larger the initial viral load, the better the opportunity to validate a high degree of virus clearance. Virus clearance was calculated in accordance with the expression

$$\log \text{clearance} = -\log \left(\frac{\text{virus detected after clearance step}}{\text{total virus load}} \right) \quad (1)$$

The PPV breakthrough curves were determined by pumping virus-spiked solutions onto the peptide columns at 0.1 mL/min. Fractions equivalent to 1 column volume were collected, for a total of 10 column volumes, and the amount of PPV in the flowthrough fractions was determined. The results are plotted in Figure 1 as the percentage of the detectable PPV as a function of column volume. Presenting the results in terms of a percentage of the detectable clearance automatically accounts for the different initial virus titers of the various batches analyzed in these experiments.

In PBS, all of the resins were able to clear completely the detectable virus available in the solutions, as shown in Figure 1. This is in contrast to the amino resin control (with no peptides, which is considered a weak ion-exchange resin) that was not able to remove any significant amounts of PPV from PBS. Clearly, the peptides were responsible for the binding of the virus, and nonspecific binding to the resin surface was ruled out.

The small peptide resins have the ability to remove viruses from simple solutions such as water, suggesting potential application to water treatment. In fact, microfiltration is being considered as an alternative to chlorine treatment of water supplies (32), but the method suffers from many of the same difficulties as nanofiltration for therapeutic processes. Small viruses, like hepatitis A virus, which has a diameter of 27–32 nm (33), and norovirus, with a diameter of 30–40 nm (34), are able to pass through many nanofiltration and all microfiltration membranes. These viruses are shed in the feces of infected humans and are common contaminants of water supplies. Small peptide ligands theoretically could be placed on microfiltration membranes to improve virus removal without the need to use membranes of small pore size which often cause fouling (8) and may require high back pressures.

To challenge the peptides for their ability to remove PPV under therapeutic processing conditions, virus-spiked 7.5% human blood plasma was used. A 7.5% human blood plasma solution contains about 5 mg/mL of protein, which is approximately the amount of protein that can be found in a therapeutic protein product. There are two general viral clearance steps in a monoclonal antibody production process, i.e., a low

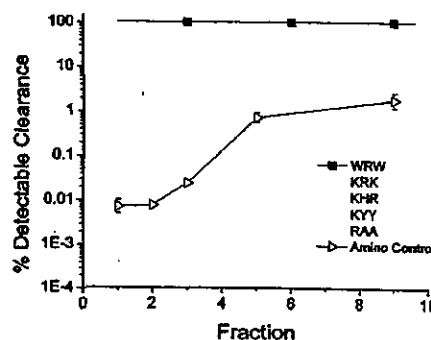


Figure 1. Binding of trimeric peptides to PPV in PBS. All ligands were able to bind 100% of the detectable PPV. The amino control, which is a weak ion-exchange resin, was able to clear less than 1% of the detectable PPV. Columns were run in duplicate, and the error bars represent the detectable clearance of each column.

pH step after cell harvest and a nanofiltration step before or during formulation (35). If effective, small peptides theoretically could be used on membranes on the nanofiltration step at the end of the process. All of the peptides were challenged with protein loads similar to those that would be found at this phase of the purification process.

Data on the removal of PPV from this complex mixture using the peptides are presented in Figure 2. All of the resins tested, except for KHR, were able to remove all of the detectable PPV in the first column volume. All of the resins had a breakthrough of PPV before the fifth column volume. It is believed that the proteins found in human blood plasma also began to nonspecifically bind to the peptides at this point, leaving less peptide available for specific binding to PPV. There may be a way to improve the selectivity of the virus over the plasma proteins by optimization of the chromatography process through changes in peptide density or buffer ionic strength.

The amino control resin had the ability to bind to some of the PPV and remove it from solutions. In the first two column volumes in PBS and 7.5% human blood plasma, the amino control achieved about 1 log clearance. This is not surprising, as anion exchange columns are often tested for their ability to clear viruses (35). It has been shown that a Q-Sepharose column was able to clear 3 log of PPV when loaded at pH 6.5 (36) and as high as 5 log of MVM when loaded in Tris buffer at pH 8.0 (14). This follows the trend that increasingly basic solutions will make the virus surface more negatively charged, which would cause increasingly stronger binding of the virus to an anion exchange column. The control in our experiments was a weak ion-exchange resin at pH 7.4, which showed clearance lower than that seen with the Q-Sepharose columns, as would be expected.

An increase in binding of virus over time was found with the amino control resin both in the presence and absence of human blood plasma (Figures 1 and 2). The PPV solution used in the experiments was cell culture supernatant that had been clarified by low-speed centrifugation and filtered through a 0.22 μ m filter, and so the solution contained cellular debris. Since the amino control is a weak ion-exchange resin, it is possible that the resin was binding the cellular debris, and the virus then bound to the debris on the resin, as many proteins in host cell proteins are known to be negatively charged and removed by anion exchange (35). Since the amino resin has a high positive charge density, it is also possible that these cellular proteins may denature onto the surface, as has been suspected in ion exchange purification (37, 38) and is known to happen when proteins adsorb to surfaces (39). Protein denaturation was also