JOURNAL OF VIROLOGY, May 1997, p. 3879–3885 Vol. 71, No. 5
0022-538X/97/$04.00+0

Phage-Displayed Peptide Targeting on the Puumala Hantavirus Neutralization Site

TUOMAS HEISKANEN,[1]* ÅKE LUNDKVIST,[2] ANTTI VAHERI,[1] AND HILKKA LANKINEN[1]

Department of Virology, Haartman Institute, University of Helsinki, FIN-00014, Helsinki, Finland,[1] *and Swedish Institute for Infectious Disease Control, S-105 21 Stockholm, Sweden*[2]

Received 20 August 1996/Accepted 7 February 1997

We have selected ligands for Puumala hantavirus, the causative agent of nephropathia epidemica, from a seven-amino-acid peptide library flanked by cysteines and displayed on a filamentous phage. To direct the selection to areas on the virus particle which are essential for infection, phages were competitively eluted with neutralizing monoclonal antibodies specific for the viral glycoproteins. The selected phage populations were specific for the same sites as the antibodies and mimicked their functions. The peptide insert, CHWMFSPWC, when displayed on the phages, completely inhibited Puumala virus infection in cell culture at the same effective concentration as the eluting antibody specific for envelope glycoprotein G2. The binding of the phage clones to the virus and inhibition of infection were not necessarily coincident; Pro-6 was critical for virus inhibition, while consensus residues Trp-2 and Phe-4 were essential for binding. The strategy described can be applied to any virus for production of molecules mimicking the effect of neutralizing antibodies.

Puumala virus belongs to the genus *Hantavirus*, family *Bunyaviridae*. Hantaviruses are enveloped and have a trisegmented, single-stranded, negative-sense RNA genome. The large, medium, and small segments are packed into nucleocapsids separately. The large segment encodes the viral RNA polymerase-transcriptase, the medium segment encodes envelope glycoproteins G1 and G2, and the small segment encodes nucleocapsid protein N (12, 36, 41). Each hantavirus is associated with a primary rodent host carrier in which it has evolved and for which it is apathogenic (33). Hantaviruses include human pathogens of two diseases with different clinical symptoms: hemorrhagic fever with renal syndrome (HFRS) caused by Hantaan, Dobrava, Seoul, and Puumala viruses (6, 18–20) and the highly lethal hantavirus pulmonary syndrome (HPS) caused by Sin Nombre and related viruses (13, 29). The pathogenesis of HFRS and HPS includes increased permeability of endothelial cells and a variety of symptoms indicative of a systemic disease and involvement of the immune system (9, 44). Development of a drug against hantavirus infections is most important, since there is no efficient treatment available for either HFRS or HPS.

Random peptide libraries displayed on a filamentous phage have been a source of many biologically active peptide ligands. The phage display method is based on affinity purification of phage clones with a target molecule and aims to select the peptide sequences which attach with the highest affinity to the binding sites of proteins. Phage display has been applied to mapping of epitopes of monoclonal antibodies (MAbs), identification of interactive peptides for many different molecules, and determination of active domains of proteins (16, 32, 37, 39). An effective way to enhance and specify the affinity purification of peptide inserts is to competitively elute the relevant clones with a high-affinity ligand that has the desired biological activity. Phage-displayed antibodies have been obtained by using fixed cells as a target and a MAb as the eluting agent (28); interactions of adenovirus proteins have also been studied by specific ligand elutions (14). We now report the isolation of peptide-carrying phage clones which attach to virus neutralization sites and show the same biological functions as the eluting MAbs. We show that selection from display libraries can be directed towards specific biological properties and that the relative importance of each amino acid residue in the displayed insert can be determined with biological assays. The residues essential for inhibition of infection are shown to be different from residues primarily determining the binding of inhibitory inserts to the virus.

* Corresponding author. Mailing address: Department of Virology, Haartman Institute, POB 21, FIN-00014 University of Helsinki, Finland. Phone: 358-9-434 6304. Fax: 358-9-434 6491. E-mail: Tuomas.Heiskanen@helsinki.fi.

MATERIALS AND METHODS

Cells, virus, antibodies, and peptides. The Puumala virus Sotkamo strain was propagated in Vero E6 cells and purified as previously described (36). Vero E6 cells were grown in Eagle's minimal essential medium supplemented with 10% fetal calf serum, glutamine, and antibiotics. In short, release of the virus into the culture medium was monitored for up to 30 days postinfection with hemagglutination tests (5) and medium was collected every second day during days 14 to 30 postinfection and stored on ice until concentrated by using a Pellicon filter system (Millipore). Final concentration of the virus suspended in 50 mM Tris-HCl (pH 7.4)–0.1 M NaCl (TN) was done by centrifugation through a 30% (wt/vol) sucrose cushion (48,000 × *g*, 3.5 h) and purification by ultracentrifugation on a 10 to 60% (wt/vol) sucrose gradient in TN containing 1 mM EDTA. The virus was inactivated with 0.05% *n*-octyl-β-D-glucopyranoside (Sigma) and stored at −70°C until use. The protein concentration of the virus preparation was determined by the Pierce bicinchoninic acid protein assay. The neutralizing Puumala virus G1 and G2 glycoprotein-specific bank vole MAbs 5A2 and 4G2, respectively, nonneutralizing G2-specific bank vole MAb 5B7, neutralizing Puumala virus G2-specific human MAb 1C9, nonneutralizing G1-specific human MAb G1-1E7-E5, and nonneutralizing nucleocapsid protein N-specific bank vole MAbs 1C12 and 5E1 used in this study were produced and purified with protein G affinity chromatography as previously described (21, 22, 25, 26). Peptides were synthesized on a 433A Peptide Synthesizer (Applied Biosystems) by using 9-fluorenylmethoxycarbonyl chemistry, cyclized in dimethyl sulfoxide as previously described (11), and purified by reverse-phase high-performance liquid chromatography. The correctness of the oxidized form of the peptide was confirmed by matrix-assisted laser desorption ionization–time-of-flight mass spectroscopy. Reduction and alkylation of cyclic peptides were done with dithiotreitol and iodoacetamide as previously described (16). Peptide concentrations were determined by using A_{280} values based on theoretically calculated absorption coefficients.

Selection of high-affinity phage population with competitive elution by neutralizing antibodies 4G2 and 1C9. The filamentous phage cyclic heptapeptide library inserted into the amino terminus of the pIII protein of phage fd-tet was kindly provided by Erkki Ruoslahti (The Burnham Institute, La Jolla, Calif.) and Erkki Koivunen (17). Affinity biopanning procedures were performed as previously described (16, 38), with modifications.

Each microtiter well was coated with 1.5 μg of purified, inactivated Puumala virus in 100 μl of Tris-buffered saline (TBS), pH 7.5, and incubated for 1 h at

37°C. The virus-coated well was blocked, control well was coated with 200 μl of TBS containing 1% bovine serum albumin (BSA), and the wells were incubated for 1 h at 37°C. After the wells were washed three times with TBST (TBS containing 0.05% Tween 20), an aliquot containing 5×10^{11} transducing units (TU) of the CX_7C library (110 TU per clone) was added to both the virus-coated and control wells in 115 μl of TBS containing 3% nonfat dry milk powder (NFDMP) and 5 mg of BSA per ml and the wells were incubated for 1 h at 37°C. Biopanning with MAb 4G2 as the eluting antibody included three antibody and two acid elution rounds. In the first panning, the wells were rinsed 10 times with 5% NFDMP-TBS, pre-eluted for 20 min at 37°C with antibody 5A2, rinsed three times, and eluted for 20 min at 37°C with MAb 4G2. The concentration of the antibodies was 40 μg/ml in 5% NFDMP. In each panning, the titer of phages was determined from the eluates and the eluate from the well with the lowest coating concentration of virus that showed a titer still over the background was amplified and used in the next panning. The second panning was done directly with MAb 4G2, whereas in the third, MAb 3H9 was used as a pre-eluting MAb. For removal of residual antibody, the phage amplification product of the third panning was treated with Prosep-A (Bioprocessing Ltd.). The fourth and fifth pannings were done conventionally with 0.1 M HCl-glycine, pH 2.2, containing 1 mg of BSA per ml as the eluent. Individual phage clones from the eluates of the third, fourth, and fifth pannings were amplified, and their binding strength and insert sequences were determined.

Biopanning with MAb 1C9 as the eluting antibody was done with the following modifications to the MAb 4G2 elutions described above. The panning included one antibody and two acid elution rounds. All solutions were made with Dulbecco's salt solution containing Ca^{2+} and Mg^{2+} (panning, washing, and antibody elution). In the first and second pannings, the phages were allowed to bind to Puumala virus in a solution containing 0.33% BSA. In the first panning, MAb 5B7 was used in the preelution, followed by MAb 1C9 elution, and the well was washed with a 1% BSA solution and then without BSA. Any residual MAb was removed from the amplification product of the first panning with Prosep A. In the second panning, the phages were eluted with a low pH. The third panning was done with Puumala virus-infected cell extract prepared as previously described (21). The extract was diluted 2:3 in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST) and 0.5% BSA and captured in a microtiter well coated with MAb 5B7 (5 μg/ml). The phages were added to the well in a solution containing 0.45% BSA and 40 μg of MAb 5A2 per ml, and the well was washed with 0.05% Tween 20. Low-pH elution was then performed.

Binding assays. The relative strengths of phage clone and population binding to the virus particle preparation were estimated by enzyme immunoassay (EIA). All incubations were performed in a 100-μl volume at 37°C. Microtiter wells were coated with 10 to 20 ng of virus diluted in PBS, set aside for 1 h, and blocked for 1 h with either 1% BSA or 5% NFDMP in PBST. Approximately 10^9 TU of phages representing either one phage clone or a phage population was added to the wells in PBST, and the plate was incubated for 40 to 60 min. The wells were washed five times with PBST, an anti-M13 antibody–peroxidase conjugate (Pharmacia) diluted 1:5,000 in PBST was added, and the plate was incubated for 30 min and washed eight times with PBST. Peroxidase activity was determined at room temperature by using an *o*-phenylenediamine dihydrochloride (Sigma) substrate, the reaction was stopped after 10 to 15 min with 100 μl of 2 M H_2SO_4, and the A_{492} was measured. In competition assays, the peptides or antibodies were added to the virus-coated wells 15 min prior to the phage clones or populations.

G1 and G2 capture assays. Microtiter wells were coated with G2-specific antibody 5B7 or G1-specific antibody G1-1E7-1E5 (100 ng per well), and approximately 200 ng of detergent-inactivated, purified Puumala virus was incubated with the antibodies overnight. The phage population eluted with MAb 4G2 (fourth panning; 10^9 TU) was allowed to react with MAb capture-bound glycoproteins. Mean values from duplicate wells were calculated.

Inhibition of Puumala virus infection. Approximately 8,000 focus-forming units (FFU) of Puumala virus in 200 μl of conditioned medium was incubated for 1 h at 37°C with 10^8 to 10^{12} TU of purified phages serially diluted in 50 or 100 μl of PBS or Hanks balanced salt solution. The volume was adjusted to 500 μl with Hanks balanced salt solution supplemented with 2 to 4% fetal calf serum and 10 to 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer (pH 7.4), and the mixture was used to infect a confluent Vero E6 cell monolayer in a six-well plate. The virus was absorbed for 1 h at 37°C, and the wells were washed once with culture medium. Cell extracts were prepared after 6 to 8 days for measurement of the expression levels of nucleocapsid protein N by antigen capture EIA essentially as previously described (23), except that a rabbit polyclonal anti-N–glutathione *S*-transferase fusion protein antibody (42), diluted 1:1,000, and an anti-rabbit antibody-peroxidase conjugate were used for antigen detection. Peroxidase activity was measured as described above. After subtraction of absorbance values of mock-infected controls, typical absorbance values for infected cell samples were 0.5 to 0.8. The mean of three parallel measurements from three microtiter wells was calculated for each sample.

Inhibition of Puumala virus infection by phages and peptides was determined by a focus reduction neutralization test (FRNT) essentially as previously described (30). Briefly, phages or peptides were serially diluted and mixed with an equal volume containing approximately 50 FFU of Puumala virus (strain Sotkamo). The mixture was incubated for 1 h at 37°C and added to confluent Vero E6 cell monolayers in six-well tissue culture plates (200 μl per well). After adsorption for 1 h at 37°C, the wells were overlaid with a mixture of agarose and Eagle's minimal essential medium. After incubation for 12 days, the cells were fixed with methanol. Human anti-Puumala virus serum, goat antibodies to human immunoglobulin G labeled with peroxidase, and a tetramethylbenzidine substrate were used to indicate virus-infected cells. Foci were enumerated.

Sequence homologies. Amino acid sequence comparisons of the isolated heptapeptides, including the flanking cysteines, with the SwissProt protein database of 50,000 entries were done by using the Blastp 1.4.9 MP program (1). The interesting matches were selected from the first 100 best-scoring sequences.

RESULTS

Selection of Puumala virus envelope glycoprotein-specific phage-displayed peptides mimicking neutralizing antibodies. Peptides were selected from a random heptapeptide library flanked by cysteines and expressed on the amino terminus of protein pIII (three to five copies) of filamentous bacteriophage fd-tet (37). This type of peptide library was used since conformationally constrained peptides are frequently reported to have higher affinities for their ligands than do linear peptides that bind to the same sites (16, 17, 31). To isolate biologically active peptide inserts, we competitively eluted the phages that attach to the same regions of Puumala virus as the neutralizing MAbs by using these MAbs. Because the amount of eluting antibody was much greater than that of phages, the relevant clones were expected to detach. Anti-G1 MAb 5A2 and then anti-G2 MAb 4G2 were used for elution of phages attached to the virus. In the otherwise identical control panning, BSA was the target.

In the 5A2 MAb eluate, there were approximately as many phages as in the control, whereas the first 4G2 eluate contained eight times as many phages as the control. Two more panning rounds with MAb 4G2 ensured homogeneity of the phage population from which high-affinity clones were then rescued in further rounds by using a low pH. Since the increase in the number of phages was low in the third panning with 4G2, any residual eluting antibody in the phage amplification product, which would inhibit binding of the phage clones to the virus, was removed before the last pannings. An alternative ligand-elution scheme was tested with anti-G2 MAb 1C9 by using antibody-captured envelope protein G2 as the target.

Sequences. Altogether, 14 different MAb 4G2-selected sequences (Table 1) and 3 MAb 1C9-selected sequences (Table 2) were obtained. The MAb 4G2-selected sequences had two motifs. The inserts in the largest group, group I, contained the motif XWXFXXX, where X is a variable residue. Within the group, there were four subgroups. Subgroups Ia and Ib had only one representative each, which shared the N-terminal HWXF motif. In subgroup Ic, the consensus was PWYF. Most clones in subgroup Ic and the only clone in Ib had Pro-6 and Trp-7. Subgroup Id had the motif SWIFWXY. Group II had the sequence LF(N/T)WP(V/A)(N/S), in which Asn-3 was accompanied by Asn-7 and Thr-3 was accompanied by Ser-7. All other sequences were distributed equally between the fourth and fifth pannings, but group II sequences were found only in the fifth. The group III sequence appeared once (Table 1) but was also represented in group IV (Table 2), the phage population obtained by elution with neutralizing human anti-G2 MAb 1C9, which has a specificity that partly overlaps that of MAb 4G2. The 1C9 group consensus was PXWWPXX. A characteristic of the sequences obtained was conserved hydrophobic residues in fixed positions in the frame provided by the terminal cysteines.

Binding specificities of sequences. The binding affinities were estimated and specificities were determined for representative phage clones of sequence groups by using a sensitive EIA-binding test based on antiphage antibody (10). As calculated from the known proportions of structural proteins in

TABLE 1. Sequences of inserts from the CX_7C library in the phage clone population eluted from different amounts of Puumala virus with neutralizing bank vole MAb 4G2

Group	Insert	No. of identical clones	Panning round	Amt of virus (ng) well
Ia	HWSFGIF	1	4	50
	HWSFGIF	2	4	25
	HWSFGIF	2	5	5
	HWSFGIF	2	5	0.56
	HWSFGIF	1	5	0
Ib	HWMFSPW	1	4	50
	HWMFSPW	2	4	25
Ic	PWYFLPW	2	4	25
	PWYFQPW	1	4	25
	PWYFQPW	1	5	0.56
	PWYFHPY	1	5	10
	PWYFFDL	1	4	25
	PWYFFGA	1	5	10
Id	SWIFWEY	1	3	250
	SWIFWQY	1	4	25
	SWIFWVY	1	5	5
II	LFNWPVN	1	5	20
	LFNWPVN	2	5	5
	LFNWPVN	2	5	0.56
	LFTWPAS	1	5	20
	LFTWPVS	1	5	5
III	PGWWPYP	1	5	5

Bunyaviridae virus particles (34), approximately 25% of the virus protein mass consists of envelope glycoprotein. Thus, 15 to 20 ng of purified Puumala virus, the amount used in the binding tests, contained 4 to 5 ng of virus glycoprotein (Fig. 1). The amount of phages needed to saturate this amount of virus on a microtiter well was in the range of 10^9 TU. All of the representative phage clones from sequence group I bound to Puumala virus significantly over the background. The binding of the most abundant clone, HWSFGIF, was lower (data not shown) than that of the less frequent clone (Fig. 1A), in accordance with a previous report (14).

The phages selected with anti-G2 MAb 4G2 could be blocked from binding to the virus by MAb 4G2 (Fig. 1A). Unexpectedly, anti-G1 MAb 5A2 also blocked the binding the 4G2-selected phage clones. Anti-G2 MAb 1C9 inhibited binding partially at 1 μg per well and almost completely (~90%) at 5 μg per well, whereas the inhibitory effects of MAbs 4G2 and 5A2 were also equal at a concentration 0.2 μg per well, at which concentration the inhibition was partial (~74%; data not shown). The nonneutralizing MAbs had no effect on binding of the phages. According to epitope mapping experiments, MAbs 5A2 and 4G2 do not cross-react, although in immunoprecipitation, MAb 4G2 gives a weak G1 band. In EIA, solid-phase 5A2-bound G1 is not detected by 4G2, or vice versa (24); thus, the G1 reactivity of 4G2 might be directed to a region that partially overlaps the epitope of 5A2. The phage population from the fourth panning with anti-G2 MAb (4G2) elution showed reactivity with both the G1 and G2 proteins when they were captured with the nonneutralizing MAbs. The overall binding in both cases was lower than with virus adsorbed on a microtiter well, which suggested that the epitope recognized by

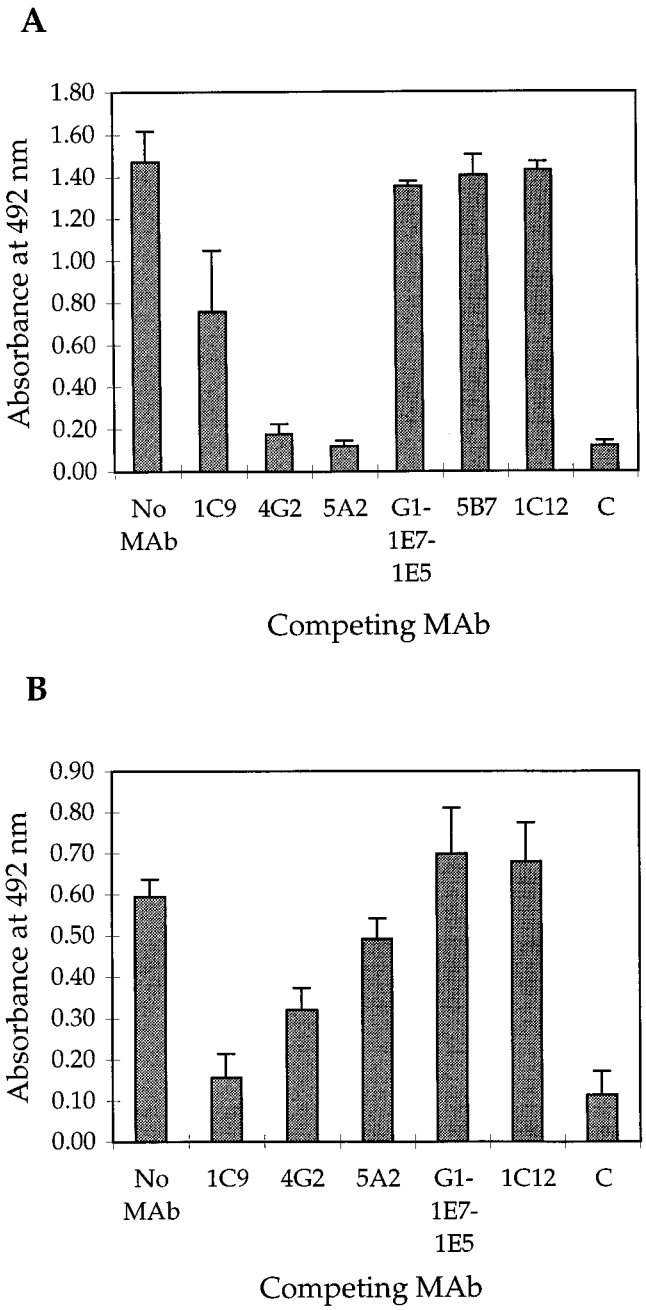

FIG. 1. Affinity and specificity of peptide-carrying phage clones for envelope glycoproteins of purified Puumala virus (20 ng) as determined by competitive EIA. The competing MAbs (1 μg per well) shown were added prior to the phage clones. (A) Binding of phage clone CHWMFSPWC selected by bank vole neutralizing MAb 4G2 (mean of three independent experiments ± standard error). (B) Binding of phage clone CPDWWPYLC selected by human neutralizing MAb 1C9. Combined results of two independent experiments are shown. The EIA background was measured in control well that did not contain virus (C).

TABLE 2. Sequences of inserts from the CX_7C library in the phage clone population eluted from envelope glycoprotein G2 with neutralizing human MAb 1C9

Group	Insert	No. of identical clones	Panning round
IV	PSWWPVN	2	3
IV	PGWWPYP	2	3
IV	PDWWPYL	1	3

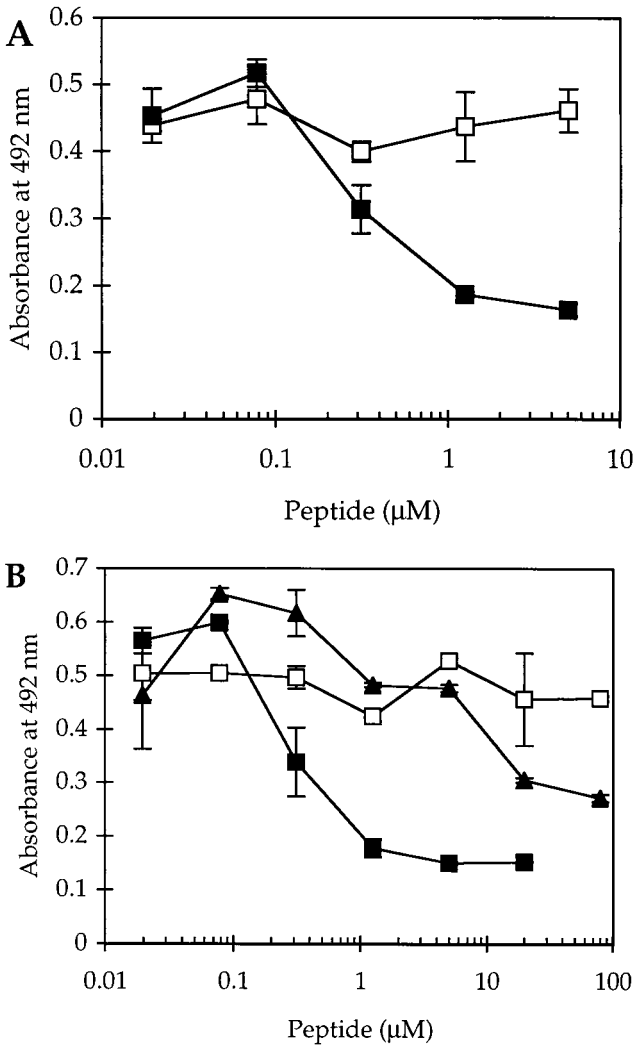

FIG. 2. Inhibition of binding of phage clones to Puumala virus by synthetic peptides. (A) HWMFSPW sequence carrying a phage clone. (B) LFNWPVN sequence carrying a phage clone. The competing cyclic peptides had the corresponding sequences CLFNWPVNC (▲) and CHWMFSPWC (■). The CVRLNSLANC peptide (□) was used as a negative control. Mean values of duplicate wells are shown. The background of 0.1 absorbance unit was not subtracted.

the phages was lost when the virus proteins dissociated. The clones that were selected with anti-G2 MAb 1C9 could be totally competed only with MAb 1C9 itself, while the effect of other neutralizing MAbs was partial (Fig. 1B).

The competition experiments showed that the selected phage clones competed for the same specific binding sites on the virus as neutralizing antibodies, since the 4G2-selected clones did not bind to MAb 4G2 (data not shown), and that neutralization sites in G1 and G2 are intimately associated, since nonneutralizing MAbs had no effect on binding of the selected phages to the virus, while all of the neutralizing MAbs had some effect. Phage populations with different binding specificities and consensus motifs could be obtained by using two different antibodies showing that antibody elution is effective.

The inserts of phage clones CHWMFSPWC and CLFNWP VNC were synthesized as peptides. Peptide CHWMFSPWC inhibited binding of the corresponding phage clone at concentrations of 1 to 5 μM (Fig. 2A), whereas control peptide CVRL NSLAC had no effect. Peptide CLFNWPVNC had only a moderate effect on the binding of the CLFNWPVNC phage clone, whereas the CHWMFSPWC peptide inhibited its binding completely (Fig. 2B). These results suggest that phage inserts CHWMFSPWC and CLFNWPVNC bind to almost the same site. As expected, competition by the cyclic form of peptide CHWMFSPWC was slightly better than if an alkylated form was used (data not shown).

TABLE 3. Inhibition of Puumala virus infection by different phage clones[a]

Peptide insert	n	$n \times 10^8$ TU	$n \times 10^9$ TU	$n \times 10^{10}$ TU	$n \times 10^{11}$ TU	$n \times 10^{12}$ TU
CHWMFSPWC	2.0	147	89	14	1	
CPWYFQPWC	1.0		126	70	35	
CPWYFHPYC	1.4		152	97	26	
CLFNWPVNC	3.5		138	105	91	
CHWSFGIFC	1.0		110	116	95	
CX_7C	1.0			121	114	101
CPSWWPVNC	4.0		142	126	126	
CSWIFWEYC	1.0		152	153	194	

[a] The percentage of the signal of expressed N compared to infection with no phage (standard deviation from the mean, 12%) is shown as a function of the amount (TU) of phages in an inhibition experiment. Phages were diluted 1:10 beginning from $n \times 10^{11}$ or $n \times 10^{12}$. The standard deviation of negative controls was 2%.

Inhibition of Puumala virus infection in cell culture. The inhibitory effects of the individual phage clones on Puumala virus cell culture infections were tested by using an FRNT (Fig. 3) and an N protein capture assay that measures the expression level of the N protein (Table 3).

In the FRNT, 4G2-eluted phage clone CHWMFSPWC inhibited Puumala virus infection completely when a virus inoculum of 50 FFU was incubated with 5×10^9 TU of phages prior to infection (Fig. 3). This corresponds to about a 1 nM concentration of the displayed insert. Antibody 4G2 inhibits infection at a concentration of ~4 nM in the FRNT (25). When the infection-inhibition efficiency of the representative peptide inserts was calculated according to the N protein expression level (Table 3), complete inhibition was obtained with 10^{11} TU of phage CHWMFSPWC. Clones CPWYFQPWC and CPWYFHPYC (Table 1) were also effective inhibitors, while clones CLFNWPVNC and CHWSFGIFC gave less inhibition (Table 3).

A phage clone from group Id (Table 1), CSWIFWEYC, stimulated infection by almost doubling it (Table 3). The CX_7C library, like most of the individual phage clones, stimulated Puumala virus infection when the phage amounts were lowered towards 10^8 to 10^9 TU. Hong and Boulanger (14) also observed stimulation of adenovirus attachment to the HeLa cell surface by their phages and proposed that phages may bind nonspecifically to the cell surface, thereby enhancing their entry. Our results show that the stimulatory effect may depend on the sequence in the insert. In contrast to the FRNT, in the N protein capture assay, no inhibition by the cyclic library was seen. Inhibition of infection by cyclic peptide CHWMFSPWC could not be consistently detected at the concentrations of ≤60 μM used.

DISCUSSION

We selected phage clones from a phage-displayed CX_7C peptide library which inhibit Puumala virus infection and bind to its neutralization epitopes. The present approach, i.e., use of

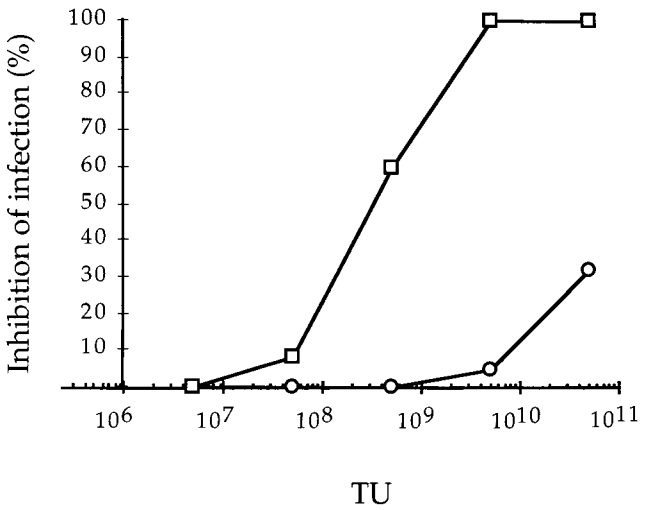

FIG. 3. Inhibition of Puumala virus infection in Vero E6 cell cultures by phages containing the CHWMFSPWC peptide insert (□) or the cyclic CX_7C library (○) as determined by an FRNT.

a neutralizing antibody for elution of phage clones binding to virus, yielded the neutralizing peptide inserts.

Our binding studies and sequence data on selected phage clones showed that the binding force and specificity of the peptide inserts in group I (Table 1) were provided mainly by Trp-2 and Phe-4. The position in the frame formed by the terminal cysteines seemed to be important for each motif, as the sequence WXF was found in only one position. In group II, the sequence LF(N/T) (positions 1 to 3) probably corresponded to WXF and determined its specificity, since its binding pattern is identical to that of other 4G2-selected clones (Fig. 1A). Clackson and Wells (7) observed that most of the binding energy at the hormone-receptor interface was provided by one or two hydrophobic contacts. This is in accordance with our findings on WXF. Besides the basic motif WXF that determines binding, the motif P(W/Y), positions 6 and 7, was found in the neutralizing inserts. Clearly, all of the peptides that bound did not neutralize, indicating that steric hindrance by the phage to the WXF site is not a sufficient mechanism for neutralization. From the sequences tested for infection-inhibiting activity, it can be concluded that Pro-6 is essential for inhibition, as it was, besides WXF, the only residue common to neutralizing peptides HWMFSPW, PWYFQP W, and PWYFHPY. Pro-6 was not needed for binding to the WXF site. Trp-7 was probably also important for neutralization, but it could be replaced at least by tyrosine. An attractive feature of phage display, as can be seen from our results, is that besides finding active reagents it is possible to do random mutagenesis at the same time and define the importance of each residue.

Puumala and Hantaan viruses elicit neutralizing antibodies to envelope glycoproteins G1 and G2, and passive immunization with neutralizing MAbs protects against virus challenge (3, 4, 20, 25, 35), which emphasizes the critical role of a neutralizing antibody response to envelope glycoproteins in the control of infection. Our data indicate that all of the Puumala virus-neutralizing MAbs used in this study bind to areas overlapping or near each other on the surface of the three-dimensional structure of the virion. The neutralizing MAbs used to competitively elute virus-binding phages have different specificities; one anti-G2 MAb (4G2) is presumably cross-reactive with the G1 protein, and the others are G1 (MAb 5A2) and G2 (MAb 1C9) specific. Some degree of overlap between the epitopes of MAbs 4G2 and 5A2 is supported by the competition of phages in groups I and II by both of these MAbs (Fig. 1A), suggesting that the neutralizing phages may bind to a conformational cleft (neotope) in the quaternary structure formed by both glycoproteins. Thus, different residues in the inserts might interact with different glycoproteins. Another possibility is that MAb 4G2 has similar binding sites in G1 and G2 corresponding to binding sites for neutralizing phages. In the case of Hantaan virus, interaction of the glycoproteins is indicated by coprecipitation of the G1 and G2 proteins by MAbs, which also applies to our MAb 4G2, and by certain neutralization escape mutants which escaped neutralization by both G1- and G2-specific MAbs (43). Both glycoproteins are needed for a protective immune response in animals (35), which also suggests the association of G1 and G2, at least in the neutralized virion. In contrast, the Puumala virus G2 epitope defined by MAb 1C9 does not show cross-reactive properties with G1 either in EIA or in experiments on MAb escape mutants, although it blocks the binding of MAb 4G2 to the G2 protein (15, 22). The overlapping of the 4G2 and 1C9 epitopes is reinforced by sequence similarity of our Puumala virus-binding phage clones in groups II and IV selected with each respective antibody (Tables 1 and 2). Anti-idiotypic antibodies against MAbs 4G2 and 1C9 reduce the infection by 32 and 51%, respectively, and stain the Vero E6 cell surface as small, distinct dots (26). It is probable that both 4G2 and 1C9 affect the interaction of the virus receptor and receptor-recognizing domains of the virus but by different mechanisms (15, 22). This is in accordance with the poor neutralization potential of the homologous phage inserts from groups II and IV (Tables 1, 2, and 3).

Puumala virus can infect many types of cells (40), but the mechanisms of virus-cell interactions are poorly understood. It has been proposed that with random-peptide phage display technology new molecular interactions could be identified. On the other hand, it has been shown that mimotopes which have only distant sequence similarity to natural ligands may be found from phage display libraries (17). For instance, tryptophans are much more common in peptides found with phage display than their natural occurrence implies. Nevertheless, because we could assign functions for separate amino acids in the heptamer insert, the sequence similarities were interesting. Proteins homologous to the peptide inserts, some of which might interact with Puumala virus, are listed in Table 4. Only a few membrane-associated proteins with similarity to the neutralizing consensus motif WXFXP(W/Y) were seen. Similarities to the integral membrane protein γ-carboxylase and different transglutaminases are of interest because infection of endothelial cells is typical of hantavirus infection. Epidermal transglutaminase attaches to the cell surface via fatty acids, and tissue transglutaminase is found in blood vessel walls. Transglutamination is proposed to be involved in entry interactions of human immunodeficiency virus (27) and stabilization of intra- and extracellular proteins by cross-linking in pathologic processes. The other similarities were to domains of two types of signal transduction-associated receptors. One homology was to amino acids conserved in a second extracellular loop of many G protein-coupled receptors, for example, in the histamine receptor. The second homology was to immunoglobulin-like domain V. The homology was strongest to human CD8 α-chain sequence SWLFQP, which is of interest since Puumala virus infects peripheral blood mononuclear cells, possibly a subpopulation, weakly (40). The WPVN homology shared between MAb 4G2- and 1C9-eluted phage clones was observed in

TABLE 4. Proteins with sequence homology to peptides isolated with MAbs 4G2 and 1C9

Protein and position of sequence	Sequences[a]
Human epidermal transglutaminase K	246 LFNPWC 251
Insert	CHWMFSPWC
Human vitamin K-dependent γ-carboxylase	235 HWLFSPF 241
Insert	CHWMFSPWC
Bovine brush border myosin heavy chain I	353 LFNWLVN 359
Insert	CLFNWPVNC
Human acrosin precursor	325 PWYFQP 330
Insert	CPWYFQPWC
Human histamine H2 receptor	82 CKWSFG--VFC 91
Insert	CHWSFG IFC
Human T-cell surface glycoprotein CD8, α chain	54 CSWLFQP 60
Insert	CPWYFQPWC
Mouse CD82	208 WPVN 211
Insert	LFNWPVN
Insert	PSWWPVN

[a] The SwissProt database was the source of the sequences. Identical (|) and similar (+) amino acids are shown.

mouse CD82 (the human sequence is WPVY), which associates with CD4 or CD8 to give costimulatory signals for the T-cell receptor–CD3 pathway.

Competitive elution of bound peptides with infection-neutralizing antibodies is an approach basically applicable to any pathogen and may yield antimicrobial peptides. One could also produce effective neutralizing antibodies by first generating a recombinant antibody library (6) from an individual having neutralizing antibody to a given pathogen and then eluting pathogen-bound phages competitively with the purified antibodies of the same individual.

We conclude that the selection from a peptide, miniprotein, or antibody library should not be directed only towards high affinity but also towards biological activity by designing an assay strategy with selection pressure towards a desired activity. We have used competitive elution with neutralizing antibodies for this purpose, a strategy which may be useful for obtaining phage-displayed peptides, antibodies, and other bioactive proteins with therapeutic potential.

ACKNOWLEDGMENTS

This work was supported by grants from the Finnish Technology Development Centre; the Sigrid Jusélius Foundation, Helsinki; and the Medical Research Council of the Academy of Finland.

We thank Erkki Koivunen for advice, Erkki Koivunen and Erkki Ruoslahti for the heptapeptide library, G. P. Smith for bacterial strains and phage vectors, and Raija Leveelahti, Pirkko Volin, and Taija Kyöstiö-Renvall for expert technical assistance.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Antoniadis, A., A. Stylianakis, A. Papa, S. Alexiou-Daniel, A. Lampropoulus, S. T. Nichol, and C. F. Spiropolou.** 1996. Direct genetic detection of Dobrava virus in Greek and Albanian patients with hemorrhagic fever with renal syndrome. J. Infect. Dis. **174:**407–410.
3. **Arikawa, J., A. L. Schmaljohn, J. M. Dalrymple, and C. S. Schmaljohn.** 1989. Characterization of Hantaan virus envelope glycoprotein antigenic determinants defined by monoclonal antibodies. J. Gen. Virol. **70:**615–624.
4. **Arikawa, J., J.-S. Yao, K. Yoshimatsu, I. Takashima, and N. Hashimoto.** 1992. Protective role of antigenic sites on the envelope protein of Hantaan virus defined by monoclonal antibodies. Arch. Virol. **126:**271–281.
5. **Brummer-Korvenkontio, M., T. Manni, S. Ukkonen, and A. Vaheri.** 1986. Detection of hemagglutination-inhibiting antibodies in patients with nephropathia epidemica and Korean hemorrhagic fever by using Puumala virus cell culture antigen. J. Infect. Dis. **153:**997–998.
6. **Brummer-Korvenkontio, M., A. Vaheri, T. Hovi, C.-H. von Bonsdorff, J. Vuorimies, T. Manni, K. Penttinen, N. Oker-Blom, and J. Lähdevirta.** 1980. Nephropathia epidemica: detection of antigen in bank voles and serological diagnosis of human infection. J. Infect. Dis. **141:**131–134.
7. **Burton, D. R., C. F. Barbas III, M. A. Persson, S. Koenig, M. Chanock, and R. A. Lerner.** 1991. A large array of human monoclonal antibodies to type 1 human immunodeficiency virus from combinatorial libraries of asymptomatic seropositive individuals. Proc. Natl. Acad. Sci. USA **88:**10134–10137.
8. **Clackson, T., and A. Wells.** 1995. A hot spot of binding energy in a hormone-receptor interface. Science **267:**383–386.
9. **Cosgriff, T. M.** 1991. Mechanism of disease in hantavirus infection: pathophysiology of hemorrhagic fever with renal syndrome. Rev. Infect. Dis. **13:**97–107.
10. **Dente, L., G. Cesareni, G. Micheli, F. Felici, A. Folgori, A. Luzzago, P. Monaci, A. Nicosia, and P. Delmastro.** 1994. Monoclonal antibodies that recognise filamentous phage: tools for phage display technology. Gene **148:** 7–13.
11. **Domingo, G. J., R. J. Leatherbarrow, N. Freeman, S. Patel, and M. Weir.** 1995. Synthesis of a mixture of cyclic peptides based on the Bowman-Birk reactive site loop to screen for serine protease inhibitors. Int. J. Peptide Protein Res. **46:**79–87.
12. **Elliott, R. M.** 1990. Molecular biology of the Bunyaviridae. J. Gen. Virol. **71:**501–522.
13. **Hjelle, B., S. Jenison, N. Torrez-Martinez, T. Yamada, K. Nolte, R. Zumwalt, K. MacInnes, and G. Myers.** 1994. A novel hantavirus associated with an outbreak of fatal respiratory disease in the southwestern United States: evolutionary relationships to known hantaviruses. J. Virol. **68:**6751–6754.
14. **Hong, S. O., and P. Boulanger.** 1995. Protein ligands of the human adenovirus type 2 outer capsid identified by biopanning of a phage-displayed peptide library on separate domains of wild-type and mutant pentamer capsomers. EMBO J. **14:**4714–4727.
15. **Hörling, J., and Å. Lundkvist.** Single amino acid substitutions in Puumala virus envelope glycoproteins G1 and G2 eliminate important neutralization epitopes. Virus Res., in press.
16. **Koivunen, E., D. A. Gay, and E. Ruoslahti.** 1993. Selection of peptides binding to α5β1 integrin from phage display library. J. Biol. Chem. **268:** 20205–20210.
17. **Koivunen, E., B. Wang, and E. Ruoslahti.** 1994. Isolation of a highly specific ligand for α5β1 integrin from a phage display library. J. Cell Biol. **124:**373–380.
18. **Lee, H. W., and P. W. Lee.** 1976. Korean hemorrhagic fever. I. Demonstration of causative antigen and antibodies. Kor. J. Intern. Med. **19:**371–394.
19. **Lee, H. W., P. Lee, L. J. Baek, and Y. K. Chu.** 1990. Geographical distribution of hemorrhagic fever with renal syndrome and hantaviruses. Arch. Virol. **1**(Suppl.)**:**5–18.
20. **Liang, M., Y. K. Chu, and C. Schmaljohn.** 1996. Bacterial expression of neutralizing mouse monoclonal antibody Fab fragments to Hantaan virus. Virology **217:**262–271.
21. **Lundkvist, Å., A. Fatouros, and B. Niklasson.** 1991. Antigenic variation of European haemorrhagic fever with renal syndrome virus strains characterized using bank vole monoclonal antibodies. J. Gen. Virol. **72:**2097–2103.
22. **Lundkvist, Å., J. Hörling, L. Athlin, A. Rosén, and B. Niklasson.** 1993. Neutralizing human monoclonal antibodies against Puumala virus, causative agent of nephropathia epidemica: a novel method using antigen-coated magnetic beads for specific B cell isolation. J. Gen. Virol. **74:**1303–1310.
23. **Lundkvist, Å., J. Hörling, S. Björsten, and B. Niklasson.** 1995. Sensitive detection of hantaviruses by biotin-streptavidin enhanced immunoassays based on bank vole monoclonal antibodies. J. Virol. Methods **52:**75–86.
24. **Lundkvist, Å., J. Hörling, and B. Niklasson.** 1993. The humoral response to Puumala virus infection (nephropathia epidemica) investigated by viral protein specific immunoassays. Arch. Virol. **130:**121–130.
25. **Lundkvist, Å., and B. Niklasson.** 1992. Bank vole monoclonal antibodies against Puumala virus envelope glycoproteins: identification of epitopes involved in neutralization. Arch. Virol. **126:**93–105.
26. **Lundkvist, Å., C. Scholander, and B. Niklasson.** 1993. Anti-idiotypic anti-

bodies against Puumala virus glycoprotein-specific monoclonal antibodies inhibit virus infection in cell culture. Arch. Virol. **132:**255–265.

27. **Mariniello, L., C. Esposito, P. Di Pierro, A. Cozzolino, P. Pucci, and R. Porta.** 1993. Human immunodeficiency virus transmembrane glycoprotein gp41 is an amino acceptor and donor substrate for transglutaminase in vitro. Eur. J. Biochem. **215:**99–104.
28. **Meulemans, E. V., R. Slobbe, P. Wasterval, F. C. S. Ramaekers, and G. J. J. M. van Eys.** 1994. Selection of phage-displayed antibodies specific for a cytoskeletal antigen by competitive elution with a monoclonal antibody. J. Mol. Biol. **244:**353–360.
29. **Nichol, S. T., C. F. Spiropoulou, S. Morzunov, P. E. Rollin, T. G. Ksiazek, H. Feldmann, A. Sanchez, J. Childs, S. Zaki, and C. J. Peters.** 1993. Genetic identification of a novel hantavirus associated with an outbreak of acute respiratory illness in the southwestern United States. Science **262:**914–917.
30. **Niklasson, B., M. Jonsson, Å. Lundkvist, J. Hörling, and E. Tkachenko.** 1991. Comparison of European isolates of viruses causing hemorrhagic fever with renal syndrome by a neutralization test. Am. J. Trop. Med. Hyg. **45:** 660–665.
31. **O'Neil, K. T., R. H. Hoess, S. A. Jackson, N. S. Ramachandran, S. A. Mousa, and W. F. DeGrado.** 1992. Identification of novel peptide antagonist for GPIIb/IIIa from a conformationally constrained phage peptide library. Proteins **14:**509–514.
32. **Pasqualini, R., E. Koivunen, and E. Ruoslahti.** 1995. A peptide isolated from phage display libraries is a structural and functional mimic of an RGD-binding site on integrins. J. Cell Biol. **130:**1189–1196.
33. **Plyusnin, A., O. Vapalahti, and A. Vaheri.** 1996. Hantaviruses: genome structure, expression and evolution. J. Gen. Virol. **77:**2677–2687.
34. **Pringle, C. R.** 1991. The Bunyaviridae and their genetics—an overview. Curr. Top. Microbiol. Immunol. **169:**1–26.
35. **Schmaljohn, C. S., Y.-K. Chu, A. L. Schmaljohn, and J. M. Dalrymple.** 1990. Antigenic subunits of Hantaan virus expressed by baculovirus and vaccinia virus recombinants. J. Virol. **64:**3162–3170.
36. **Schmaljohn, C. S., S. E. Hasty, J. M. Dalrymple, J. W. LeDuc, H. W. Lee, C.-H. von Bonsdorff, M. Brummer-Korvenkontio, A. Vaheri, T. F. Tsai, H. L. Regnery, D. Goldgaber, and P. W. Lee.** 1985. Antigenic and genetic properties of viruses linked to hemorrhagic fever with renal syndrome. Science **227:**1041–1044.
37. **Scott, J. K., and G. P. Smith.** 1990. Searching for peptide ligands with an epitope library. Science **249:**386–390.
38. **Smith, G. P., and J. K. Scott.** 1993. Libraries of peptides and proteins displayed on filamentous phage. Methods Enzymol. **217:**228–257.
39. **Stephen, C. W., P. Helminen, and D. P. Lane.** 1995. Characterisation of epitopes on humans p53 using phage-display peptide libraries: insights into antibody-peptide interactions. J. Mol. Biol. **248:**58–78.
40. **Temonen, M., O. Vapalahti, H. Holthöfer, M. Brummer-Korvenkontio, A. Vaheri, and H. Lankinen.** 1993. Susceptibility of human cells to Puumala virus infection. J. Gen. Virol. **74:**515–518.
41. **Vapalahti, O., H. Kallio-Kokko, M. Brummer-Korvenkontio, E.-M. Salonen, and A. Vaheri.** 1992. Cloning and sequencing of Puumala virus Sotkamo strain S and M segments: evidence for strain variation in hantaviruses and expression of the nucleocapsid protein. J. Gen. Virol. **73:**829–838.
42. **Vapalahti, O., H. Kallio-Kokko, A. Närvänen, I. Julkunen, Å. Lundkvist, A. Plyusnin, H. Lehväslaiho, M. Brummer-Korvenkontio, A. Vaheri, and H. Lankinen.** 1995. Human B-cell epitopes of Puumala virus nucleocapsid protein, the major antigen in early serological response. J. Med. Virol. **46:**293–303.
43. **Wang, M., D. G. Pennock, K. W. Spik, and C. S. Schmaljohn.** 1993. Epitope mapping studies with neutralizing and non-neutralizing monoclonal antibodies to the G1 and G2 envelope glycoproteins of Hantaan virus. Virology **197:**757–766.
44. **Zaki, S. R., P. W. Greer, L. M. Coffield, C. S. Goldsmith, K. B. Nolte, K. Foucar, R. M. Feddersen, R. E. Zumwalt, G. L. Miller, A. S. Khan, P. E. Rollin, T. G. Ksiazek, S. T. Nichol, B. W. J. Mahy, and C. J. Peters.** 1995. Hantavirus pulmonary syndrome, pathogenesis of an emerging infectious disease. Am. J. Pathol. **146:**552–579.