European Journal of Pharmaceutics and Biopharmaceutics 70 (2008) 577–581

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Non-specific translocation of peptide-displaying bacteriophage particles across the gastrointestinal barrier

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ARTICLE INFO

Article history:
Received 11 December 2007
Accepted in revised form 9 June 2008
Available online 17 June 2008

Keywords:
Phage display
Peptide library
Drug delivery
M13 phage

ABSTRACT

Phage display technology could provide a rapid means for the discovery of novel peptides and proteins from genetically engineered variants which may act as specific vehicles for drug delivery particularly through the intestinal barrier. In this work, we utilized *in vivo* phage display in order to study the sequences which may be responsible for transmucosal transport. We hypothesized that the introduction of a library of peptide displaying phages into the intestine may lead to the identification of sequences that could induce transport. A biopanning protocol was performed by applying a 7-mer random amino acid phage library to mice by gavage and then assessing their absorption via phage recovery from the spleen and blood. Following the isolation of 77 different phages, the sequences of the displayed peptides were identified. Statistical treatment of the obtained sequences did not support the notion that the GI translocation depends on the presence of any particular peptide sequence fused on the pIII coat proteins of the M13 phages. There are, however, some residue types underrepresented which could be due to specific GI selection mechanisms and/or their effects on the amplification rate for phages bearing those residues.

1. Introduction

The intestinal barrier regulates the transport and host defense mechanisms at the mucosal interface with the outside world. Transcellular and paracellular fluxes are tightly controlled by membrane pumps, ion channels and tight junctions, adapting permeability to physiological needs [1]. One of the major factors which affect the oral bioavailability of therapeutic agents is the absorption through the GI epithelial cell membrane, which is especially important for peptide and protein drugs. Protein-based drugs have become the drugs of choice for the treatment of numerous diseases, because of their incredible selectivity and their ability to provide effective and potent action. However, oral administration of these therapeutics is not generally feasible. The main reasons for their low oral bioavailability are presystemic enzymatic degradation and poor penetration through the intestinal membrane [2]. There are lines of evidence that some viruses and bacteria are able to pass through the mucosal barrier. This is specifically reported for rotavirus, *Vibrio Cholerae* and *Salmonella typhimurium* to mention few [3,4]. The pathogens use different mechanisms to invade the mucosal cells. For example, they secrete factors enabling them to bind to epithelial cells, such as M-cells. Then, by remodeling the host cell cytoskeleton, these pathogens penetrate into the cells [5]. M-cells possess a highly transcytotic capacity, and are able to transport a variety of materials including macromolecules, inert particles and microorganisms [3,5]. The development of delivery systems to transport the therapeutic agents across the intestinal barrier has been considered challenging. Phage display technology is a remarkably versatile tool for the discovery of novel peptides and proteins from genetically engineered variants, and it has been proven to be a powerful tool for isolating ligands for drug discovery, affinity chromatography, studying protein–protein interactions, epitope mapping of antibodies, isolating antibody fragments, engineering the binding affinity of displayed proteins and identifying peptides that target specific organs or tissues [6]. This technology shows promise for therapeutic applications either by the discovery of phage displayed-derived peptides or the phage particles themselves. For the former case, the technique provides novel peptides as therapeutic alternative to antibodies [7], as intracellular drug delivery vehicles [8] and as modulators of receptors and enzymatic systems [9–11]. A phage itself can be used as the therapeutic agent. For example, M13 bacteriophage was used successfully to treat a bacterial infection via delivering DNA encoding for a bactericidal protein [12]. Yacoby and coworkers demonstrated the possibility of targeting antibacterial agents using drug-carrying bacteriophages [13,14].

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doi:10.1016/j.ejpb.2008.06.005

Filamentous phage was also used as an immunogenic carrier to elicit antibody responses to the synthetic peptide B2.1 [15]. There are examples where this method was used in order to identify the peptide sequences that induce the transport of phages across the gastrointestinal mucosal barrier [3]. The identification and use of such peptides have a great impact on the delivery of pharmaceuticals with poor GI absorption. To this end, we examined the possibility of using the phage display method to isolate peptides responsible for transmucosal transport of phage particles, which then can be used for facilitating the GI delivery of pharmaceuticals. Oral absorption of the M13-based library of peptide displaying phages was used in this study, and the results are discussed in view of the peptides' observed non-specific GI translocation.

2. Materials and methods

2.1. Materials

The Ph.D.-7 phage display peptide library kit, constructed based on M13 bacteriophage, was purchased from New England Biolabs (Berverly, MA, USA). The kit also includes the *Escherichia coli* host strain ER2738, a robust F+ strain with a rapid growth rate. The displayed peptides are fused at the N-terminus of the minor coat protein pIII, resulting in the display of five identical peptides per phage particle. The complexity of the library is $\sim 2.8 \times 10^9$ tranformants. 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), tetracycline, isopropyl-thio-β-D-galactoside (IPTG) and agar were from AppliChem (Darmstadt, Germany). Bacto-trypton, yeast extract and agarose were from Sigma–Aldrich. Polyethylene glycol (PEG) 8000 was obtained from Scharlau (Barcelona, Spain). The PCR purification kit was from Corebio, Korea. All other chemicals were of biological grade. The primer for sequencing was ordered from MWG and all samples for sequencing were also sent to MWG, Germany.

2.2. Treatment of animals and tissue preparation

Swiss albino male mice weighing 25 g were procured from Central Animal House Facility of Tabriz University of Medical Sciences and used throughout this study. Animals were housed in an air-conditioned room in polypropylene cages and had free access to a pellet diet and water ad libitum. The animals were kept at room temperature of 24 °C (±2 °C) and were exposed to alternate cycles of 12 h light and dark. All animal experiments were in accordance with the guide for the care and use of laboratory animals [DHEW Publication No. (NIH) 78-23, revised 1987]. Translocation of phages from the GI barrier of mouse was evaluated using a single-round panning protocol. We utilized total of two mice in this study. Each animal received about 2×10^{12} pfu of the phage library in 0.5 mL saline delivered by gavage. Blood samples were collected via orbital bleeds in heparinized tubes 1 h after phage administration. Subsequently, the animals were sacrificed by cervical dislocation and the spleen was immediately removed, washed in ice-cold saline (0.9% NaCl) and the extraneous material was removed. Whole spleen was homogenized in 2 mL PBS using a Potter S (B Braun Biotech International) glass:glass homogenizer on ice [16]. The homogenate was centrifuged and the supernatant was used for phage titration and colony isolation.

2.3. Phage titration

To provide an initial estimate of the number of phage, specimens were serially diluted up to five orders of magnitude using LB (Luria–Bertani) medium and subsequently bio-assayed according to the supplier's instructions. Briefly, 10 μl of diluted samples (blood serum or tissue extract) was added to 190 μl of cells of *E. coli* ER2738 grown overnight (on LB supplemented with 10 μg/ml of tetracycline). After brief vortexing, cells were incubated for 5 min and mixed with 3 ml of pre-melted (45 °C) 1% LB agarose. The mixture was immediately layered on the top of pre-warmed LB agar plate supplemented with 40 μg/ml of X-gal and 50 μg/ml of IPTG. Plates were incubated overnight at 37 °C. Blue plaques were counted and the phage number in each sample was estimated after considering the dilution factor.

2.4. Phage amplification and isolation

Overnight *E. coli* (ER2738) culture was diluted 1:100 in LB and then 3 mL of the diluted culture was dispensed into a fresh tube, one for each clone to be sequenced. Using a sterile pipette tip, a distinct plaque was transferred into the tube containing the diluted culture. The culture was incubated at 37 °C for 4.5 h while shaking. At this time the culture volume was increased to 50 mL by adding diluted culture. The incubation was continued for additional 1.5 h before removing the cells by centrifugation at 3000*g* for 20 min. Clarified supernatant was precipitated overnight at 4 °C with 1/6 the volume of 20% PEG 8000 in 2.5 M NaCl. Precipitated phage particles were harvested by centrifugation at 3000*g* at 4 °C for 30 min. The pellet was resuspended in 1.0 mL of Tris-buffered saline (TBS buffer; containing 50 mM Tris and 150 mM NaCl, pH 8.0) and re-precipitated overnight with 1/6 the volume of PEG at 4 °C. Phage particles were removed by centrifugation at 20,000*g* at 4 °C for 20 min. The pellet was resuspended in 200 μl of TBS. Before preparing phage DNA for sequencing a sub-sample was removed for titration.

2.5. DNA extraction and sequencing

The phage product as prepared above was used to extract and purify single stranded genomic DNA. An equal volume of a disruption buffer (containing 100 mM Tris, 10 mM EDTA, 2% SDS, 2% β-mercaptoethanol, pH 8.0) was added to phage suspension and incubated at 65 °C for 30 min. After cooling to room temperature, half the volume of 3.0 M potassium acetate, pH 5.5, was added and placed at −20 °C for 10 min. Precipitates were removed by centrifugation at 10,000*g* for 10 min. The supernatant was further precipitated with an equal volume of iso-propanol while kept at −20 °C for 1.0 h. The DNA pellet was recovered by centrifugation at 20,000*g* for 20 min. After rinsing with 70% ethanol and air-drying, the product was resuspended in 200 μl of sterile water. To prepare the template for sequencing, 100 μl of DNA product was further purified using a spin column containing a silica-based membrane (PCR purification kit, Corebio, Korea) according to the manufacturer's recommendations. The final product was eluted using 80 μl of pre-warmed Tris buffer. A 20 μl sample from this purified single stranded DNA was sent for sequencing using the reverse primer 5′-CCC TCA TAG TTA GCG TAA CG-3′.

3. Analysis of peptide sequences

The BLASTP server (http://www.ncbi.nlm.nih.gov/BLAST) was used to search for the homologous proteins to those peptide sequences identified in this study (Table 1). Secondary structures for the displayed sequences were predicted using GOR algorithm developed by Garnier et al. directly from the website at http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html [17]. Three commonly used hydrophobicity scales, namely Carugo [18], Kyte-Doolittle [19] and Abraham [20] scales, were used to calculate the hydrophobicity of the displayed sequences. The amino acid hydrophobicity values of Carugo's consensus scale have a zero

Table 1
Peptide sequences displayed on pIII coat proteins of the isolated phages from spleen (1–59) and blood (60–77) samples

1	AHVELMM	21	VLTTRLA	41	QSPAAQP	61	WHLPRPI
2	LTLAGAS	22	WSPLHNT	42	KLPPSFP	62	DVPAPKP
3	NVDGAVS	23	ALLDPTV	43	MTSHTSG	63	IRSPASY
4	IAPTNGP	24	YPGYFTK	44	LPKPWLN	64	DNRHSTP
5	AENITTK	25	YSKPSQM	45	GEVRTHA	65	SNYAVLL
6	QQYDPMH	26	YHYKTTS	46	GQFKLTK	66	HQNHNLS
7	ALNMAPH	27	HNFNVPF	47	AETVESC	67	AVYSPSR
8	NTSPVPK	28	SDNVKWN	48	STAGPVG	68	TLPTIFH
9	SFQLFHG	29	AATPSEG	49	NAEGVRL	69	AAGNRLP
10	TWPYYPN	30	FTSSPSP	50	SIRLPSP	70	SYTPYQP
11	SAFDNPY	31	TSTVTHV	51	NNSMPGP	71	NPPHLAS
12	TRLNIPP	32	AADNTSG	52	NTRLPVI	72	FTSQAPT
13	HSPPAMR	33	ASSLRSV	53	SKTDIPN	73	DSLTARL
14	FNTLNSI	34	SFKPAMH	54	KSPPLMQ	74	LPLHPVH
15	QLDTRLL	35	NDPWQFH	55	AQVDVTV	75	THLMSPI
16	LPLNPLL	36	TATDLSP	56	HPSSYWT	76	STVSSMR
17	MLPHPTP	37	MYTSPLS	57	FKMPLIS	77	DHLVTAP
18	AETVKVV	38	NMSGPLP	58	LQTNWYS		
19	STPRNSS	39	ADAITIG	59	RAPTPPF		
20	SPQQASA	40	HAYATFQ	60	APLLSSK		

mean and a standard deviation of unity. In order to be able to compare the results of hydrophobicity analyses by different methods, the amino acid hydrophobicity values of Kyte-Doolittle and Abraham-Leo were rescaled to also have a zero mean and unit standard deviation. Statistical analyses were performed by SPSS (SPSS for Windows, ver. 11.5) and Excel (Microsoft Office 2003) programs.

4. Results and discussion

One hour after administration of the M13 phage (2×10^{12} pfu) by gavage to the mouse, its passage through the intestinal barrier was evaluated by investigating its presence in the samples prepared from blood (~84,300 ± 5200 pfu/mL) and spleen (10,400 ± 808 pfu in total). (The results of our preliminary study indicated the recovery of M13 from blood 30 min after administration by gavage.) The spleen was chosen for analysis because of well-documented evidence for the non-specific accumulation of phage in this organ [3,21–23]. Obtaining evidence for phage particles in the blood and spleen of mice that had been fed phage indicates translocation of the phage particles from GI into the systemic circulation. This could be the result of either specific transporter mediated process, a totally non-specific event, or a combination of both the mechanisms. The sequences of peptides displayed on the pIII (minor coat protein encoded by gene 3 of M13 genome) coat protein of 77 different isolated clones of phages are listed in Table 1. Number of occurrences of different residue types within a given position for the total of 77 isolated clones and those for clones isolated from spleen and blood samples are shown in Table 2. The theoretically expected and observed frequencies of finding different residue types in the available positions are also shown in the same table. The expected frequency for a given residue type is the number of codons for that residue divided by 32, the total number of available codons for all residue types, represented as percentage. The observed frequency for a residue type is equal to its number of occurrences divided by the total number of residues present in all positions, also expressed as a percentage. The results of this study indicate that there are no appreciable similarities among the different peptides displayed on the isolated phages. The lack of similarity is also apparent when comparing the sequences for the phages isolated either from spleen or blood samples. This is evident from the sequences shown in Table 1. There are, however, a few residue types underrepresented which could be due to specific GI selection mechanisms and/or their effects on the amplification rate of phages bearing those residues. Performing the BLAST search as well as the PROSCAN pattern search on the obtained sequences resulted in identification of unrelated proteins, which do not share any commonality of sequence. The results of hydrophobicity analyses on the displayed sequences are given in Fig. 1. Three different hydrophobicity scales were used in this study. In Carugo's consensus scale, the amino acid hydrophobicity values have a zero mean and a standard deviation of unity, and so for the purpose of comparison the hydrophobicity values of Kyte-Doolittle and Abraham-Leo were also rescaled to have a zero mean and a standard deviation of unity. The hydrophobicity analyses using different scales are comparable (Fig. 1). The mean hydrophobicities for the residues found in each of positions I to VII are not different from each other, statistically (p-value > 0.05). The mean hydrophobicity values in different positions are also

Table 2
Number of occurrences, observed and expected frequencies of different residue types at different positions of the identified peptides displayed on pIII coat proteins of the isolated phages from blood and spleen samples

Amino Acid	Number of occurrences of amino acids (total, spleen, blood) in different positions of displayed peptides								Observed frequency (%) for total, spleen and blood	Expected frequency (%)
	I	II	III	IV	V	VI	VII	Sums for total, spleen, blood		
Ala	14,11, 3	9, 8, 1	2, 2, 0	5, 3, 2	9, 6, 3	3, 1, 2	3, 3, 0	45, 34, 11	8.2, 8.2, 8.7	9.4
Arg	1, 1, 0	2, 1, 1	3, 2, 1	2, 2, 0	5, 3, 2	2, 1, 1	3, 1, 2	18, 11, 7	3.3, 2.7, 5.6	9.4
Asn	8, 7, 1	5, 3, 2	4, 3, 1	6, 5, 1	5, 4, 1	1, 1, 0	4, 4, 0	33, 27, 6	6.0, 6.5, 4.8	3.1
Asp	4, 0, 4	3, 3, 0	3, 3, 0	6, 6, 0	0, 0, 0	0, 0, 0	0, 0, 0	16, 12, 4	2.9, 2.9, 3.2	3.1
Cys	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	1, 1, 0	1, 1, 0	0.2, 0.2, 0.0	3.1
Gln	3, 3, 0	5, 4, 1	2, 2, 0	2, 1, 1	1, 1, 0	3, 2, 1	2, 2, 0	18, 15, 3	3.3, 3.6, 2.4	6.2
Glu	0, 0, 0	4, 4, 0	1, 1, 0	1, 1, 0	1, 1, 0	1, 1, 0	0, 0, 0	8, 8, 0	1.5, 1.9, 0.0	3.1
Gly	2, 2, 0	0, 0, 0	2, 1, 1	4, 4, 0	1, 1, 0	2, 2, 0	6, 6, 0	17, 16, 1	3.1, 3.9, 0.8	6.2
His	5, 4, 1	5, 2, 3	0, 0, 0	6, 2, 4	1, 1, 0	3, 3, 0	6, 4, 2	26, 16, 10	4.8, 3.9, 7.9	3.1
Ile	2, 1, 1	1, 1, 0	0, 0, 0	2, 2, 0	3, 2, 1	2, 2, 0	4, 2, 2	14, 10, 4	2.6, 2.4, 3.2	3.1
Leu	5, 4, 1	7, 6, 1	10, 4, 6	7, 6, 1	6, 5, 1	9, 6, 3	5, 3, 2	49, 34, 15	9.0, 8.2, 11.9	9.4
Lys	2, 2, 0	2, 2, 0	3, 3, 0	2, 2, 0	2, 2, 0	1, 0, 1	5, 4, 1	17, 15, 2	3.1, 3.6, 1.6	3.1
Met	3, 3, 0	1, 1, 0	1, 1, 0	3, 2, 1	0, 0, 0	6, 5, 1	2, 2, 0	16, 14, 2	2.9, 3.4, 1.6	3.1
Phe	4, 3, 1	2, 2, 0	3, 3, 0	0, 0, 0	2, 2, 0	4, 3, 1	2, 2, 0	17, 15, 2	3.1, 3.6, 1.6	3.1
Pro	0, 0, 0	8, 5, 3	14,11, 3	12, 9, 3	15,12, 3	11, 8, 3	15,10, 5	75, 55, 20	13.7, 13.3, 15.9	6.2
Ser	12, 9, 3	8, 7, 1	9, 7, 2	5, 3, 2	7, 3, 4	13,10, 3	9, 7, 2	63, 46, 17	11.5, 11.1, 13.5	9.4
Thr	6, 4, 2	9, 7, 2	11,10, 1	6, 4, 2	9, 8, 1	8, 7, 1	3, 2, 1	52, 42, 10	9.5, 10.2, 7.9	6.2
Trp	2, 1, 1	1, 1, 0	0, 0, 0	1, 1, 0	2, 2, 0	2, 2, 0	0, 0, 0	8, 7, 1	1.5, 1.7, 0.8	3.1
Tyr	3, 3, 0	2, 1, 1	5, 3, 2	2, 2, 0	3, 2, 1	1, 1, 0	2, 1, 1	18, 13, 5	3.3, 3.1, 4.0	3.1
Val	1, 1, 0	3, 1, 2	4, 3, 1	5, 4, 1	5, 4, 1	5, 4, 1	5, 5, 0	28, 22, 6	5.1, 5.3, 4.8	6.2
Sum	77	77	77	77	77	77	77	539, 413, 126	98.7, 100, 100	102.7

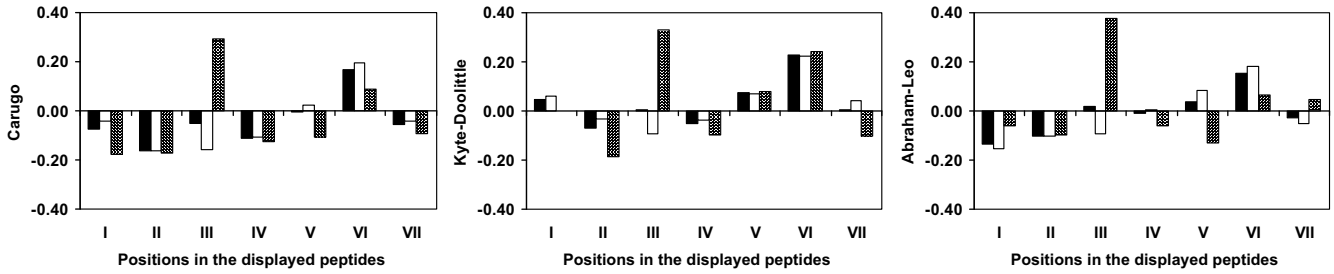

Fig. 1. Results of the hydrophobicity analyses on the displayed peptide sequences. The filled, white and gray rectangles represent the mean hydrophobicity values for the residues present in different positions (I–VII) of the displayed peptides at the N-terminus of pIII coat protein for total of 77 isolated phages, phages isolated from spleen (59 clones), and blood (18 clones) samples, respectively. The results indicate that the presence of residue types in different positions is totally random with no hydrophobicity preferences.

not statistically different from zero (p-value > 0.05). The average hydrophobicities of zero for the residues found in different positions are indicative of a random distribution of the residue types without any hydrophobicity preferences on these positions implied by the GI barrier on the translocated phages via their displayed sequences. The same analyses done separately on the sequences displayed by the phages isolated either from spleen or blood samples led to the similar results. There were also no statistically significant differences between the mean hydrophobicity values for the residues found in each position in the comparison between the spleen and blood samples. The results of the secondary structure prediction for the displayed sequences using GOR program revealed that they do not have the propensity to form regular secondary structures: they conform mostly to random coil or extended conformations. The N-terminal end of the major coat protein of M13 (i.e., pVIII) consists of mostly hydrophilic residues adopting a random coil conformation [24]. The secondary structure similarities between the displayed sequences and those of the aqueous-exposed N-terminal section of the major coat proteins of M13 may have a structural significance. As stated earlier, a study reported by Duerr et al. [3] suggested a novel model of macromolecular transport for phages bearing a specific peptide, and the authors further showed that M13 phage is unable to pass the intestinal mucosal barrier in rats [3]. There are, however, many reports that indicate the existence of GI translocation of different types of phage particles in mice and other animals [25,26]. In spite of a few similar peptides among the identified sequences in this study, it seems that the majority of the peptides are not related significantly and distribution of the residues in different positions of the displayed peptides was totally random. This may indicate that the translocation of the phage particles through the GI barrier does not depend on the displayed peptide and its sequence. This is in contrast to the finding of Duerr et al., who related the GI translocation of M13 phages to the displayed peptides [3]. They concluded that the sequence similarity of these peptides to different parts of the HIV gp120 protein is the main reason for their recognition by the same carrier system responsible for the translocation of the HIV viruses from the GI tract. This is, however, quite hard to believe as they draw their conclusions based on only seven isolated phage colonies. Large number of clones that we have isolated enabled us to perform a statistical analysis on the identified displayed sequences. Our sequence analysis did not result in any consensus. This is also true for the sequences obtained by Duerr et al., although it is not realistic to expect to see consensus based on such limited data unless few rounds of *in vivo* panning were used [3]. From a morphological point of view, it seems that the phage particles may translocate across the GI tract according to their size. Generally, filamentous phages such as M13 are approximately 6.5 nm in diameter and 930 nm in length which are comparable with nanoparticles [27]. It has been demonstrated that nanoparticles as big as 500 nm can translocate the GI tract with different mechanisms (Transcellular transport and paracellular transport) [28,29]. There are many reports where nanoparticle vehicles were used for the oral delivery of therapeutics, such as vaccines, peptides, proteins and anticancer drugs to mention a few [30–32]. There is also evidence that shows that phages can pass the intestinal barrier and enter the peripheral blood [26] and accumulate non-specifically in some organs such as in the spleen [21,22]. The random nature of amino acid distribution of the displayed peptides in the isolated phages in our study indicates that the GI translocation of these M13 phages is primarily related to their intrinsic capability to do so. Even if there are specific transport systems facilitating the translocation of some of the isolated phages via a specific interaction with the displayed peptides, it is not at all clear if this is a major route. However, our results cannot rule out the possibility of existence of such transport systems completely. It seems that the presence of a random heptapeptide at the N-terminus of the pIII coat proteins of M13 phage do not affect its penetration through the GI barrier. In a study by Schubber et al., M13 DNA was found in the blood samples of mice fed by wild type M13 [33]. In contrast to the findings by Schubber et al. [33], and our present study, Duerr and his coworkers demonstrated that wild type M13 phages cannot pass the GI in rat [3]. These controversial results may be explained in light of interspecies differences between mouse and rat.

Although we were not successful in isolating specific peptides which could have been used as oral drug delivery vehicles, the results demonstrate that M13 can pass the GI barrier and therefore can be used as a delivery system, e.g., by linking a drug to its coat proteins and delivering genes for the cytotoxic proteins. However, one of the important points that needs to be considered when the phages are being used as the pharmaceuticals is their complex and unique pharmacokinetic behavior [34,35].

In summary, we have demonstrated that M13 phages can cross the intestinal barrier after oral administration, enter the blood stream and accumulate in a tissue like spleen. This finding, in conjunction with the lack of any consensus in the variable part of the large number of isolated phages (i.e., the displayed peptide region), may lead us to the conclusion that the translocation of the phage particles in mice is not, in the most part, a specific process mediated via a specific carrier(s) which recognizes a particular sequence. The most probable mechanisms for the observed translocation of phages may be those involved in the translocation of viruses, bacteria and nanoparticles.

Acknowledgements

The authors thank the Research Office of Tabriz University of Medical Sciences for providing financial support. Thanks go to Bret Church for critical reading of the manuscript.

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