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A peptide mimetic of Gal-α1,3-Gal is able to block human natural antibodies

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Abstract

The carbohydrate of Gal-α1,3-Gal is thought to be the major antigenic epitope present on pig vascular endothelium. The peptides that mimic the binding of antigenic epitope (Gal-α1,3-Gal) to lectin BS-I-B4 were identified from screening a filamentous phage-displayed random library. A phage bearing the peptide NCVSPYWCEPLAPSARA has been identified to bind the lectin strongly. Melibiose was able to inhibit the binding of the human natural anti-αGal antibody to the peptide competitively. Our experiments show that the peptide mimetic of Gal-α1,3-Gal is able to inhibit the agglutination of pig RBCs by human natural antibody or lectin BS-I-B4. The peptide inhibitor of human natural antibodies may prove useful in pig-to-human xenotransplantation.

Keywords: Peptide mimetics; Natural antibodies; Gal-α1,3-Gal; Xenotransplantation

The primary barrier of pig-to-human xenotransplantation is hyperacute rejection (HAR), which is believed to be initiated by the binding of human natural anti-α-galactosyl antibodies to the αGal epitope on the pig vascular endothelium [1,2]. In vitro evidence indicates that HAR may be prevented by blocking the interaction of human natural anti-αGal antibodies with antigenic epitope Gal-α1,3-Gal present on pig tissues [3]. In vivo experiments also support this conclusion. The cytotoxicity of baboon serum to pig kidney PK15 cells can be reduced through intravenous infusion of melibiose(Gal-α1,6-Glc) and arabinogalactan (a glycan terminating with αGal) [4]. However, these carbohydrate infusions had toxic effects on the kidneys and lungs of the recipient baboon [4]. Given the high cost and complexity of carbohydrate synthesis, the prospect of finding peptides that are able to block or remove human natural antibodies may simplify the development of potent therapeutic agents.

Random peptides displayed on the phage surface offer a rich source of molecular diversity in search for ligand peptides that bind to an antibody, receptor or other binding proteins [5]. We attempted to identify peptides using a phage-displayed library XCX15. A peptide was identified that appears to bind the lectin BS-I-B4 at the binding site of antigenic epitope Gal-α1,3-Gal. Our experiments have demonstrated that the peptide mimetic can block the human natural antibody-mediated agglutination of pig RBCs.

Materials and methods

Materials. A phage-displayed random peptide library (XCX15) and *Escherichia coli* Kank91 were kindly provided by Scott and Smith [5]. *Bandeiraea simplicifolia* isolectin B4 (BS-I-B4) and melibiose were obtained from Sigma. Synthetic peptide was obtained from Chinese Peptide Company, Hangzhou, China. Rabbit anti-M13 antibody and anti-M13-HRP were made by Dr. Ke-yi Wang's laboratory (Shanghai Institute of Biochemistry, China). Human sera for in vitro studies were obtained from healthy donors at Sir Run-Run Shaw Hospital of Zhejiang Province, China. All other chemicals were obtained from Sigma.

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Selection of lectin-binding phage (biopanning). Streptavidin (10 μg/ml) was coated on microtiter wells at 4 °C overnight. Wells were saturated with 5% BSA for 2 h and washed with 0.2% TBST [Tris-buffered saline (TBS) supplemented with 0.1% Tween 20 (TBST)]. The biotinylated BS-I-B4 was added and reacted for 1 h before panning. In the first panning, the phage library was incubated at room temperature for 1 h with slow shaking. Nonbinding phages were removed by extensive washing with 0.2% TBST. The bound phages were eluted with 100 mg/ml of melibiose. Phages were amplified in *E. coli* (Kank91). Affinity selection was repeated for three times and individual phages were isolated from each round of amplification [5,6].

Micropanning to determine the specificity of target phages. In order to test the melibiose inhibition of peptide binding to BS-I-B4, different concentrations of melibiose were mixed with the phage solution before the phage was added into the lectin-coated wells. All other procedures were same as the above method.

Detection of binding of peptide to human anti-αGal antibodies by ELISA. A flat-bottomed microtiter plate was coated for 48 h at 4 °C with 100 μl of the synthesized peptide. Wells were then saturated with 5% BSA and then washed with PBS (pH 7.4) three times. 50 μl of 1% heat-inactivated human serum (containing natural anti-αGal antibodies) and 50 μl of different concentrations of melibiose were added into each well and incubated for 1 h. The wells were washed with 0.2% TPBS (PBS containing 0.2% Tween 20) and PBS to remove unbound antibodies, and then protein A-HRP was added into each well and incubated at room temperature for 30 min. Unbound rabbit anti-M13-HRP was removed by washing with 0.2% TPBS and PBS eight times. The substrate 3,3′, 5,5′-tetramethylbenzidine (TMB) was used for development and the reaction was carried on for 30 min and then terminated by adding 100 μl of 1 M H_3PO_4. Solute optical density in each well was measured at 450 nm by a microplate reader.

Inhibition assay of pig RBC agglutination. Freshly collected pig RBCs (3%) were washed and resuspended in PBS. The assay was carried out on an agglutination plate. The system of agglutination reaction included 40 μl of peptide with different dilutions in PBS, 40 μl of heat-inactivated human serum (containing natural anti-αGal antibodies) and 40 μl of 3% pig RBCs. The reaction complex was mixed by gently shaking for minutes and then left for 1 h to observe the agglutination of pig RBCs. To test the peptide inhibition of pig RBC agglutination by BS-I-B4, 40 μl of 5.0 μg/ml BS-I-B4 was added into the reaction system to replace the human serum. Agglutination was visible as homological distribution of RBC clumps on bottom of the well. In contrast, RBCs were precipitated as a small dot on the bottom indicated no agglutination. Agglutination was also verified as clumps of RBCs under microscope while the plate was gently agitated, and in the case of no agglutination, the RBCs were homogeneously suspended in the media [7].

Sequencing of the phage DNA. The random peptide portions of a population of biopanned phage were sequenced to identify consensus motifs. The single-stranded phage DNA was extracted and purified according to Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, NY, 1982). The primer 5′-CTGAAGAGAGTCAAAAGC-3′ was used for sequencing. Peptide sequences were then deduced by DNA sequencing.

Results

Affinity purification of target phage clones

The phage-displayed random peptide library XCX15, consisting of approximately 10^9 independent phage recombinants, was screened in three cycles of panning, elution, and amplification against lectin BS-I-B4 on microtiter plates. The 96-well plate was washed with 0.1% TBST to remove the unbound phage in the three rounds. The result is indicated in Fig. 1. The enrichment of positive phage clones was observed. The yield of the third round was approximately 20 times is more than that of the first round.

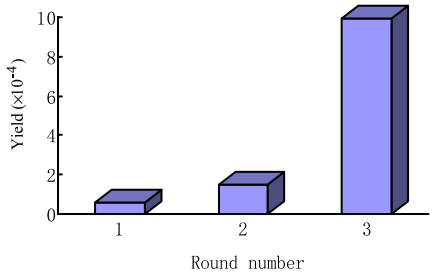

Fig. 1. Enrichment of positive phage clones after three rounds of biopanning with BS-I-B4. Yield was calculated as the total number of phage clones following elution (output) divided by the number of input phage clones.

After biopanning, 18 clones were randomly picked and screened. On four out of them was performed the ELISA again to compare the binding activity (Fig. 2). All clones selected were able to bind the lectin BS-I-B4. Among them, clone 2 and clone 3 showed stronger binding abilities than clone1 and clone 4, and therefore were chosen for the competitive binding assay with melibiose.

The binding to BS-I-B4 of phage 2 and phage 3 was completely inhibited by melibiose at the concentration of 50 mg/ml (Fig. 3). This result suggested that phage 2

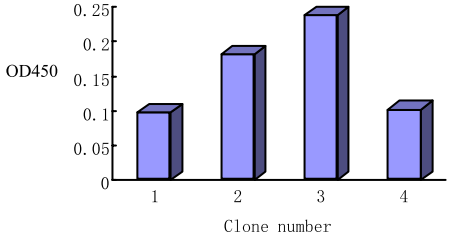

Fig. 2. Identification of phage clones by phage binding ELISA. Optical density at 450 nm (OD450) was calculated as average of two duplicates.

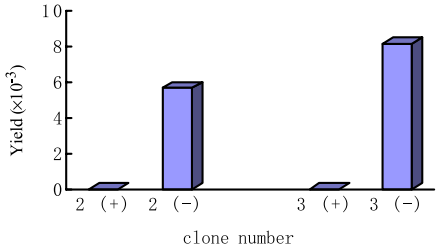

Fig. 3. Melibiose inhibition of peptide binding of BS-I-B4. Yield was calculated as the total number of output phage clones divided by the number of input phage clones. (+) and (−) indicate that phages were eluted in the presence of melibiose (50 mg/ml) and in the absence of melibiose.

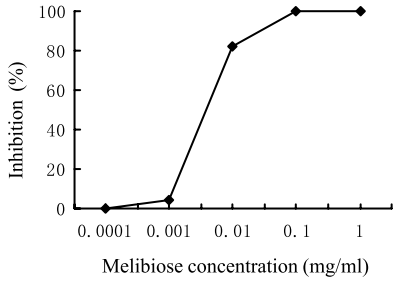

Fig. 4. Melibiose competitive inhibition of peptide binding of human anti-αGal antibodies. Inhibition ratio (%) was calculated as (OD450 in presence of melibiose minus OD450 of control) divided by OD450 of control.

and phage 3 were bound to BS-1-B4 at the melibiose binding site.

Sequencing of the peptide

The reverse primer 5′-CTGAAGAGAGTCAAAAG C-3′ was used for sequencing the single-stranded phage f88.4. The common sequence of phages was AAT TGT GTG TCT CCG TAT TGG TGT GAG CCG CTT GCT CCG TCT GTG AGG GCG, which encodes the corresponding sequence of a 17-amino acid peptide Asn Cys Val Ser Pro Tyr Trp Cys Glu Pro Leu Ala Pro Ser Ala Arg Ala.

Inhibition of binding of antibody to peptide by melibiose

The binding to human natural anti-αGal antibodies of the synthetic peptide was competitively inhibited by melibiose at the concentration range of 0.0001–1.0 mg/ml. The 50% inhibition concentration of melibiose (IC50) was approximately 0.005 mg/ml (Fig. 4). This result suggested that the peptide mimetic was specifically bound to BS-I-B4 at the melibiose binding site.

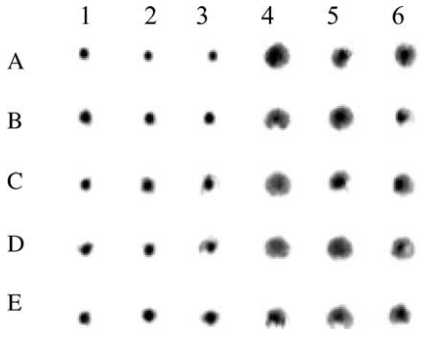

Fig. 5. The inhibition of agglutination of pig RBCs by human serum with different concentrations of the peptide and melibiose. A1–3: PBS control, no agglutination; A4–6: positive control, agglutination; B1–6 and C1–6: concentration range of melibiose from 1.563 to 0.049 mM/L with twice dilution one by one. Agglutination in previous 3 wells was inhibited. D1–6 and E1–6: the peptide concentration was 7.126×10^{-3}, 3.563×10^{-3}, 1.782×10^{-3}, 0.891×10^{-3}, 0.446×10^{-3}, and 0.223×10^{-3} mM/L, respectively. RBC agglutination in previous 3 wells was inhibited.

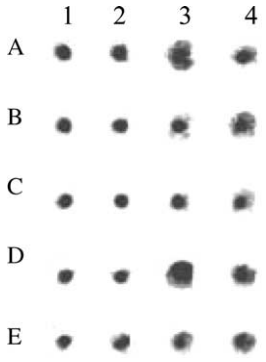

Fig. 6. The inhibition of agglutination of pig RBCs by BS-I-B4 with different concentration of melibiose, and the peptide mimetic. A1–2: PBS control, no agglutination A3–4: positive control, agglutination B1–4 and C1–4: concentration of melibiose was 1.563, 0.391, 0.098, and 0.029 mM/L, respectively, in each well. Agglutination in previous 2 wells was inhibited. D1–4 and E1–4: peptide concentration was 0.214, 0.054, 0.014, 0.004 mM/L, respectively. RBC agglutination in previous 2 wells was inhibited.

RBC agglutination assay

The utility of the peptide in binding xenoreactive antibodies was demonstrated by the pig RBC agglutination assay [7]. The assay consisted of addition of heat-inactivated human serum or BS-I-B4 with varying concentrations of melibiose, the synthetic peptide, and pig RBCs. After incubation for 1 h at room temperature, agglutination was assessed by gross and microscopic examination. The result has showed that the peptide can block the natural antibody-mediated or lectin-mediated agglutination of pig RBCs (Figs. 5 and 6).

Discussion

Millions of short peptides can be easily surveyed for binding to an antibody, receptor or other binding protein using a phage-displayed peptide library. The library is a large mixture of filamentous phage clones, each displaying one peptide sequence on the virion surface. The affinity-purified phages that display tight-binding peptides are able to propagate in *E. coli*. The sequences of the peptides displayed on the surface protein of phage are then determined by sequencing the specific coding region in the viral DNAs. The phage-display system has been effective in the discovery of various ligands [8], including sugar-mimicking peptides [9]. A hexapeptide mimetic of Gal-α1,3-Gal was found by screening the hexapeptide library with the lectin BS-I-B4, which can block the binding of BS-I-B4 to pig aortic endothelial cells [7].

In this article, we report the identification of a 17-amino acid peptide, NCVSPYWCEPLAPSARA, that binds to lectin BS-I-B4 at the same binding site of Gal-α1,3-Gal. This was demonstrated in several assays. In the first assay, we have demonstrated that melibiose can inhibit the binding of the peptide to BS-I-B4. Since the lectin can specifically recognize and bind to Gal-α1,3-Gal, the peptide acted as a mimetic. If it is true, the peptide should also bind to human natural antibodies. This has been proved in the second assay by analyzing the capability of the peptide to block human natural antibody-mediated agglutination of pig RBCs. In order to verify that the peptide was a conformational mimetic of Gal-α1,3-Gal, we showed the peptide was also able to block the pig RBC agglutination by BS-I-B4, which has already been shown to bind the structure of Gal-α1,3-Gal [7].

The peptide motif is significantly different from the hexapeptide SSLRGF reported by Kooyman et al. [7]. The strategy to prevent HAR by removing or blocking human natural anti-αGal antibodies has been proven effective [10]. Removal of human natural antibodies prolongs discordant xenograft survival and has been performed in several ways such as plasmapheresis, removal by perfusion through pig organs [11], or by binding to glycans with an αGal terminal residue [12]. In previous experiments, it has been demonstrated that in vivo intravenous infusion of carbohydrates terminating with αGal was effective at eliminating or reducing the cytotoxicity of baboon serum to pig kidney cells. However, these commercially inexpensive carbohydrates will prove not optimal for clinical usage due to their low affinity of human natural antibodies and their toxic effects on the kidneys and lungs [4]. Identification of a peptide that blocks human natural antibodies provides several applications. First, the peptide can be attached to a matrix and employed as a column to remove human natural antibodies specifically. Attachment of peptides to various matrices is much easy and inexpensive. Second, the peptide and its conjugate might be directly used in vivo as a blocking agent or serve as a guide in designing new therapeutic agents.

Acknowledgments

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