Isolation of peptides useful for differential diagnosis of Crohn’s disease and ulcerative colitis

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Background: Phage displayed random peptide technology has been utilised to identify binding epitopes of antibodies or receptor ligands.

Aim: To isolate peptides from a phage library which are specifically recognised by antibodies in serum from patients with Crohn’s disease (CD).

Methods: A phage displayed random peptide library composed of nine amino acids was established and sequentially screened using serum immunoglobulin G obtained from CD patients.

Results: Five different CD specific peptides were isolated from the phage library. No homology in amino acid sequences was observed among four (CDP-1, -3 to -5) of the five peptides exhibiting different binding characteristics with each CD patient’s serum. In contrast, two peptides (CDP-1 and -2) had similar amino acid sequences and similar binding characteristics. Four multiple antigenic peptides (MAP, CDP-1, -3 to -5) were synthesised, and an enzyme linked immunosorbent assay (ELISA) using the four peptides was developed to detect serum antibodies against them. Fifty two of 92 CD patients (56.5%) were detected by ELISA, none of 20 ulcerative colitis (UC) patients, only one of 25 duodenal ulcer patients, and only three of 48 healthy subjects.

Conclusions: ELISA using the four peptides isolated in this study may be useful for the differential diagnosis of CD and UC.

Inflammatory bowel disease (IBD) is characterised by chronic gastrointestinal tract inflammation of unknown aetiology. Immunological, environmental, infectious, and genetic factors have been postulated to increase the risk for the development of IBD. Signs and symptoms of IBD are often non-specific and it is often a clinical challenge to distinguish IBD from other disorders of childhood and adolescence, such as chronic diarrhoea, recurrent abdominal pain, and acute infectious colitis. Currently, the diagnosis of IBD requires a combination of typical clinical signs and symptoms, exclusion of other diseases, in addition to radiographic, endoscopic, and histological features consistent with IBD. Despite careful clinical evaluation, it has been reported that 10–15% of children with colitis have an indeterminate form and 2–4% of patients undergoing colectomy for the treatment of ulcerative colitis (UC) are ultimately determined to have Crohn’s disease (CD). Thus specific serological markers which could accurately distinguish CD from UC would be clinically useful.

Recently, several candidate serological tests have been described for patients with IBD, including those with anti-Saccharomyces cerevisiae antibodies (ASCA), perinuclear antineutrophil cytoplasmic antibodies (pANCA), antiperinuclear antibodies, antineutrophilic antibodies, antibacterial/permeability increasing protein antibodies, and anti-p40 antibodies. ASCA and pANCA have been studied most extensively. However, the sensitivity and specificity of ASCA and pANCA have been reported to be insufficient, especially for differentiation between CD and UC.

Phage displayed random peptide libraries have been widely used to select peptides that bind to target molecules such as antibodies and receptors. This approach is very useful for identifying ligands for disease specific antibodies as it requires only a phage displayed random peptide library, serum from patients with a target disease, and serum from normal individuals or patients to be differentiated. Thus it is particularly suitable for the study of autoimmune diseases whose aetiopathological antigens are largely unknown. In fact, several studies have been performed using phage displayed peptide libraries for autoimmune diseases such as rheumatic arthritis, type 1 diabetes, and autoimmune thrombocytopenia.

We suspected that it may be possible to develop a specific serological test method for the differential diagnosis of CD and UC by selecting peptides from a phage displayed random peptide library using serum from CD patients and those from UC patients. In this study, we used this strategy to identify peptides from a nonapeptide phage library recognised by serum antibodies from CD patients but not those from UC patients.

MATERIALS AND METHODS

Reagents

To screen for CD specific peptides and detect antibodies to multiple antigenic peptides (MAPs), the following substances and reagents were prepared. Phagemid vector (pTV119N) was purchased from Takara Suyou (Shiga, Japan). Ampicillin, isopropyl-β-D-thiogalactopyranoside (IPTG), and kanamycin were from Wako Pure Chemical (Osaka, Japan). Oligonucleotides were synthesised by Amersham Pharmacia Biotech (Tokyo, Japan). Anti-human immunoglobulin G (IgG) was from Biodesign (Kennebunk, Maine, USA). Bovine serum albumin, horseradish peroxidase, and anti-human immunoglobulin G were purchased from Sigma (St Louis, Missouri). Rabbit polyclonal antibodies to human MAP were purchased from Biodesign (Kennebunk, Maine, USA).

Abbreviations: CD, Crohn’s disease; UC, ulcerative colitis; DU, duodenal ulcer; IBD, inflammatory bowel disease; pANCA, perinuclear antineutrophil cytoplasmic antibodies; ASCA, anti-Saccharomyces cerevisiae antibodies; EUSA, enzyme linked immunosorbent assay; HRP, horseradish peroxidase; MAP, multiple antigenic peptide; IPTG, isopropyl-β-D-thiogalactopyranoside; BSA, bovine serum albumin; D-PBS, Dulbecco’s phosphate buffered saline; LD, lura-Bertani; TMB, 3′,5′-tetramethylbenzidine; OD, optical density; ROC, receiver operating characteristic.
albumin (BSA) was from Seikagaku Kogyo (Tokyo, Japan). Casein was from Calbiochem (La Jolla, California, USA). Anti-M13 phage monoclonal antibody was from Amersham Pharmacia Biotech (Buckinghamshire, UK). Magnetic beads (tosylactivated Dynabeads M-450) were from Dynal (Oslo, Norway). Ninety six well microtitre plates for affinity selection and Phage ELISA (Immunoplate Maxisorp) were from Nalge Nunc International (Rochester, New York, USA) and those for MAP enzyme linked immunosorbent assay (ELISA) (Coster) were from Corning Incorporated (Corning, New York, USA).

**Immunoglobulin immobilised magnetic beads or microtitre plates for affinity selection**

Magnetic beads immobilised with antihuman IgG were prepared according to a standard method. Monoclonal antibodies were purchased from Corning Incorporated (Corning, New York, USA). Immunoglobulin immobilised magnetic beads or microtitre plates were from Nunc International (Rochester, New York, USA) and those for MAP enzyme linked immunosorbent assay (ELISA) (Coster) were from Corning Incorporated (Corning, New York, USA).

**Serum samples**

To isolate CD specific peptide displayed phages, 20 serum samples from CD patients, 20 samples from UC patients, and 20 samples from asymptomatic healthy subjects were collected. After isolation and development of an ELISA using the isolated peptides, an additional 72 CD samples, 25 duodenal ulcer (DU) samples, and 28 asymptomatic healthy samples were collected to evaluate the clinical usefulness of the ELISA. The diagnoses of CD, UC, and DU were made based on results of clinical, radiological, histological, and endoscopic examinations at the Department of Internal Medicine, Division of Gastroenterology, Hyogo College of Medicine. In addition, CD patients were divided into three groups based on the principal location of inflammation: a colon CD group (n=11) including nine patients with a small inflammatory area in the small bowel, a small bowel CD group (n=32) including 31 patients with a small inflammatory area in the colon, and a colon/small bowel CD group (n=49) with large inflammatory areas in both the colon and small bowel.

**Random peptide displayed phage library**

In order to establish a phage library displaying random nonamer peptides fused with a major coat protein (pVIII) of the filamentous M13 phage, a pVIII protein displayed phagemid vector was constructed according to a standard method, and random 27-mer oligonucleotides were inserted into the near N terminal portion of the pVIII gene by converting the SacI and BamHI sites with a site directed mutagenesis method. The polymerase chain reaction template for random 27-mer oligonucleotides was 5'-CTGCTGAGGTCGGTCTGGCTC (NNK), GGCGATCCGGCAAACGCCTTT3'. The N in the template represents any nucleotide while K represents either G or T. The random oligo DNA library was transformed to Escherichia coli JM109 competent cells by electroporation and infected transformed E. coli JM109 cells together with M13KO7 helper phage to establish a phage displayed random peptide library. The phage library was incubated with magnetic beads immobilised with IgGs of UC patients to exclude phages associated with UC (pre-absorbed phages).

**Affinity selection using patient serum**

Pooled serum samples (30 µl) from three randomly selected CD patients (pooled serum A and B) were incubated with 2x10⁸ anti-hu-IgG magnetic beads overnight at 4°C. After washing 10 times with panning buffer (Dulbecco’s phosphate buffered saline (D-PBS) containing 0.1% BSA and 0.1% Tween 20), the magnetic beads were incubated with 1x10⁹ pre-absorbed phage particles overnight at 4°C and washed. The phages bound to the magnetic beads were eluted with phage elution buffer (100 mM hydrochloric acid adjusted to pH 2.2 with glycine, containing 1% BSA and 0.1% phenol red) and then neutralised with 1 M Tris (hydroxymethyl) aminomethane chloride buffer (pH 9.1). E. coli JM109 was prepared and infected with the phages eluted. The infected E. coli was then superinfected with M13KO7 helper phage and incubated in Luria-Bertani (LB) media containing 150 µg/ml of ampicillin, 100 µg/ml of kanamycin, and 0.1 mM IPTG overnight at 37°C with vigorous shaking to amplify the eluted phages (first panning). After amplifying the eluted phages, the phages were panned again with the same pooled serum samples or individual serum samples immobilised on anti-hu-IgG plates (second panning). After washing, the phages bound to the plate were eluted with the phage elution buffer and infected E. coli JM109. Cells were plated on an LB agar plate containing 150 µg/ml of ampicillin and 1% of glucose and incubated overnight at 37°C. Two hundred colonies were randomly picked up from each affinity selection and incubated independently for three hours at 37°C in LB media containing 150 µg/ml ampicillin with vigorous shaking. Cells were infected with M13KO7 helper phage in LB media containing 150 µg/ml ampicillin, and sequentially mixed with 100 µg/ml kanamycin and 0.1 mM IPTG. After overnight incubation at 37°C with vigorous shaking, the immunoreactivity of each amplified phage solution was evaluated by ELISA (phage ELISA).

**Phage ELISA**

Ninety six well microtitre plates were coated with anti-M13 phage monoclonal antibody. Each phage solution (10 µl) and 90 µl of phage ELISA buffer (D-PBS containing 0.5% BSA, 0.05% Tween 20, and 10% normal goat serum) were added to each well of the plates and incubated for one hour at 37°C. After washing four times with PBST (D-PBS containing 0.05% Tween 20), 100 µl of serum samples diluted 1:100 in Phage ELISA buffer were added and incubated for one hour at 37°C. After washing with PBST, 100 µl of horseradish peroxidase (HRP) conjugated goat antihuman IgG (in house) diluted 1:20000 was added and incubated for one hour at 37°C. After washing, 100 µl of substrate solution containing 3,3',5,5'-tetramethylbenzidine (TMB) were added and incubated at room temperature for 10 minutes for colour development. Colour development was stopped by adding 100 µl of 1 N sulphuric acid. Optical density (OD) was measured at 450 nm.

**Screening of target phage clone**

Two hundred phage clones from each affinity selection were screened by phage ELISA with the serum sample from a CD patient used for second panning (positive screening). The phage clones selected by positive screening were then screened by phage ELISA using the pooled serum from 10 randomly selected healthy individuals (negative screening). The phage clones selected by negative screening were assayed by phage ELISA using serum samples from 20 CD patients, 20 UC patients, and 20 healthy subjects. The phage clones which exhibited a positive reaction with several CD samples but no reaction to the UC samples and the healthy samples (phagotopes) were finally selected (final screening).

The DNA sequences of the phagotopes selected by final screening were determined by the dideoxynucleotide chain termination method. The deduced DNA sequences were converted to amino acid sequences.

**MAP synthesis**

Multiple antigenic peptides (MAPs) were synthesised according to the Fmoc strategy starting from MAP (8) wing resin (Watanabe Chemical, Hiroshima, Japan) using an ACT-357 peptide synthesiser (Advanced ChemTech, Tokyo, Japan). Confirmation of each MAP synthesis was performed by amino acid analysis after hydrolysis.
Table 1  Amino acid sequences of specific peptides selected by binding to antibodies from patients with Crohn’s disease

<table>
<thead>
<tr>
<th>Clone</th>
<th>pVIII sequence</th>
<th>Random region</th>
<th>pVIII sequence</th>
<th>Serum used for panning</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDP-1</td>
<td>AEGEL</td>
<td>GLVQGMDY</td>
<td>ADPA</td>
<td>Pooled serum A</td>
</tr>
<tr>
<td>CDP-2</td>
<td>AEGEL</td>
<td>RLVVQQVMQ</td>
<td>GDPA</td>
<td>Pooled serum A</td>
</tr>
<tr>
<td>CDP-3</td>
<td>AEGEL</td>
<td>GGYQQOLVS</td>
<td>GDPA</td>
<td>Pooled serum B</td>
</tr>
<tr>
<td>CDP-4</td>
<td>AEGEL</td>
<td>YWYFPPSSA</td>
<td>GDPA</td>
<td>Pooled serum A</td>
</tr>
<tr>
<td>CDP-5</td>
<td>AEGEL</td>
<td>RGSDGVQAM</td>
<td>GDPA</td>
<td>Pooled serum A</td>
</tr>
</tbody>
</table>

The amino acid sequences are inserted on the common motif of pVIII protein. The nucleotide of the clone CDP-1 contains a single nucleotide exchange (GGG to GCG) in the position corresponding to the Gly, resulting in a Gly>Ala substitution in the amino acid sequence.

RESULTS

Selection of phagotopes

To isolate CD specific phagotopes, serum samples from 20 CD patients, 20 UC patients, and 20 healthy subjects were collected. Six CD samples were randomly selected and two pooled serum samples (pooled serum A and B) were prepared, each by mixing of three of these samples. In the affinity selection using pooled serum A and B, respectively, two phagotopes (CDP-1 and -2) and one phagotope (CDP-3) were isolated. When the reactivity of the remaining 14 CD patient samples not selected for preparation of the pooled serum samples was examined using the three phagotopes, eight CD samples exhibited no reactivity to the phagotopes. Additional phage selections were then performed by combined use of pooled serum A and the eight individual samples (C-J). As a result, two additional phagotopes (CDP-4 and -5) were isolated from the phage library with combined use of pooled serum A and two individual samples (serum C and E) (table 1, fig 1).

Each phagotope isolated exhibited positive reactions for only some of the 20 CD patients, but no positive reactions were observed for the 20 UC patients or 20 healthy subjects on phage ELISA (fig 1). These results suggest that the antibodies detected using the phagotopes isolated are strongly associated with CD. Similar immunoreactivity was observed between CDP-1 and CDP-2 but the remaining phagotopes exhibited different characteristics related to reactivity (fig 1). The amino acid sequences of the peptides displayed on the five phagotopes are shown in table 1. CDP-1 and CDP-2 isolated from the same combination of serum samples as used for affinity selection exhibited sequence similarity, with three identical amino acids and two related amino acids. In contrast, the amino acid sequences of the remaining three peptides (CDP-3, -4, and -5) exhibited no homology among themselves.

MAP ELISA

MAP mixture (mixture of CDP-1, -3 to -5) was coated on wells of microtitre plates. Serum samples (100 µl) diluted 1:100 with MAP ELISA buffer (100 mM Tris (hydroxymethyl) amino-methane chloride buffer (pH 8.0) containing 0.5 M sodium chloride, 1.5% casein, 0.2% Tween 20, and 2% normal goat serum) were incubated on MAP plates for one hour at 25°C with shaking. For quantitative assay using the MAP mixture (cocktail MAP ELISA), a series of dilutions of the calibrant prepared from positive serum samples (300, 100, 50, 25, and 0 units/ml, in arbitrary units) were also incubated in each plate. After washing, plates were incubated with HRP conjugated goat antihuman IgG. After washing, 100 µl of substrate solution containing TMB was added and incubated at room temperature for 15 minutes for colour development. Colour development was stopped by adding 100 µl of 1 N sulphuric acid. OD was measured at 450 nm. For cocktail MAP ELISA, antibody titres of samples were calculated using a calibration curve prepared by OD of calibrants measured at the same time. Samples calculated to be over 300 unit/ml were further diluted and assayed to obtain actual antibody levels.

ASCA ELISA

ASCA in serum samples were measured using a commercially available ELISA kit (Medipan Diagnostica, Selchow, Germany) according to the manufacturer’s instructions.

Figure 1  Immunoreactivity of each Crohn’s disease (CD) associated phage to sera from each CD patient (n=20), ulcerative colitis (UC) patient (n=20), and healthy subject (n=20) in phage ELISA. Reactivity (black bars) was expressed as actual optical density (OD) at 450 nm.

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and none with CDP-1 (-2). Because there were no relationships among the four peptides (CDP-1, -3, -4, and -5), higher sensitivity was expected when the four MAPs were used as cocktail antigens. Therefore, the four peptides were synthesised as MAPs and a cocktail MAP ELISA was developed using plates coated with a mixture of the four MAPs.

**Cocktail MAP assay**

Cocktail MAP ELISA was performed on 92 CD samples, including the 20 samples used for affinity selection, 20 UC patients used for phage ELISA, 25 DU patients, and 48 healthy subjects, including the 20 samples used for phage ELISA. A typical standard curve and actual OD of the calibrants, including blank calibrant, are shown in fig 2. The receiver operating characteristic (ROC) curve for the cocktail MAP assay and ASCA assay obtained by calculation using 92 CD samples and 48 healthy samples are shown in fig 3. A better ROC curve was obtained with the cocktail MAP assay than with the ASCA assay (fig 4B).

Among the three groups classified by location of inflammation in CD (colon CD group, small bowel CD group, and colon/ small bowel CD group), no significant differences were observed in cocktail MAP ELISA (fig 5). In a comparison between the colon CD group and UC patients, a small but significant difference \( (p=0.0004) \) was observed between the two. In addition, one of the two CD patients who had inflammation only in the colon exhibited very high antibody titres on cocktail MAP ELISA \( (1019.3 \text{ unit/ml}) \). These results suggest that the cocktail MAP ELISA is capable of detecting antibodies which are associated with CD both in the colon and small bowel.

In a comparison of the results of ASCA ELISA and cocktail MAP ELISA for CD patients, no correlation \( (r=0.050, p=0.6362 \text{ by Fisher’s } r \text{ to } z \text{ transformation test}) \) was observed (fig 6). Among the 92 CD patients, there were 17 double positive patients, 11 patients single positive for ASCA ELISA, 35 patients single positive for cocktail MAP ELISA, and 29 double negative patients. Because there was no correlation between the results of the two ELISAs, sensitivity was increased to 68.5% when the two results were combined.

**DISCUSSION**

The diagnostic tests currently used to diagnose IBD and to differentiate CD from UC are invasive and expensive. Despite the use of such invasive and expensive diagnostic methods, some colitis patients cannot be easily classified as having CD or UC at the initial diagnosis. Non-invasive tests are thus needed for timely and accurate diagnosis of UC and CD. Several candidate serological tests, including ASCA and pANCA, have been studied. Although results of ASCA and pANCA assays have been demonstrated to be associated with CD and UC, respectively, poor sensitivity and specificity have been reported, particularly in Japan. Serological tests more specific for CD and UC are thus required.

In order to develop a specific serological test for CD, we utilised phage display technology to identify peptides associated with CD. After absorption of UC associated phages from a phage displayed peptide library by incubation of the phages with magnetic beads immobilised with serum IgGs of UC patients, CD associated peptides were screened from the phage
Figure 4 Antibody titres in serum from patients with Crohn’s disease (CD), ulcerative colitis (UC), duodenal ulcer (DU), and healthy subjects measured by cocktail multiple antigenic peptide (MAP) enzyme linked immunosorbent assay (ELISA) [A] and anti-Saccharomyces cerevisiae antibody (ASCA) ELISA [B]. Each cut off value is indicated as a broken line. Data were analysed using the Mann-Whitney test. **p<0.001, ***p<0.0001.

Figure 5 Comparison of antibody titres among the colon Crohn’s disease (CD) group, small bowel CD group, and colon/small bowel CD group compared with ulcerative colitis (UC) patients. The cut off value is indicated as a broken line. Data were analysed using the Mann-Whitney U test.

Figure 6 Correlation between serum antibody titres measured by cocktail multiple antigenic peptide (MAP) enzyme linked immunosorbent assay (ELISA) and those measured by anti-Saccharomyces cerevisiae antibody (ASCA) ELISA. Serum antibody titres of Crohn’s disease patients (n=92) were used for this analysis, and Fisher’s r to z transformation test was applied.

that commercially available ASCA kits vary in sensitivity and specificity. Further comparison of results obtained using different ASCA kits is thus necessary.

Higher reactivity of CD patient samples was observed in the cocktail MAP ELISA than in the phage ELISA, probably because peptides can be directly coated on microtitre wells in the MAP ELISA while the peptides displayed on phages have to be captured by antiphage antibody coated on microtitre wells.

Among the three groups of CD patients classified by location of inflammation, no significant differences were observed in antibody titres. Location of inflammation in CD patients thus did not affect MAP ELISA results.

Two (CDP-1 and -2) of the five peptides isolated were similar in amino acid sequence and exhibited similar immunoreactivity characteristics. Immunoreactivity to each of these two peptides was completely inhibited by the other peptide (data not shown), proving that these two peptides detect the same antibody, and suggesting that they mimic the same region of a protein. The remaining three peptides and CDP-1 (or -2) exhibited no homology among themselves in amino acid sequence and exhibited similar immunoreactivity characteristics. We therefore speculate that these peptides mimic different proteins or different regions of the same protein. Searching of protein databases revealed no significant similarity between the amino acid sequences of the peptides and known CD related
autoantigens or even unrelated proteins of any known organism. As the ability to mimic a natural epitope does not necessarily correspond to strong sequence homology, additional information such as key amino acids recognised by serum antibodies of CD patients will be required to identify proteins mimicked by the peptides identified in this study.

In conclusion, we have developed a novel serological test for Crohn’s disease using a new strategy with the phage displayed random peptide technique. This serological test was confirmed to be very specific for Crohn’s disease, and should be useful for differential diagnosis between Crohn’s disease and ulcerative colitis. As variation in the prevalence of serological markers, such as ASCA and pANCA, has been observed worldwide, multicentre prospective studies of the ELISA developed in this study need to be conducted.

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REFERENCES