Peculiar antibody reactivity to human connexin 37 and its microbial mimics in patients with Crohn's disease


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Abstract

Background/aims: We found that pooled Crohn's disease (CD) sera strongly react with a human gap-junction connexin 37 (Cx37) peptide and tested for anti-Cx37 antibody reactivity in sera from CD patients and controls. We also investigated whether peptide-recognition is due to Cx37/microbial molecular mimicry.

Methods: The PSI-BLAST program was used for Cx37121–135/microbial alignment. Reactivity to biotinylated human Cx37121–135 and its microbial mimics was determined by ELISA using sera from 44 CD, 30 ulcerative colitis and 28 healthy individuals.

Results: Anti-Cx37121–135 reactivity (1/200 dilution) was present in 30/44 (68%) CD cases and persisted at 1/1000 dilution. Database search shows that Cx37121–135 contains the –ALTAV– motif

Abbreviations: aa, amino acid; CAV24, coxsackievirus A24; CAV9, coxsackievirus A9; CBV4, coxsackievirus B4; Cx37, connexin 37; CD, Crohn's disease; ECV, enterovirus C; IBD, inflammatory bowel disease; LACLA, Lactococcus lactis; MTUB, Mycobacterium tuberculosis; OD, optical density; PLV, poliovirus; RBL, rubella virus.
1. Introduction

Epidemiological, clinical, serological and experimental studies support the notion that immune-mediated intestinal destruction in Crohn’s disease (CD) commences when genetically susceptible individuals are exposed to microbial stimulants, and that the microbial/host immune encounter is essential in triggering, initiating or modulating the course or progression of inflammatory bowel disease (IBD).\(^1\) Essential in triggering, initiating or modulating the course or progression of IBD is the microbial/host immune encounter, which involves a genetic susceptibility of the host to microbial triggers in the immunopathogenesis of CD, but also as a tool to identify CD-specific autoantigens.\(^1\)\(^-\)\(^9\) With regard to Cx37, there is no published data to suggest that it is involved in the immunopathogenesis of CD. Nevertheless, a recent study demonstrated increased gene expression and altered protein expression of Cx37 in newly purified enterocytes with active coeliac disease, suggesting that this connexin may be involved in enterocyte-specific immunopathological processes.\(^10\) On the basis of these emerging data, we have considered that Cx37's antibody recognition by pooled CD sera was worthy of a more detailed study. Intriguingly enough, the diabetes-specific phogrin peptide with a striking similarity (see later discussion) to Cx37 peptide, and included in the same peptide library, was totally unreactive, suggesting a Cx37-specific reactivity of the pooled CD sera.

The goal of the present study was to test for anti-Cx37-specific antibody responses in a well-defined population of CD patients. Having found that the great majority of these patients contain high-titres of anti-Cx37 antibodies, and that this peptide is highly homologous to phogrin and the phogrin-mimicking microbial peptides previously implicated in the pathogenesis of insulin-dependent diabetes mellitus\(^31\)\(^-\)\(^37\), we have also assessed whether microbial and Cx37 peptides are targets of cross-reactivity antibody recognition in patients with CD.

2. Material and methods

2.1. Patients

Serum samples from 44 patients with CD (mean age 41, range 21–76 years, 23 female), attending the outpatient clinic of Hepatogastroenterology Unit, Attikon Hospital, University of Athens, Greece, were tested. The diagnosis of CD was based on clinical, endoscopic, radiological, and histological criteria. Table 1 summarizes the demographic and clinical characteristics of the patients enrolled in the study. Thirty five patients had ileocolonic disease, 8 had colonic disease and 1 had isolated small bowel disease. At the time of serum collection, 41 patients were on treatment (Table 1), and 7 patients had undergone at least 1 surgery for CD. With regard to disease severity, 21 patients had quiescent disease, 2 had mild disease, 13 had moderate and 8 had severe disease as classified using the Harvey–Bradshaw activity index.\(^38\)

Demographically matched sera from 30 patients with well-characterized UC (mean age 39, 39±15.4 years, range, 19–79 years, 22 men) were included as pathologic controls.
All diagnoses of UC were supported by endoscopic and histologic findings. At the time of serum collection, 10 patients were on systemic steroid treatment (prednisolone), and 3 patients were on azathioprine.

Twenty-eight sera from healthy volunteer staff members (mean age, 38.2 ± 7.1 years; range, 19–52 years; 16 men) also were tested as normal controls.

Experimental work complied with the principles laid down in the Declaration of Helsinki. Participating individuals gave informed consent to the work.

The project was approved by the Attikon Hospital research ethics committee.

### 2.2. Protein database search and analysis

The computer-assisted protein sequence alignment Basic Local Alignment Search Tool (BLASTp) program (NCBI, BLAST server available at http://www.ncbi.nlm.nih.gov/BLAST, matrix: BLOSUM62) was used to search for microbial sequences that share with the human Cx37_{121–135}, the –ALTAVE– motif or other related motifs as deposited in the nr protein database (EBI-EMBL, Cambridge, UK).

### 2.3. Three dimensional modelling

Three dimensional modelling of the human coxsackie B4 mimicking sequence was performed by analysing the corresponding known structure of the conserved human coxsackie B3 polyprotein (Molecular Modelling Database [MMDB] code: 17225; PDB:1JEW_1) using the Cn3D visualization tool (http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3dtut.shtml).

### 2.4. Peptide construction

Ten 15-meric peptides containing human Cx37_{121–135} and 9 mimicking peptides (Fig. 1) were synthesized commercially and supplied at >75% purity (Mimotopes, Clayton, Victoria, Australia). A 15-mer peptide encoding a randomly generated sequence of amino acids (-YVNQSLRPTPLEISV-) was used as negative control peptide.11,39,40

### 2.5. Anti-peptide antibody reactivity by ELISA

Antibody binding to the peptides was determined by ELISA, as previously described.11–14,22,24,40–43 The optimal concentrations of reagents at various steps of the immunoassay were determined in preliminary experiments by checkerboard titration.

Serum samples were tested at various dilutions ranging from 1/100 to 1/100,000 to define the dilution giving the lowest background noise, and the optimal anti-peptide binding value. Similarly, different concentrations of individual peptides were tested (0.1, 1, 5 and 15 μg/ml). The optimal coating concentration for individual peptides was 5 μg/ml and the optimal serum dilution was 1/200. Absorbance (optical density) was read in a microplate reader (MRX; Dynex Technologies, West Sussex, UK) at 490 nm. On each plate, 2 wells were used as blanks in which serum and peptide were omitted, and 2 additional wells were used for positive and negative controls. The positive control consisted of a liver kidney microsomal type 1-positive serum (titre 1/

<table>
<thead>
<tr>
<th>Sequence</th>
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<th>Protein</th>
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<tr>
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<td>991-1005</td>
</tr>
<tr>
<td>2</td>
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<td>121-135</td>
</tr>
<tr>
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<td>AFISMALTAVYIGIV</td>
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</tr>
<tr>
<td>10</td>
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<td>388-402</td>
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Figure 1: Sequence alignment of the immunodominant autoepitope on human connexin 37 (aa 121–135) and its mimicking sequences. Amino acids appear in standard single letter code. Bold letters indicate identical residues between human connexin 37 and the corresponding sequence; italic letters indicate conservative substitution. aa, amino acid.
2.3. Anti-peptide antibody reactivity to connexin 37

Reactivity to native human connexin 37 was tested by immunoblotting using a human whole cell lysate from colorectal adenocarcinoma cells (COLO 320DM, Santa Cruz Biotechnology Inc, Santa Cruz, California, USA). For immunoblotting, 20 μg COLO 320DM cell lysate was resolved in a 10% acrylamide mini-gel using 200 μl SDS-PAGE Western blotting buffer and then transferred onto a polyvinylidene fluoride (PVDF) membrane in a semi-dry electrophoretic transfer cell, in transfer buffer, at 25 V constant voltage for 2 h. To inhibit non-specific reactivity, the strips were incubated with blocking buffer (5% skimmed milk in PBS 0.1% Tween 20) for 1 h at room temperature and were immunoblotted for 2 h at room temperature with individual sera (dilution 1/200) from 12 anti-Cx37_{121–135} antibody positive sera (8 CD, 2 UC and 2 healthy controls) and 8 Cx37_{121–135} antibody negative sera (5 CD and 2 UC and 1 healthy control).

A purified rabbit polyclonal antibody specific for human connexin 37 raised using as an immunogen a polypeptidl sequence corresponding to aa 108–157 of the protein (Abcam, Cambridge, UK) was used as a positive control. Washing followed by the addition of goat horse radish peroxidase-conjugated anti-human IgG (1/5000) for the serum samples or anti-rabbit IgG (1/10,000) for the polyclonal anti-Cx37 antibody for 1 h at room temperature. The reaction was detected using a non-radioactive enhanced chemiluminescent system and developed on an X-OMAT film.

2.7. Inhibition studies

Reactive peptides were used as solid phase inhibitors to absorb out anti-specific antibody reactivity, as previously described.\(^\text{12,14,42–45}\) Briefly, 200 μl of individual microbial and human Cx37_{121–135} double reactive sera (n = 5) diluted 1/500 were added to the first well of an ELISA plate pre-coated with individual peptides at final concentration of 15 μg/ml. After 20 min incubation at room temperature, the serum was sequentially transferred to wells 2–12 (pre-coated with the same antigenic preparation), with a 20-minute incubation in each well. The same experiments were carried out for each of the reactive peptides and the control peptide or PBS alone. One hundred microliters of absorbed serum were then tested for reactivity against Cx37_{121–135} or the microbial mimics by ELISA, and was compared with reactivity before absorption.

2.8. Statistical analysis

Results are presented as the mean ± standard deviation (SD) or as percentages. Comparisons between categoric values were made using the t test, \(\chi^2\) test or the Fisher exact as appropriate. Correlations between variables were assessed using the Pearson’s correlation coefficient. A 2-tailed \(P\) value of less than 0.05 was considered significant. Statistical analyses were performed using SPSS (SPSS Inc., Chicago, IL) statistical package.
and M. tuberculosis mimics or the beta cell human phogrin (Fig. 2 and Table 2).

Overall (Table 2), 30 (68%) CD cases reacted with Cx37121–135 and at least one microbial mimic including 2 (2/44, 4.5%) reacting with Cx37121–135, human enterovirus C, L. lactis, coxsackievirus A24; 22 (22/44, 50%) with Cx37121–135, human enterovirus C, L. lactis, coxsackievirus A24 and B4; 4 (4/44, 9%) with Cx37121–135, human enterovirus C, L. lactis and coxsackievirus A24; 1 (1/44, 2.2%) with Cx37121–135, human enterovirus C, L. lactis and coxsackievirus B4; and 1 (1/44, %) with Cx37121–135, human enterovirus C and L. lactis.

There were no differences in biochemical, clinical or other parameters between CD patients with or without microbial/human Cx37 double reactivity.

3.4. Inhibition studies

Inhibition results of anti-peptide antibody binding by pre-incubation with human Cx37121–135 and at least two of the most reactive microbial mimics (coxsackie B4 or L. lactis) in 5 representative cases are shown in Fig. 4. Antibody binding to human Cx37121–135 was inhibited by 89–93% after pre-incubation with Cx37121–135; by 79–88% after pre-incubation with coxsackievirus B4; by 85–90% after pre-incubation with L. lactis mimic as solid phase competitor in all 5 cases tested. The control peptide did not abolish reactivity to human Cx37121–135 or the microbial mimics.

3.5. Anti-peptide reactivity and inhibition studies in pathological and normal controls

Reactivity to at least one Cx37-mimicking microbial mimic was present in 15/30 (50%) patients with UC (mean OD±SD:0.28±0.17) and in 16/28 (57%) of normal (0.29±0.15) controls (p>0.05). The most reactive microbial mimics in both groups were those of coxsackievirus A24 and L. lactis (50% for both), coxsackievirus B4 (43% for UC and 50% for healthy controls) and human enterovirus C (43% for UC and 46% for healthy controls). Prevalence and absorbance values of anti-peptide reactivity did not differ between CD and UC

Table 2  Anti-peptide antibody reactivity patterns against human connexin 37 (Cx37) and its mimicking sequences in 44 patients with Crohn’s disease.

| Cases reacting with Cx37 and at least one microbial mimic (n = 30) | 22 reactive with Cx37 and ECV, CBV4, CAV24, LACTA |
| Cases reactive only to microbial mimics (n = 1) | 4 reactive with Cx37, CAV24, LACTA, |
| Cases reactive only to Cx37 (n = 0) | 2 reactive with Cx37 and ECV, CAV24, CBV4, CAV9, LACTA |
| Cases unreactive to Cx37 and any of the mimics (n = 13) | 1 reactive with Cx37 and ECV, CAV24, CBV4, CAV9, LACTA |
| Total numbers of cases (n = 44) | 1 case reactive with CAV9 |

ECV, enterovirus C; CBV4, coxsackievirus B4; CAV24, coxsackievirus A24; LA CLA, Lactococcus lactis; CAV9, coxsackievirus A9.

Figure 2  Antibody reactivity to human connexin 37121–135 and its mimicking sequences in 44 patients with Crohn’s disease. OD, optical density.

Figure 3  Antibody reactivity to human connexin 37121–135 at various dilutions ranging from 1/100 to 1/10,000 in 8 representative cases with Crohn’s disease. OD, optical density.
or normal controls ($p>0.05$). Inhibition experiments have been performed in 5 microbial/Cx37 double reactive cases (3 UC and 2 healthy): anti-Cx37$_{121-135}$ was inhibited by 77–91% after pre-incubation with Cx37$_{121-135}$ in all cases; by 29–71% after pre-incubation with coxsackie virus B4; and by 27–67% after pre-incubation with L. lactis. Overall, the microbial coxsackie virus B4 and L. lactis mimics were unable to significantly inhibit (~35%) reactivity to Cx37 mimic in 3 (2 UC and 1 healthy) cases.

3.6. Antibody reactivity to native human connexin 37

All but one anti-human Cx37$_{121-135}$ reactive sera immunofixed a band of ~37 kDa corresponding to human connexin 37 by immunoblot using colon adenocarcinoma cell lysate. The reactive sera included all CD (n=2) and UC (n=2) patients and one of the two healthy controls. Amongst the 8 (5 CD and 3 UC) Cx37$_{121-135}$ antibody negative cases, one CD serum immunofixed a 37 kDa band. Results of the immunoblotting experiments of representative cases are shown in Fig. 5.

3.7. Three dimensional modelling

Local sequence homology modelling of the human coxsackie B4 virus polyprotein, using as template the known homologous coxsackie B3 3D-model predicts that the human connexin 37$_{121-135}$ mimicking coxsackie B4 sequence is located in a solvent-accessible surface region of the protein (Fig. 6).

4. Discussion

We report evidence that antibody reactivity to a human Cx37 peptide is highly prevalent in patients with CD, being present in all but a few cases. The great majority of them also recognise microbial mimics from human enteroviruses and L. lactis. The prevalence of reactivity to these mimics (up to...
68%) is higher than that reported for antibodies to *Saccharomyces cerevisiae* (ASCA), porin protein-C or other CD-serological markers, none of which seems to exceed a prevalence of 50%.46

While the relevance of the present findings to the immunopathogenesis of CD is yet to be determined, some findings are intriguing and warrant further investigation.

Why human Cx37 is targeted by autoantibodies in CD is not clear. Cx37 belongs to the family of connexins, the proteins assembled into gap junction channels. Although connexins serve diverse functions, they are best known for their role in cell differentiation and proliferation.25 These proteins are selectively expressed in monocytes, and current evidence suggests that they play an important role in mediating intracellular signalling by cells of the immune system, and participate in immunopathological inflammatory processes.25,27–29 A recent report based on a variety of techniques including microarray, real time PCR and immunohistochemical analysis demonstrated that connexin 37 is upregulated in enterocytes from patients with active coeliac disease, when compared to patients with treated coeliac disease, as well as controls.30 The possible involvement of this protein in an immune-mediated gastrointestinal disease is intriguing and warrants further investigation.30 Studies on atherosclerosis, a disease recently appreciated to have a strong autoimmune component to its pathogenesis, clearly demonstrate that Cx37 expression changes location as the disease progresses, and this reallocation influences the outcome of the disease.47–52 This was also the case in a murine model of asthma where it has been shown that Cx37 is localized in epithelial layers around the bronchioles before the onset of the disease but disappears in allergen-induced asthmatic lungs.53

Whether altered Cx37 expression, (especially at sites making it accessible to antibodies) provides the initial stimulus for the induction of an autoimmune response against this specific protein needs to be determined.

Interestingly, antibody reactivity was not limited to Cx37121–135 but also included four of its nine microbial mimics, and in particular those originating from the enteroviruses coxsackie B4, A24 and C and one from *L. lactis* (Fig. 1 and Table 2). The fact that the reactive peptides share with Cx37 the -ALTAV- motif implies that microbial sequences with this motif are highly immunogenic, and induce anti-self responses by virtue of molecular mimicry with self-antigens, such as phogrin in the case of diabetes or Cx37 in Crohn’s disease. Indeed, three dimensional prediction analysis (Fig. 6) clearly indicates that corresponding mimicking sequences such as that of coxsackie virus B4 are exposed to the surface of the molecule, a finding compatible with antibody recognition. None of the CD sera, however, reacted with the -ALTAV- containing phogrin autoepitopic region. Moreover, rubella, poliovirus and *M. tuberculosis* mimics were totally unreactive while reactivity to coxsackie virus A9 was found only in few cases (Fig. 2).

Collectively these data indicate that some of the observed reactivities are peptide/microbial-specific, and that the presence of the -ALTAV- motif per se is not sufficient for antibody binding. An explanation might lie in different patients having a different history of microbial exposure, leading to a case-specific reactivity to different outlying residues, in addition to the core -ALTAV- epitope. In support of this is the finding of a case-by-case pattern and magnitude of reactivity to the respective peptides (Table 2). Certainly, the inhibition patterns and dissimilar proportion of absorbed reactivities for the five representative cases (Fig. 4) do suggest that relative reactivity is peptide-specific and not exactly the same for each serum.

Those concluding that responses to microbial mimics are irrelevant to disease pathogenesis because they appear to react in patients and controls need to take into account that the Cx37/phogrin-mimicking microbial peptides injected in animals are able to induce autoimmune responses to their phogrin mimic, as well as the full-length phogrin antigen.31,32 This has been demonstrated to induce pancreatic beta cell destruction indistinguishable from that seen in patients with insulin-dependent diabetes mellitus.31,32 Interestingly, pancreatic autoantibodies directed against the major zymogen granule membrane glycoprotein 2 are frequently seen in patients with CD.54 GP2 autoantibodies appear to be directed against conformational epitopes while anti-Cx37 antibodies recognise a short linear sequence of the protein.54 Epitope mapping studies based on overlapping peptides and mammalian expressed polypeptidyl constructs are needed to identify most of the (linear or conformational) epitopes that are recognized by Cx37-specific antibodies. This investigation will facilitate a better understanding of the potential pathogenic role of microbial/Cx37 molecular mimicry in CD. The fact that sera reacting with native human Cx37 in immunoblot experiments also react with Cx37121–135 (Fig. 5) clearly indicates that this short sequence is an immunodominant epitope.

In relation to Cx37, antibody responses were less prevalent in pathological (UC patients) and healthy controls compared to CD patients, but this did not reach statistical significance. Our inability to inhibit reactivity to Cx37 by the microbial mimics in three of the 5 control cases tested is intriguing and may suggest microbial/self reactivity may be cross-reactive in CD cases but not in Cx37/microbial double reactive controls. This and other antibody characteristics, such as the IgG subclass distribution, may set CD sera apart from the other groups. These characteristics have not yet been explored.

Despite the lack of disease specificity, cross-reactive immune responses to microbial/human connexin 37 homologues may indeed be pathogenetically meaningful but only in those genetically susceptible individuals who are prone to develop autoimmune disease because of impaired safe-guard mechanisms. Our findings require external validation in larger human studies and ideally in an appropriate animal model setting.

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**Authorship contribution**

AK performed experiments and analyzed the data; DP collected clinical material and information and edited the paper; ZT collected clinical material and performed
experiments; DS helped to draft the paper; GK and KT collected clinical material and information; EIR performed statistical analysis; AP and DV helped to draft the manuscript; SDL was responsible for the general supervision of the project, collected clinical material and edited the paper; DPB had the original idea, performed sequence alignment and 3-dimensional prediction analysis, designed the study and wrote the paper.

Conflict-of-interest disclosure

The authors declare no competing financial interests.

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