Comparison of epitope specificity of anti-heat shock protein 60/65 IgG type antibodies in the sera of healthy subjects, patients with coronary heart disease and inflammatory bowel disease

George Füst · Katalin Uray · László Bene ·
Ferenc Hudecz · István Karádi · Zoltán Prohászka

Received: 5 July 2011 / Revised: 5 October 2011 / Accepted: 6 October 2011 / Published online: 29 October 2011
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Abstract Previously, we reported on the presence of antibodies to linear epitopes of human and mycobacterial 60 kD heat shock proteins (HSP) in the sera of healthy blood donors. Since many recent findings indicate that the levels of these antibodies may be altered in coronary heart disease (CHD) and also inflammatory bowel diseases (IBD), it seemed worthwhile to compare the epitope specificity of the anti-HSP60 and anti-HSP65 antibodies in the sera of patients with these diseases to those in healthy subjects. The multipin enzyme-linked immunosorbent assay method was applied with a large overlapping set of

synthetic 10-mer peptides covering selected regions of human HSP60 and *Mycobacterium bovis* HSP65. Sera of 12 healthy persons (HP), 14 CHD, and 14 IBD patients with the same concentration of total anti-HSP60 and HSP65 IgG antibodies were tested. We have identified CHD-specific epitopes in the equatorial domain of the HSP60 protein but in neither region of the HSP65 molecule, indicating that the formation of anti-HSP60 antibodies is not or only partially due to the cross-reaction between human HSP60 and bacterial HSP65. IBD-specific epitopes were found in many regions of the HSP60 and in even more regions of the HSP65 molecule including an IBD-specific T cell epitope in region X as well. These findings indicate that the epitope specificity of the anti-human and anti-mycobacterial HSP60 antibodies associated with various diseases is different.

Electronic supplementary material The online version of this article (doi:10.1007/s12192-011-0301-7) contains supplementary material, which is available to authorized users.

G. Füst (✉) · I. Karádi · Z. Prohászka
3rd Department of Internal Medicine, Semmelweis University,
Kútvölgyi út 4,
1125 Budapest, Hungary
e-mail: fustge@kut.sote.hu

K. Uray · F. Hudecz
Research Group of Peptide Chemistry,
Hungarian Academy of Sciences, Eötvös L. University,
Budapest, Hungary

L. Bene
Hospital of Péterfy Sándor,
Budapest, Hungary

F. Hudecz
Institute of Chemistry, Eötvös L. University,
Budapest, Hungary

Z. Prohászka
Research Group of Inflammation Biology and Immunogenomics,
Hungarian Academy of Sciences and Semmelweis University,
Budapest, Hungary

Keywords Coronary heart disease · Inflammatory bowel disease · Human HSP60 · Mycobacterial HSP65 · Epitope analysis

Introduction

Heat shock proteins are highly conserved proteins found in prokaryocytes and eukaryocytes (Lindquist and Craig 1988). Antibodies against the 60 kD heat shock protein (HSP60) are present in the sera of most healthy persons; these antibodies can be considered as natural autoantibodies (Pozsonyi et al. 2009).

Data including our previous studies indicate that serum levels of anti-HSP60 antibodies and/or against their prokaryote analogue HSP65 are increased or decreased in different diseases as compared to the healthy subjects. A

significant part of the studies on the presence and pathological significance of the immune response to heat shock proteins was performed in animal models of atherosclerosis and patients with atherosclerotic vascular diseases [reviewed recently by Ayada et al. (2009)]. Elevated levels of anti-HSP60 or anti-HSP65 antibodies were found to be associated with increased intima-media thickness (Xu et al. 1993) and the development (Wick and Xu 1999) of carotid plaques as well as with coronary artery disease (Birnie et al. 1998; Burian et al. 2001; Hoppichler et al. 1996; Prohaszka et al. 1999; Veres et al. 2002) and can be considered as risk factors for morbidity and mortality of coronary artery disease (Ayada et al. 2009).

Although the changes in the levels of anti-HSP60 antibodies in autoimmune diseases—as reviewed recently (Wu and Tanguay 2006)—in general are rather controversial, in children with type I diabetes mellitus, the levels of anti-HSP60 antibodies are increased as compared to the healthy controls (Horvath et al. 2002). In addition, we have reported on the changes of the anti-HSP65 antibodies in patients with inflammatory bowel disease (IBD) (Bene et al. 2002; Huszti et al. 2004).

Like other polyclonal antibodies, anti-HSP antibodies are directed to many different B-cell epitopes, and the anti-HSP cellular immune response is oriented to several T cell epitopes. Therefore, the analysis of epitope specificity of these antibodies in health and disease is most important. Such analysis was performed in different infections [*Rickettsia tsutsugamushi* (Lachumanan et al. 1993), *Porphyromonas gingivalis* (Maeda et al. 2000), *Chlamydia trachomatis* (Yi et al. 1993), *Helicobacter pylori* (Yamaguchi et al. 2000)], picornaviruses (Harkonen et al. 2000), and other diseases including adjuvant arthritis (Ulmansky et al. 2002), carotid atherosclerosis (Metzler et al. 1997; Perschinka et al. 2003), acute coronary syndrome (Wysocki et al. 2002), children with type 1 diabetes mellitus (Horvath et al. 2002).

We were the first to report on the epitope specificity of antibodies to 60 kD heat shock proteins in healthy adults (Uray et al. 2003). Since our group was active in the study of the connection between antibodies against different heat shock proteins and coronary artery disease (Burian et al. 2001; Kocsis et al. 2003; Lu et al. 2010; Prohaszka et al. 1999; Veres et al. 2002), we carried out B cell epitope analysis with sera of coronary artery disease. As to our best knowledge, only T cell epitopes of HSP60 have been analyzed until now in IBD (Puga Yung et al. 2009). We aimed also to carry out B cell epitope analysis in this disease. Given that vaccination with one or more HSP epitopes was considered in both atherosclerotic vascular diseases (George et al. 2001; Harats et al. 2002; Lu et al. 2010; Maron et al. 2002; Maron et al. 2002) and IBD (Puga Yung et al. 2009), the results of these analyses can be of practical importance as well.

Therefore, the aim of the present work was to describe and compare the B cell epitope pattern of human and mycobacterial 60 kD HSP. We hypothesized that there are differences between healthy subjects and patients with CHD and IBD not only in the levels of anti-HSP60 and anti-HSP65 antibodies but in their epitope specificity as well.

Materials and methods

Study subjects

Serum samples from 12 healthy persons, 14 patients with severe coronary heart disease (CHD), and 14 patients with inflammatory bowel disease (IBD) were included in the analysis. All CHD patients had severe CHD and underwent coronary by-pass operation (Prohaszka et al. 2001). All 14 IBD patients had Crohn's disease (Bene et al. 2002). The patients were selected from our previous studies, where details on clinical diagnosis and inclusion/exclusion criteria were described (Bene et al. 2002; Prohaszka et al. 2001; Uray et al. 2003). In order to exclude the effect of the quantitative differences of specific IgG, sera with the same reactivity of total anti-HSP60 and total HSP65 antibodies were selected from all the three groups of subjects.

Synthesis of peptides

Decapeptides overlapping by five amino acid residues were synthesized on β -alanyl-glycine functionalized polyethylene pins on two blocks (Mimotopes) with Fmoc/tBu chemistry according to Geysen's method (Geysen et al. 1984), as described before (Uray et al. 2003). Briefly, we used *t*Bu (Thr, Ser, Tyr), *O**t*Bu (Asp, Glu), *Acm* (Cys), *Pmc* (Arg), and *Boc* (His, Lys) as side-chain protecting groups. The Fmoc α -amino protecting group was removed with 20% piperidine: DMF (v/v). The coupling was performed with DIC/HOBt methodology and monitored with bromophenol blue added to the coupling mixture (Krchnak et al. 1988). The peptides were acetylated at the N-terminus, and then, the side-chain protecting groups were removed with TFA/EDT/anisole 38:1:1 (v/v/v), but the unprotected peptides remained covalently attached to the pins.

Localization of the tested peptides within the HSP60 and HSP65 molecules

The 46 peptides we tested were present in ten regions (regions I to X) of the human HSP60 molecule and the corresponding regions of the mycobacterial HSP65 molecule (Fig. 1).

As we have described previously (Uray et al. 2003), we have obtained three-dimensional models of Hsp60 and

Fig. 1 Three-dimensional model of human Hsp60 monomer based on the tertiary structure of *E. coli* Hsp60. The sequences marked by the ribbon structure represent the localization of the ten regions of synthetic peptides. The insert shows the localization of a GroEL monomer in the oligomeric structure of GroEL-GroES machinery consisting of 14 GroEL monomers and an additional ring of seven GroES monomers (*cap*)

Hsp65 proteins from the Swiss-Model Automated Protein Modeling Server (<http://www.embl-heidelberg.de/predictprotein/>; Guex et al. 1999; Guex and Peitsch 1997; Peitsch 1996; Schwede et al. 2000). The models were based on the homology in the primary protein sequence between human and *Escherichia coli* GroEL (Hsp60 homologue) and between *M. bovis* Hsp65 and GroEL. Based on these models, we have located the recognized peptide sequences within their respective proteins. The monomer protein consists of three parts, the apical domain, the equatorial domain where the two heptamer rings connect, and the intermediate domain functioning as a hinge during the conformational changes of the functional multimeric protein.

The homologous peptide sequences in region I on both HSP60 and HSP65 peptide blocks are located in the equatorial domain of the intact monomeric proteins and form exposed loops stabilized by β -strand structures, but in the tetradecamer heat shock protein, the strands participate in binding the neighboring monomers together. The C-terminal part of the region adopts α -helical structure and is less exposed in the monomer. The sequence of the region II peptides consists of a loop surrounded by α -helices on both N- and C-termini and is located at the base of the monomer (equatorial domain) binding both to the lower barrel of the tetradecamer and the neighboring monomer.

Region III is located on the outer surface of the intermediate domain of the protein forming two α -helices with a loop structure between. Region IV contains 11 overlapping peptides. Its N-terminal part is in the intermediate domain of HSP60. After a loop a β -strand follows consisting of the second and third peptides of the region,

which is exposed in all forms of the protein. An approximately 20-residue long unstructured part follows, located in the apical domain, part of which (the sixth and seventh peptides in the region) participates in connecting the monomer to its neighbor in the tetradecamer, but is exposed in the monomer. The rest of the region is partly buried in the multimer, except the N-terminal part of the region, which is on the “top” of the protein adopting helical conformation, exposed not only in the monomer, but in the open form of the multimeric HSP as well, but can be masked in the closed form by the “GroES” cap. Region V is still located in the apical domain; its structure is a loop edged by an unordered and a helix-like structure, close to the neighboring monomer within the multimer, directed to the inside of the barrel, but exposed in the monomer. The peptide sequences within region VI of the protein (intermediate domain) consist of a helix followed by a loop structure, in proximity to the next monomer in the barrel.

Region VII, forming part of the intermediate domain, mostly adopts β -strand structure (antiparallel to the region IV strand) edged by unordered structures and then short helices on both termini, HSP60 peptide 399–408 covers the sequence connecting the two domains; a large part of this region is exposed both in the monomeric and multimeric protein. Region VIII is in the equatorial domain of the molecule, partly buried, partly exposed even in the multimeric structure. Region IX consists of a partly buried helix followed by an exposed loop and then another helix, after that another loop and a β -strand follows. The second helix is participating in connecting the upper and lower barrel in the multimeric form; the loop and the C-terminal end of the strand are also proximal to the neighboring monomer, but a

large part of the region is exposed in the monomer. Peptides of region X are located in the equatorial domain forming an amphipathic helix and the C-terminal unstructured part of the HSP65, participating strongly in the binding of the neighboring monomers building up the multimer, but partially exposed in the monomer protein.

Antibody binding to the immobilized human HSP60 or *M. bovis* HSP65 peptides

Antibody binding to peptides immobilized on polyethylene pins was detected using a modified enzyme-linked immunosorbent assay (ELISA). After blocking the non-specific binding sites [phosphate-buffered saline (PBS)/0.5% gelatin], pins were incubated with 150 µl of 1:500 diluted sera in PBS/0.5% gelatine/0.05% Tween 20 for 1 h at room temperature. Binding of anti-HSP peptide antibodies was determined using rabbit anti-human IgG peroxidase-labeled antibodies (Dako) and peroxide/*o*-phenylene-diamine (Sigma) detection system. The optical density was measured at $\lambda=490$ nm (reference at $\lambda=620$ nm), and means of duplicates were calculated. Pins were used repeatedly after thorough cleaning by sonication in disruption buffer (PBS, 1% sodium dodecyl sulfate and 0.1% 2-mercaptoethanol). The results were normalized by the following formula: $OD/OD_{\min}$ (where $OD_{\min}$ is the optical density of the peptide showing minimum OD values in the given experiment; in most experiments, the HSP60 peptide 480–489 showed the minimum reactivity and was used for this purpose) was designated as *reactivity index*. The reactivity index was considered significant when it differed significantly ($p<0.05$) from 1.0 as tested by the non-parametric Wilcoxon signed rank test.

Statistical analysis

The non-parametric Mann–Whitney test was used for group comparisons; correlation between the different variables was calculated with the non-parametric Spearman test. Categorical data were compared using the Fisher's exact test. All the tests were two tailed. Statistical analysis was performed using the GraphPad Prism 3.0 (GraphPad Software Inc., San Diego, CA, USA, www.graphpad.com) software.

Results

Differences in the epitope specificity of the anti-human HSP60 antibodies between HP and patients with CHD and IBD

Reactivity to peptides in ten regions of the human HSP60 with the three groups of sera was measured, and the amount

of IgG type antibodies bound to these peptides were compared (Table 1).

Differences between CHD and HP sera

All but one peptide (122–131) in region II reacted significantly stronger with CHD sera than with those of healthy persons (HP), and the reactivity of peptide 122–131 was also stronger in the CHD sera. When the reactivity indices of the whole region II were compared (Fig. 2a), a significantly elevated reactivity was found in the CHD sera. No difference in the same direction was observed with any other peptide of either region.

Reactivity to three overlapping peptides (213–222, 218–227, and 223–232) in region IV was higher in the HP than in the CHD sera.

There was no significant difference between the HP and CHD sera for the other peptides of HSP60.

Differences between IBD and HP sera

Many peptides, such as almost the whole regions I and II (Fig. 2c), 2–2 overlapping peptides (218–227, 223–232 and 248–257, 253–262, respectively) in region IV, both peptides of region V, two out of three peptides in region VI, as well as one–one peptide in regions VII and IX reacted more strongly with IBD sera than HP sera.

The reactivity in the IBD sera was weaker than in the HP sera with five single peptides in regions III, VII, VIII, and IX.

Comparison of all three groups of sera

Although the reactivity index showed antibody binding, there were no differences among the sera of the three groups in the reactivity to some peptides (52–61 in region I, 122–131 in region II, 208–217, 228–237, as well as two overlapping peptides 238–247 and 243–252 in region IV, peptide 378–387 in region VI, peptides 436–445 and 441–450 in region VIII, as well as for all peptides of region X).

Finally, we found several peptides in regions III, IV, and IX to which no significant ($p<0.05$ by the Wilcoxon signed test) reactivity was observed in either type of sera.

Differences in the epitope specificity of the anti-mycobacterial HSP65 antibodies between HP and patients with CHD and IBD

Binding to the mycobacterial HSP65 peptides in different regions with the three groups of sera was measured, and the amounts of IgG type antibodies bound to these peptides were compared (Table 2).

Table 1 Epitope specificity of the anti-HSP60 antibodies in healthy persons (HP), coronary heart disease (CHD) and inflammatory bowel disease (IBD)

Region	Peptide ^a		Reactivity index median (IQ range) values		
			Healthy persons (<i>n</i> =12)	CHD patients (<i>n</i> =14)	IBD patients (<i>n</i> =14)
I	26–35	KVTLGPKGRN	2.54 (1.99–4.01)	2.24 (1.40–2.58)	5.61 (4.25–5.90)***
	31–40	PKGRNVVLEK	3.53 (2.80–4.48)	2.43 (1.47–3.16)++	9.52 (8.15–10.95)***
	36–45	VVLEKKWGAP	3.16 (2.67–3.86)	2.80 (1.80–3.25)	8.14 (6.96–9.36)***
	41–50	KWGAPTINTND	1.74 (1.42–2.50)	1.26 (0.80–1.41)++	9.92 (8.48–11.40)***
	46–55	TITNDGVSIA	1.53 (1.27–1.77)	1.20 (0.70–1.57)+	1.52 (1.43–1.80)**
II	91–100	TVLAQALVRE	2.72 (2.12–3.68)	2.67 (1.96–3.17)	4.05 (3.41–5.07)**
	96–105	ALVREGLRNV	2.86 (2.32–3.30)	2.42 (1.83–3.46)	5.16 (4.34–6.46)***
	101–110	GLNRVAAGAN	2.12 (1.82–2.63)	1.98 (1.19–3.78)	7.44 (6.09–8.49)***
	106–115	AAGANPLGLK	1.91 (1.72–2.20)	1.59 (0.98–2.77)	6.64 (5.44–7.59)***
	111–120	PLGLKRGIEK	2.74 (1.96–3.47)	2.14 (1.46–2.49)	4.47 (3.79–5.56)***
III	136–145	VETKEQIAAT	1.50 (1.33–1.88)	1.21 (0.79–1.46)++	2.02 (1.64–2.40)*
	141–150	QIAATAAISA	1.69 (1.26–2.44)	1.66 (1.40–2.20)	1.79 (1.47–2.14)
	146–155	AAISAGDQSI	1.13 (1.00–1.78)	1.00 (1.00–1.00)	0.91 (0.81–1.06)
	151–160	GDQSIGDLIA	1.28 (1.13–1.78)	1.12 (0.86–1.28)	1.03 (0.91–1.20)
IV	176–185	ESNTFGLQLE	2.43 (1.65–4.05)	2.12 (1.95–2.49)	2.32 (1.89–3.24)
	181–190	GLQLELTEGM	3.04 (1.61–3.86)	3.25 (2.82–4.01)	4.80 (4.11–7.22)***
	186–195	LTEGMRFDKG	3.06 (1.83–3.86)	2.78 (2.47–3.75)	4.80 (4.11–7.22)***
	191–200	RFDKGYISGY	3.43 (2.98–4.24)	2.83 (2.38–3.75)+	4.78 (4.04–6.29)**
	196–205	YISGYFVTDP	3.43 (3.04–4.21)	3.19 (2.32–3.84)	4.78 (4.04–6.29)***
	201–210	FVTDPERQEA	1.94 (1.50–2.57)	2.03 (1.55–2.30)	1.84 (1.42–2.20)
	206–215	ERQEAVLEDP	1.96 (1.60–2.57)	2.02 (1.75–2.39)	1.84 (1.42–2.20)
	211–220	VLEDPYILLV	2.41 (2.14–2.96)	2.89 (2.47–3.24)	2.13 (1.77–3.23)
	216–225	YILLVSSKVS	2.34 (2.07–2.65)	1.94 (1.73–2.96)	2.13 (1.77–3.23)
	221–230	SSKVSTVKDL	2.25 (1.86–2.89)	2.20 (1.77–3.96)	3.48 (3.14–4.43)
V	226–235	TVKDLLPLLE	2.28 (1.80–2.61)	2.09 (1.60–2.35)	3.48 (3.14–4.43)***
	276–285	PGFGDRRKAM	2.49 (2.02–3.10)	2.30 (1.73–3.22)	11.88 (10.78–14.19)***
	281–290	RRKAMLQDMA	2.51 (1.85–3.23)	2.55 (1.79–3.84)	4.49 (3.81–6.00)***
VI	341–350	GRVAQIRQEI	2.15 (1.71–2.45)	2.04 (1.33–2.76)	3.83 (3.26–5.12)***
	346–355	IRQEIENSDS	1.48 (1.36–2.19)	1.20 (0.95–1.35)+++	2.66 (2.26–3.56)***
	351–360	ENSDDYDRE	1.84 (1.71–2.22)	1.30 (1.15–1.46)+++	1.33 (1.25–1.68)++
VII	366–375	LAKLAGGVAV	2.66 (2.21–3.42)	2.07 (1.67–2.82)+	2.25 (2.09–3.29)
	371–380	GGVAVIKAGA	1.93 (1.64–2.60)	1.97 (1.33–2.32)	2.70 (2.37–3.44)**
	376–385	IKAGAATEVE	2.19 (1.91–2.61)	2.04 (1.61–2.59)	2.52 (2.11–2.97)
VIII	408–417	IVAGGGVTLL	2.48 (2.00–2.96)	2.19 (1.42–2.93)	3.27 (2.14–4.08)
	413–422	GVTLLQAAPT	2.56 (2.02–3.20)	2.47 (1.82–3.18)	3.27 (2.14–4.08)
	418–427	QAAPTLDELK	1.51 (1.28–1.89)	1.15 (0.88–1.30)+++	1.30 (1.14–1.46)+
IX	441–450	VAEAPLKQI	1.76 (1.58–2.03)	1.88 (1.70–2.05)	1.71 (1.65–1.98)
	446–455	PLKQIAFNSG	1.72 (1.53–1.97)	1.46 (1.18–2.29)	2.15 (1.99–2.37)**
	451–460	AFNSGLEPGV	1.44 (1.00–1.79)	1.00 (1.00–1.00)	1.76 (1.63–1.94)*
	456–465	LEPGVVAEKV	1.72 (1.38–2.04)	1.63 (1.38–2.03)	1.89 (1.65–2.05)
	461–470	VAEKVRNLPA	1.74 (1.40–2.41)	1.50 (1.17–2.40)	1.89 (1.65–2.05)
	466–475	RNLPAGHGLN	1.94 (1.56–2.60)	1.82 (1.33–3.05)	1.80 (1.69–2.09)
	471–480	GHGLNAQTGV	1.48 (1.22–1.72)	1.53 (1.30–1.75)	1.38 (1.29–1.60)
X	502–511	NAASIAGLFL	2.51 (1.70–3.18)	2.83 (2.05–3.28)	3.23 (2.87–3.99)**
	507–516	AGLFLTTEAV	2.60 (2.10–3.26)	2.49 (2.02–3.23)	3.65 (2.77–4.46)*
	512–521	TTEAVVADKP	2.13 (1.86–2.47)	1.92 (1.61–2.63)	3.09 (2.34–3.78)**

Overlapping peptides of ten regions of the HSP60 molecule were used as antigens. Antibody binding to peptides immobilized on polyethylene pins was detected using a modified ELISA. Reactivity index was defined as OD/OD_{min}, where OD_{min} is the optical density of the peptide showing minimum OD values in the given experiment

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, significantly elevated; + $p < 0.05$; ++ $p < 0.01$; +++ $p < 0.001$, significantly decreased

^aNumbering according to the human HSP60 sequence (Heat shock 60 kD protein, isoform 1, NP_955472.1, 573 amino acids). Difference to the Healthy persons group calculated by Mann–Whitney test. Significantly elevated: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, significantly decreased + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$

Fig. 2 Comparison of the reactivity of the region II of the HSP60 (*left panels*) and HSP65 (*right panels*) in CHD patients vs healthy persons (*upper panels*) and IBD patients vs. healthy persons (*lower panels*) P vales for comparison with two-way ANOVA are indicated

Differences between CHD and HP sera

None of the peptides reacted stronger with the CHD than with HP sera (Fig. 2b).

By contrast, three of five peptides in region I, two out of three peptides in region VI, as well as some single peptides in regions III, IV, VII, and VIII reacted weaker with the CHD than the HP sera.

Differences between IBD and HP sera

All peptides in regions I, II (Fig. 2d), V, and X, two overlapping peptides in region VI, a large part of region IV, two overlapping peptides in region IX, as well as single peptides in regions III, IV, and VII exhibited higher reactivity with the IBD than with the HP serum samples.

One peptide each in regions VI and VIII, respectively, reacted weaker with the IBD than HP sera.

Comparison of all three groups of sera

There were no differences among the sera of the three groups in the binding activity to peptides in large part of regions IV (201–230) and IX (456–480) and in case of several other single peptides in regions III, IV, VII, and VIII.

In contrast to anti-HSP60 antibodies, we found peptides that did not significantly bind anti-HSP65 antibodies from either type of the sera only in case of two peptides in region III.

Similarities in the epitope specificity of the anti-human HSP60 and anti-HSP65-antibodies between HP and patients with CHD and IBD

In spite of the marked differences in the anti-HSP60 and anti-HSP65 epitope specificity of the three types of serum antibodies studied and detailed above, the overall pattern of the reactivity was similar: Almost all peptides bound antibodies from all types of sera or did not bind from either type of sera (Table 3). There were only a few peptides in which case the HSP65 peptides were recognized with significant reactivity index but HSP60 peptides were not recognized by all types of serum antibodies: These were HSP60 (172–186)–HSP65 (146–160) of region III, HSP60 (203–212)–HSP65 (176–185) of region IV as well five peptide pairs from region IX.

Differences in the reactivity of the corresponding HSP60 and HSP65 peptides in the HP, CHD, and IBD sera

We have compared separately the reactivity of the three types of sera with the HSP60 and the corresponding HSP65

Table 2 Epitope specificity of the anti-HSP65 antibodies in healthy controls, in patients with coronary heart disease (CHD) and with inflammatory bowel disease (IBD)

Region	Peptide ^a		Reactivity index median (IQ range) values		
			Healthy persons (n=12)	CHD patients (n=14)	IBD patients (n=14)
I	26–35	KVTLGPKGRN	2.54 (1.99–4.01)	2.24 (1.40–2.58)	5.61 (4.25–5.90)***
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	36–45	VVLEKKWGAP	3.16 (2.67–3.86)	2.80 (1.80–3.25)	8.14 (6.96–9.36)***
	41–50	KWGAPTINTND	1.74 (1.42–2.50)	1.26 (0.80–1.41)++	9.92 (8.48–11.40)***
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	101–110	GLNRVAAGAN	2.12 (1.82–2.63)	1.98 (1.19–3.78)	7.44 (6.09–8.49)***
	106–115	AAGANPLGLK	1.91 (1.72–2.20)	1.59 (0.98–2.77)	6.64 (5.44–7.59)***
	111–120	PLGLKRGIEK	2.74 (1.96–3.47)	2.14 (1.46–2.49)	4.47 (3.79–5.56)***
III	136–145	VETKEQIAAT	1.50 (1.33–1.88)	1.21 (0.79–1.46)++	2.02 (1.64–2.40)*
	141–150	QIAATAAISA	1.69 (1.26–2.44)	1.66 (1.40–2.20)	1.79 (1.47–2.14)
	146–155	AAISAGDQSI	1.13 (1.00–1.78)	1.00 (1.00–1.00)	0.91 (0.81–1.06)
	151–160	GDQSIGDLIA	1.28 (1.13–1.78)	1.12 (0.86–1.28)	1.03 (0.91–1.20)
IV	176–185	ESNTFGLQLE	2.43 (1.65–4.05)	2.12 (1.95–2.49)	2.32 (1.89–3.24)
	181–190	GLQLELTEGM	3.04 (1.61–3.86)	3.25 (2.82–4.01)	4.80 (4.11–7.22)***
	186–195	LTEGMRFDKG	3.06 (1.83–3.86)	2.78 (2.47–3.75)	4.80 (4.11–7.22)***
	191–200	RFDKGYISGY	3.43 (2.98–4.24)	2.83 (2.38–3.75)+	4.78 (4.04–6.29)**
	196–205	YISGYFVTDP	3.43 (3.04–4.21)	3.19 (2.32–3.84)	4.78 (4.04–6.29)***
	201–210	FVTDPERQEA	1.94 (1.50–2.57)	2.03 (1.55–2.30)	1.84 (1.42–2.20)
	206–215	ERQEAVLEDP	1.96 (1.60–2.57)	2.02 (1.75–2.39)	1.84 (1.42–2.20)
	211–220	VLEDPYILLV	2.41 (2.14–2.96)	2.89 (2.47–3.24)	2.13 (1.77–3.23)
	216–225	YILLVSSKVS	2.34 (2.07–2.65)	1.94 (1.73–2.96)	2.13 (1.77–3.23)
	221–230	SSKVSTVKDL	2.25 (1.86–2.89)	2.20 (1.77–3.96)	3.48 (3.14–4.43)
V	226–235	TVKDLLPLLE	2.28 (1.80–2.61)	2.09 (1.60–2.35)	3.48 (3.14–4.43)***
	276–285	PGFGDRRKAM	2.49 (2.02–3.10)	2.30 (1.73–3.22)	11.88 (10.78–14.19)***
	281–290	RRKAMLQDMA	2.51 (1.85–3.23)	2.55 (1.79–3.84)	4.49 (3.81–6.00)***
VI	341–350	GRVAQIRQEI	2.15 (1.71–2.45)	2.04 (1.33–2.76)	3.83 (3.26–5.12)***
	346–355	IRQEIENSIDS	1.48 (1.36–2.19)	1.20 (0.95–1.35)+++	2.66 (2.26–3.56)***
VII	351–360	ENSDDSYDRE	1.84 (1.71–2.22)	1.30 (1.15–1.46)+++	1.33 (1.25–1.68)++
	366–375	LAKLAGGVAV	2.66 (2.21–3.42)	2.07 (1.67–2.82)+	2.25 (2.09–3.29)
	371–380	GGVAVIKAGA	1.93 (1.64–2.60)	1.97 (1.33–2.32)	2.70 (2.37–3.44)**
VIII	376–385	IKAGAAATEVE	2.19 (1.91–2.61)	2.04 (1.61–2.59)	2.52 (2.11–2.97)
	408–417	IVAGGGVTLL	2.48 (2.00–2.96)	2.19 (1.42–2.93)	3.27 (2.14–4.08)
	413–422	GVTLTQAAPT	2.56 (2.02–3.20)	2.47 (1.82–3.18)	3.27 (2.14–4.08)
IX	418–427	QAAPTLDLKL	1.51 (1.28–1.89)	1.15 (0.88–1.30)+++	1.30 (1.14–1.46)+
	441–450	VALEAPLKQI	1.76 (1.58–2.03)	1.88 (1.70–2.05)	1.71 (1.65–1.98)
	446–455	PLKQIAFNISG	1.72 (1.53–1.97)	1.46 (1.18–2.29)	2.15 (1.99–2.37)**
	451–460	AFNSGLEPGV	1.44 (1.00–1.79)	1.00 (1.00–1.00)	1.76 (1.63–1.94)*
	456–465	LEPGVVAEKV	1.72 (1.38–2.04)	1.63 (1.38–2.03)	1.89 (1.65–2.05)
	461–470	VAEKVRNLPA	1.74 (1.40–2.41)	1.50 (1.17–2.40)	1.89 (1.65–2.05)
	466–475	RNLPAHGHLN	1.94 (1.56–2.60)	1.82 (1.33–3.05)	1.80 (1.69–2.09)
	471–480	GHGLNAQTGV	1.48 (1.22–1.72)	1.53 (1.30–1.75)	1.38 (1.29–1.60)
X	502–511	NAASIAGLFL	2.51 (1.70–3.18)	2.83 (2.05–3.28)	3.23 (2.87–3.99)**
	507–516	AGLFLTTEAV	2.60 (2.10–3.26)	2.49 (2.02–3.23)	3.65 (2.77–4.46)*
	512–521	TTEAVVADKP	2.13 (1.86–2.47)	1.92 (1.61–2.63)	3.09 (2.34–3.78)**

Overlapping peptides of ten regions of the HSP65 molecule were used as antigens. Antibody binding to peptides immobilized on polyethylene pins was detected using a modified ELISA. Reactivity index was defined as OD/OD_{min} where OD_{min} is the optical density of the peptide showing minimum OD values in the given experiment

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, significantly elevated; + $p < 0.05$; ++ $p < 0.01$; +++ $p < 0.001$, significantly decreased

^a Numbering according to the *M. bovis* HSP65 sequence (60 kDa chaperonin 2 GroEL2 (protein CPN60-2) (65 kDa antigen) (heat shock protein 65) (cell wall protein A) (*Mycobacterium bovis* AF2122/97). GenBank: CAD93311.1. 540 amino acids). Difference to the healthy persons group calculated by Mann–Whitney test

peptides (Online Tables 1, 2 and 3, Online Fig. 2). There were fewer differences between the IBD and HP than between CHD and HP sera:

- (a) When we compared *HP* and *IBD* sera HSP60 dominant peptides (by our definition, peptides of which both HSP60 and HSP65 homologues are recognized by serum antibodies, but the HSP60 homologue is recognized significantly more strongly) were infrequently found. Only two peptides fell into this category and exhibited such pattern of reactivity in either and both type of sera: HSP60 peptide 72–81 in region I and peptide 470–479 in region IX, both located in the equatorial domain of the protein and both partially buried. It should be noted that while peptide 72–81 is highly homologous with its HSP65 counterpart (50% identical, 80% similar), in case of peptide 470–479, the homology is small (30% identical and 40% similar).

The number of the HSP65 dominant peptides (serum antibodies reacting weaker with the HSP60 than with the corresponding HSP65 peptides) was higher with HP and IBD sera (Online Tables 1 and 3), such peptides were found in all regions of the HSP65 with HP sera and in all regions but VIII with IBD sera. The preference for HSP65 peptides was higher in case of IBD patients' sera, the HSP65 dominant peptides were recognized 1.94 times higher in average (1.2–3.2) than the HSP60 peptides, compared with the 1.5 (1.1–2.3) times higher average values in case of HP sera.

- (b) The autoantibodies of the group of *CHD* patients showed a different pattern of recognizing HSP60 or HSP65 peptides. In the *CHD* sera (Online Table 2) peptide 72–81 did, while peptide 470–479 did not react more strongly with the HSP60 than the corresponding HSP65 peptide. On the other hand, many other HSP60 dominant peptides in half of all regions, peptide 52–61 and the overlapping peptides 67–76 and 72–81 in region I, peptide 132–141 in region II, two overlapping peptides (peptide 162–171 and 167–176) in region III, peptides of the sequence 373–387 in region VI as well as two single peptides in region VIII were found to be HSP60 dominant. This dominance is only partly owing to the enhanced serum antibody binding to the HSP60 peptides; partly, it may be due to lower HSP65 peptide recognition compared to the recognition of the antibodies of healthy persons (Table 2).

HSP65 dominant peptides were found mainly in region four (all except two peptides in this region), two overlapping peptides and a single one in region IX (HSP65 peptides 466–475, 471–480, and 456–465), and three further single peptides, 96–105 from region

II, 341–350 from region VI, and 502–511 from region X. All HSP65-dominant peptides in *CHD* are also HSP65 dominant with HP sera as well.

- (c) It is interesting to note that the HSP60 dominant peptides recognized by HP and IBD sera are located exclusively in the equatorial domain and in case of *CHD* in the equatorial (regions I, II and VIII) and also in the intermediate domain of HSP (regions III and VI). On the other hand, the HSP65-dominant sequences can be found in all parts of the protein, a large part of the apical domain (region IV) is HSP65 dominant in all type of sera, and region V peptides are also HSP65 dominant in IBD and HP sera.

Anti-HSP60 and anti-HSP65 antibody binding to an IBD-specific T cell epitope in the IBD and HP sera

Recently, several HSP60 T cell epitopes specific for IBD patients were described (Puga Yung et al. 2009). One of these [peptide VASLLTTAEVVVTEIP (535–550)] was tested as potential B cell epitope using two overlapping decamers [peptide 536–545 (HSP60 ASLLTTAEVV) and peptide 541–550 (HSP60 TAEVVVTEIP)] by us, too. In case of these HSP60 peptides, we did not observe higher IBD than HP serum recognition, but in case of their HSP65 counterparts, we detected significantly higher IBD serum binding (Tables 1 and 2).

Discussion

In accordance with our previous results obtained with IVIG preparation and individual sera of healthy subjects (Uray et al. 2003), we found IgG antibody reactivity against synthetic peptides in almost all studied regions of human HSP60 and mycobacterial HSP65 (Table 3). There were only two consecutive peptides (172–181 and 177–186 of HSP60 and the corresponding 146–155 and 151–160 of HSP65 in region III, which did not significantly bind antibodies from either type of sera. These peptides were non-reactive in our previous study (Uray et al. 2003) as well. This sequence is localized in the equatorial domain of HSP; it consists of two α -helices surrounding a loop structure. The loop participates in binding the protein to its neighboring monomer within the multimer, but in the monomer, the sequence is exposed. Apart from peptide 470–479, HSP60 peptides of the region IX were non-reactive in all the three types of sera; however, strong binding was observed with the two peptides corresponding to the 475–489 sequence (HSP65 446–455 and 451–460) of the same region of HSP65. The recognized sequences overlap in five amino acids, but they are not identical; in

Table 3 Similarities in the over-all reactivity for different HSP60 and HSP65 peptides among sera of HP (healthy persons), CHD patients and IBD patients

Region	HSP60 peptide	HP	CHD	IBD	HSP65 peptide	HP	CHD	IBD
I	52–61	+	+	+	26–35	+	+	+
	57–66	+	+	+	31–40	+	+	+
	62–71	+	+	+	36–45	+	+	+
	67–76	+	+	+	41–50	+	–	+
	72–81	+	+	+	46–55	+	–	+
II	117–126	+	+	+	91–100	+	+	+
	122–131	+	+	+	96–105	+	+	+
	127–136	+	+	+	101–110	+	+	+
	132–141	+	+	+	106–115	+	+	+
	137–146	+	+	+	111–120	+	+	+
III	162–171	–	+	+	136–145	+	–	+
	167–176	+	+	+	141–150	+	+	+
	172–181	–	–	–	146–155	–	–	–
	177–186	–	–	–	151–160	–	–	–
	203–212	–	–	–	176–185	+	+	+
IV	208–217	+	+	+	181–190	+	+	+
	213–222	+	+	+	186–195	+	+	+
	218–227	+	+	+	191–200	+	+	+
	223–232	+	+	+	196–205	+	+	+
	228–237	+	+	+	201–210	+	+	+
	233–242	+	+	+	206–215	+	+	+
	238–247	+	+	+	211–220	+	+	+
	243–252	+	+	+	216–225	+	+	+
	248–257	+	+	+	221–230	+	+	+
	253–262	–	–	+	226–235	+	+	+
V	303–312	+	+	+	276–285	+	+	+
	308–317	+	+	+	281–290	+	+	+
VI	368–377	+	+	+	341–350	+	+	+
	373–382	+	+	+	346–355	+	–	+
	378–387	+	+	+	351–360	+	+	+
VII	394–403	+	+	+	366–375	+	+	+
	399–408	+	+	+	371–380	+	+	+
	404–413	+	+	+	376–385	+	+	+
VIII	436–445	+	+	+	408–417	+	+	+
	441–450	+	+	+	413–422	+	+	+
	446–455	+	+	+	418–427	+	–	+
IX	470–479	+	+	+	441–450	+	+	+
	475–484	–	–	–	446–455	+	+	+
	480–489	–	–	–	451–460	+	–	+
	485–494	–	–	–	456–465	+	+	+
	490–499	–	–	–	461–470	+	+	+
X	495–504	–	–	–	466–475	+	+	+
	500–509	–	–	–	471–480	+	+	+
	531–540	+	+	+	502–511	+	+	+
	536–545	+	+	+	507–516	+	+	+
	541–550	+	+	+	512–521	+	+	+

(+) Significant ($p < 0.05$) reactivity; (–) not significant (≥ 0.05) reactivity index. The reactivity index was considered significant when it differed significantly ($p < 0.05$) from 1.0 as tested by the non-parametric Wilcoxon signed rank test

HSP65, the more exposed loop is also recognized, while in HSP60, only the less exposed helical region (Online Fig. 2). The unordered part of this sequence is in close proximity to the other barrel in the multimer.

Although the overall qualitative pattern of the reactivity was similar (Table 3), we identified several *disease-* and *HSP-specific differences* at the comparison of the epitope specificity of the IgG-type antibodies against HSP60 and HSP65 in three types of sera (healthy persons, patients with severe coronary heart disease, and patients with inflammatory bowel disease) tested.

Disease-specific differences

Comparing the epitope specificity of the HSP60 and HSP65 antibodies in the CHD sera vs. the HP sera several marked differences were detected. Peptides corresponding to one of the ten regions (region II) reacted significantly more strongly with the anti-HSP60 antibodies in the CHD than those in the HP sera, indicating that the patients' sera contained higher amount of antibodies recognizing epitopes in this region of the HSP60 molecule. The sequence of this region consists of a loop surrounded by α -helices and is located in the equatorial domain of the monomer binding to the other barrel of the tetradecamer and the neighboring monomer within the barrel. With other peptides, we did not observe significant differences between the CHD and HP sera. To our best knowledge, only one report (Wysocki et al. 2002) dealt with the epitope specificity of the anti-HSP60 antibody in coronary heart disease. Unfortunately, the CHD-specific epitope (409–424) of HSP60 described by the paper of Wysocki et al. (2002) was only partly represented among the peptides we used (five amino acid overlap in peptide 404–413). This peptide was not found to be CHD specific in our analysis.

As it was mentioned above, abundant experimental and clinical data indicate the role of the anti-HSP60 antibodies in the pathomechanism of CHD. For example, in earlier studies of Foteinos et al. (2005), antibodies to human HSP60 that were isolated from the blood of CHD patients were injected to apoE-deficient mice. The antibodies induced an increased atherosclerosis as compared to the controls. In another study (Perschinka et al. 2003), authors produced antisera specific for HSP60-HSP65 cross-reactive epitopes by immunizing rabbits with peptides derived from human HSP60. These epitopes were found to be present in the arterial wall of young subjects during the earliest stages of the disease.

Based on the above studies, it seems most probable that the antibodies that cross-react to different epitopes of human HSP60 and bacterial HSP65 are of pathogenetic significance. It was demonstrated that the higher is the titer of these antibodies in the blood of patients the higher extent

of atherosclerosis can be found (Birnie et al. 2005; Prohaszka et al. 2001; Zhang et al. 2008). One crucial, not yet resolved question is the mechanism that leads to the increased titers of the anti-HSP60 antibodies. According to the “immunological cross-reaction” hypothesis of Georg Wick's group (Wick and Xu 1999)—due to high degree of antigenic homology that exists between microbial (viral, bacterial, parasitic) and human HSP60—protective cellular and humoral immunity against bacteria may lead to pathological reactions to human HSP60 that is expressed by endothelial cells of stressed arteries and result in atherosclerosis. Therefore, it seemed interesting to study whether the CHD-specific region found in our present study is represented among the HSP65 cross-reactive epitopes revealed by Wick's group (Metzler et al. 1997; Perschinka et al. 2003, 2007) as well as among the HSP60-homologue epitopes of different microorganisms such as *C. trachomatis* (Campanella et al. 2009; Yi et al. 1993), *R. tsutsugamushi* (Lachumanan et al. 1993), or *P. gingivalis* (Maeda et al. 2000). Region II, which was found to be CHD specific in the present study, contained the HSP65 homologue epitope 1 (aa 97–109) of Metzler et al. (1997) and HSP60 epitope 2 of Perschinka et al. (aa 90–102) (Perschinka et al. 2003), which were recognized by high titer sera of patients with atherosclerosis and anti-HSP60/65 sera. Apparently, this observation supports the hypothesis of Wick's group. However, the CHD sera did not react more strongly with corresponding HSP65 peptides of the same region than the HP sera, and in case of some peptides, even lower reactivity was observed, which could indicate that other mechanisms than the cross-recognition to bacterial infections are also responsible for the pathological anti-HSP60 immune response, which is most important in the pathomechanism of the atherosclerotic vascular diseases. This conclusion is in line with our previous observations on the difference of the anti-human HSP60 and anti-bacterial HSP65 antibody response in these diseases: Previously, we found that HSP60–anti-HSP60 immune complexes effectively activated the complement system, while HSP65–anti-HSP65 immune complexes did not (Prohaszka et al. 1999). In addition, we found that the level of anti-HSP60 antibodies was significantly higher in patients with severe CHD as compared with patients with negative coronarography, while no such difference was found with the anti-bacterial HSP65 antibodies (Prohaszka et al. 1999). Moreover, serum concentrations of anti-*H. pylori* antibodies significantly correlated with those of anti-HSP65 and anti-GroEL antibodies, but they did not correlate with the anti-HSP60 antibodies (Prohaszka et al. 2001).

Since several observations from our (Pozsonyi et al. 2009; Prohaszka et al. 2001; Varbiro et al. 2010) and other groups (Cohen and Young 1991; van Eden et al. 2003) as well indicate the natural autoantibody nature of the anti-

human HSP60 autoantibodies, we provided a new explanation (Prohaszka et al. 2001; Varbiro et al. 2010) for the association of the elevated anti-HSP60 levels and atherosclerotic vascular diseases. We assumed that the carriage of *high anti-Hsp60 autoantibody levels is an inherited trait*, and carriers of this trait are more prone to these diseases than the non-carriers. According to our recent studies (Pozsonyi et al. 2009), a significant part of subjects with high IgM type anti-HSP60 antibody levels carries the HLA-DR*1501 antigen as well.

Comparing the epitope specificity of the HSP60 antibodies in the IBD and HP sera, even more marked differences were found (Table 1). Many HSP60 peptides, but mainly those in regions I, II, IV, V, and VI, reacted significantly more strongly with serum antibodies in the IBD than with those in the HP sera, indicating that the patients' sera contained higher amount of antibodies against epitopes within these but not all regions of the HSP60 molecule. Similar findings were obtained with the HSP65 molecule as well; peptides, mostly those in the same regions as well as in region X, were recognized by more antibodies from the IBD than from the HP sera (Online Fig. 1). Most of the peptides recognized by IBD sera are exposed on the surface of the monomeric HSP, although they are participating in the connections of the monomers within the tetradecamer protein. HSP65 peptide sequences Aa111–120 and Aa502–511 recognized by IBD sera of regions II and X may form a discontinuous sequential epitope on the protein surface for IBD serum antibodies.

The observed stronger reaction to different HSP60 and HSP65 peptides in IBD vs. HP sera appears to be in contradiction with our previous results (Bene et al. 2002; Huszti et al. 2004) on the impaired antibody response of different bacterial HSP65 in IBD patients. This contradiction, however, may exist only, since we selected sera with the same levels of HSP60 and HSP65 antibodies from all the three groups for the present study; therefore, the differences we observed in the present study reflect qualitative differences only in the HSP65 antibody response between healthy individuals and IBD patients.

The only work on the epitope specificity of the anti-HSP60 immune response in Crohn's disease was published recently by Puga Yung et al. (2009). These authors studied the possible pathogenetic significance of the cellular immune response against some HSP60 epitopes as well. They have stimulated colonic biopsies with HSP-derived peptides from children with Crohn's disease (CD) and measured different inflammatory responses. They identified two epitopes that were able to sustain tumor necrosis factor alpha and interferon gamma induction. Since these responses correlated to the disease activity, the authors concluded that anti-HSP cellular immune responses contribute to the mucosal inflammation in CD. In our present

study, two overlapping HSP65 peptides (peptides 507–516 and 512–521 in the region X), covering one of the IBD-specific T cell epitopes described (Puga Yung et al. 2009), reacted more strongly with serum antibodies. Therefore, this region can also be considered as IBD-specific B cell epitope, which binds anti-HSP60 antibodies *in vivo*. It is tempting to speculate that this binding may have pathogenetic consequences, too.

HSP-type specific differences

HSP60-dominant peptides (by our definition, there is antibody reaction with both the corresponding HSP60 and HSP65 peptides, but significantly stronger with the HSP60) were found relatively infrequently in the HP and IBD sera (Online Fig. 2). Only one peptide (HSP60 peptide 72–81 in region I) behaved so in all the three types of sera tested. Peptide 72–81 was found to be HSP60 specific in our previous study (Uray et al. 2003), and it is 100% identical with the corresponding putative autoimmune epitope of the *C. trachomatis* in the recent study of Campanella et al. (2009). By contrast, in the CHD sera many peptides behaved as HSP60-dominant exclusively (Online Fig. 2), indicating again the dominant role of the anti-HSP60 immune response in this disease. Among these, both HSP60 peptide 67–76 (region I) and peptide 132–141 (region II) reacted much more strongly with serum antibodies than their HSP65 counterparts in our previous study (Uray et al. 2003) as well. All HSP60 dominant sequences are located either in the equatorial or in the intermediate domain.

In contrast to the HSP60-dominant peptides that were located mainly in the regions I, II, and VIII, true HSP65-dominant reactions (reaction with the HSP65 peptide, no reaction with the corresponding HSP60 peptide) in all the three types of sera were found mainly in regions IV and IX (Online Table 1, 2, and 3, Table 3, Online Fig. 2). There are two recognized sequences in region IV: the first is the β -strand on the surface of the protein in the intermediate domain, and the other is on the "top" of apical domain of the protein adopting helical conformation, exposed in the open form of the multimeric HSP as well, but masked in the closed form by the "GroES" cap. As for the region IX, the recognized sequences overlap in five amino acids, but they are not identical; in HSP65, the more exposed loop is also recognized, while in HSP60, only the less exposed helical region is recognized, which may explain the HSP65 dominant nature of this region.

In conclusion, we have found two human HSP60-dominant sequences, which is a characteristic of the healthy persons' group, which are located in the inside of the equatorial part of the HSP monomer, as if the immune system were able to attack certain autoimmune epitopes

only if the protein is denaturated or disintegrated. This pattern of HSP60 dominance does not change in case of IBD patients. In case of CHD patients' sera, more and partly different equatorial domain sequences were found to be HSP60 dominant than with HP sera; these are also partly buried sequences. On the other hand, there are two HSP60-dominant sequences located in the intermediate domain, and these are exposed in monomers and multimers alike.

Several HSP65 peptides were recognized significantly more strongly than their HSP60 counterparts in all three groups, especially by the sera of IBD patients, but many of them, especially the peptides derived from the exposed apical domain, show little disease specificity.

In addition, several new HSP65 epitopes have been recognized in IBD compared to HP; these latter epitope sequences are exposed in the monomer proteins, although some of them are participating in connecting the monomer units within the multimer. These epitopes may have arisen upon the immune system's encounter with the intact HSP proteins of pathogens, indicating the connection of bacterial infection and Crohn's disease.

The dissimilar localization of the HSP60-dominant epitopes and the HSP65-specific or HSP65-dominant epitopes also indicate that, in general, the HSP60 and HSP65 antibody response is qualitatively at least partly different in the serum samples tested. These findings could be utilized perhaps for the development of "peptide-cocktails" for diagnosis and/or monitoring the presence/progress or even more, for vaccination against relevant diseases.

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