Human Hsp60 could be a possible target of immune response triggered by Hsp60 of Salmonella Enteritidis – a preliminary study

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Abstract
Hsp60 of Salmonella Enteritidis appears to be involved in pathogenesis of infectious processes and host immune responses. Because of the similarities between microbial and human Hsps, a humoral response against microbial Hsps may be destructive for the host due to antigen mimicry leading to an autoimmune response. We have performed a preliminary study to investigate whether the homology between Hsp60 of Salmonella Enteritidis and human Hsp60 really exists. ELISA tests were done with success to show that polyclonal antibodies developed against Hsp60 of Salmonella Enteritidis recognize and cross-react with human Hsp60 and one of its seven synthetic peptides used in the study. It was revealed that the antigenic epitopes, which both the proteins have in common, are located between 409 and 424 amino acid residues of human Hsp60 molecule, and are determined by the amino acid sequence of human Hsp60 (409-424) synthetic peptide, the same sequence which was found by other authors to be an immunodominant epitope in patients with acute coronary syndromes. The results show the presence of an immunological and sequence similarity between bacterial Hsp60 and its human counterpart, and suggest that human Hsp60 could be a possible target of immune response triggered by Hsp60 of Salmonella Enteritidis. Salmonella Enteritidis Hsp60 might be potentially involved in autoimmune mechanisms operating in humans. This conclusion must be considered preliminary and hypothesis-generating.

Key words: Hsp60, Salmonella Enteritidis, Hsp6s homology, antigenic mimicry, immunological similarity.


Introduction
Microbial heat shock proteins (Hsps) appear to be involved in pathogenesis of infectious diseases and host immune responses [1-11]. There is a strong evidence that Hsps of the GroEL class (Hsp60 class) act as immunodominant antigens of many pathogenic microorganisms [5, 12-15]. Despite being conserved, they are also strongly immunogenic and their involvement in autoimmunity has been examined extensively [8, 16-20]. Due to a considerably high degree of sequence homology between bacterial and human heat shock proteins, this protein might be involved in autoimmune disease mechanisms operating in humans [7, 10, 11, 21-30]. Although many studies of Hsps have been conducted with different pathogenic bacteria [2, 6, 12, 13, 31, 32], including some with Salmonella Choleraesuis, Salmonella Typhimurium and Salmonella Typhi [1, 3, 5, 33-36], there have been only a few studies with Salmonella Enteritidis done [37]. It was revealed that Hsp60 of Salmonella Enteritidis is highly immunogenic. The important role of this protein as a target of the immune response was reported for the laying hens from Salmonella Enteritidis naturally infected flocks – with acute infection (flock I) as well as with occasional bacteria excretion (flock II) [37]. The levels of Hsp60 specific immunoglob-
ulins in egg yolks, especially during the acute phase of infection were high and related to those against lipopolysaccharide and flagellin, the antigens of the established immunological importance in *Salmonella Enteritidis* infections. The values obtained from flock II were lower than those for the flock I, being, however, considerably higher than results obtained from the control flock (III). Within *Salmonella Enteritidis*-negative hens (flock III), the anti-Hsp60 antibody concentrations were consistently low. To this time still little is known about Hsp60 produced by *Salmonella Enteritidis* organisms and its possible role in the autoimmune processes. To our knowledge, there has been no published report on the similarity between Hsp60 of *Salmonella Enteritidis* and human Hsp60. We have thus performed a preliminary study to investigate whether the homology between both these proteins really exists. In this paper we report the identification of one immunogenic 16-mer peptide within human Hsp60 following the screening of a synthetic peptide library with anti-*Salmonella Enteritidis* Hsp60 serum. Enzyme-linked immunosorbent assays were done successfully to show that polyclonal antibodies developed against Hsp60 of *Salmonella Enteritidis* recognize and interact with human Hsp60 and one (aa 409–424) of its seven synthetic peptides used in the study. These results show the presence of an immunological and sequence similarity between bacterial Hsp60 and its human homologous protein, and suggest that human Hsp60 could be a possible target of immune response triggered by Hsp60 of *Salmonella Enteritidis*.

### Material and methods

**Salmonella Enteritidis Hsp60 preparation**

The experimental details of the *Salmonella Enteritidis* Hsp60 preparation were earlier described by Dera-Tomaszewska et al. [37]. In the purification process, protein which was found to have a molecular weight of approximately 60 kDa under reducing conditions was obtained. Sodium dodecyl sulphate polyacrylamide gel electrophoresis was used to evaluate the protein concentration and purity. The protein identity was estimated by monoclonal antibodies against *Escherichia coli* GroEL protein (SPA-870, Stressgen, Victoria, BC, Canada) in the Western immunoblot analysis.

**Human Hsp60 fragments synthesis**

A total of seven human Hsp60 fragments were synthesized in the Faculty of Chemistry, University of Gdański, Gdańsk, Poland. The experimental details associated with synthesis were described by Wojciechowska et al. [38]. Briefly, knowing the sequence from literature [39] and using the special computer program [40], potential surface oriented regions of human Hsp60 were identified. To define potential antigenic determinants acrophilicity and hydrophilicity profiles of this protein were constructed. Based on the obtained results, seven potential immunogenic human Hsp60 fragments were selected for immunological tests (Table 1). They were of 12, 13, 14, 15, and 16 amino acids in length. The peptides were synthesized by the solid phase method [41] in a 0.5 mmol scale. The Fmoc/But procedure for synthesis was used. The peptides were homogenous on HPLC and revealed the expected amino acid composition and molecular weight.

**Anti-Salmonella Enteritidis Hsp60 polyclonal antibodies induction**

Polyclonal antibodies against Hsp60 of *Salmonella Enteritidis* were obtained as a result of rabbit hyperimmunization. Before immunization, a blood sample from the marginal vein of the rabbit ear was taken to determine whether the serum contains antibodies against Hsp60. The animal was free from spontaneous antibodies. The rabbit received antigen (20–30 µg per injection) subcutaneously four times at intervals of two weeks. The primary injection was given in complete Freund’s adjuvant, while all boosts were done in incomplete adjuvant. The rabbit was bled ten days after the last injection. After collection, blood was allowed to clot for 30-60 min at 37°C. The clot was then separated from the sides of the collection vessel using a Pas-

<table>
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<th>Table 1. Synthetic peptides of human Hsp60 protein</th>
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<td><strong>Peptide</strong></td>
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<td>Hsp60 (119–131)</td>
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<td>Hsp60 (301–314)</td>
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<td>Hsp60 (409–424)</td>
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ExpertPlus, AS YS Hitach GmbH, Austria). Each test was performed in triplicate. Results were taken from two used data at 492 nm using an ELISA Reader (microELISA Reader). The difference between the mean reactivity value of the antibody control (the mean value, calculated for each antigen, was used as a negative control). The difference between the mean reactivity value of six measurements for each antigen and the proper negative control plus three standard deviations was plotted in the corresponding graphs.

**Competition ELISA**

The competition ELISA was performed to confirm the specificity of binding of serum antibodies to human Hsp60. In this assay, one of the ELISA steps described above was modified. The rabbit antiserum against Salmonella Enteritidis Hsp60 was diluted 1:1000 in the blocking solution and incubated at 37°C for 3 h with additional 5 μg/ml of Salmonella Enteritidis Hsp60, or 5 μg/ml of recombinant human Hsp60, or 10 μg/ml of human Hsp60 (aa 409-424) synthetic peptide, and then added to the corresponding wells. The rabbit antiserum absorbed with Hsp60 of Salmonella Enteritidis was tested with three antigens for which the positivity level of absorbance in the ELISA test was indicated, i.e., Salmonella Enteritidis Hsp60, recombinant human Hsp60 and the human Hsp60 (aa 409-424) synthetic peptide. *Salmonella Enteritidis* Hsp60 was used as a solid phase antigen to examine the antibody-competing effect of recombinant human Hsp60 and synthetic peptide. The assay was continued according to the procedure described above. Each test was performed in triplicate. Two independent experiments were done. The antibody binding to the proper solid phase antigen in the presence and absence of competing Hsps or synthetic peptide was compared.

**Results**

The polyclonal antibodies developed against Hsp60 of *Salmonella Enteritidis* recognized and reacted with *Salmonella Enteritidis* Hsp60, as well as with the human homologous protein and one of its synthetic fragments. Of these seven synthetic peptides used in the study, only 16 amino-acid fragment located between 409 and 424 amino acid residues of human Hsp60 gave ELISA signals above cut-off value (Fig. 1). Specificity of binding of anti- *Salmonella Enteritidis* Hsp60 antibodies to human Hsp60 and its 16-mer synthetic peptide (aa 409-424) was confirmed by inhibition of immunoenzymatic reaction by means of absorption of serum antibodies with the excess amount of a given antigen. The reactivity of rabbit antiserum to the human Hsp60 and human Hsp60 (aa 409-424) synthetic peptide was completely lost, and to *Salmonella Enteritidis* Hsp60 highly reduced (to a very low level), when serum was absorbed with *Salmonella Enteritidis* Hsp60 protein (Fig. 2). The test revealed also that synthetic fragment
of human Hsp60 and human recombinant Hsp60 markedly inhibited (over fivefold and over threefold, respectively) the binding activity of rabbit anti-Salmonella Enteritidis Hsp60 antibodies to the solid phase bound Hsp60 of Salmonella Enteritidis (Fig. 3).

**Figure 2.** ELISA results of reactivity of anti-Salmonella Enteritidis Hsp60 serum (1) and serum after absorption with homologous Hsp60 to the three following antigens: Salmonella Enteritidis Hsp60, human Hsp60 (409-424) synthetic peptide and human Hsp60.

**Figure 3.** ELISA results of reactivity of no-absorbed anti-Salmonella Enteritidis Hsp60 serum (1) and serum after absorption with human Hsp60 (409-424) synthetic peptide (2) and human Hsp60 (3) to Hsp60 of Salmonella Enteritidis.

**Discussion**

On a global scale, Salmonella is responsible for an estimated 3 billion human infections annually. Additionally, data concerning non-typhoidal Salmonella serovars are difficult to obtain, as most patients do not need to consult the health service. The estimates suggest that there are at least three additional cases for every reported case. Most Salmonella enterica serovars cause illness in humans. The epidemiology of human diseases is currently dominated, however, by only a few serovars. Salmonella Enteritidis is the predominant etiologic agent of human salmonellosis in numerous countries worldwide [42]. Salmonella Enteritidis mainly tends to cause a gastroenteritis, which is usually self-limiting, but it is also included to the group of these non-typhoidal Salmonella serovars which may cause human invasive salmonellosis. Salmonellosis has also been associated with long-term and sometimes chronic sequelae. Although the decreasing trend has been currently observed in Salmonella Enteritidis human infections [43, 44], this serovar still remains the “number one” within epidemic Salmonella serovars in many countries and still makes the real health problem. All aspects associated with its pathogenicity, including the possibility of Hsp60 involvement in the human autoimmune response, are worth to be elucidated.

Heat shock proteins constitute a subject of research of many scientists through years. These proteins are induced in prokaryotic as well as eukaryotic species under various conditions of stress [45]. They are traditionally grouped into families of homologous proteins depending of their molecular weight [46]. They are strongly immunogenic and highly conserved, and their involvement in autoimmunity has been examined extensively. Heat shock proteins have been shown to elicit a strong humoral and cellular immune responses during infection by a variety of pathogens. The 60 kDa heat shock proteins family is a focus of interest as a potential antigen in autoimmune diseases. Humoral immune responses to Hsp60 have been found in a number of human autoimmune diseases [7, 47]. Many studies revealed that antibodies against Hsp specifically bind to target tissue of the autoaggressive response. Accumulating evidence from epidemiological or immunopathological studies indicates a role of gastrointestinal tract associated bacteria in the induction and/or perpetuation of autoimmune diseases [7, 27, 28, 48-50]. Anti-self-Hsp60 antibodies could be induced by pathogenic, e.g., Helicobacter pylori [51-53], as well as commensal bacteria like Escherichia coli [47], due to the molecular mimicry. Local inflammatory and immune responses against Helicobacter pylori in the stomach can also induce systemic immune reactions that promote the pathogenesis of extra-gastrointestinal diseases such as atherosclerosis [46, 54]. The association of antibodies against Hsp60 of Helicobacter pylori production with the development of cardiovascular disease (CDV) was demonstrated by Okada et al. [53]. Furthermore, it was revealed that the 20 amino acid residues (Glu141-Leu160) of Helicobacter pylori Hsp60 might be predominant CVD-associated epitopes that induce anti-human Hsp60 autoantibodies, whose location was predicted in the tertiary structure of human Hsp60. Hirata and colleagues [48] have found that anti-Hsp60 antibodies in patients with rheumatoid arthritis as well as other connective tissue disorders are
Human Hsp60 could be a possible target of immune response triggered by Hsp60 of Salmonella Enteritidis – a preliminary study

raised by infection with intestinal microorganisms. Antibodies against human Hsp60 cross-reacting with *Escherichia coli* Hsp60, which significantly exceeded the titers found in normal controls, have been detected in patients with rheumatoid arthritis. These antibodies react preferentially with *Escherichia coli* Hsp60 compared with *Mycobacterium tuberculosis* Hsp65 or human Hsp60. The antibodies to different enterobacterial Hsp60s are suggested to be associated with the inflammatory process and initiation of ankylosing spondylitis (AS) [27, 50]. It has been demonstrated that patients with AS (HLA-B27 positive) have high titers of antibodies to the 60 kDa heat shock protein of *Klebsiella pneumoniae*, the Gram-negative bacteria frequently found in the human gut [49]. It has been postulated that Hsp60s of bacteria from the gastrointestinal tract may be involved in the onset of Kawasaki disease (KD) [28], and that an interaction between bacterial and self-Hsp antibody responses could play a role in vascular damage characteristic of KD. Based on Nagata et al. [28] findings, the most likely candidates for bacteria associated with the KD pathogenesis might be Gram-negative microbes, such as *Neisseria mucosa* or *Acinetobacter lwofii*, which have been isolated from KD patients with vascular involvements. The incidence rate of coronary lesions in patients with KD may depend upon how strongly causative agents can induce the initial immune activation and, importantly, how much self-Hsp molecules they can evoke from the cytoplasm or mitochondria to the vascular surface. Interestingly Gram-negative bacteria appeared to trigger more self-Hsp than Gram-positive cocci [28]. The intestinal bacteria may represent another example of autoimmune responses triggered by antigen mimicry of host proteins to microbes. *Salmonella* Enteritidis should be taken into consideration, especially that in infected patients, the transient intestinal carriage of these Gram-negative, pathogenic bacteria occurs and can last even several months. Some patients may harbour these organisms for up to a year or longer.

The Hsps are antigenic, and the recognition of specific epitopes on such highly conserved antigens may have pathological autoimmune consequences [7]. Because of the similarities between microbial and human Hsps, a humoral response against microbial Hsps may be destructive for the host due to antigen mimicry leading to an autoimmune response. The results of experiments presented in this paper, confirmed that a homology between Hsp60 of *Salmonella* Enteritidis (Gram-negative, intestinal pathogenic bacteria) and human Hsp60 exists. Enzyme-linked immunosorbent assays were done with success to show that polyclonal antibodies developed against Hsp60 of *Salmonella* Enteritidis recognize and cross-react with human Hsp60 and one of its seven synthetic peptides used in the study. Their specificity of binding was confirmed by absorption experiments with the proper antigens. It was revealed that the antigenic epitopes which both the proteins have in common are located between 409 and 424 amino acid positions of human Hsp60, and are determined by the amino acid sequence of human Hsp60 (aa 409-424) synthetic peptide: Thr-Ser-Asp-Val-Glu-Val-Asn-Glu-Lys-Lys-Asp-Arg-Val-Thr-Asp-Ala. This sequence shows a great overlap with that one presented by Boog and colleagues [55], as containing the cross-reactive epitope between bacterial GroEL and human cognate, which was located by one (LK2) of two monoclonal antibodies generated against human Hsp60. The LK2 antibody reacted with its homologous protein, and additionally showed reactivity with mycobacterial Hsp60 as well as corresponding proteins present in cell extracts of some other bacteria, i.e., GroEL protein of *Treponema innocens*, *Treponema hyodysenteriae*, *Salmonella Typhimurium*, *Yersinia enterocolitica* and *Escherichia coli*. The epitope recognized by LK2 antibody is formed by amino acid residues within the sequence 383-419 of the human Hsp60 molecule. Epitopes of bacterial proteins structurally similar to self-antigens present in the host may induce antibodies against self protein structures. It means, that *Salmonella* Enteritidis Hsp60 potentially might induce autoantibodies. An immune response originally triggered by bacterial antigen might induce an autoimmune reactivity and even mild infections are able to cause cross-reactive response and, as a consequence, the autoimmune disease. The methods used in the performed study and presented in this paper allow to determine explicitly that Hsp60 of *Salmonella* Enteritidis meets the requirements of molecular mimicry. Due to a sequence homology between *Salmonella* Enteritidis heat shock protein 60 and its human counterpart, this protein might be potentially involved in autoimmune mechanisms operating in humans. That conclusion must be considered preliminary and hypothesis-generating than hypothesis-proving.

The Hsp60 (409-424) domain, recognized by polyclonal antibodies raised against *Salmonella* Enteritidis Hsp60, was also recognized by serum antibodies of patients with acute coronary syndromes (ACS) [56]. Sera from groups of patients with unstable angina (UA) and myocardial infarction (MI) reacted against this Hsp60 fragment with significant positive reactivity values relative to the reactivity of other peptides tested. The control group showed a much lower average response to this peptide than the UA and MI groups. Hence, it was concluded by Wysocki and colleagues [56] that peptide Hsp60 (409-424) behaved as an immunodominant epitope in patients with ACS. This suggests that amino acids sequence determined by this peptide might be this antigenic determinant of human Hsp60 which induces production of autoantibodies responsible for development of atherosclerotic lesions.

Serum specimens from the above patients were used for a screening study to determine in ELISA tests if *Salmonella* Enteritidis Hsp60 specific antibodies are present. All samples from patients with UA and MI showed relatively high levels of anti-*Salmonella* Enteritidis Hsp60 antibodies (mean value OD = 0.418) in comparison to the control individuals (OD = 0.146) (data not shown). It is tempting to specu-
late that Hsp60 of Salmonella Enteritidis, due to the common epitopes with human Hsp60, can potentially generate anti-Hsp60 antibodies that may be responsible for an autoimmune reaction associated, for example, with an accelerated development of atherosclerotic lesions (?). Future studies should clarify whether Hsp60 of Salmonella Enteritidis contributes to the initiation and/or perpetuation of diseases by an autoimmune reaction. The results obtained in this study make a considerable contribution to the research on the heat shock proteins and Salmonella Enteritidis pathogenicity.

The authors declare no conflict of interest.

References


