A role for anti-HSP60 antibodies in arthritis: a critical review

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Abstract

Introduction
As a result of the high sequence similarity between 60-kDa heat shock proteins (HSP60), found in both prokaryotic and eukaryotic cells, it has been suggested, but never concluded, that anti-HSP60 antibodies could be of importance in the pathology of arthritis diseases explained by a concept named molecular mimicry. In a number of clinical studies, antibodies to both human and bacterial HSP60 have been detected in serum from patients with different inflammatory diseases, but divergent results have been published. Recent progress, however, has increased the specificity in tests used to determine the humoral response to HSP60. In this review, these new findings are compared with old questioning the durability of molecular mimicry as a hypothesis for arthritis pathogenesis.

The current literature was examined through a thorough PubMed search using appropriate keywords in relation to anti-HSP60 antibodies and arthritis-related disease. Main research articles were selected for review.

Discussion
Recent reports have added new information to the interpretation of the humoral immune response to HSP60. By introducing determination of IgG subclasses by the ELISA (enzyme-linked immunosorbent assay), HSP60-specific antibodies were shown to be predominantly of the IgG1 and IgG3 isotypes for bacterial and human HSP60, respectively, thereby improving the strategy to test the hypothesis of cross-reaction.

Conclusion
The hypothesis of molecular mimicry comes from attempts to link bacteria to the development of arthritis. However, based on a critical investigation of the literature, it is here argued that investigation on the role of HSP60 in the development of arthritis in addition to anti-HSP60, serology should include a more functional study on how HSP60 can reach the extracellular locations and whether it is tolerogenic. Second, studies are needed on how the different subclasses of IgG-specific human HSP60 may stimulate the immune environment into an anti- or pro-inflammatory state.

Introduction
A disease-related role for antibodies against 60-kDa heat shock proteins (anti-HSP60) in arthritis was suggested in 1984 by Lakomek et al.¹. The group demonstrated by the use of immunofluorescence staining that antibodies from a significant amount of sera from ankylosing spondylitis (AS) patients (39% of 62 patients) did bind antigens present in the puffs generated in polytene chromosomes of Drosophila melanogaster by heat shock. These antigens were induced from a specific locus (93D) by heat shock and thus the proteins were called HSPs².

In bacteria, HSP60 is also called GroEL, in which the L stands for large as it represents the larger subunit of a complex between two rings of 7 HSP60 subunits joined together with a ring of GroES, the smaller subunit of the complex (S for small) (Figure 1). Similarly, in eukaryotic cells, HSP60 is associated with HSP10, and the complex is located within the mitochondrial matrix. The primary role of HSP60 is to facilitate protein folding inside the mitochondria. Therefore, they have also been termed molecular chaperones, and HSP60 has recently been renamed HSPD1 in a new nomenclature³. In this review, however, we will use bacterial and human HSP60 throughout the article.

Proteins within the HSP60 family are highly conserved, which from alignment of the amino acid (aa) sequence shows close to 50% identity between bacterial and human HSP60. The high homology led to the hypothesis that bacterial HSP60 might induce a break in immune tolerance as a result of molecular mimicry leading to autoimmunity⁴. Cross-reactivity between bacterial and human antigens was first suggested by Ebringer et al. who in an attempt to link the presence of antibodies to Klebsiella and recurrent inflammation in AS, suggested that linear or conformational epitopes common to microbial antigens and host cell molecules would give rise to autoimmunity through an immune cross-reaction⁵. In the case of Ebringer et al., it was suggested that the cross-reaction was between a component of gram-negative bacteria and human leucocyte antigen B27 (HLA-B27) as this antigen was found to be a major susceptibility gene for AS⁶, HLA-B27

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Figure 1: The structure of the E. coli GroEL-GroES protein complex. The structure of the E. coli GroEL-GroES protein complex has been solved by X-ray crystallography (PDB 1AON)\(^2\). (A) Its barrel-like appearance is created by 14 identical subunits of relative molecular mass of 58 kDa (GroEL also known as HSP60) assembled as two heptameric rings stacked back to back (green and blue, respectively). On top of the hollow barrel, in which protein folding is executed through specific interaction between polypeptide and hydrophobic side groups, a heptameric ring or ‘cap’ (gray) of seven identical 10 kDa subunits (GroES also known as HSP10) associates to create the GroEL-GroES complex. The association of the HSP10 heptamer comes with the cost of ATP and has an allosteric effect on the complex assuring proper folding of the encapsulated polypeptide. Electron microscopy of the human GroEL-GroES protein complex illustrates that human in contrast to the E. coli complex, might be formed from one ring of 7 HSP60 subunits\(^2\). As for now, the mammalian HSP60/HSP10 structure has not been solved.

Discussion

The authors have referenced some of their own studies in this review. These referenced studies have been conducted in accordance with the Declaration of Helsinki (1964), and the protocols of these studies have been approved by the relevant ethics committees related to the institution in which they were performed. All human subjects, in these referenced studies, gave informed consent to participate in these studies.

Human HSP60

The gene encoding human HSP60 is located on chromosome 2\(^9\). The protein has a mitochondrial leader sequence in its N-terminal end, indicating its role as an intra-mitochondrial protein. Human HSP60, most likely released from damaged cells, is found outside cells where it is able to interact with cells of both the innate and the adaptive immune system (Figure 2)\(^9\). This suggests a multifunctional role for HSP60 and consequently introduces the concept of moonlighting proteins reviewed in the literature\(^10\). However, a defined mechanism for the transport of human HSP60 to the membrane and across, has yet to be determined in a healthy mammalian cell. Some suggest that human HSP60 can be transported in extracellular vesicles called exosomes with the involvement of lipid rafts\(^11\). However, because exosomes have long been seen as ‘cell garbage’, it is still a new topic and not completely understood\(^12\). Also, a large part of HSP60 secretion has been observed from cancer cells, in which alternative forms of HSP60 transcripts are suggested to lead the protein through specific internal cellular membrane structures referred to as the endoplasmic reticulum (ER)-Golgi pathway\(^13\). However, we would suggest not extracting conclusive content from cancer research and apply it on normal homeostasis and the secretary routes of proteins.

A more clearly suggested mechanism is the release of human HSP60 from damaged or dead cells. Death of cells can be either harsh or gentle to the extracellular environment termed necrosis or apoptosis, respectively. Apoptosis is a well-defined process, whereby the cell, as a result of an unbalance between pro- and anti-apoptotic members of the Bcl-2 family, actively triggers a cascade of events including mitochondrial cytochrome C release and caspase-proteolysis\(^14\). This ultimately leads to cell shrinking and fragmentation into small membrane-bound apoptotic bodies. Compared with necrosis, the process is gentle to the surroundings without eliciting an inflammatory response.
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human HSP60 have a link to a bacterial trigger, it must be a distant role.

The epitope recognised by antibodies on bacterial HSP60 consists of a three-dimensional structure presented on the protein surface (also named a conformational epitope). Before such antibodies can be generated, an antigen-presenting cell must take up the HSP60 protein through a scavenger receptor, process it and present a linear peptide in MHC class II to a CD4+ T cell (Figure 2d). Hereby HSP60-specific CD4+ T cells are amplified. In parallel, B cells presenting antibodies specific for HSP60 on their surface will take up HSP60, process it and present a linear fragment within MHC class II. This in turn can lead to CD4+ T cell-dependent activation of the antigen-specific B cell. B cell activation is executed through antigen recognition and costimulatory molecules by which plasma cells producing Ig molecules of different classes are generated through somatic hypermutation and switching (Figure 2e). In that way, the IgG antibodies are produced. IgG can be divided into four subclasses, IgG1-4, which are defined by a structural difference in the Fc receptor. In a recent study\(^\text{21,22}\), HSP60-specific antibodies were only present in the IgG1 and IgG3 isotypes, as discussed in the next section.

**Cross-reaction of interspecies HSP60**

To investigate the hypothesis of molecular mimicry and a diagnostic potential of anti-HSP60 in arthritis, several studies have used enzyme-linked immunosorbent assay (ELISA) in which detection of serum antibodies against bacterial and human HSP60 was measured for a disease group and compared with a healthy control group (the most important findings are shown in Table 1). In the majority of these publications, indirect ELISA is used. In indirect ELISA, the antigen is immobilised and targeted with serum antibodies followed by detection with conjugated antibodies specific for the class of interest (e.g. IgA, IgM, IgG) (Table 1). Measurements are carried out by optical density (OD) readings, and the ELISA is optimised for the best dynamic range, with or without quantification. A significant correlation to disease or difference between disease and control of measured antibody levels has generated information regarding the role of anti-human HSP60 antibodies in a number of inflammatory diseases (Table 1). However, in our opinion, such results based on ELISA can be questioned as will be discussed.

The first significant attempt to assess the like of autoimmunity as a result of cross-reaction between bacterial (Mycobacterium bovis MbHSP65) and human HSP60 was carried out by Handley et al. in 1995\(^\text{23}\). The use of a plasmid (pRSET) that contained a 4.5 kDa fusion peptide with a polyhistidine tail made it possible to produce a pure recombinant protein using metal ion (Ni\(^{2+}\)) affinity chromatography. By generating specific antisera in rabbits towards probability-selected

### Table 1 Study overview: cross-reaction as a potential trigger in arthritis

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<td>Gelsing Carlsen et al.(^\text{25})</td>
<td>Sandwich ELISA</td>
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AS, Ankylosing spondylitis; CD, Chron’s disease; Mb, mycobacterial; Ra, rheumatoid arthritis; ReA, reactive arthritis; SpA, spondylo arthritis; SLE, systemic lupus; TB, active tuberculosis; TFI, tubal factor infertility; UC, ulcerative colitis.

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surface-associated epitopes delivered subcutaneously as a peptide conjugate, they were able to separate an IgG<sub>total</sub> response to bacterial HSP60 from human HSP60. Also, they could demonstrate that in serum IgG<sub>total</sub> antibodies to human HSP60 were significantly higher than IgG<sub>total</sub> antibodies toMbHSP65 in both normal persons and in patients with Ra. Finally, they demonstrated that serum from Ra patients did not show higher levels of such antibodies than was found in normal serum samples. These findings are regarded as the first report questioning a bacterial triggered HSP60 autoimmunity.

Probably because M. bovis cannot induce rheumatoid arthritis (Ra), several groups have hypothesised cross-reactivity based simply on ELISA. IgG<sub>total</sub> measurements have revealed a relationship between HSP60 antibodies and Ra, whether it comes from an indirect, sandwich or competitive ELISA (C-ELISA<sup>23-27</sup>). Such results are often inconclusive because determination of IgG<sub>total</sub> may mask differences that can be determined using subclass determination. The lack of IgG subclass determination has in several cases shown correlations and thereby leads to the assumption of molecular mimicry. In one of these studies, antibodies to human HSP60 correlated to antibacterial HSP60 antibodies (r<sup>2</sup> = 0.735). More importantly, the correlation could be inhibited from a C-ELISA in six different sera (not from the same disease group) using soluble bacterial HSP60 (E. coli) as a competitive antigen<sup>28</sup>. Had the authors determined IgG subclass antibodies to HSP60, they may have changed their conclusions. Not least because it was noticed from the assay that the inhibition of anti-bacterial antibodies was far better with bacterial HSP60 antigen than with human HSP60, suggesting that a minority of the antibodies are cross-reacting.

An important part of generating recombinant antigens for ELISA is the conformation of the antigen. Most recombinant antigens are until 2009 purified under denaturing conditions<sup>29</sup> leaving the purified antigen misfolded (Table 1). This will cause problems if the epitopes recognised by the antibodies are conformational.

In the case of HSP60, the folding of the protein is important for binding of antibodies (Figure 3). Carlsen et al. described a purification method without prior denaturation of the antigen<sup>22</sup>. To test the sensitivity of the ELISA using a purified antigen, we

![Figure 3: Comparison of ELISA results using either denatured or native purified HSP60. A difference in antibody affinity for denatured (a) and non-denatured (b) HSP60 was evaluated from a serial two-fold dilution (1/20–1/80) of serum treated with different concentrations of enzyme-conjugated sheep-anti-human IgG1 (1/2500–1/80,000). Each dot represents the mean of two measurements supplied with the standard deviation. The serum sample was taken from an axial SpA patient, who was tested seropositive for Campylobacter jejuni HSP60. The ELISA plate was coated with 4 mg/ml denatured and non-denatured C. jejuni HSP60. Coating was prepared on the same plate to reduce intra-assay variation.](image-url)
compared the native version of *C. jejuni* HSP60 (A) with the denatured protein (B) using dilution series of a seropositive serum from a patient with SpA. The measurements were carried out in duplicates on the same plate to diminish intra-assay variation. Results presented in Figure 3 show from the lines highlighted by arrows that more sensitivity (based on the slope steepness between two measurements) is achieved from the native antigen, and this is important for obtaining the best resolution and interpretation of ELISA measurements.

Because *E. coli* is used for producing recombinant HSP60, it has been speculated that *E. coli*-derived lipopolysaccharide (LPS) contamination could cause false-positive results in the HSP60 ELISA. This, however, has been ruled out from several experiments reviewed in the literature. It is now generally accepted that LPS can be completely removed or reduced to insufficient levels using polymyxin B (an LPS-binding antibiotic) and therefore has no influence on ELISA result. Moreover, the most widely used *E. coli* clone for the production of recombinant proteins, BL21 is a rough mutant missing the O-chain of LPS seen in wild type *E. coli*. Therefore, the LPS is not immunogenic, but the lipid-A part is present, so it can activate toll-like receptor 4 (TLR-4) in cellular assays.

In the publications by Hjelholt et al. and Carlsen et al., it was found that the dominant subclass for human HSP60 in SpA is IgG3, while antibodies to bacterial HSP60 were dominated by IgG1 (Figure 4). In both studies, there was a significant correlation between SpA and IgG1 and IgG3 antibodies to human HSP60 but either weak or no correlation of SpA to antibodies to bacterial HSP60. It was therefore concluded that while antibodies against human HSP60 are associated with SpA and are predominantly of the IgG3 subclass, antibodies against bacterial HSP60 are predominantly of the IgG1 subclass, indicating that there is no cross-reaction, and a role for anti-human HSP60 based on the ELISA results cannot support molecular mimicry as a pathogenic mechanism for arthritis diseases. The study by Hjelholt et al. showed in addition a weak correlation between anti-human HSP60 IgG3 and one of the several disease parameters. This was not seen in cohort study in which antibodies were determined overtime comprising 40% of the samples analysed, probably due to the reduced number of patients participating in this study.

The association between elevated levels of antibodies against human HSP60 and SpA probably reflects a general activation of the immune system combined with increased appearance of human HSP60 outside inflammatory-associated damaged cells in the joints of patients with SpA.

Our studies showed that generation of antibodies to human HSP60 was independent of presence of antibodies to bacterial HSP60 and therefore the hypothesis of molecular mimicry could not be supported.

**Conclusion**

The hypothesis of molecular mimicry comes from the attempt to link bacteria to the development of arthritis, but it is argued here that investigation on HSP60 serology should be switched to a more functional study on how HSP60 begins to appear in the extracellular locations and whether it is tolerogenic. Second, it remains to be investigated how the different subclasses of IgG specific for human HSP60 may stimulate the immune environment into an anti- or pro-inflammatory state.

**Abbreviations list**

AS, ankylosing spondylitis; CD, chron disease; CD4, cluster of differentiation 4; C-ELISA, competitive enzyme-linked immunsorbent assay; DC, dendritic cell; HLA-B27, human leukocyte antigen B27; HSP, heat shock protein; Ig, immunoglobulin; LPS, lipopolysaccharide; OD, optical density; PAMPs, pathogen-associated molecular patterns; Ra, rheumatoid arthritis; ReA, reactive arthritis; SpA, spondyloarthritis; TB, active tuberculosis; TFI, tubal factor infertility; TLR, toll-like receptor; UC, ulcerative colitis.

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