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> Antibodies against C-reactive protein cross-react with 60-kilodalton heat shock proteins
Antibodies against C-reactive protein may cross-react with 60 kD heat shock proteins

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Abstract

C-reactive protein is an acute-phase reactant frequently used in histochemistry as a marker of ongoing inflammation. Furthermore, CRP is a powerful biomarker for prediction of coronary artery disease risk. Heat-shock protein 60 and CRP are complement activating molecules and we aimed to study the effect of their interactions on the regulation of complement activation. However, during the first experiments we learned that polyclonal anti-CRP antibodies cross-react with Hsp60. Therefore, the aim of our present study was to analyze the cross-reactivity of anti-CRP antibodies with Hsp60 in solid-phase enzyme immune assays, in epitope studies using series of overlapping synthetic peptides and in Ouchterlony analyses. We found that three different commercial rabbit polyclonal, and two monoclonal (9C9, CRP-8) anti-CRP antibodies specifically recognize recombinant human Hsp60 and recombinant *M. tuberculosis* Hsp65, respectively. Hsp60 was found to inhibit the binding of anti-CRP polyclonal Ab to Hsp60. Six epitope regions of Hsp60 were recognised by the anti-CRP antibodies and one region (AA218-232) was recognised by monoclonal antibody CRP-8. This epitope region of Hsp60 displays 26.6% amino acid identity to CRP AA77-90 region. These data suggest that the shared B-cell epitopes between CRP and Hsp60 give rise for a true mimicry based cross-reaction. Furthermore, commercial anti-CRP preparations contain antibodies to Hsp60 which might be explained by the fact that complete Freund’s adjuvant was used during the immunization process. Our study underlines the importance of thorough study design and careful interpretation of results while using polyclonal anti-CRP antibodies for histochemistry especially at low dilutions.

Key words: C-reactive protein, heat shock protein 60, anti-CRP antibodies, cross-reaction, epitope analysis
Introduction

C-reactive protein (CRP) is an acute phase reactant in humans, rabbit, and a number of other mammalian species. It is expressed and secreted primarily by hepatocytes but recently local production at sites of inflammation by monocytes has also been reported (18). The serum level increases up to 1000-fold concentration during the acute phase reaction or inflammation. CRP has been shown to activate the classical complement pathway by C1q binding (5). Furthermore, CRP is active in opsonization, lymphocyte modulation, natural killer cell, macrophage, neutrophil, and platelet responses (3). It has been reported that human CRP may exist in two antigenically distinct forms, which are known as native CRP (nCRP) and as a modified-CRP (mCRP). Neoepitopes are expressed on mCRP when the native pentameric form of CRP is dissociated into free subunits (7). Commercial anti-CRP antibody preparation may possess a significant proportion of specificities (up to 16% of the total reactivity) directed against CRP neoepitopes (11).

Heat-shock proteins (Hsps) are ubiquitous, phylogenetically highly conserved stress proteins, which have an essential role in cell survival (2). Hsps are often immunodominant antigens recognized in bacterial, fungal, and parasitic infections, therefore capable of inducing strong humoral and cellular immune responses in mammals (1). Immunization schedules often involve administration of the antigen in Freund’s complete adjuvant (CFA), followed by booster injections of antigens. CFA is an emulsion of mycobacteria in oil, and Hsp65 is an immunodominant antigen of mycobacteria. It is hardly surprising that Hsp65-reactive T-cells and antibodies develop in response to CFA, indeed this has been demonstrated in rats (10) and rabbits (16).

We have previously showed strong activation of the classical pathway by human Hsp60 (8). Since expression of Hsp60 and the complement-activating acute-phase reactant CRP is increased at sites of inflammation we aimed to determine whether Hsp60 is able to form complexes with CRP. However, we learned during the first experiment that anti-CRP polyclonal antibodies recognize human Hsp60. Hence, the aim of the present study was to characterize the anti-Hsp60 activity present in anti-CRP antibody preparations. Since anti-CRP antibodies are widely used for immuno-histochemistry our data may be of importance for the evaluation and discussion of those studies.
Materials and Methods
Proteins and antibodies used

Recombinant human Hsp60 and recombinant *M. tuberculosis* Hsp65 antigens were obtained from Lionex Ltd., Braunschweig, Germany. Rabbit polyclonal anti-Hsp65 antibodies were produced by immunizing two New Zealand white rabbits by standard protocol. Briefly, following prebleeding to collect pre-immune serum, 0.2 mg of antigen was injected intradermally to the rabbits with Freund’s complete adjuvant (Sigma-Aldrich, St. Louis, MO, USA). Quintuple booster immunizations, with 0.2 mg antigen and Freund’s incomplete adjuvant were given to the rabbits intradermally every two weeks following the initial immunization. Animals were bled a week after the second and fourth booster injections to determine titers. A week after the final booster injections the animals were exsanguinated under deep anaesthesia. All experimental procedures were approved by the Animal Care and Ethics Committee of the Faculty of Veterinary Science, Szent István University and complied with the Hungarian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Purified human C-reactive protein (Code: C-4063) was obtained from Sigma. Goat anti-CRP (Autokit CRP-HS R2, WAKO Chemicals GmbH, Neuss, Germany), rabbit anti-CRP (Code: C-3527 Sigma-Aldrich, Steinheim, Germany) and rabbit anti-CRP (Code: A0073 DAKO, Glostrup, Denmark) polyclonal antibodies were purchased. The anti-CRP monoclonal antibody panel (clones I-15-1D6, II-15-2C10, III-26-8C10, IV-13-3H12, IV-26-9C9 and IV-13-12D7), the phosphatidyl-cholin conjugated to keyhole-lympet haemocyanin (PC-KLH) and the recombinant modified CRP were produced as described previously (19). Anti-CRP monoclonal antibody CRP-8 was obtained from Sigma (Code: C-1688).

Enzyme-Linked Immunosorbent-Assay (ELISA)

ELISA plates (Greiner, Germany) were coated with 100 µl/well recombinant human Hsp60 (2 µg/ml) or recombinant *M. tuberculosis* Hsp65 (2 µg/ml) in 0.1 M bicarbonate buffer (pH=9.6) overnight at 4C and uncoated plates were used as controls. Plates were washed with PBS containing 0.05% Tween 20 and blocked with 0.15M PBS (pH = 7.2) containing 0.5% gelatine for 1 hour in room temperature. The wells were incubated with 100 µl serial dilutions of goat anti-CRP polyclonal (WAKO) or two different rabbit anti-CRP polyclonal (Sigma and DAKO) antibody preparations; and mouse anti-CRP monoclonal antibodies diluted in PBS.
0.5% gelatine and 0.05% Tween 20 (pH=7.2). Binding was determined using horse radish peroxidase labelled anti-goat IgG (Atlantic Antibodies, Stillwater, MN, USA), anti-rabbit IgG (Sigma) or anti-mouse IgG (Southern Biotechnology Associates, Birmingham, USA) antibodies and o-phenylene-diamine (OPD, Sigma) detection system. The optical density was measured at \( \lambda = 490 \) nm (reference at \( \lambda = 620 \)nm) and mean value of two parallel measurements was calculated.

The same ELISA system was applied to assess the inhibition of binding of rabbit anti-CRP polyclonal antibody and of 9C9 anti-CRP monoclonal antibody to human Hsp60 or \textit{M. tuberculosis} Hsp65 coated onto the ELISA plates by free inhibitors (Hsp60, Hsp65 and native CRP). In these competitive experiments the ELISA plates were coated with 1 \( \mu \)g/ml Hsp60, Hsp65 and indirectly with native CRP through PC-KLH (1:1000). The antibodies with the serial dilutions of inhibitors were preincubated for 1 hour at room temperature before the addition of the mixture to the Hsp-coated wells. Anti-rabbit or anti-mouse IgG horse radish peroxidase labelled antibodies were added and binding was determined by OPD, as described above.

Ouchterlony analysis

Double diffusion plates were prepared with 1% agarose (Reanal, Hungary) in 0.2 M K\(_3\)PO\(_4\) buffer (pH 7.8, Merck, Darmstadt, Germany). Wells were filled with 4 \( \mu \)l of samples and precipitation patterns were read after 24 hours incubation at room temperature by detection with 0.2% amido-black stain after differentiation.

Synthesis of Peptides on the Tips of Polyethylene Pins

Decamer peptides overlapping by 5 amino acid residues were synthesized using Fmoc-\( \beta \)-alanine-glycine ester derivatized pins obtained from Chiron Technologies (Australia) as previously described (14). Briefly, Fmoc/\( t \)-Bu technique was used, the Fmoc protecting group was removed by 20 % piperidine/DMF (v/v). The coupling was performed with DIC/HOBut methodology and monitored with bromophenol blue added to the coupling mixture. The peptides were acetylated at the N-terminus, 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.
Enzyme-Linked Immunosorbent-Assay for Epitope Scanning of anti-CRP antibodies

Binding of anti-CRP polyclonal (Sigma) and monoclonal CRP-8 antibodies to the human Hsp60 and *M. tuberculosis* Hsp65 peptides immobilized on polyethylene pins was detected by using modified ELISA. After blocking (PBS, 0.5% gelatine) pins were incubated with 150 μl 1:500 diluted antibodies in PBS 0.5% gelatine and 0.05% Tween 20 for 1 hour at room temperature. Binding of antibodies was determined using anti-rabbit IgG horse radish peroxidase labelled antibodies and OPD detection system. The optical density was measured at λ = 490 nm (reference at λ = 620 nm) and means of duplicates were calculated. Pins were used repeatedly after thorough cleaning by sonication in disruption buffer (PBS, 1% SDS, 0.1% 2-mercapto-ethanol).

Statistical analysis
Data are presented as means of parallel measurements with standard errors of mean. Binding characteristics of different antibodies were compared by the analysis of variance method. A p value less than 0.05 was considered as significant. GraphPad Prism 3.0 (www.graphpad.com) was used for data presentation and statistical analysis.
Results

Binding of anti-CRP antibodies to 60 kDa heat shock proteins

In the first experiment we aimed to determine the formation of potential complexes between microplate coated Hsp60 and soluble CRP (Fig. 1). However, we learned that the polyclonal rabbit anti-CRP antibodies fixed to the Hsp60 coated plate irrespective of previous addition of CRP. Thus, we hypothesized that anti-CRP antibody cross-reacts with Hsp60 or contain anti-Hsp60 activity. Therefore in the upcoming experiments we repeated this observation with different anti-CRP preparations and intended to characterize this reaction. ELISA plates were coated with recombinant human Hsp60 or recombinant *M. tuberculosis* Hsp65, respectively and were incubated with polyclonal antibody preparations against CRP. All of the three polyclonal antibody preparations showed dose dependent binding to heat shock protein (Fig. 2). Binding of the DAKO and WAKO anti-CRP preparations to Hsp60 and Hsp65 was detected at high concentrations whereas the Sigma antibody preparation showed a marked binding to both Hsps even at 1:400 dilution.

The origin of anti-Hsp60 antibodies in anti-CRP preparation might be true immunological cross-reaction based on antigenic mimicry or contamination. Both of these possibilities were investigated in our experiments. The potential of cross reaction of anti-CRP antibodies with Hsp60 was further studied using a panel of mouse anti-CRP monoclonal antibodies. Seven different anti-CRP monoclonal antibodies (clones 1D6, 2C10, 8C10, 3H12, 9C9, 12D7 and CRP-8) were included in these experiments. MAb 1D6 react only with the native, pentameric form of CRP, MAb 2C10 reacts with both native and modified CRP molecules, whereas MAbs 8C10, 3H12, 9C9, 12D7 and CRP-8 are specific for the modified CRP molecule (19), (15). An anti-Hsp60 specific monoclonal antibody (clone LK2) was used as a positive control and uncoated plates served as negative controls. Anti-CRP monoclonal antibodies derived from clone 9C9 and CRP-8 were found to bind to the solid phase heat shock proteins, however, other CRP monoclonal antibodies were not reactive (Fig. 3).

In a separate experiment, the interaction between the polyclonal anti-CRP antibody (Sigma) and heat shock proteins was studied in a double diffusion analysis. Polyclonal anti-CRP antibody was added into the central well, and different dilutions of Hsp60, Hsp65, native and modified CRP were added to the peripheral wells. Recombinant human Hsp60 and *M. tuberculosis* Hsp65 was precipitated by rabbit polyclonal anti-CRP antibody as well as native and modified-CRP (Fig. 4). Furthermore it can be also seen that every precipitin bands join at
their closest ends and fuse which means that the antigen epitopes are identical and cross-reactive.

Inhibition of binding of anti-CRP antibodies to Hsp60, Hsp65

To test whether anti-CRP polyclonal and monoclonal (9C9) antibodies cross-react with human Hsp60 and *M. tuberculosis* Hsp65, a competitive inhibition ELISA was performed next. Polyclonal anti-CRP (Sigma) or monoclonal 9C9 antibodies were preincubated with Hsp60, Hsp65, or CRP before reaction with solid phase 60 kDa heat shock proteins in the ELISA plate. The inhibition of anti-CRP antibody binding to CRP was also measured as control. Hsp60 inhibited the binding of anti-CRP polyclonal Ab to Hsp60 (Fig. 5, panel A). The inhibition was pronounced at high concentration of the competitive protein (10 μg/ml). The inhibition of anti-CRP antibody binding to Hsp60 by Hsp65 and native CRP was very weak. Similar reaction was observed on Hsp65 coated plates (Fig. 5, panel B). However, on CRP coated plates the binding of anti-CRP antibody was markedly inhibited by soluble CRP, whereas the inhibitory effects of heat shock proteins were very weak, although statistically significant in case of Hsp65 (Fig. 5, panel C).

Next, the monoclonal antibodies to CRP (clones 9C9 and CRP-8) were preincubated with Hsp60 and added to Hsp60 coated plates. Hsp60 was found to inhibit the binding of the 9C9 and CRP-8 monoclonals to Hsp60 (Fig. 5, panels D and E).

Epitope scanning of the binding sites for polyclonal and monoclonal (CRP-8) anti-CRP antibodies on human Hsp60

Data of the binding experiments documented that antibodies specific for Hsp60 are present in the anti-CRP antibody preparations. Therefore, we aimed to determine the epitope specificity of these antibodies using a set of overlapping synthetic peptides in the next experiments. Potential antigenic sites were predicted based on hydrophility and secondary structure, and selected for synthesis as described earlier (14). A total of 46 decamer peptides covering 50 % of human Hsp60 were synthesized. The results of the DAKO rabbit anti-CRP antibody reacting with peptides are presented in Table 1. (similar results were obtained for the Sigma anti-CRP antibody, as well). For reference, the purified IgG preparation of a rabbit immunized with *M. tuberculosis* Hsp65 was applied. Six epitope regions of Hsp60 were recognised by the anti-CRP antibodies, whereas anti-Hsp65 IgG cross-reacted with 11 epitope
sites of Hsp60. Most importantly 4 out of the 6 regions recognized by anti-CRP antibodies were exactly the same as the sites recognized by anti-Hsp65 antibodies; the remaining two sites were partly overlapping.

The binding sites for monoclonal antibodies against CRP (CRP-8 and 9C9) on human Hsp60 were also analysed (Fig. 6). One epitope region located in 218-232 on Hsp60 was found to be recognized strongly by CRP-8 monoclonal and weakly by anti-CRP antibodies. The corresponding region of Hsp65 was also recognized by the monoclonals being 9C9 stronger. This region was found to be 26.6% similar (amino acid identity) to CRP AA77-90 region compared with 17.4% similarity between the complete molecules analyzed by ClustalW program (13). The similarity (nucleotide identity) in the nucleotide sequences of the same regions was 40.48 % between the two molecules (Fig. 6).
Discussion

The major novel observation of this study is the description and characterization of anti-60 kD heat shock protein antibodies present in commercial polyclonal C-reactive antibody preparations. These IgG antibodies are able to recognize human Hsp60 and *M. tuberculosis* Hsp65 in solid-phase binding experiments and in Ouchterlony precipitation assay, as well. The anti-Hsp antibody binding from anti-CRP preparations seems to be specific since its binding is dose dependent and can be competitively inhibited by free Hsp60 and Hsp65. In line with this observation, two out of seven monoclonal anti-CRP antibodies were found to cross-react with 60 kD heat shock proteins.

There are two possibilities for the induction of these antibodies: cross-reaction based on mimicri mechanism and non-specific induction during immunization by the adjuvant. Here, in this study we provide evidences for both possibilities. Importantly, in our experiments, competitive inhibition of anti-CRP binding to CRP was weakly but statistically significantly inhibited by heat shock proteins. Therefore, true immunological cross reaction, between Hsp60 and CRP seems probably. Supporting this assumptions are the following observations. First, two monoclonal anti-CRP antibodies among the tested seven monoclonal antibodies did show specific binding to Hsp60. Furthermore, the binding of monoclonal anti-CRP antibodies to Hsp60 was detected only at high concentrations, but this binding was competitively inhibited by Hsp60 (Fig. 5, panels D and E). In addition, we were able to show an epitope region on human Hsp60 (AA223-236) recognised by the CRP-8 and 9C9 monoclonal antibodies, representing a potential site of cross-reaction. Finally, according to Clustal W homology search there is a region in the CRP molecule (AA 77-90), with 26.6% amino acid identity to Hsp60 (Fig. 6.). Thus, some antibodies in anti-CRP antibody preparations may show true, homology based cross-reaction with Hsp60.

The presence of anti-Hsp60 antibodies in anti-CRP preparations could also be explained by the method of immunization with CRP. All of the rabbit polyclonal anti-CRP antibodies were raised using complete Freund’s adjuvant (CFA) in the animals (DAKO Ltd. and Sigma Chemical Co., personal communication). Since one major fraction of the protein components of CFA is the 65 kD antigen (Hsp65) of *Mycobacterium tuberculosis*, which shows 45% amino acid identity with the human Hsp60 homologue, it is plausible to presume that anti-Hsp65 antibodies cross-reacting with human Hsp60 are induced by CFA in the animals. In line with this assumption Hajeer and Bernstein (4) observed in commercial anti-lactoferrin
and anti-albumin the presence of antibodies to mycobacterial Hsp65. Since lactoferrin failed to inhibit anti-Hsp65 reactivity, but Hsp65 itself did, the authors concluded that commercial anti-lactoferrin antibodies are contaminated with antibodies to Hsp65, possibly because of the usage of CFA during immunization.

Several observations of the present study indicate that the anti-Hsp60 reactivity present in the polyclonal anti-CRP preparations is raised mainly by the method of immunization, namely the application of CFA. First, the anti-Hsp60 antibodies in anti-CRP preparations seem to be specific antibodies with dose-dependent binding activity. Second, the binding of anti-Hsp60 reactive antibodies to Hsp60 can be inhibited by free Hsp60, but the inhibitory activity of free CRP on this reaction is very little if any (Fig. 5, panels A and B). Finally, complete Freund’s adjuvant is known to induce anti-Hsp65 antibodies in immunized rabbits, as shown previously in the study of Xu et al. (16) and proposed by Hajeer and Bernstein (4). B-cell epitope analysis was carried out to provide further evidence of the induction of anti-Hsp60 reactivity present in anti-CRP preparation by CFA immunization. Specificities of antibodies induced by Hsp65 (in CFA) immunization and the anti-Hsp60 reactivity present in anti-CRP preparations were compared. An overlapping set of synthetic peptides representing predicted epitope sites of human Hsp60 was used in these experiments. The immunization of a rabbit with Hsp65 yields a preparation with reactivity to 11 epitope regions on Hsp60 whereas the anti-Hsp60 antibodies present in the anti-CRP preparation recognized six epitope regions on Hsp60. Most importantly 4 out of the 6 regions were exactly the same as the sites recognized anti-Hsp65 antibodies; the remaining two sites were partly overlapping (Table 1). These data strongly support our assumption that the induction of anti-Hsp60 antibodies in animals immunized with CRP may be a consequence of the usage of CFA. All of the above observations support that anti-Hsp60 and anti-CRP antibodies exist in parallel in polyclonal anti-CRP preparations. There might be important practical consequences of our results. The polyclonal antibodies characterized in this study are widely used reagents in immunohistochemistry in comparable concentrations to our experiments (9), (6), (20), (12). Special attention should be paid for the interpretation of studies using the above reagents. Although some eminent studies apply appropriate controls to rule out undesirable cross reactions and provide data on the characterization of the antibodies applied, there are several studies reporting deposition/presence of CRP using antibodies even in low concentrations for detection. The cross-reaction of monoclonal anti-CRP antibodies were detected at far lower concentration as compared to the concentrations applied for immunohistochemistry, therefore the observed homology-based cross-reaction (although theoretically interesting) is most probably without
practical consequences in immunochemistry studies. However, since elevated levels of serum soluble Hsp60 were reported in atherosclerosis (17), it can not be excluded that sHsp60 might interact with high-sensitivity CRP measurements if Hsp60 cross-reacting monoclonal is applied in the turbidimetric assay.

A potential limitation of the present study is the lack of inhibition data by the Hsp60 peptides. This is due to the fact, that the overlapping peptides were covalently synthesized onto polyethylene pins and could not be used for inhibition. Thus, the strength of antibody binding to CRP and Hsp60 epitopes could not be compared directly.

In conclusion, the preparation raised against CRP using CFA characterized in this study and the anti-lactoferrin antibodies (4), both contaminated by anti-Hsp65 IgG cross reacting with the human homologue protein, call special attention for the appropriate design and interpretation of those studies using similar, polyclonal antibody reagents, raised with CFA.
Acknowledgement

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Figure Legends

Figure 1. Binding of polyclonal anti-CRP antibody to solid phase Hsp60 in the presence or in the absence of CRP. Negative controls were uncoated, while positive controls were CRP coated plates. Each bar represents mean ± SEM OD values. Results are the sum of three independent experiments each performed in duplicate.

Figure 2. Interaction of polyclonal anti-CRP antibodies with 60 kDa heat shock proteins. Binding of different dilutions of goat (WAKO, panel A) and two different rabbit (DAKO, panel B, SIGMA, panel C) anti-CRP polyclonal antibodies to recombinant human Hsp60 (2 μg/ml, -■-) or recombinant M. tuberculosis Hsp65 (2 μg/ml, -▲-) target antigens as detected by ELISA. Control wells were uncoated (-▼-). Optical density was measured at λ = 490 nm, mean ± SEM OD values are shown. Results are the sum of three independent experiments each performed in duplicate.

Figure 3. Binding of anti-C reactive protein monoclonal antibodies (1D6, 2C10, 8C10,3H12, 9C9, 12D7, CRP-8) to adsorbed human Hsp60 (A) and mycobacterial Hsp65 (B) target antigens. Supernatant antibodies were used with serial dilutions from 1:2, except CRP-8 which is a purified antibody (serially diluted from 1:200). LK-2 monoclonal anti-Hsp antibody was used as positive, uncoated plates were used as negative controls (panel C). Each point represents mean ± SEM value of two parallel measurements. The experiment for which results are shown on Fig 3 was performed three times in duplicate; the variance between assays was less than 10%.

Figure 4. Precipitation of 60 kDa heat shock proteins with polyclonal anti-CRP antibodies. Ouchterlony analysis of the interaction between Hsp60, Hsp65, native- and modified-CRP and polyclonal anti-CRP antibody (well B: 2 mg/ml native-CRP diluted in K3PO4 buffer (pH 7.8), wells C and D: Hsp60 (1mg/ml and 0.5 mg/ml), well E: mCRP (2 mg/ml), wells F and A: Hsp65 (1mg/ml and 2 mg/ml)). Central well: polyclonal anti-CRP antibody (DAKO) diluted 1:10 in the K3PO4 buffer (pH 7.8). Experiments were performed in 1% agarose.

Figure 5. Inhibition of rabbit anti-C reactive protein polyclonal (A, B, C) and monoclonal (D) antibody binding to solid phase adsorbed Hsp60 (A), Hsp65 (B) and native CRP (C) by preincubation with soluble Hsp60 (-■-), Hsp65 (-▲-) and CRP (-▼-), (-▼- antibody alone, -
○- antibody on uncoated plate) in competitive ELISA. In panels D and E the interaction of mouse 9C9 or CRP-8 anti-CRP monoclonal antibodies and soluble Hsp60 are shown (- ▼ - antibody alone, ■ - antibody+Hsp60, 10 μg/ml, ☣ - antibody+BSA, 10 μg/ml, ○ - antibody on uncoated plate). The antibodies (polyclonal and CRP-8 monoclonal: 1:200, 9C9 monoclonal 1:50) and inhibitors (10 μg/ml) were preincubated for 1 h at room temperature and diluted on the plate thereafter. Values represent mean ± SEM of parallel measurements. Results are the sum of three independent experiments each performed in duplicate. Two-way analysis of variance (ANOVA, p value), panel A: Hsp60 <0.0001, Hsp65 0.0002, CRP 0.0013; panel B: Hsp60 <0.0001, Hsp65 0.0004, CRP 0.048; panel C: Hsp60 0.089, Hsp65 0.0157, CRP <0.0001; panel D: Hsp60 <0.0001, panel E: Hsp60 <0.0001, as compared to ‘antibody alone’.

Figure 6. Binding of anti-CRP monoclonal antibodies to 60 kDa heat shock protein peptides. Epitope analysis of human Hsp60 (panel A and B) and M. tuberculosis Hsp65 (panel C and D) protein for the binding sites of monoclonal antibody against C-reactive protein (clone CRP-8, clear bars; clone 9C9 black bars). Bars represent mean ± SEM OD values of different overlapping peptides, corrected with background OD values. Background reactivity is indicated by the horizontal line. The experiment was repeated three times with similar results. Sequence homology between CRP and the Hsp60 223-236 region analyzed by ClustalW program is indicated. Identical residues are marked with an asterisk and similar residues are marked with dots.

Table1. Reactivity of rabbit polyclonal anti-C reactive protein antibodies with synthetic peptides representing regions of human heat shock protein 60.
References


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<th>Region</th>
<th>Human Hsp60 peptides</th>
<th>Reactivity&lt;sup&gt;2&lt;/sup&gt;(OD/OD control&gt;2)</th>
<th>Rabbit anti-CRP polyclonal antibody</th>
<th>Rabbit anti-Hsp65 polyclonal antibody</th>
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| I      | 52AVTMGPKGRT<sup>61</sup>
57PKGRTVIIEQ<sup>66</sup>
62VIIEQSWGSP<sup>71</sup>
67SWGSPKVTKD<sup>76</sup>
72KVTKDGVTV<sup>81</sup> | -                                       | +                                   |
|        |                      | -                                       | -                                   |
|        |                      | +                                       | -                                   |
| II     | 117TVLARSIAKE<sup>126</sup>
122SIAKEGFEKI<sup>131</sup>
127GFEKISKGAN<sup>136</sup>
132SKGANPVEIR<sup>141</sup>
137PVEIRRGVML<sup>146</sup> | +                                       | +                                   |
|        |                      | -                                       | -                                   |
|        |                      | -                                       | -                                   |
| III    | 162VTTPEEIAQV<sup>171</sup>
167EIAQVATISA<sup>176</sup>
172ATISANGDKE<sup>181</sup>
177NGDKEIGNII<sup>186</sup> | -                                       | -                                   |
|        |                      | +                                       | +                                   |
|        |                      | -                                       | -                                   |
| IV     | 203DGKTLENDELE<sup>212</sup>
208DNELEIIEGMI<sup>217</sup>
213IIEGMKFDRG<sup>222</sup>
218KFDRGYISPY<sup>227</sup>
223YISPYFINTS<sup>232</sup>
228FINTSKGQKC<sup>237</sup>
233KGQKCEFQDA<sup>242</sup>
238EFQDAYVLLS<sup>247</sup>
243YVLLSEKKIS<sup>252</sup>
248EKIISSIQSI<sup>257</sup>
253SIQSIVPALE<sup>262</sup> | -                                       | -                                   |
|        |                      | -                                       | -                                   |
| V      | 303PGFGDNRKNQ<sup>312</sup>
308NRKNQLKDMA<sup>317</sup> | -                                       | +                                   |
| VI     | 368EKRIQEIIEQ<sup>377</sup>
373EIIEQLDVTT<sup>382</sup> | -                                       | -                                   |
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<td>480</td>
<td>AKNAGVEGL&lt;sup&gt;489&lt;/sup&gt;</td>
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<tr>
<td>485</td>
<td>VEGSLIVEKI&lt;sup&gt;494&lt;/sup&gt;</td>
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<td>-</td>
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<tr>
<td>490</td>
<td>IVEKIMQSSS&lt;sup&gt;499&lt;/sup&gt;</td>
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<tr>
<td>495</td>
<td>MQSSSEVGYD&lt;sup&gt;504&lt;/sup&gt;</td>
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<tr>
<td>500</td>
<td>EVGYDAMAGD&lt;sup&gt;509&lt;/sup&gt;</td>
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<td>IX</td>
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<tr>
<td>531</td>
<td>DAAGVASLLT&lt;sup&gt;540&lt;/sup&gt;</td>
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<tr>
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<td>541</td>
<td>TAEVVVTEIP&lt;sup&gt;550&lt;/sup&gt;</td>
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<sup>1</sup>Pepitides were immobilized on polyethylene pins at their C-terminus. Numbers represent the position of the sequence in the protein.

<sup>2</sup>Reactivity was defined as follows: ratios of OD of the given peptide /OD of control peptide (background) for each peptide were calculated, ratios >2 were considered +. OD/OD background ratios obtained for each peptide with anti-CRP + anti-rabbit IgG antibody conjugated with peroxidase or anti-Hsp65 + anti-rabbit IgG antibody conjugated with peroxidase were corrected with ratios obtained in the control experiment (anti-rabbit IgG antibody conjugated with peroxidase only).
Figure 1
Figure 2

A

Anti-CRP antibody dilution WAKO

B

Anti-CRP antibody dilution DAKO

C

Anti-CRP antibody dilution SIGMA
Figure 3

A Coat: Hsp60 (2 mg/ml)

B Coat: Hsp65 (2 mg/ml)

C Dilution

mAb bound (OD490)

Dilution

mAb bound (OD490)

mAb bound (OD490)

mAb bound (OD490)

1D6

2C10

8C10

3H12

9C9

12D7

CRP-8

LK2-Hsp5

LK2-Hsp60

uncoated

LK2-Hsp60

uncoated
Figure 4
Figure 5

A. Plate: Hsp60 (1 μg/ml)

B. Plate: Hsp65 (1 μg/ml)

C. Plate: nCRP (1 μg/ml)

D. Plate: Hsp60 (1 μg/ml)

E. Plate: Hsp60 (1 μg/ml)
Figure 6

Human CRP: 77 K T M R F F I F W S K D I G 90
Human Hsp60: 223 Y I S P Y F I N T S K G Q K 236
Human CRP: 229 aag aca atg aga ttc ttc ata ttt tgg tct aag gat ata gga 270
Human Hsp60: 667 tat att tct cca tac ttt att aat aca tca aaa ggt cag aaa 708