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Autoantibodies to heat shock protein 90 in the human natural antibody repertoire

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Abstract

The present study demonstrates the presence of natural autoantibodies of the IgG isotype directed against heat shock protein 90 (HSP90). The binding properties of affinity-purified anti-HSP antibodies were compared with those of natural antibodies specific for other self antigens, including anti-thyroglobulin and anti-myoglobin autoantibodies, by using semiquantitative immunoblotting, with solubilized proteins from normal liver tissue as antigens, and cross-blot analysis using purified self proteins. Affinity-purified anti-HSP90 antibodies were polyreactive and the non-HSP90-specific fraction of normal IgG was depleted in its natural autoantibody content. We further observed that self antigens including HSP, myosin, tubulin and aldolase with highly conserved structures show similar patterns of binding with natural antibodies, and form a well-defined cluster as demonstrated by cluster analysis of immunoreactivity data, whereas the less-conserved self and non-self antigens remained unclustered. The results favor the hypothesis that HSP90 belongs to a subset of highly conserved and immunodominant self antigens that are the primary target for natural autoantibodies in normal human IgG.

Introduction

Natural self-reactive antibodies of the IgG, IgM and IgA isotype are present in the serum of healthy individuals and animals (1). Recent studies have shown that B cells can be subject to positive selection and maintained on the basis of their autoreactivity (2). The autoantibodies recognize a limited set of self antigens that are highly conserved in evolution (3), shared between individuals and conserved throughout life (4–6). An analogous set of conserved T cell reactivities has also been documented in healthy mice (7). It has been proposed that the antigens recognized by natural antibodies and T cells are essential for the selection of natural B and T cell repertoires, and for the maintenance of tolerance to self (8,9).

Heat shock proteins (HSP) are highly conserved self antigens. Bacterial and human HSP share considerable structural homology (10,11). Antibodies and T cells that recognize microbial HSP as immunodominant, often cross-react with human HSP antigens (12). Antibody reactivity to bacterial and homologous HSP is present in the serum of healthy individuals and patients with autoimmune diseases (13–15). T and B cell reactivity to HSP60 has been involved in the pathogenesis of experimental arthritis, diabetes and atherosclerosis (16–18). However, in mice, HSP60-specific T cells also appear as a part of the normal inflammatory response (19) and protect from adjuvant arthritis under certain conditions (20); the presence of HSP60-reactive T cells in patients with juvenile rheumatoid arthritis correlates with remission of the disease (21). Whereas HSP60 primarily represents an inducible antigen, HSP90 is a highly conserved (22) and constitutively expressed antigen in a large number of tissues (23). Its expression may further be up-regulated as a consequence of noxious stimuli and mitogenic signals (24). HSP90 is an immunodominant antigen recognized by antibodies occurring in several mycotic infections (25). It is recognized by autoantibodies in the serum of healthy individuals and patients with autoimmune diseases (13).

In the present study, we show that antibodies to HSP90 are predominant within the natural IgG autoantibody repertoire of healthy individuals. We further show that HSP90, together with

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other highly conserved self antigens, represents a primary target for the binding of natural antibodies. The results support the hypothesis that epitopes within the HSP90 molecule have a key role in shaping natural self-reactive B cell repertoires.

Methods

Reagents

Human thyroglobulin (TG) (Biogenesis, Poole, UK), bovine muscle actin, myosin, brain tubulin, calf thymus DNA, horse muscle myoglobin, horseradish peroxidase (HRP), lactoperoxidase (LP) (Sigma, St Louis, MO), aldolase, pancreatic RNase, chymotrypsinogen A (CTA), spinach phosphorybulokinase (PRK) (Pharmacia Biotech, Uppsala, Sweden) and tetanus toxoid (TT) (generously provided by Dr L. A. Hanson, Gothenburg University, Sweden) were obtained as indicated. The RGD sequence-containing peptide AVTGRGDSPA was synthesized by Neosystems (Strasbourg, France), the FAS–GST fusion protein was a generous gift from Dr G. Ruberti (Rome, Italy). Normal polyspecific IgG for therapeutic use (IVIg) was a kind gift from the Central Laboratory of the Swiss Red Cross (Bern, Switzerland). $F(ab')_2$ fragments were prepared from IVIg by pepsin digestion and chromatography on Protein A–Sepharose (Pharmacia Biotech). $F(ab')_2$ fragments were free of detectable Fc fragments as assessed by ELISA.

Purification of HSP90

HSP90 was purified from spleen obtained from the Pathology Department of the Medical University in Sofia. The tissue was frozen within 30 min of splenectomy in aliquots of 50 g. An extract of cytosolic proteins was prepared by homogenizing 50 g of tissue in 150 ml of 0.03 M carbonate buffer, pH 7.1, containing 5 mM PMSF and 5 mg/ml aprotinin. The homogenate was cleared by ultracentrifugation and subjected to ammonium sulfate precipitation at 50% after which the supernatant was brought to 70% saturation. The precipitate was dissolved and dialyzed extensively against 0.02 M phosphate buffer, pH 7.4, 1 mM EDTA and 0.2 M NaCl. The solubilized proteins were then subjected to ion-exchange chromatography on a MonoQ column (Pharmacia Biotech) using a gradient of NaCl from 0.2 to 0.6 M in phosphate buffer, pH 7.4. The fractions with the highest content of protein that migrated at ~90 kDa upon SDS–PAGE, were pooled and further fractionated on a hydroxyapatite column using a gradient of 0.02–0.3 M phosphate buffer, pH 7.0, containing 0.1 mM EDTA and 15 mM 2-mercaptoethanol. The fractions were analyzed by SDS–PAGE and Western blotting using rat mAb to human HSP90 (StressGen Biotechnologies, Victoria, BC, Canada). Fractions containing HSP90 at apparent homogeneity were pooled, dialyzed against PBS, aliquoted and kept at –70°C until use. Purified HSP90 stained as a single protein band of ~85–90 kDa. The band specifically stained with anti-human HSP90 mAb. The yield of the purification procedure was 5 mg antigen/50 g wet weight of starting material.

ELISA

Microtiter plates (Nunc, Roskilde, Denmark) were coated with 1.0 μg/well of HSP90 purified as described above. The plates were incubated overnight at 4°C with the source of IgG to be tested at concentrations ranging between 0.05 and 7 mM. Bound antibodies were revealed using biotinylated secondary antibodies to human Fcγ (Sigma) and to subclasses of human IgG (Sigma) followed by ExtrAvidine conjugated to alkaline phosphatase (Sigma).

Affinity purification of antibodies

Immunoadsorbent columns were prepared with antigens of interest coupled to cyanogen bromide-activated Sepharose (Pharmacia Biotech). Two milligrams of protein were used for coupling to 1.5 ml of bed volume of CNBr-activated Sepharose. One gram of IVIg in 100 ml of PBS was loaded on the immunoadsorbent column and run twice on the column at a speed of 1 ml/min at room temperature, followed by washing with PBS until the absorbance of the flow-through at 280 nm reached baseline values. Bound antibodies were eluted using glycine–HCl (0.1 M) buffer of pH 2.8, 2 M NaCl followed by PBS and then diethanolamine (0.1 M) buffer, pH 11, 2 M NaCl. The eluates obtained at different pH were brought to pH 7.0 and pooled. Two milliliters of the flow-through fractions were allowed to run through the sorbents for two more cycles and further used as effluent fractions. Eluates and effluents were dialyzed against PBS.

Immunoblot and cross-blot

For semiquantitative immunoblotting, we have used a method that has been described in detail elsewhere (5). The source of self antigens was a soluble extract of normal human liver. Proteins were subjected to preparative 10% SDS–PAGE and transferred onto nitrocellulose (Schleicher & Schuell, Dassel, Germany) for 60 min at 0.8 mA/cm^2 using a semi-dry Electroblotter A (Ancos, Hoejby, Denmark). Membranes were blocked with PBS/Tween.

For cross-blotting, a prewetted PVDF or nitrocellulose membrane was inserted in the miniblotter and incubated with the antigens overnight at 4°C.

In both settings, after extensive washing, membranes were blocked with TBS/Tween for 30 min at 37°C. The PVDF membrane was further blocked with TBS/Tween, 1% BSA. Antibodies to be tested were then incubated with the membranes following the addition of one sample per slot in a cassette Miniblot System (Immunetics, Cambridge, MA) overnight at 4°C. For the cross-blot the direction of the slots was perpendicular to that of the imprints of the slots containing the antigens during the first incubation step. In the case of the cross-competition assay the antibodies were preincubated with the inhibitor for 48 or 72 h at 4°C.

After washing, the membranes were incubated with secondary goat anti-human Fcγ antibody coupled to alkaline phosphatase (Southern Biotechnology, Birmingham, AL). The reactivities were revealed using the nitroblue tetrazolium/bromo-chloro-indolyl-phosphate chromogenic substrate. Quantitation of bound antibodies was performed by densitometry in reflective mode using a SNAPScan 600 scanner (Agfa Gevaert, New York, NY) linked to a Power Mac G3 computer. Data were analyzed using a ImageTool version 2.0 for Windows (UTHSCSA, San Antonio, TX). Migration distances (*x*-axis) and light

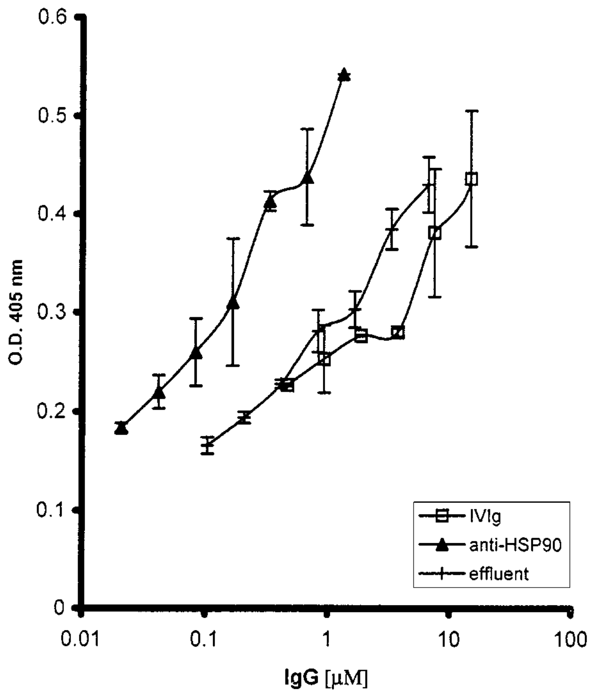

Fig. 1. Binding of anti-HSP90 isolated from IVIg to HSP90. ELISA plates were coated with 1 μg/well of HSP90, blocked and incubated with affinity-purified anti-HSP90 antibodies or unfractionated IVIg followed by goat anti-human IgG antibody coupled to alkaline phosphatase. In parallel, the binding of the same concentrations of antibodies was tested on wells that contained no antigen. The results are obtained as a difference between the OD of the specific binding and the background for each concentration, and are represented as means of duplicate wells ± SD; open squares, anti-HSP90 antibodies; filled triangles, IVIg.

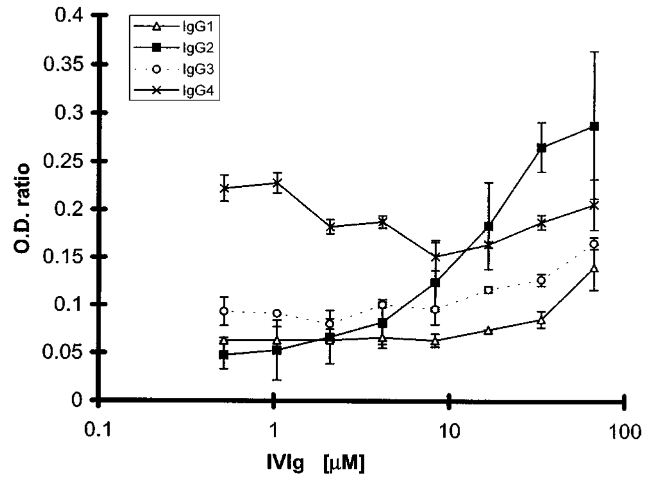

Fig. 2. Binding of IgG subclasses in IVIg to HSP90. The conditions for the ELISA are as in Fig. 1, except that biotin-coupled antibodies specific for the different subclasses of human IgG were used as secondary antibodies. The plateau level reached in binding to total IgG was used to standardize the values from the experiment on the binding to HSP90. The ratio of these two OD levels is presented.

absorption (*y*-axis) were expressed as arbitrary units. For quantitation of cross-blots, we used the densitometric profiles of the lanes corresponding to the antigens. The background defined by secondary antibody alone was subtracted from the densitometric profile. The intensity of the staining was quantified as the average levels of gray of the stained regions.

Statistical analysis

The binding data from the polyreactivity assays was subjected to cluster analysis using STATISTICA for Windows (StatSoft, Tulsa, OK) after standardizing by dividing the value for each antibody by the average value of binding for the antigen of interest. This data was subjected to cluster analysis using tree clustering with Euclidean distances and a single-linkage algorithm. The amalgamation schedule was studied for sharp increases in the linkage distance which signifies clustering when an irregularly big distance is spanned in order to unite the lower level clusters. The same data was subjected further to clustering using the *k*-means algorithm for a predetermined number of clusters, defined by the first method, in order to confirm the composition of the clusters.

Results

Affinity purification of autoantibodies to HSP90 from normal human IgG

Antibodies to HSP90 were purified from IVIg as described in Methods. Affinity-purified IgG bound to immobilized HSP90 in a dose-dependent fashion, as assessed by ELISA. There was approximately one order of magnitude of difference in the concentration of affinity-purified antibody required for 50% binding to HSP90, as compared with unfractionated IVIg (Fig. 1).

Anti-HSP90 reactivity of IVIg was further probed by ELISA using antibodies to subclasses of IgG as revealing antibodies. Significant binding to HSP90 was only observed with the anti-IgG2 reagent (Fig. 2). Similar results were obtained when probing affinity-purified anti-HSP90 antibodies using a dot-blot technique, in that anti-HSP90 antibodies were found to be predominantly of the IgG2 subclass.

Polyreactivity of anti-HSP90 autoantibodies

The polyreactivity of affinity-purified anti-HSP90 IgG was assessed using immunoblot and cross-blot as described in Methods. Polyreactivity of anti-HSP90 IgG was compared with that of antibodies to other self antigens that had been affinity purified from the same pool of IgG. For immunoblotting, antibodies were used at a concentration of 200 μg/ml. Solubilized glycoproteins from normal liver tissue were used as a source of self antigens. We observed a high-intensity binding to liver antigens of affinity-purified anti-HSP90 IgG as well as of affinity-purified anti-actin, anti-aldolase and anti-TG antibodies (Fig. 3a). The binding of the affinity-purified autoantibodies was much higher than that of unfractionated IVIg which, in turn, was higher than that of the effluents of the HSP90, actin and aldolase affinity columns. Actually most of the reactivities with antigens in the liver were ablated following

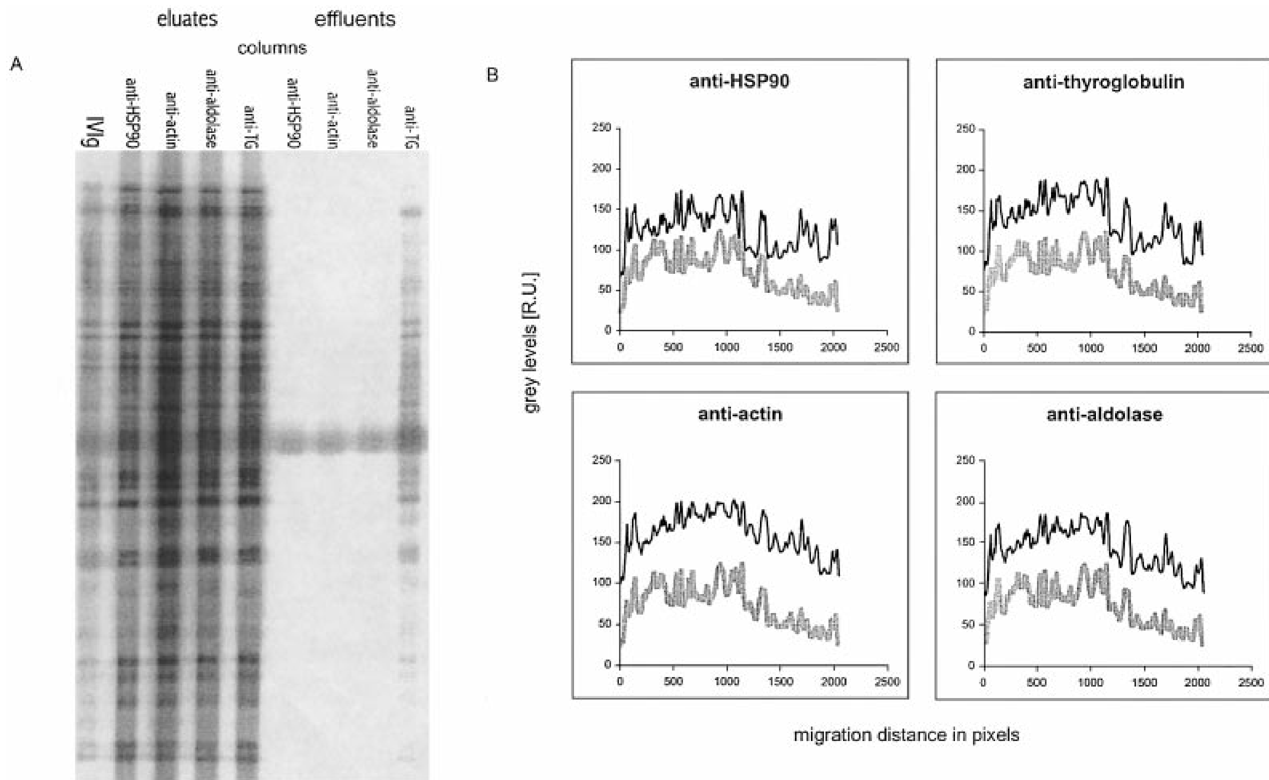

Fig. 3. Immunoblot analysis of the reactivity of antibody repertoire of IVIg, anti-HSP90, anti-actin, anti-aldolase and anti-TG antibodies isolated from IVIg, and of the corresponding effluent fractions towards antigens extracted from liver tissue. (A) The antibodies were incubated with the membrane at a concentration of 0.2 mg/ml for 4 h at room temperature. After washing, the membranes were incubated with secondary goat anti-human Fcγ antibody coupled to alkaline phosphatase (Southern Biotechnology). Immunoreactivities were revealed using the nitroblue tetrazolium/bromo-chloro-indolyl-phosphate chromogenic substrate. (B) Densitometric data of the lanes IVIg (the grey curve in all plots), anti-HSP90, anti-thyroglobulin, anti-aldolase and anti-actin from the immunoblot shown in (A). The grey level of the image of the immunoreactivities expressed on a scale of 0–255 arbitrary units (the ordinate) is plotted against migration distance.

selective removal of anti-HSP, anti-actin or anti-aldolase antibodies. The pattern of reactivity with liver antigens of affinity-purified anti-HSP90 antibodies was similar to that of affinity-purified anti-TG, anti-actin and anti-aldolase antibodies, and to that of unfractionated IVIg (Fig. 3b).

We also used native purified proteins in a cross-blot assay to investigate the polyreactivity of anti-HSP90 IgG and that of affinity-purified antibodies to other self antigens. Serial dilutions of the antibodies to be tested were incubated with a PVDF membrane loaded with the relevant native antigen. A miniblot system was used for incubating antibodies with the respective antigens (Fig. 4). Reactivities were measured by densitometry. All affinity-purified autoantibodies tested were found to be polyreactive in that they bound to all antigens tested. Affinity-purified antibodies to TT, however, did not bind to HRP.

We then quantified the staining by measuring the average gray level of stained regions by densitometry in the cross-blot (Fig. 5). The binding curves of all affinity-purified autoantibodies were superimposable when HSP90 was the target antigen (Fig. 5). In contrast, the binding curves of affinity-purified autoantibodies varied considerably from each other when TT and HRP were used as target antigens. In the latter case, the highest binding was observed with affinity-purified anti-TT and anti-HRP antibodies respectively, and differing patterns of binding were observed in the case of antibodies to the other self antigens. In the case of TG as antigen, the highest binding was observed with affinity-purified anti-TG antibodies, whereas all other antibodies tested bound with a lower intensity although with a similar intensity. The binding to HSP90 of affinity-purified anti-HSP90 IgG was similar to that of IVIg. Taken together, the data indicate that anti-HSP90 autoantibodies are present in large amounts in IVIg. The results also show that affinity-purified antibodies to self antigens are polyreactive, bind to a similar extent to HSP90 and recognize the same subset of antigens among glycoproteins solubilized from normal liver tissue.

The natural autoantibody repertoire contains antibodies with strong reactivity towards the constituents of the cytoskeleton (26–28). In order to compare the binding of natural autoantibodies to HSP90 with that to cytoskeletal proteins, a second cross-blot experiment was performed using as antigens HSP90, actin, myosin and tubulin along with aldolase, LP, RNase, CTA, PRK and DNA. These antigens were probed with anti-HSP90, anti-actin, anti-aldolase, anti-TG, anti-Fas and anti-RGD antibodies that had been affinity purified from IVIg. A membrane coated with the specified antigens was treated with the affinity-purified antibodies in duplicate lanes.

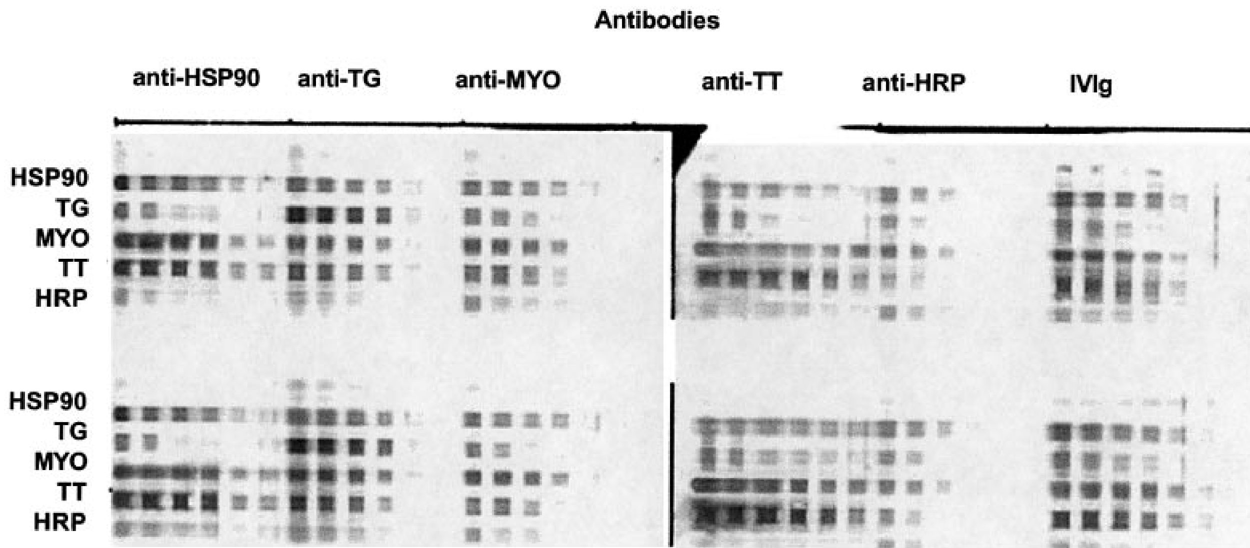

Fig. 4. Quantitative cross-blot for estimating the polyreactivity of the isolated fractions of IV Ig. The antigens were deposited on PVDF membrane by incubating 100 μl of solution containing 2 μg/ml of the antigen of interest in the slots of a miniblotter for 30 min at 37°C followed by washing of the slots and blocking with PBS/Tween for 30 min followed by further 30 min blocking with 0.5% cold water fish skin gelatin (Sigma) in PBS/Tween for an additional 30 min at room temperature. The membranes were incubated in miniblotter with antibodies 4 h at room temperature. The direction of the slots was perpendicular to that of the imprints of the slots containing antigen solutions during the first incubation. After further extensive washing, the membranes were incubated floating in solution of secondary goat anti-human Fcγ antibody coupled to alkaline phosphatase (Southern Biotechnology). Immunoreactivities were revealed using the nitroblue tetrazolium/bromo-chloro-indolyl-phosphate chromogenic substrate.

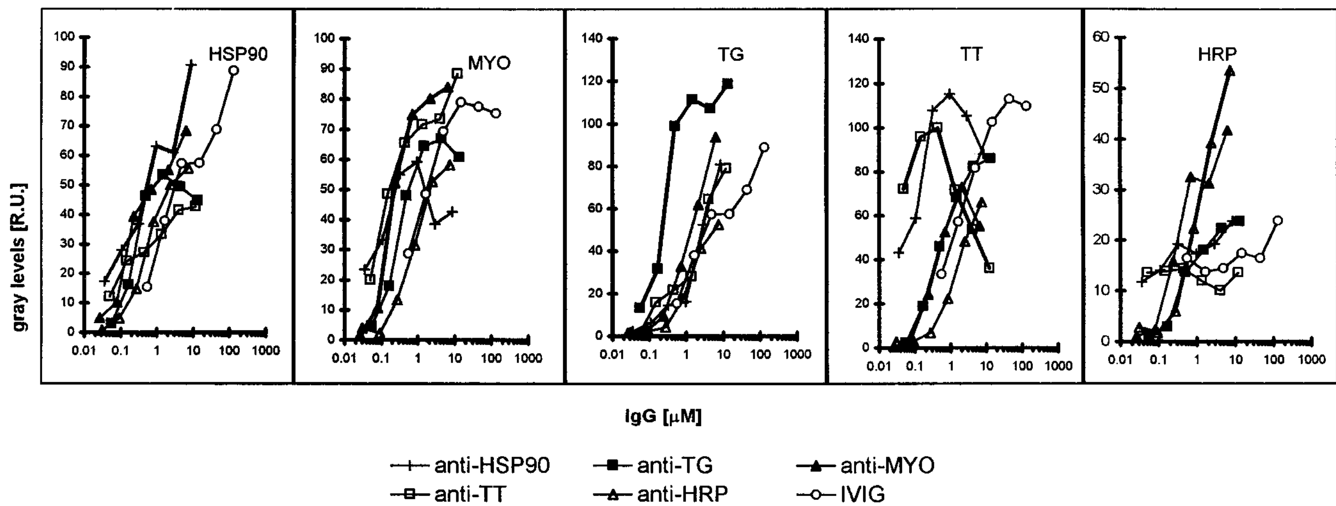

Fig. 5. Densitometric data from the cross-blot shown on Fig. 4. The profiles were treated numerically to subtract the background. The concentration of IgG in mM is represented on the abscissa and the mean optical density over a square of constant area at the crossing of two channels after subtraction of the background is depicted on the ordinate. Each plot shows the data for one antigen presented as the average of duplicate spots. The thick line in each plot represents the binding of the antibodies specific for the respective antigen.

The reactivities were quantitated by densitometry. In order to make the values comparable across antigens, the intensity of staining of the spots was further divided by the value of the average intensity of all the spots, representing the reactivities of different antibodies with the given antigen. The binding data was then subjected to cluster analysis to compare the patterns of binding of different antibodies with the panel of antigens. The analysis showed distinct clusters at the sixth step of the clustering procedure. At that point, one cluster containing HSP90, myosin, tubulin, RNase and aldolase, and another that included PRK and CTA (Fig. 6) could be distinguished. Actin and TG appeared similar in binding pattern with the first cluster, but failed to cluster together at the sixth step. The other antigens remained unclustered. These results indicate that self antigens may be divided into those, including HSP90 and cytoskeleton proteins, to which natural antibodies exhibit a

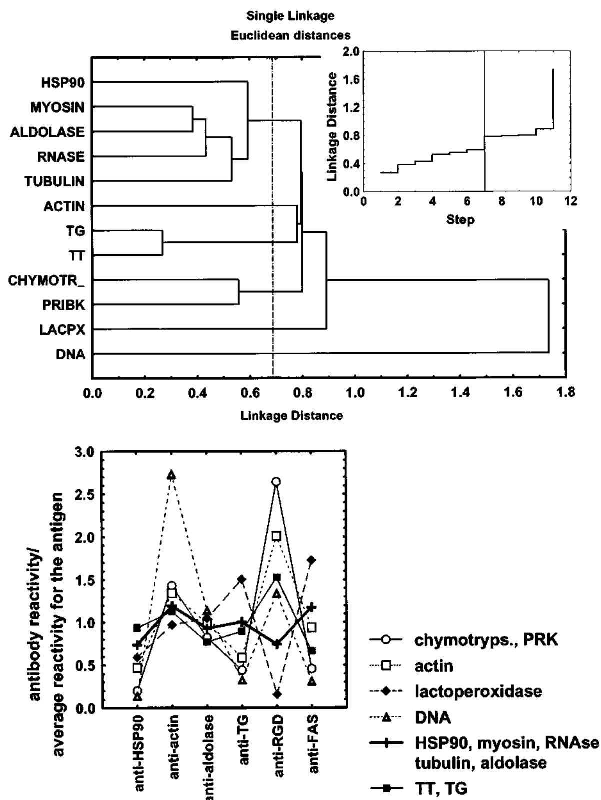

Fig. 6. Cluster analysis of the binding data from cross-blot experiments. The reactivity of antibodies with different antigens was visualized using alkaline phosphatase-conjugated second antibody and chromogen substrate. The membranes were scanned and subjected to digital densitometry. The reactivities of a given antigen with the different antibodies were standardized by dividing by the average reactivity for that antigen. The data was subjected to cluster analysis using tree clustering with Euclidean distances and a single-linkage algorithm. On the upper panel, tree clustering of the antigens based on the reactivity pattern with the panel of antibodies used is shown. The inset shows the amalgamation schedule which signifies clustering of equally spaced data points up to the seventh step when an irregularly long distance is spanned in order to unite the lower level clusters. The finding indicates the existence of at least one cluster of points in the data which has been identified at the sixth step of the clustering. This stage of the clustering is marked by a broken vertical line in the tree plot. The lower panel shows the results from cluster analysis of the same data using the *k*-means algorithm for a predetermined number of six clusters. This method confirms the clustering shown in the tree plot. The mean reactivity ratios for each cluster are plotted. The analysis shows the existence of one cluster of similar antigens as opposed to the rest of the antigens studied, which are diverse. This cluster, containing HSP90, myosin, RNase, tubulin and aldolase, is characterized by uniform binding of all antibodies used. Note the highly scattered values for the binding of anti-RGD compared to the clustered values for the binding of anti-HSP90.

typical homogenous pattern of reactivity with self components and those to which natural antibodies display varied, non-homogeneous patterns of reactivity.

The polyreactivity of anti-HSP90 autoantibodies with immunodominant autoantigens was further studied by cross-competition assay. The inhibition of the binding of anti-HSP90 natural autoantibodies to HSP90, myosin, RNase and actin by these same antigens was tested in cross-blot. Anti-HSP90 autoantibodies were preincubated with HSP90, myosin, RNase and actin in different concentrations for 72 h at 4°C. The antigens were deposited onto PVDF membrane and further incubated with the preincubated mixtures of antibodies and inhibitors. The results of the densitometric quantitation of the blot is shown in Fig. 7. There was an inhibition of binding of anti-HSP90 natural autoantibodies to HSP90, myosin, RNase and actin in 12 out of 16 combinations. Inhibition of binding of anti-HSP90 natural autoantibodies to myosin and RNase by actin was weak as compared to other combinations. These results further indicate the polyreactive nature of affinity-purified self-reactive antibodies.

Discussion

In this study, we demonstrate the presence of natural polyreactive autoantibodies in normal human IgG that recognize HSP90. We investigated the reactivity of anti-HSP immunoaffinity purified from pooled normal IgG with HSP and other self and non-self antigens, and compared the binding characteristics of anti-HSP antibodies with those of affinity-purified natural antibodies directed against other self antigens. Several self antigens including HSP90, myosin, tubulin and aldolase with highly conserved structures showed similar patterns of reactivity with natural IgG antibodies. Less conserved self antigens like chymotrypsin, and non-self antigens such as TT, remained unclustered upon analysis of data using tree clustering with Euclidean distances and a single-linkage algorithm. Taken together, the results suggest the presence of abundant autoantibodies to HSP in normal human IgG, and favor the hypothesis that HSP90 belongs to a subset of highly conserved and immunodominant self antigens that is the primary target of natural autoantibodies.

Natural self-reactive antibodies belong to IgG, IgM and IgA isotypes (29). Here we found that natural IgG antibodies to HSP90 are predominantly of the IgG2 isotype. This may be of interest in view of previous findings indicating that autoantibodies specific for DNA in healthy individuals are of IgG2 subclass, while in systemic lupus erythematosus (SLE) patients they are mostly IgG1 (30).

Natural IgG antibodies were immunoaffinity-purified from pooled normal IgG on matrices carrying HSP90 or other self proteins to compare their binding properties to various antigens. The binding of anti-HSP90, anti-actin, anti-aldolase and anti-TG to the proteins solubilized from normal human liver tissue in immunoblot, showed that all the tested natural antibodies are polyreactive and demonstrated that the natural antibodies bind to the same set of self antigens with only quantitative differences among the antibodies tested. The effluents of the HSP90 affinity column, i.e. the fraction of IgG that does not interact with HSP90 was almost totally devoid of immunoreactivity with antigens present in the liver tissue extract. Such an absence of natural antibody activity in the non-HSP-specific fraction of IgG suggest that HSP is an immunodominant self antigen. The binding curves to HSP90 of most of the affinity-purified autoantibodies, specific for various self antigens, exhibited almost superimposable profiles. These results together with the observation of a differential binding of the anti-HSP90 IgG fraction to other self antigens

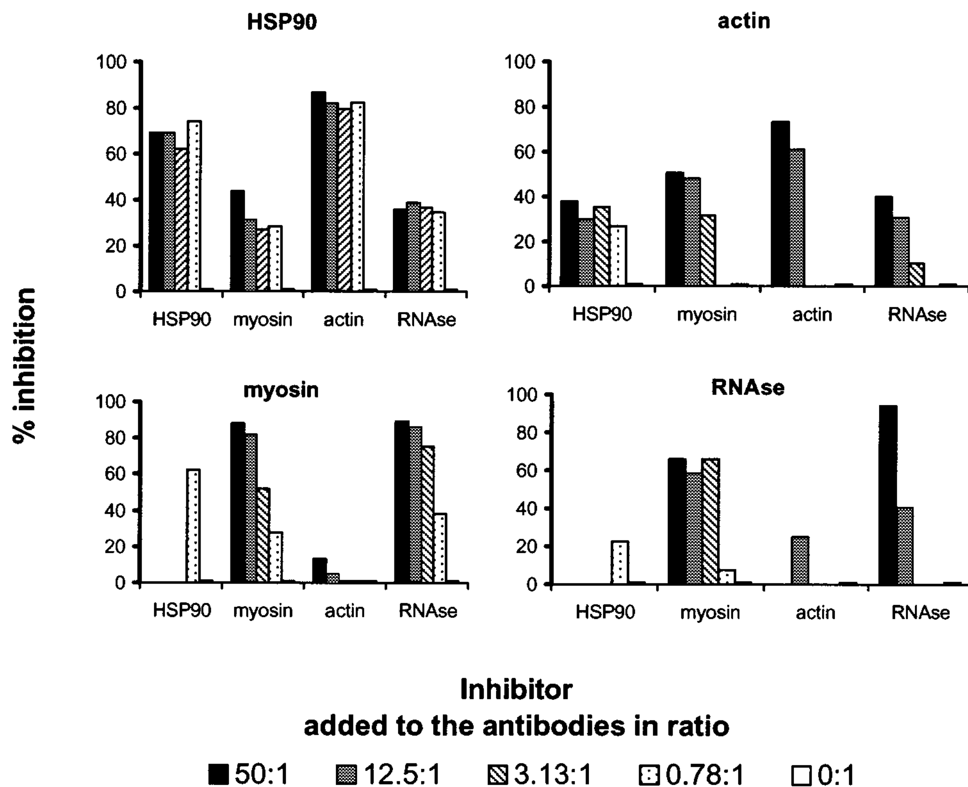

Fig. 7. Inhibition of the binding of anti-HSP90 natural autoantibodies to HSP90, myosin, RNase and actin by the respective antigens tested in cross-blot followed by densitometry. Anti-HSP90 autoantibodies at 0.02 mg/ml were preincubated with myosin, RNase and actin at inhibitor to antibodies ratios of 50:1, 12.5:1, 3.13:1 and 0.78:1, and with HSP90 at ratios of 1.5:1, 0.75:1, 0.33:1 and 0.17:1, as well as with no inhibitor, for 72 h at 4°C. The antigens were deposited onto PVDF membranes, and further incubated with the preincubated mixtures of antibodies and inhibitors.

may be explained by the existence of a set of public epitopes on HSP90 that is preferentially targeted by natural antibodies. The cluster analysis on data of immunoreactivity of natural antibodies of different specificity to a panel of different self and non-self antigens further confirmed a similar and uniform pattern of binding to highly conserved self antigens including HSP90. The reactivity of anti-HSP90 autoantibodies with other highly conserved antigens was confirmed also by cross-competition assay. HSP90, myosin, actin and RNase all inhibited the binding of anti-HSP90 autoantibodies to HSP90 and actin while only myosin and RNase inhibited the binding to these same two antigens. HSP90 rather increased the binding of anti-HSP90 autoantibodies to myosin and RNase which most probably is due to the formation of complexes or a prozone effect.

Another explanation for the polyreactivity of the autoantibodies purified on HSP90 affinity column could be the presence of a variety of peptides attached to the HSP90 molecules isolated. Although this impurity could not be excluded, it is probably of minor importance for the following reasons. During elution process of the Sepharose columns, part of these peptides would be stripped. In addition, the binding properties of the natural antibodies, that were isolated after several rounds of elution at pH 2.7 and 11, and cleaning of the column with 3 M urea, did not change while these treatments should have greatly reduced the content of the peptides still associated with HSP90.

The data from the present study are in line with the concept of a subset of immunodominant self antigens referred to as immunological homunculus, that is essential for maintenance of tolerance to self (9,31). Whereas the original concept dealt with a restricted set of T cells recognizing homuncular antigens, evidence has now accumulated extending the concept to the B cell compartment (32). Thus, natural autoantibodies within the IgM and IgG fractions of human plasma recognize a restricted set of self antigens that is highly conserved between individuals and throughout life (4,5). It has been proposed that human HSP is one of the prime candidates for such dominant homuncular antigens in the T cell compartment (11). Thus, at a high frequency, non-adherent mononuclear cells in human cord blood were found to proliferate in response to mycobacterial 65-kDa HSP. In comparison, a 10–100 times lower frequencies to purified protein derivative of *Mycobacterium* were found in non-adherent cord blood mononuclear cells than in adult peripheral blood mononuclear cells (35). Our data further identify HSP90 as a privileged target for natural autoantibodies.

Natural antibodies recognize mostly simple epitopes of low specificity. Thus, for instance, natural antibodies bind mostly to multiple epitopes in the light meromyosin which exhibits a repetitive helical secondary structure (36) and highly charged sequences block that binding (37). Polyreactive anti-histone autoantibodies were found to bind to poly-L-lysine (38) and protamine-reactive natural autoantibodies were shown to bind

to RRR epitopes (39). HSP90 contains several highly charged helical regions with compositional bias in favor of lysine and/or glutamate. Autoantibodies to self-HSP90 found in patients with candidiasis, aspergillosis and SLE recognize epitopes that include repetitive charged sequences including KKIK, GLELPE and the highly conserved epitope KILKVIRK (40). Tchernychev *et al.* have proposed recognition of proline-rich regions as the structural basis for the polyreactivity of natural antibodies (41). When compared to eight other proteins, including three cytoskeletal proteins, HSP90 showed the highest number of stretches with high similarity to sequences in the other tested proteins (data not shown). Similar findings have been reported with regard to the homology between HSP60 and self antigens (42). It is important to note that the sequence similarities found are mostly within regions with many repetitions of charged amino acids. We thus speculate that such structures are involved in the binding interaction between natural antibodies, HSP and other homuncular antigens. It is possible that idiotopes of the germline antibodies themselves contain regions belonging to this set of simple epitopes. The latter possibility is supported by the existence of self binding autoantibodies and the epibody phenomenon (43–45). Idiotypic interactions between the B and T cell repertoires have also been demonstrated (46). Thus, it is possible that the same simple and conserved structures exist in the preimmune repertoires of the receptors of adaptive immunity and in the self antigens, ensuring the connectivity in the system (3,33,34).

The present study demonstrates the presence of abundant anti-HSP90 antibodies in the normal human IgG repertoire. It also supports that HSP, together with highly conserved self antigens, is a primary target of natural self-reactive antibodies. Identifying the molecular basis of recognition of self antigens by natural autoantibodies may enhance our understanding of the processes involved in the positive selection of the normal self reactive B repertoire and be relevant to the design of novel approaches for immunomodulation of autoimmune disease.

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Abbreviations

CTA	chymotrypsinogen A
HRP	horseradish peroxidase
HSP	heat shock protein
IVIg	intravenous immunoglobulin G
LP	lactoperoxidase
PRK	phosphorybulokinase
SLE	systemic lupus erythematosus
TG	thyroglobulin
TT	tetanus toxoid

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