Characterization of the anti-DnaJ monoclonal antibodies and their use to compare immunological properties of DnaJ and its human homologue HDJ-1

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Abstract *Escherichia coli* DnaJ (Hsp40) is suspected to participate in rheumatoid arthritis (RA) pathogenesis in humans by an autoimmune process. In this work a set of 6 anti-DnaJ monoclonal antibodies (mAbs) was raised and localization of the epitopes recognized by the mAbs was investigated. Western blotting and enzyme-linked immunosorbent assay (ELISA) experiments showed that the mAbs efficiently bound only native antigen. Using DnaJ mutant proteins with deletions of specified domains and ELISA, we found that AC11 mAb reacted with the best conserved in evolution N-terminal J domain, whereas BB3, EE11, CC5, CC8, and DC7 bound to the C-terminal part after residue 200. Mapping performed with the use of a random peptide library displayed by filamentous phage indicated that (1) AC11 mAb bound to a region between residues 33–48, including D-34 which belongs to the HPD triad, present in all DnaJ homologues, (2) BB3 recognized residues localized in the 204–224 region, (3) EE11 recognized the 291–309 region, (4) CC5—the region 326–359, and (5) CC8—the 346–366 region. All these mAbs, as well as the polyclonal antibodies against the N- or C-terminal domain, bound efficiently to HDJ-1, human Hsp40. These results show the presence of a significant immunological similarity between bacterial DnaJ and human HDJ-1, which is not restricted to the evolutionarily conserved parts of the proteins, and suggest that HDJ-1 could be a possible target of immune response triggered by DnaJ.

INTRODUCTION

Escherichia coli DnaJ heat shock protein is a member of the DnaJ (Hsp40) family of chaperone proteins that function together with Hsp70 chaperones in a variety of cellular processes (reviewed in Bukau and Horwich 1998; Cheetham and Caplan 1998). In the full-length *E. coli* DnaJ protein there are 4 domains formed by a 375-amino acid sequence. The amino-terminal 75 residues of DnaJ constitute an evolutionarily highly conserved motif, the J domain, which together with the adjacent region, rich in glycine and phenylalanine (Gly/Phe motif), is essential for DnaJ's interactions with DnaK (reviewed in Kelley

1999). The third domain, rich in cysteine residues, binds 2 zinc ions and together with the least conserved C-terminal region functions through an unknown mechanism to bind substrate proteins (Martinez-Yamout et al 2000 and citations therein).

E. coli DnaJ is suspected to participate in autoimmune response and pathogenesis of rheumatoid arthritis (RA) in humans. It has been suggested that the immune response directed against the bacterial protein cross-reacts with the human homologous protein(s) (Albani et al 1995; Albani and Carson 1996; Kurzik-Dumke et al 1999). Several human DnaJ homologues have been identified: HDJ-1, HDJ-2, HSJ-1, HLJ-1 (reviewed by Cheetham and Caplan 1998), HDJ-3 (Andres et al 1997; Edwards et al 1997), Hsc40 (Chen et al 1999), and HEDJ (Yu et al 2000); however, it is not known which homologue may be in-

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volved in RA etiology. HDJ-1 is the best-studied eukaryotic DnaJ homologue containing the conserved J and G/F domains but not the Zn-binding domain (Cheetham and Caplan 1998).

One of the approaches aimed at comparing immunological properties of the *E. coli* DnaJ and its human homologues is to use well-characterized monoclonal antibodies (mAbs) raised against the *E. coli* DnaJ and to test their reactivity with the human proteins. In this work, a panel of 6 anti-DnaJ mAbs was prepared and characterized. We used mutant DnaJ proteins, carrying specified domains to tentatively localize epitopes recognized by the anti-DnaJ mAbs, and then a filamentous fd phage library displaying 15-residue random peptides to map the epitopes more precisely. The characterized mAbs and also polyclonal antibodies against defined DnaJ domains were used to investigate immunological similarity of DnaJ and HDJ-1.

MATERIALS AND METHODS

Bacteria, plasmids, and media

E. coli K91 strain was used for filamentous phage growth (Parmely and Smith 1988). *E. coli* B178 (pDW19dnaJΔ77–107) (Wall et al 1995), *E. coli* B178 (pDW19dnaJΔ144–200) (Banecki et al 1996), *E. coli* BL21(DE3) (pAED4dnaJΔ1–199) (Doering 1992), *E. coli* DH5α (pWK100dnaJΔ110–375) (Guzman et al 1995), *E. coli* B178 (pMOB45dnaJ⁺) (Żylicz et al 1985), and *E. coli* BL21(DE3) (pET21HDJ-1) (Freeman et al 1995) strains were used for overproduction of DnaJΔ77–107, DnaJΔ144–200, DnaJ742, DnaJ12, DnaJ wt, and HDJ-1, respectively. Bacterial strains used for protein overproduction were grown in Luria-Bertani (LB) medium with either ampicillin (50 µg/mL) or tetracycline (20 µg/mL). Phage-inoculated *E. coli* K91 was grown in 2× YT medium with tetracycline (20 µg/mL). LB and 2× YT media were as described by Sambrook et al (1989).

Expression and purification of proteins

Overexpression of DnaJ, DnaJΔ77–107, DnaJΔ144–200, DnaJ742, and HDJ-1 proteins in *E. coli* cells, transformed with appropriate plasmids, was induced at OD₅₉₅ of 0.5 by 1 mM isopropyl-L-thio-β-galactoside for 3 hours. DnaJ, DnaJΔ77–107, and DnaJΔ144–200 proteins were purified as described previously in Żylicz et al (1985). In the case of DnaJ742, the purification procedure was modified, by decreasing KCl concentration by half during all purification steps, to achieve proper binding of DnaJ742 protein to ion exchange resins. The *E. coli* DnaJ12 protein was overexpressed in *E. coli* DH5α cells, transformed with pWK100dnaJΔ110–375 plasmid, and induced with 0.1% arabinose. DnaJ12 was purified using a modified method of Karzai and McMacken (1996). HDJ-1 was purified ac-

cording to the DnaJ purification protocol with 1 exception: HDJ-1 was eluted from the hydroxyapatite column with 100 mM potassium phosphate buffer, pH 8.6, instead of a 150 mM buffer, used for DnaJ preparation. The endogenous DnaJ protein was still bound to the hydroxyapatite under these conditions (not shown), resulting in HDJ-1 not contaminated with *E. coli* DnaJ.

Protein assay, electrophoresis, and Western blotting

Protein concentration was estimated by Bradford method and spectrophotometric measurements, as described in Sambrook et al (1989). Proteins were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970), using 10% or 12.5% (w/v) acrylamide. Native gel electrophoresis was performed in SDS-depleted Laemmli system, without stacking gel, in 7% resolving gels.

Western blots were performed on nitrocellulose type BA83 (Schleicher & Schuell, Dassel, Germany) as described previously (Lipińska et al 1990), using anti-DnaJ antibodies as the primary antibodies. Secondary antibodies were alkaline phosphatase-conjugated goat anti-rabbit immunoglobulins (Roche, Mannheim, Germany) (for polyclonal primary antibodies), horseradish peroxidase-conjugated goat anti-mouse immunoglobulins (Roche) (for IgG mAbs), or horseradish peroxidase-conjugated goat antibodies against mouse heavy µ chain (Sigma, Poznan, Poland) (for IgM mAbs). Tetramethylbenzidine (TMB; Sigma) was used as the substrate for horseradish peroxidase.

Preparation of anti-DnaJ mAbs

Female, 5- to 6-week-old BALB/c-PZH mice were primed with 40 µg DnaJ dissolved in 0.2 mL of sterile phosphate-buffered saline (PBS) emulsified with 0.3 mL of complete Freund's adjuvant. On days 14, 35, and 56 after the priming, immunizations with the same amount of antigen in 0.5 mL sterile PBS were repeated. Finally, 80 µg DnaJ was injected on day 63. All immunizations were done intraperitoneally. Three days after the final booster injection, somatic cell fusion to generate antibody-producing hybridomas was performed by standard methods (Peters and Schulze 1985), using the mouse myeloma cell line P3X63Ag8.653 from the European Collection of Animal Cell Cultures (Salisbury, UK) as a fusion partner. The standard hypoxanthine aminopterin thymidine medium was used to select hybrids. For large-scale culture of mouse hybridomas, Ex-Cell 610 medium (JHS Biosciences, Andover, UK) was used. Cloned hybridomas were expanded either in serum-free culture medium or as ascites fluid in pristine-primed BALB/c mice (Schulze and Baumgarten 1985). Antibodies from culture supernatants and ascitic fluids were partially purified by 50% ammonium sulfate precipitation and dialyzed against

PBS. Their isotypes were determined by enzyme immunoassays with mouse mAbs typing kits (Pierce, Rockford, IL, USA).

Preparation of anti-DnaJ mAbs coupled with horseradish peroxidase

Anti-DnaJ mAbs were purified using Hi-Trap affinity columns (Pharmacia Biotech, Uppsala, Sweden). Conjugating horseradish peroxidase (Roche) with the anti-DnaJ mAbs was carried out using the periodate method as described by Wilson and Nakane (1978).

Polyclonal anti-DnaJ antibodies

Rabbit anti-DnaJ, anti-DnaJ742, and anti-DnaJ12 polyclonal antibodies were prepared according to the method described by Szewczyk and Harper (1994) and tested by Western blotting with appropriate antigens.

Phage selection from the fd library displaying random peptides

The filamentous phage library used in this study displays random peptide sequences of 15-amino acid residues at the N-terminus of the minor coat protein III (Scott and Smith 1990) due to a 45-bp random insertion into the *Sfi*I site of phage fUSE5 (Smith and Scott 1993). In a biopanning procedure, performed as described in Böttger et al (1996), the library (5×10^{11} phage/mL) was panned against each of 6 anti-DnaJ mAbs, AC11, BB3, CC5, CC8, DC7, and EE11, immobilized on polystyrene petri dishes (Falcon 3001). Three rounds of biopanning were carried out to select higher from lower affinity clones, and after the third round, specifically bound phage were eluted. Independent phage clones were tested for their affinity for a mAb in ELISA, and positive clones were used for phage amplification. From the amplified phage (500 μ L) single-stranded deoxyribonucleic acid (DNA) was extracted using Wizard M13 DNA Purification System (Promega, Southampton, UK). DNA was used as a template for automated sequencing by ABI PRISM 310 Genetic Analyzer (Perkin-Elmer, Buckinghamshire, UK), with a primer 5' CCC TCA TAG TTA GCG TAA CG 3' (Cambridge Bioscience Ltd, Cambridge, UK).

Enzyme-linked immunosorbent assays

ELISAs were performed as described by Crowther (1995). Assay plates (Costar 3590) were coated with serial dilutions of DnaJ or HDJ-1 proteins (25–0.012 μ g/mL), and then 50 μ L of diluted anti-DnaJ mAb was added to the wells. The mAbs were used at constant dilutions as follows: AC11—1:6000; BB3, CC8, and EE11—1:2000; DC7—1:500; and CC8—1:200. After 90 minutes of incubation, goat anti-mouse immunoglobu-

lins coupled with horseradish peroxidase were added to each well. Bound conjugates were detected colorimetrically, after 60 minutes of incubation, by using horseradish peroxidase substrate—TMB. When the rabbit polyclonal anti-DnaJ sera were tested, horseradish peroxidase-conjugated goat anti-rabbit IgG were used. For ELISAs with denatured antigen, the DnaJ protein was treated with 6 M guanidine hydrochloride for 60 minutes at 30°C. Each assay was repeated 3 times.

Competition ELISAs

Direct competition ELISAs were carried out according to Crowther (1995). Briefly, assay plates (Costar 3590) were coated with 75 μ g DnaJ protein, and then 50 μ L of diluted anti-DnaJ mAb coupled with horseradish peroxidase was added to the wells. Conjugates were used at constant dilutions as follows: AC₁₁, BB₃, and CC₅—1:400; EE₁₁ and CC₈—1:200; and DC₇—1:100. At the same time, competing unlabeled anti-DnaJ mAbs were added at dilutions 1:50, 1:100, 1:500, or 1:1000. After 2 hours of incubation, bound conjugates were detected colorimetrically by using TMB. Each experiment was repeated 3 times. The competition was calculated as described in Crowther (1995). The significance of competition levels was estimated according to Jacobs et al (1990).

Phage ELISA

The assays were performed as described by Böttger et al (1996). Briefly, polystyrene assay plates (Falcon 3912) were coated with 50 μ L mAb (AC11, BB3, CC5, CC8, DC7, or EE11) at 4 μ g/mL. Fifty microliters of phage suspension from each round of biopanning was then added, and after 2 hours of incubation at room temperature, bound phage was reacted with 50 μ L horseradish peroxidase-labeled mouse anti-M13 antibody (Pharmacia LKB, Uppsala, Sweden) diluted 1:1000 for 1 hour at room temperature, followed by reaction with a substrate—50 μ L *o*-phenylenediamine (Sigma Aldrich)—for 30 minutes. As the positive control, an anti-p53 mAb DO-1 was used, whose epitope sequence had been defined using the same phage display library (Stephen et al 1995).

Aligning sequences and calculating the *P* value

Nucleotide sequences of phage DNA inserts were translated to amino acid sequences using the evaluation version of OMIGA 2.0 software. Sequences of the 15-amino acid peptides selected by each mAb were analyzed by multiple alignments using Clustal W algorithm (OMIGA 2.0). Consensus peptide sequences recognized by the mAbs were then aligned with the DnaJ amino acid sequence using slow pairwise alignment of Clustal W algorithm and identity matrix to score protein alignments

Table 1 Regions of similarity between the DnaJ amino acid sequence and the consensus sequences of the peptides binding to the anti-DnaJ monoclonal antibodies (mAbs)

Monoclonal antibody	Number of phage clones used for the determination of consensus sequences	Consensus sequences of the peptides bound by the mAbs	DnaJ regions bearing similarity to the consensus sequences of the peptides (amino acid residues)	Efficiency of Western blotting (native)	Efficiency of Western blotting (denatured)
AC11 (IgG)	12	xDxPQxxAEAxAFxF	33–48	+++	+/-
BB3 (IgM)	18	NVxxLxVxPLVDxF	204–222	++	—
CC5 (IgM)	19	VxxPxxPxNYxxIPS	178–227, 326–359	—	—
CC8 (IgM)	10	SGxxLxPxxSxxxSC	346–366	+	—
DC7 (IgM)	11	QCxLAlxxxxxxLPY	67–118 (?), 224–269 (?)	—	—
EE11 (IgM)	10	XVxGxYSTxxxFRVx	291–309	++	—

+++ very strong reaction; ++ strong reaction; + weak reaction; +/- very weak reaction; — no reaction.

Fig. 1. Binding of the anti-DnaJ monoclonal antibodies (mAbs) to native and denatured DnaJ proteins. Reactivity of the anti-DnaJ mAbs with native DnaJ and DnaJ denatured by treatment with 6 M guanidine hydrochloride was assayed using ELISA. Panels present ELISA results obtained with AC11 (diluted 1:6000), BB3 (diluted 1:2000), CC5 (diluted 1:200), CC8 (diluted 1:2000), DC7 (diluted 1:500), and EE11 (diluted 1:2000). Antigen concentration was 25 μ g/mL. N—native DnaJ, D—denatured DnaJ, and C—control with BSA (25 μ g/mL). Mean values of 3 independent assays are presented.

(OMIGA 2.0) (Thompson et al 1994; Myers and Miller 1998). *P* values for alignments were calculated according to Chen et al (1996). Numeric values of *P* indicate an estimated frequency of obtaining the sequence match when comparing 2 random sequences 375 and 15 amino acids long (Chen et al 1996).

RESULTS AND DISCUSSION

mAbs against DnaJ recognize nonlinear epitopes localized in various domains of DnaJ

Six hybridoma cell lines producing mAbs against *E. coli* DnaJ were isolated and characterized first by Western blotting performed under denaturing and nondenaturing conditions. Whereas DC7 and CC5 were negative, AC11, BB3, CC8, and EE11 reacted much better with native than with denatured DnaJ (Table 1). All 6 mAbs were positive in ELISAs, and again native DnaJ was better recognized than the guanidine hydrochloride-treated protein (Fig 1).

These findings suggested that conformational epitopes of DnaJ were recognized.

To localize the epitopes, the ELISA tests were repeated using a set of truncated DnaJ proteins. The DnaJ variants are schematically presented in Figure 2, and their analysis by SDS-PAGE is shown in Supplementary Material (available online) (Fig S-1). Each of the mAbs reacted with each of the variants with a distinct kinetics, indicating that they recognize distinct epitopes (Supplementary Material—Fig S-2). Whereas the AC11 epitope was localized in the J domain, the other 5 were in the C-terminal domain after amino acid 200 (Fig 3).

Epitope mapping using peptide library displayed by filamentous phage

The fd phage library displaying 15-residue random peptides was used to select phage clones binding to each of 6 anti-DnaJ mAbs. The clones were further an-

Fig. 2. Schematic representation of DnaJ protein variants and localization of the anti-DnaJ monoclonal antibodies (mAbs). The schemes show the *Escherichia coli* DnaJ wild-type protein with its domain structure marked, the deletion mutants of DnaJ protein used in this study, and the regions in which the DnaJ residues binding the mAbs were identified.

alyzed by phage DNA extraction and by sequencing the nucleotide inserts coding the 15-amino acid peptides. Deduced amino acid sequences of all the tested clones are shown in Supplementary Material (Table S-1), and the consensus sequences of the peptides-epitopes bound by the mAbs are presented in Table 1. Search of the DnaJ protein sequence for regions of similarity to the consensus epitope sequences using 2 independent computer programs OMIGA 2.0 and Antheprot 4.9 gave unambiguous results for AC11, BB3, CC8, and EE11, whereas for the CC5 and DC7, 2 widely spaced regions of similarity were found in each case (Table 1; Supplementary Material—Fig S-3). Because previously obtained ELISA results indicated that the epitopes recognized by the CC5 and DC7 were located after amino acid 200, for CC5 we tentatively chose the region of similarity formed by the residues 326(V)–359(S) and for DC7 we chose the region 224(R)–269(N).

In the case of peptides bound by AC11, the consensus sequence between D-2 and F-13 contained a match of 6 of 12 amino acids with residues 34–46 of DnaJ (Supplementary Material—Fig S-3). The estimated frequency of obtaining the sequence match when comparing 2 random sequences 375 and 15 amino acids long is very low ($P = 0.0058$), which means that the match could not be accidental and indicates that the matching resi-

dues participate in the formation of a discontinuous AC11 epitope. This localization is consistent with the results of epitope mapping using mutant DnaJ proteins (Fig 3). It is worth noting that D matching position 34 was conserved in 11 out of 12 peptides (Table S-1) and that D-34 belongs to the HPD triad, conserved in all DnaJ homologues and participating in interactions between DnaJ and DnaK (Hsp70) chaperones (Cheetham and Caplan 1998; Kelley 1998). Solution of the nuclear magnetic resonance (NMR) structure of the J domain (Pellecchia et al 1996) enabled us to locate the amino acids of the AC11 epitope in the 3-dimensional structure of this domain (Fig 4). All the residues are easily accessible, except for the F-46 whose side chain participates in the formation of a hydrophobic contact surface between helices II and III (Pellecchia et al 1996). On the basis of this analysis, we conclude that D-34, Q-37, E-41, A-42, and A-44 belong to AC11 epitope, whereas F-46 participation is not evident.

Peptides selected by panning against BB3 contained a match of 6 of 16 amino acids with residues 204–222 of DnaJ. In addition, there were 2 conservative substitutions in the peptides for DnaJ residues (N for R-204 and L for A-217). The estimated frequency of obtaining such sequence match is low ($P = 0.1141$). The data indicate that BB3 recognizes a discontinuous epitope localized in the

Fig. 3. Binding of the anti-DnaJ monoclonal antibodies (mAbs) to DnaJ protein variants and to HDJ-1. Reactivity of the anti-DnaJ mAbs with the DnaJ full-length protein, DnaJ12, DnaJ742, DnaJΔ144–200, DnaJΔ77–107, and HDJ-1 was assayed using ELISA. Panels present ELISA results obtained with (A) AC11 (diluted 1:6000), (B) BB3 (diluted 1:2000), (C) CC5 (diluted 1:200), (D) CC8 (diluted 1:2000), (E) DC7 (diluted 1:500), and (F) EE11 (diluted 1:2000). Antigen concentration was 25 μ g/mL. Mean values of at least 3 independent assays are presented.

Fig. 4. Localization of the AC11 epitope in the J domain 3-dimensional structure. Panels (A–D) show different views of the J domain surface. Amino acids participating in the binding of AC11 monoclonal antibody are shown in green (green patches on the surface of the J domain). N designates the N-terminal Ala1 and C designates Gly78, the C-terminus of the J domain. Models were prepared using 1XBL sequence, PDB Viewer, and POV-Ray programs.

205–220 region. The fact that the mutant protein DnaJ742, comprising residues 200–375, bound BB3 less efficiently than the DnaJ wt (Fig 3; Supplementary Material—Fig S-2) could be explained by a different conformation of the isolated C-terminal part of DnaJ when compared with its native conformation in full-length DnaJ. Such difference would be quite probable at the N-terminus of the truncated protein, where the proposed epitope is located (Huang et al 1999).

Peptides selected because of their ability to bind to EE11 showed a match of 6 out of 18 amino acids with residues 291–309 of DnaJ. In addition, there were 2 conservative substitutions in the peptides for DnaJ residues (Y for T-296 and S for Q-297) that increased the similarity of the peptides to this DnaJ region. The data suggest that EE11 recognizes a discontinuous epitope localized in the 291–309 region, which is consistent with the results of epitope mapping using mutant DnaJ proteins (Fig 3).

Alignment of the peptides selected by CC5 with the DnaJ sequence showed in region 326–359, 5 matches in 2 groups of 2 and 3 separated by a stretch of 20 residues. In addition, in 8 out of 19 peptides bound by CC5 there was a conserved Val-5, which matches V-330 of DnaJ, and in 9 out of 19 peptides there was a conserved N-12, which matches N-355 of DnaJ (Supplementary Material—Fig S-3, Table S-1). Taking into account these 2 additional matches, we arrived at a statistically significant *P* value (*P* = 0.0268). These data indicate that the CC5 epitope is nonlinear and suggest that the matching residues participate in CC5 binding. This finding is in agreement with

previous results, showing that isolated C-terminal domain bound CC5 (Fig 3). However, this binding was significantly less efficient when compared with the DnaJ wt. This might be caused by a difference in conformation of the isolated C domain vs C domain that is a part of a full-length DnaJ or by the existence of additional residues binding CC5 and localized outside the C-terminal part (or by both). The latter is probable because (1) there is another region of similarity among the peptides and DnaJ, between residues 178–227 (Table 1), and (2) DnaJ Δ 144–200 bound CC5 much less efficiently than did the DnaJ wt (Fig 3; Supplementary Material—Fig S-2). In conclusion, our data suggest that the CC5 epitope is composed of the residues from region 326–359 and that some residues from region 178–227, localized in the Zn-binding domain, may also participate.

Peptides selected by panning against CC8 had a match of 4 out of 16 residues with amino acids 346–366 of DnaJ (Table 1; Supplementary Material—Fig S-3). Localization of the CC8 epitope in this region was supported by competition assays.

Competition assays

ELISAs, in which the anti-DnaJ mAbs competed with each other in binding to immobilized DnaJ, showed inhibition of CC5 binding by CC8 (and vice versa) (Table 2), which was in agreement with previous results, indicating that the CC5 and CC8 epitopes partially overlapped (Table 1; Supplementary Material—Fig S-3). Pres-

Table 2 Results of competition assay

Competing mAbs	Competition (%)					
	AC11	BB3	CC5	CC8	DC7	EE11
AC11	61	8	3	3	3	3
BB3	8	54	7	2	6	6
CC5	3	7	57	21	5	11
CC8	3	4	21	50	3	11
DC7	3	6	5	3	49	5
EE11	3	6	11	11	5	56

mAb, monoclonal antibody.

Competition between monoclonal antibodies (mAbs) for DnaJ binding was determined by competitive ELISA. DnaJ was immobilized on microtiter plates, and each mAb conjugated with horseradish peroxidase was incubated with dilutions of the unlabeled competing mAb. Competing mAbs were at dilution 1:1000. Conjugates were used at constant dilutions as follows: AC11, BB3, and CC5—1:400; CC8, and EE11—1:200; DC7—1:100. After preincubation, binding of the labeled mAb to DnaJ was assayed and competition (%) was calculated as described in Materials and Methods. Significant competition values are in boldface.

ence of competition between EE11 and CC8 as well as between EE11 and CC5 was in agreement with localization of the EE11 epitope in close proximity to the CC8 and CC5 epitopes (Table 1; Supplementary Material—Fig S-3). Taking into consideration the size of the IgM mAbs, some steric interactions are highly probable, assuming that the EE11 epitope is only 16 residues apart from CC5 and 36 residues apart from CC8 epitope. Lack of competition between DC7 and BB3 or EE11 was contrary to our earlier assumption that the DC7 epitope might be localized in the region comprising residues 224–269. Precise localization of DC7 epitope is not possible at this stage of work.

Taking into consideration all presented data, we propose the localization of the epitopes recognized by the anti-DnaJ mAbs as shown in Figure 2.

Polyclonal antibodies against full-length DnaJ, N-terminal DnaJ domain, and C-terminal DnaJ domain recognize HDJ-1

We have overexpressed human HDJ-1 in *E. coli* cells and purified it (Supplementary Material—Fig S-1). HDJ-1 was produced in *E. coli* cells because there are no indications of its being posttranslationally modified in eukaryotic cells (Terada and Mori 2000). Results of ELISA showed that HDJ-1 reacted significantly with the rabbit polyclonal antibodies against the full-length DnaJ, against the N-terminal domain (DnaJ12), and

A.

B.

C.

Fig. 5. Reaction of polyclonal anti-DnaJ antibodies with HDJ-1 and DnaJ. Binding of the rabbit polyclonal antibodies anti-DnaJ (panel A), anti-DnaJ12 (panel B), and anti-DnaJ742 (panel C) to HDJ-1 and DnaJ proteins was assayed using ELISA. Assay plates were coated with HDJ-1, DnaJ, DnaJ12, or DnaJ742 protein (25 µg/mL), and then

diluted anti-DnaJ polyclonal serum was added to the wells (anti-DnaJ—1:20 000, anti-DnaJ12—1:1000, and anti-DnaJ742—1:4000).

also with the antibodies raised against the C-terminal part of DnaJ (DnaJ742) (Fig 5). Because the N-terminal domains of DnaJ and HDJ-1 bear 45% homology at the amino acid level (amino acids 1–76 of DnaJ and HDJ-1 were compared), some degree of immunological similarity could be expected. However, the C-terminal parts of DnaJ and HDJ-1 have only 12% homology of the amino acid sequence (amino acids 200–375 of DnaJ and 164–339 of HDJ-1 were compared), and their immunological similarity was surprising. To confirm results obtained with the polyclonal antibodies, we performed ELISAs using the mAbs described in this work.

The mAbs recognizing epitopes in N- and C-terminal DnaJ domains bind to HDJ-1

Using ELISA, we have found that the mAbs AC11, BB3, EE11, CC5, CC8, and DC7 bound efficiently to the HDJ-1 (Fig 3; Supplementary Material—Fig S-2). It is not surprising that the mAb AC11, whose epitope is localized in the J domain, best conserved among DnaJ proteins, recognized HDJ-1. Of the whole DnaJ region binding AC11 (residues 33–48), 6 conserved residues are present in HDJ-1 and 2 have conservative substitutions; furthermore, 3-dimensional structures of the J domains of DnaJ and HDJ-1, solved by NMR analysis, are almost identical (Pellicchia et al 1996; Qian et al 1996; Kelley 1998).

In the case of the mAbs BB3, CC5, CC8, and EE11, recognizing epitopes localized in various parts of the C-terminal domain, efficiency of binding to HDJ-1 was comparable with the efficiency of binding to the isolated C-terminal domain of DnaJ, although lower than binding to the full-length DnaJ (Fig 3). In the case of DC7, binding to HDJ-1 was even more efficient than binding to the original antigen DnaJ (Fig 3). These data show that the C-terminal domains of DnaJ and HDJ-1, despite very low sequence homology, bear immunological similarity.

Our finding that there exists significant immunological similarity between DnaJ and HDJ-1 and that it is not restricted to the conserved J domains indicates that immune response against the bacterial DnaJ antigen could be aimed against human HDJ-1 and that it could involve a wide spectrum of antibodies. This supports the hypothesis that infection with bacteria, triggering anti-DnaJ reaction, could in the course of the autoimmune process participate in pathogenesis of RA (Albani et al 1995; Albani and Carson 1996). We have found recently that patients with RA had approximately 3- and 2.5-fold higher levels of anti-DnaJ and anti-HDJ-1 antibodies, respectively, when compared with healthy controls; furthermore, patients with high response against DnaJ and HDJ-1 had higher levels of antibodies against DnaJ's C-terminal domain than the control (unpublished results). Because there are several human DnaJ homologues known,

their immunological similarity to DnaJ needs to be tested in future to identify the best candidate that could be a target in this disease.

The above-described immunological similarity suggests that the C-terminal, poorly conserved regions of DnaJ and HDJ-1 may have a similar 3-dimensional structure. To date, the structure of the C-terminal, substrate-binding region of *E coli* DnaJ has not been solved. Within the Hsp40 family of proteins, only the C-terminal domain of the yeast Sis1 protein has been crystallized and characterized (Sha et al 2000).

Apart from the studies on immunological similarity of Hsp40 proteins, the anti-DnaJ mAbs described in this work, or their derivatives (Fab fragments or single-chain variable regions), could be used in research on DnaJ interactions with DnaK or with the substrates.

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