Pakistan Journal of Pharmacology
Vol. 18, No.2, July 2001, pp.35-42

PRODUCTION AND CHARACTERISATION OF ANTI-MYCOBACTERIAL DNAJ (HEAT SHOCK PROTEIN) MONOCLONAL ANTIBODIES

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ABSTRACT

DnaJ, a heat shock protein, of *Mycobacterium tuberculosis* was cloned and expressed in *E.coli*. In order to study the importance of DnaJ in bacteria, anti-DnaJ monoclonal anibodies (m-Ab) have been produced. Mice were immunised with the recombinant mycobacterial-DnaJ. DnaJ was found to be highly immunogenic in mice.

A large number of anti-DnaJ m-Abs clones were produced. All the m-Abs were characterised using ELISA and Western Blotting Immunostaining. All the antibodies were found to recognise mycobacterial-DnaJ. A total of eight good m-Abs were produced and with exception of CD2, all of them were belong to IgG1 subclass. One of the m-AB AB7 was produced in large quantity in mice. The m-Abs AB7 was found to reacts with a numbers of Gram negative bacterial culture sonicates including *Escherichia, Salmonella, Klebsiella* and *Yersinia* species, indicating that DnaJ is highly conserved protein. It is interesting that AB7 anti-DnaJ m-Ab recognises only DnaJ antigen in Gram negative bacteria and not in Gram positive bacteria tested.

INTRODUCTION

Clinical expression of tuberculosis with *M. tuberculosis* is quite varied and dependent on various factors. Among generally healthy individuals, infection is usually asymptomatic (about 90%). Immunity to this infection is cell-mediated. Knowledge of the structure of mycobacterial antigen is fundamental to the understanding of the host immune response to the organism. The number of antigenic specificities represented within the organism is immense. Many antigens of mycobacteria are widely shared throughout the genus and some more widely throughout the microbial world.

Heat-shock (stress) proteins (hsp) are highly conserved homologous proteins with respect to structure and function in both prokaryotes and eukarytoes. The amount of stress proteins increased under stress condition particularly at elevated temperature (Lindquist 1986; Lindquist & Craig 1988), absence of oxygen or presence of reactive oxygen metabolites which are commonly found in inflammation (Polla, 1988). Different kinds of stress promote denaturation of intracellular proteins which in turn signals the induction of the hsp response (Ananthan *et al.* 1986). Most hsps are present as constitutive molecules in normal active cells (Linquist, 1986; Ellis, 1987; Pelham, 1988).

The most prominent hsps have a molecular weight of about 70 kDa and constitute the hsp 70 family. In *E. coli,* the hsp 70 is a product of a single gene, DnaK. Genetic and biochemical studies show that the DnaK protein functions in association with two other hsps, DnaJ and DnaGrpE, which can act by direct modulation of the ATPase activity of DnaK (Lathigra *et al.*

1991). Genes encoding the DnaK, GrpE and DnaJ hsps occupy adjacent positions on the chromosome of *Mycobacterium tuber-culosis*. This arrangement is distinct from other bacteria (Georgopoulos *et al.* 1990). The identification of the hsp 70 family of mycobacteria arose from immunological studies designed to identify prominent antigens involved in antibody and T cell mediated immune responses to infection (Young *et al.* 1990).

DnaJ is a stress protein induced at an elevated temperature. It has a homologous sequence with *E. coli* DnaJ (Lathigra et al. 1992). Recently it has been suggested that species-specific determinants may predominate over cross-reactive determinants in the antibody response to DnaJ. It has recently been reported that a major antigen of the malarial parasite-the ring infected erythrocytes surface antigen, RESA has sequence features indicative of a relationship with the DnaJ family (Bork *et al.* 1992). Importance of DnaJ has been discussed by Ahmad 1995. Its role in survival of pathogen has been reported by Ahmad *et al.* 1997. However, the role of the 43 kDa hsp is yet not clear.

Mycobacterium tuberculosis was found to be a cause of some cases of infective arthritis. This has suggested a possible role of *M. tuberculosis* also in rheumatoid arthritis (Mateo *et al.* 1992). The amino acid sequence of DnaJ contains an 11 amino acid segment that is homologous to the third hypervariable region of HLA DW4, the part of the Class II molecule that carries susceptibility to rheumatoid arthritis. Antibody to DnaJ reacts with HLA DW4-homologous B lymphoblastoid cells, but not with HLA DW2, DR1, DR3 and DR7 homologous cells (Albani *et al.* 1992a, b). This sequence data suggest that immunological cross-reactivity might exist between HLA DR4 and DnaJ. This raises the possibility that induction of an antibody and/or T cell responses to DnaJ, which is a stress protein of many bacteria, might be implicated in an autoimmune process in which the DRB 10401 haplotype (DR4) is involved. This haplotype is strongly associated with severe, erosive forms of rheumatoid arthritis.

MATERIALS AND METHODS

Five female Balb/c mice were immuniscd with recombinant mycobacterial antigen DnaJ with FCA by the intraperitoneal route. The mice were tail bled after the primary boost and the antibody titres were determined by ELISA (Catty and Raykundalia 1989).

Fusion (Brown and Ling, 1989):

Mice showing good antibody response were killed by cervical dislocation. The spleen was removed aseptically and cells were obtained. Myeloma cell line X63-Ag8.653 cells were counted. Cells were taken for fusion at a ratio of 5:1 of spleen to myeloma cells. Each preparation was centrifuged at 1700 rpm (400g) for 5 minutes. Supernatants were withdrawn by pasteur pipette and discarded. Each cell suspension was resuspended in 10 ml of RPMI-1640 and mixed. It was centrifuged at 1850 rpm (500g) for 7 minutes and the supernatant was again sucked off with a pasteur pipette. The pellet was warmed to 37°C in the water bath, and 0.8 ml of PEG 1500 (50% in distilled water) was added quickly. It was warmed further in the water bath at 37°C for 2 minutes, with regular rotation. The PEG was slowly diluted with 2 ml of prewarmed RPMI over 2 minutes. The 20 ml of prewarmed RPMI was added over a further 5-6 minutes. The final cell suspension was centrifuged at 1500 rpm (350g) for 6 minutes. The supernatant was removed by pasteur pipette and the cells were resuspended in 30 ml of HAT medium. One drop of cell suspension was added to each well of a Linbro 96 well plate, 6 trays of each fusion. Trays were incubated at 37°C in a CO_2 incubator with adequate water to maintain humidity. Trays were inspected daily. 5 days after fusion 50µl of HAT medium was added. Medium was changed by

day 9. By 12 days post fusion, hybridoma colony growth was evident and the medium turned yellow.

Culture supernatants were removed and screened by RIA (Goding 1986). When the cell growth was vigorous the medium was regularly replaced. Cells were subcultured progressively from Linbro wells to 24 well plates and then to flasks.

Classess and subclasses of monoclonal antibodies were determined by using the double immunodiffusion (Ouchterlony) technique (Catty and Raykundalia, 1989). Antisera to mouse IgG1, IgG2a, IgG2b, IgG3, IgA and IgM were used. These were raised in sheep, prepared as IgG fractions and kindly donated by the departments Immunodia-gnostic Research Laboratory. Code Numbers: X1291F, X711H, X1307D, X1056D and X1343B respectively. The neat antisera (5µ) were applied to the outer wells of each pattern. A hybridoma culture supernatant 20µl was placed in the middle well of each pattern. The reaction was allowed to proceed overnight in a humid chamber at room temperature. Next day the plates were washed in distilled water and dried with filter paper. Dried plates were put into Coomassie Blue stain for 10 minutes, washed with water and put in the destainer until the background was clear. The plates were observed carefully for the lines of precipitation. A single precipitation line against one anti-isotype system clearly indicated the isotype of each monoclonal antibody.

Production of ascitic fluid:

Mice were primed 3-60 days before required with 0.2 ml pristane i/p. Hybridomas were injected i/p using 2.5-5 x10^6 cells/mouse in 0.5 ml of sterile PBS. When mice showed evidence of ascites, they were killed and ascitic fluid was removed. Heparin was used to collect the fluid in universals. The ascitic fluid was centrifuged at 1800 rpm (450g) for 10 minutes at room temperature; the supernatant was taken off and frozen at -20°C. The pelleted cells were either frozen down or injected into further mice.

Characterisation of monoclonal antibodies:

Monoclonal anti-DnaJ antibodies were characterised using ELISA and Western Blotting Immunostaining. The purified recombinant DnaJ antigens (10µg/well) were run on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) along with the prestained molecular weight markers (Sigma). Bacteria were grown, sonicated and protein contents were determined by Lowery`s method. 10µg of each bacterial sonicate was also run on SDS-PAGE. The antigens were transferred onto nitrocellulose paper with a wet electroblotting system (Bio Rad).

Bacteria used in the study:

Mycobacterium tuberculosis, Streptococcus pyogenes, Staphylococcus aureus, Campylobacter jejuni, Bacillus spp., *Escherichia coli, Salmonella typhi, Shigella dysenteriae, Klebsiella pneumoniae* and *Yersima enterocolitica.*

Table-I
Titre of antibodies after primary and final boosts of Mice

Mice injected with	Mouse No.	Titre after primary boost	Titre after final boost
DnaJ	1	1:3200	1:12800
	2*	1:12800	1:25600
	3	1:3200	1:12800
	4	1:6400	1:25600
	5	1:12800	1:25600

*Selected for fusion experiment

Immunostaining:

A series of strips of 4mm were cut and were blocked in 3% BSA (Sigma) or in 0.1% PBS-Tween-20 overnight at 4°C. Antibody dilutions (1:100) were made in 0.05% PBS-Tween and the antibody layer was applied to the separate strips for 1 hour at room temperature. The strips were washed 5 x 15 minutes with 0.05% PBS-Tween. HRP sheep anti-mouse-lg conjugate (diluted 1:2000) was prepared in 5% non-fat milk and added to the strips and incubated for 1 hour at room temperature on a shaker. The strips were washed 1 x 60 minutes and 4 x 5 minutes with 0.1% PBS-Tween on a shaker. The strips were developed with 50mg of DAB in 100 mls of DAB substrate buffer (filtered and added 25 µl of 30% H2O2). The reaction was stopped by washing in 0.1% PBS-Tween 20.

RESULTS

The antisera and normal mouse serum were titrated in a series from 1:100 to 1:51200. The antibody titre was determined as the highest dilution which gives a higher reading than OD+ SD of the lowest dilutioin of normal mouse serum. The titration result of each animal`s serum taken from the group of mice immunised with DnaJ are shown in Table-I.

These data show that DnaJ is highly immunogenic in mice. After fusion, clones were screened for monoclonal antibody production by RIA. The results are shown in Table-II. Clones which were giving high cpm were selected and transferred to 24 well plates and then to tissue culture flasks. Clones were examined at each step by RIA. The good clones, which were showing continuous production of m-Ab, were injected intraperitoneally into pristane-primed mice for ascites production.

Two anti-Dnaj m-Ab clones, AB7 and CC3 retained their binding to DnaJ antigens. These clones retained binding activity to the antigen on solid phase RIA when grown *in vitro,* first in 96-well microtitre plates and then in tissue culture flasks. Normal mouse serum was used as a control.

Ascitic fluid was raised by injecting 3 X 10^6 cells intraperitoneally per mouse, which had previously been primed by pristane.

Anti-DnaJ m-Abs were characterised for their isotype. Seven of the eight m-Abs are IgG_1 and one is IgG_2a. The results are shown in Table-III.

Both m-Abs and polyclonal antibody,

Table-II
Screening of Hybridoma culture supernatant by Radioimmunoassay (RIA)

Anti-DnaJ Clones	CPM binding to DnaJ	Anti-DnaJ Clones	CPM binding to DnaJ	Anti-DnaJ Clones	CPM binding to DnaJ	Anti-DnaJ Clones	CPM binding to DnaJ
Polyclonal	1532	CH8	186	HG5	278	AG3	256
AB7*	456	AB10	215	EG5	234	BC1	302
CC3*	356	BC8	289	AH11	212	DC6	268
CD2	326	CG9	183	DD5	158	EG7	335
NMS	135	NMS	143	BB8	211	Back-ground	69

NMS = Normal mouse serum
Note:-Culture supernatants giving more than 200 cmp were regarded as positive but many clones initially positive lost binding activity on further culture.
*=Good clones continued to produced monoclonal antibodies, while others lost this property.

obtained from immunised mouse, were found to recognised DnaJ expressed in *E. coli*. While normal mouse serum and anti-SOD m-Ab did not show any band on the blots.

DnaJ is a heat shock protein, and hsps are highly conserved molecules in both eukaryotes and prokaryotes and are induced by elevated temperature. To study this conserved nature of DnaJ in different species, reactivity of the anti-DnaJ m-Abs was studied against different bacteria, available in the department, grown at 42°C. Their sonicates were prepared, and were analysed by Western blotting by using 1:100 dilution of anti-DnaJ m-Ab. Results are shown in Table-IV.

DnaJ heat-shock protein was detected in *M. tuberculosis, E. coli, S. typhi, K. pneumoniae* and *Y. enterocolitica,* indicating DnaJ is a highly conserved protein. Howeer it could not be detected in *S. dysenteriae, S. aureus, S. pyogenes* and *C. jejuni*. It is interesting that AB7 anti-DnaJ m-Ab recognises only DnaJ antigen in the Gram negative bacteria and not in the Gram positive bacteria tested. This could be due to the absence of this gene or its uninducibility by heat shock in this group of organisms. Alternatively the protein in these species may lack the epitope recognised by the monoclonal antibody

DISCUSSION

The introduction of monoclonal antibody technology (Kohler and Milstein 1975) has revolutionised identification, purification and characterisation of single antigens or epitopes of specific molecules. Progress in research on mycobacterial diseases has been slow because of the complex antigenic structure and slow growing nature of the mycobacteria and it is difficult to obtain sufficient quantities of pure antigens for immunological or biochemical studies by conventional isolation procedures. Numerous genes encoding protein antigens have been cloned in recent years by Yong *et al* (1992) and many other groups from the slow growing pathogenic mycobacteria. A combination of recombinant DNA technology and application of monoclonal antibodies is having a great impact on mycobacterial research. This approach has yielded considerable information about the nature of many important antigens found in mycobacterial species.

Young and his colleagues (Young et al. 1985a; 1985b; Thole et al. 1985) have constructed genomic libraries of mycobacteria using different vector systems, particularly the λgt11 expression library system (Young and Davies 1983). An

Table-III
The isotypes of anti-DanJ m-Abs in hybridoma culture supernatants by ouchterlony technique.

No.	Anti-DnaJ m-Abs	Isotype
1	AB7*	IgG1
2	CC3*	IgG1
3	CD2	IgG12a
4	AG3	IgG1
5	BC1	IgG1
6	DC6	IgG1
7	EG7	IgG1
8	HG5	IgG1

important mycobacterial recominant antigen, DnaJ heat-shock protein, has been produced by Dr. T. Garbe at the Hammersmith hospital in London.

DnaJ is an example of a heat shock protein produced by many bacteria and mammalian cells under stress conditions. The function is unknown. Its role in pathogenesis of human diseases and infections is under debate. There is much debate on the role of mycobacterial heat shock proteins in the pathogenesis of rheumatiod arthritis. Recently DnaJ, a 43 kDa hsp, has been identified and suggested to have some role in RA.

DnaJ of M. *tuberculosis* was found to be highly immunogenic in mice. In view of their highly immunogenic nature, DnaJ antigen was thought to be helpful in developing a reliable diagnostic test for tuberculosis at an early infection stage and also in understanding the mechanisms of pathogenesis of tuberculosis and rheumatoid arthritis. In order to achieve these goals, the first aim was to produce monoclonal antibodies to this immunodominant antigen of *M. tuberculosis*.

Albani *et al.* (1992) have reported sequence homology between *E. coli* DnaJ and the human DR4 haplotype by using a polyclonal antiserum and implicated some relationship between DnaJ and rheumatoid arthritis. Recently immune response to *E. coli* DnaJ in juvenile RA has been reported (Albani *et al.* 1994). Since DnaJ is a hsp, it would be of interest to see whether DnaJ is involved in immunopathology or autoimmunity due to cross-reaction or molecular mimicry (Young 1990).

We have prepared m-Abs to DnaJ and found that this antigen is also present in many bacteria, particularly Gram negative bacteria No monoclonal antibody specific for mycobacterial DnaJ has been reported. Earlier we have reported that serum immune complexes of a few rheumatoid arthritis patients possess the DnaJ/cross-reactive antigens which is not seen in normal subject (Ahmad *et al.* 1997). Synthetic peptides based on the *M. tubrculosis* DnaJ sequence should be made to localise both T cell and B cell (antibody) epitopes on this molecule.

Table-IV
Presence of DnaJ antigen in different bacterial species
Detected by anti-mycobacterial DnaJ monoclonal antibody (AB7)

Bacteria	*DnaJ*
Mycobacterium tuberculosis	+
Streptococcus pyogenes	-
Staphylococcus aureus	-
Campylobacter jejuni	-
Bacillus *spp*	-
Escherichia coli	+
Salmonella typhi	+
Shigella dysenteriae	-
Klebsiella pneumoniae	+
Yersinia enterocolitica	+

ACKNOWLEDEMENT

DnaJ, a 43 kDa recombinant protein of *M. tuberculosis* was kindly supplied by Dr. T. Grab, Hammersmith Hospital, London.

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