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Hsp70: a carrier molecule with built-in adjuvanticity

G. Del Giudice

*World Health Organisation-Immunology Research and Training Centre, Department of Pathology, University of Geneva, CH-1211 Geneva 4, and *Institute of Biochemistry, University of Lausanne, Chemin des Boveresses 155, CH-1066 Epalinges (Switzerland)*

Abstract. One problem associated with the development of subunit vaccines is their limited immunogenicity, due to their physico-chemical structure, their inability to encounter the correct MHC restriction element, or the need for strong adjuvants to be delivered along with them. These problems are usually solved by conjugating target epitopes (peptides or oligosaccharides) with carrier proteins which provide a source of T-cell epitopes recognised by a large proportion of the vaccinated individuals. We have shown that mycobacterial hsp65 and hsp70 exert a strong helper effect in vivo when conjugated to synthetic peptides or oligosaccharides. Interestingly, this helper effect did not require the need for any adjuvant, either in mice or in monkeys. The helper effect mediated by the hsp65 required that animals were previously primed with either live BCG or the hsp65 alone; on the other hand, such a priming was not required when the hsp70 was used in the conjugates. Similar results were obtained with HSP molecules from *Escherichia coli*. This may suggest that the adjuvant-free helper effect observed applies not only to mycobacterial HSP, but also to HSP from other prokaryotes. These findings suggest that microbial hsp70 could be considered for the design of conjugated vaccine constructs for eventual human use.
Key words. Heat shock proteins; mycobacteria; vaccines.

Synthetic vaccines: the need to enhance their immunogenicity

Much research is currently being devoted to the improvement of existing vaccines and to the development of new vaccines. Synthetic peptides and recombinant proteins are now considered as promising vaccine candidates for several viral, bacterial, and parasitic diseases. However, although synthetic peptides offer several advantages over classical approaches to vaccine development (e.g. attenuation or inactivation of the pathogens), such as low cost, easy production, and better definition of the vaccine composition, they encounter a major limitation in their poor immunogenicity. In fact, in many instances synthetic peptides may lack the structural elements required for their binding to major histocompatibility complex (MHC) molecules, which is necessary for the initiation of immune responses triggered by specific T lymphocytes. This is also the case for other B-cell epitopes, such as polysaccharide antigens. The problem of the MHC-controlled T cell recognition has often been overcome by chemically linking these poorly immunogenic epitopes to carrier molecules, which provide the constructs with the T-cell epitopes necessary for specific recognition by T cells, and for subsequent optimal priming of B cells. The use of carrier molecules conjugated either to synthetic peptides or to oligosaccharides is now actively being pursued in the development of vaccines against several infectious diseases. Some of these constructs are at a very advanced stage of development, and a few of them are becoming commercially available[6].

However, the use of carrier molecules only partially solves the problems related to the degree of immunity provided by a particular vaccine construct. In fact, most of these constructs require strong adjuvants in order to confer protection in experimental animal models or in humans. For several decades, aluminium salts have remained the only adjuvants admitted for human use. Despite their wide use and acceptance, the use of aluminium salts entails a series of problems (unpredictability of adsorption, no lyophilisation, associated pathology, etc.)[13], which make it urgent to find new or improved vaccine adjuvants better suited for use in humans[6].

Mycobacterial antigens as carrier molecules

Besides the use of Bacillus Calmette-Guérin (BCG, attenuated *Mycobacterium tuberculosis* var. *bovis*, widely used as a vaccine against tuberculosis) to enhance non-specifically the immune response to co-injected antigens[10], Lachmann and co-workers originally reported that the need for adjuvants for the induction of a strong antibody response to selected B-cell epitopes was overcome in mice that were first sensitised with BCG, and then immunised with the B-cell epitope conjugated with the purified protein derivative PPD (currently used in humans for diagnostic skin testing of previous contacts with *M. tuberculosis* or vaccination with BCG)[19,20].

In our laboratory we had previously shown that only mice with the MHC H-2^b haplotype were able to mount a specific T- and B-cell response to the synthetic

polypeptide $(NANP)_{40}$, which reproduces the entire repetitive region of the *Plasmodium falciparum* circumsporozoite (CS) protein[7, 38], and envisaged as a potential vaccine against malaria[35]. All other murine MHC haplotypes tested were consistently unresponsive. Antibody response to the $(NANP)_{40}$ synthetic peptide could be induced in all the mouse strains, irrespective of their H-2 haplotypes, when mice were immunised with the peptide conjugated to a carrier molecule, e.g. KLH (ref. 7). However, both peptide alone and conjugates required strong (Freund's) adjuvants for the induction of detectable antibody responses in immunised mice.

The model of immunisation using PPD as a carrier molecule was then used to enhance the immunogenicity of synthetic malaria peptides. When mice of various H-2 haplotypes were sensitised with BCG and subsequently immunised in the absence of adjuvants with the $(NANP)_{40}$ malaria peptide conjugated to PPD, anti-peptide specific IgG antibodies were found at titres comparable to those observed in groups of mice immunised with the same conjugate in Freund's adjuvant. This antibody response lasted for at least 20 weeks and was boosted by a re-injection of the conjugate in the absence of adjuvant. Interestingly, the anti-$(NANP)_{40}$ antibodies raised with this model of immunisation were functionally active, since they inhibited the penetration and development of *P. falciparum* sporozoites in primary cultures of liver cells[26].

More recently, the same model of immunisation has been proved to be successful in the New World non-human primates, *Saimiri sciureus* (squirrel monkeys), which were first primed with BCG and then immunised subcutaneously with the PPD-$(NANP)_{40}$ conjugate in the absence of adjuvants[32]. It is worthy of note that both in mice and in monkeys the adjuvant-free carrier effect of PPD for the induction of antibody responses to the conjugated malaria peptide was observed only when animals had previously been primed with BCG.

Hsp65 as carrier molecule for conjugated B-cell epitopes

PPD consists of several mycobacterial antigens which are very poorly characterised[20]. However, some observations made in this model of immunisation contributed to the definition of the antigens present in the PPD which were mediating the helper effect observed in vivo. The PPD in the conjugate could not be replaced by non-mycobacterial antigens. The in vivo adjuvant-free helper effect of PPD for the induction of anti-(NANP) IgG antibodies was only observed when the mice were sensitised with live BCG, but not when they received heat-killed or sonicated BCG. Surprisingly, the adjuvant-free helper effect of PPD was also observed in groups of mice previously primed with live *Salmonella typhimurium* or *Leishmania tropica*, which are intracellular parasites of the macrophages[25].

These findings strongly suggested that mycobacterial heat shock proteins (HSP) would be likely to mediate the in vivo helper effect observed with PPD-based conjugates in the absence of adjuvants. In fact, epitopes of hsp65 were known to be present in the PPD[36] and the expression of microbial HSP is known to increase after uptake to live microorganisms by macrophages[5]. HSP are well conserved throughout phylogeny[23].

Using conjugates consisting of purified recombinant *M. bovis* hsp65 and the $(NANP)_{40}$ malaria synthetic peptide to immunise mice in the absence of adjuvants, it was found that anti-(NANP) IgG antibodies could be induced in mice previously primed with live BCG, at titres similar to those obtained in groups of mice receiving PPD-based conjugates[25]. It is noteworthy that no epitope-specific suppression was observed in mice which had high titres of anti-hsp65 antibodies at the time of immunisation with the conjugate, unlike mice receiving tetanus toxoid-peptide conjugates and previously immunised with tetanus toxoid alone[2]. The in vivo adjuvant-free helper effect of the mycobacterial hsp65 was also observed in *Saimiri sciureus* monkeys immunised subcutaneously with hsp65-$(NANP)_{40}$ conjugates without adjuvants[32]. The helper effect of the hsp65 was also exerted for the induction of IgG antibodies to the oligosaccharide of group C *Neisseria meningitidis* after immunisation of mice in the absence of adjuvants[2].

It is remarkable that in all cases, both in mice and in monkeys, the adjuvant-free helper effect of the *M. bovis* hsp65 was detectable only in animals previously sensitised with live BCG[2, 25, 32], in agreement with what was previously found after immunisation with conjugates containing PPD[26].

More recent data from our laboratory have shown that this effect is not peculiar to the mycobacterial hsp65, but is shared by proteins from the same HSP family. In fact, a similar in vivo helper effect in the absence of adjuvants has been observed after immunisation of mice with conjugates containing the purified GroEL hsp of *Escherichia coli*, which is highly homologous with the *M. bovis* hsp65 (ref. 15). Such a helper effect was, again, only observed in mice previously sensitised with live BCG (ref. 2a). These findings suggest that the adjuvant-free carrier behaviour of *M. bovis* hsp65 and *E. coli* GroEL protein observed after priming with BCG may be a characteristic of GroEL-like molecules, also those from other microorganisms.

Preliminary data have shown that injection of mice with BCG induces hsp65-specific IFN-γ-producing T cell precursors at a high frequency, which increases following immunisation with hsp65-based conjugates. This would suggest that priming of mice with live BCG (or immunisation with hsp65 alone[2]) activates hsp65-specific T cells, which undergo clonal expansion following immunisations with conjugates containing this HSP, and provide the help to B lymphocytes for their differentia-

tion and production of antibodies for the conjugated peptides or oligosaccharides. This hypothesis would be in line with observations made by others on the high frequency of T cell precursors specific for the mycobacterial hsp65[17,18], and on the high frequency of T-cell clones, obtained from subjects vaccinated with BCG or with a *M. leprae* vaccine, that recognise the hsp65 in the context of most of the HLA-DR molecules[31].

Hsp70: a carrier molecule with built-in adjuvanticity

Further investigations have shown that the adjuvant-free helper effect observed in vivo is not peculiar of the *M. bovis* hsp65. In fact, mice also mounted a strong anti-(NANP) IgG antibody response when previously primed with BCG and then immunised, in the absence of adjuvants, with conjugates consisting of purified recombinant *M. tuberculosis* hsp70[28] and the malaria synthetic peptide $(NANP)_{40}$ (ref. 25). A similar carrier effect was observed when mice were immunised with hsp70 conjugates containing the oligosaccharide of group C *N. meningitidis*[2]. However, it was surprising to observe that, unlike the mice receiving the hsp65-based conjugates, those immunised with hsp70-$(NANP)_{40}$ or with the hsp70-oligosaccharide conjugates did not require a previous priming with BCG in order to mount an IgG antibody response to the peptide or the oligosaccharide following immunisation with the conjugates in the absence of adjuvants[2] (fig.). Interestingly, a strong anti-peptide IgG antibody response was also obtained in *Saimiri sciureus* monkeys immunised subcutaneously with hsp70-$(NANP)_{40}$ conjugate in the absence of adjuvant, irrespective of a previous sensitisation of the monkeys with BCG[32].

As in the case of GroEL-type HSP, in this model of immunisation also the mycobacterial hsp70 could be replaced in the conjugates by the purified, highly homologous *E. coli* DnaK protein[12], which induced a strong antibody response to the $(NANP)_{40}$ peptide conjugated to it, in the absence of adjuvants and of previous priming of the animals with BCG (ref. 2a). Data discussed so far support the concept that an adjuvant-free helper effect can be mediated by GroEL-type and DnaK-type HSP from different prokaryotic microorganisms. However, it does not appear that all mycobacterial HSP can mediate this in vivo helper effect: in fact, it was not observed in mice immunised with conjugates consisting of the *M. leprae* 18 kDa hsp (ref. 21) and the $(NANP)_{40}$ peptide[25] without adjuvant.

The fact that the helper effect of the *M. tuberculosis* hsp70 and *E. coli* DnaK proteins did not require any adjuvants to be co-injected along with the conjugate, nor a previous sensitisation of the animals with live BCG, strongly suggests that the mechanisms behind this helper effect of the hsp70 differs from those responsible for the in vivo helper effect exerted by the hsp65.

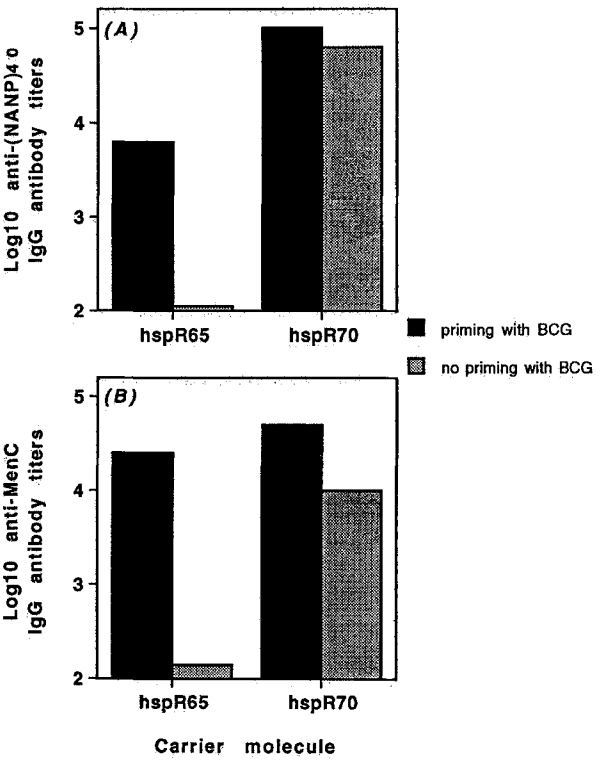

Figure. Adjuvant-free carrier effect of mycobacterial recombinant heat shock proteins of 65 and 70 kDa. On day 0 groups of BALB/c mice received 10^6 colony forming units of BCG in PBS i.p. Control mice only received PBS. 14 and 35 days later mice were immunized i.p. in the absence of adjuvants with recombinant 65 kDa hsp (hspR65) or 70 kDa hsp (hspR70) conjugated to the synthetic peptide $(NANP)_{40}$ from *Plasmodium falciparum* (*A*) or with the capsular oligosaccharide of group C *Neisseria meningitidis* (MenC) (*B*). One week after the last immunization, anti-$(NANP)_{40}$ and anti-MenC IgG antibodies were titered by ELISA, using the $(NANP)_{40}$ peptide or MenC as solid phase, respectively.

The simplest explanation for the in vivo helper effect of hsp70 in the absence of previous priming by BCG would be a non-specific stimulation of B lymphocytes through bacterial LPS, possibly contaminating the preparation of the recombinant protein. However, three lines of evidence obtained in vivo do not support this hypothesis: 1) the anti-peptide or anti-oligosaccharide IgG antibody titres induced in this manner, both in mice and monkeys, were boosted upon re-immunisation with the hsp70-based conjugates without adjuvants[2,25,32]; 2) the hsp70-mediated in vivo helper effect for the induction of antibody responses to conjugated antigens was not observed in athymic *nu/nu* mice, but it was strong in C3H/HeJ mice, which are genetically resistant to LPS (ref. 2); 3) the helper effect of hsp70 was also observed in *Saimiri* monkeys[32], whose B lymphocytes do not respond to LPS (ref. 11).

These findings, instead, strongly suggest that the helper effect provided by the mycobacterial hsp70 depends on the presence of an intact T-cell compartment, specifically stimulated after immunisation with hsp70-based

conjugates in the absence of adjuvants. This is in line with the observation that the in vivo helper effect of the hsp70 is only observed when the hsp70 and the antigen are covalently cross-linked together (ref. 2a), a requirement met in classical hapten-carrier systems where the activation of carrier-specific T cells is a prerequisite for the activation and differentiation of hapten-specific B cells[22].

One could hypothesise that the adjuvant-free carrier effect of the hsp70 in the absence of previous priming with BCG may be mediated by a 'natural' priming of hsp70-specific T cells, for example through HSP derived from the saprophytic intestinal flora. Experiments are now in progress in germ-free mice in order to test this hypothesis. The recent finding that murine intestinal intraepithelial T lymphocytes respond to the mycobacterial hsp70, but not to the hsp65 (ref. 3), would be in agreement with this hypothesis. At present it is not possible to completely rule out that the peculiar in vivo helper effect of the hsp70 might be exerted through its function as a molecular chaperone[14]. However, recent data from our laboratory do not seem to support this hypothesis.

HSP in conjugated vaccine constructs and autoimmunity?

HSP are molecules which have been well conserved during evolution[23]; however, despite this, prokaryotic HSP are very strong immunogens[17,18]. These observations, together with others on T cells and antibodies against the mycobacterial hsp65 cross-reacting with the human homologue, have led several authors to suggest a potential role for microbial HSP (and especially for the hycobacterial hsp65) in the induction of autoimmune phenomena[9,34].

It would, then, be logical to raise the question of whether or not the use of microbial HSP in conjugated vaccine constructs would be able to trigger immune responses cross-reacting with self-HSP, or with other self proteins sharing some limited homologies with microbial HSP[16].

Some data from our laboratory, although limited to the antibody responses triggered by hsp65-based conjugates given in the absence of adjuvants after previous priming with BCG, do not appear to be in support of the induction of autoimmune phenomena following this model of immunisation. For example, we have found (ref. 2b) that anti-mycobacterial hsp65 antibodies induced after immunisation with conjugates without adjuvants cross-reacted with the *E. coli* GroEL protein, but not with the recombinant human hsp60, nor with the 60 kDa hsp expressed by murine cells, which is virtually identical to its human counterpart[39]. Furthermore, it is known that priming to HSP occurs after a wide variety of infections[27,40], as well as after vaccination with vaccines consisting of inactivated whole-cell microorganisms, such as BCG or *M. leprae* vaccine[31]. We have shown that a similar priming to microbial HSP takes place in healthy 4 month-old infants following vaccination with DTP vaccine against diphtheria, tetanus, and pertussis. This vaccine consists of diphtheria and tetanus toxoids, and of inactivated whole-cell *Bordetella pertussis*. In fact, anti-hsp65 and hsp70 IgG antibodies were detectable in almost 90% of infants vaccinated with DTP[8]. Anti-HSP antibodies in these infants were induced by the whole-cell pertussis vaccine present in the DTP, since they were not found in age-matched infants vaccinated with DT plus an acellular recombinant pertussis vaccine[33]. Interestingly, the anti-hsp65 antibodies induced by the whole-cell pertussis vaccine cross-reacted with the *E. coli* GroEL protein, but not with the human hsp60 homologue[8].

Data discussed above suggest, then, that priming of the immune system to microbial HSP can be considered as a common phenomenon taking place in healthy individuals, even very early in life. It thus becomes questionable whether such a priming could contribute to the induction of autoimmune disorders. This consideration should also be taken into account with respect to the dual role reported for the mycobacterial HSP, and for the hsp65 in particular, on the one side in the pathogenesis and on the other side in the prevention of autoimmunity in animal models. It is in fact known that immunisation of mice and rats with the *M. bovis* hsp65 (or with defined synthetic peptides from it) prevents the appearance of autoimmune disorders, such as arthritis or diabetes mellitus[9]. Importantly, this beneficial effect has also been reported for the human hsp60 expressed in vaccinia virus, despite the antibody and T-cell responses induced against this hsp in immunised rats[24].

Conclusions

The use of microbial HSP as carrier molecules for the triggering of immune responses to conjugated antigens may offer several advantages. In fact, high and long-lasting titres of specific and functional antibodies are induced in primates after subcutaneous immunisation with HSP-based conjugates in the absence of adjuvants[32]. Some elements of this model of immunisation (e.g. HSP-antigen conjugation via gluataraldehyde, sensitisation with BCG) are already commonly used. Moreover, hsp70-based conjugates do not even require any previous priming with BCG. Several T-cell epitopes have been identified on the mycobacterial hsp65 (ref. 4) and hsp70 (ref. 1), both in mice and in humans, where they have been shown to associate with a wide variety of HLA-DR molecules[31]. Finally, the presence of HSP-specific T cell populations in healthy individuals[29,30] may speak in favour of the use of HSP-based vaccine constructs in a wide proportion of individuals, besides those individuals already sensitised to mycobacteria

through natural contact with *M. tuberculosis* or through vaccination with BCG.

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* Address for correspondence.

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