A synthetic 10-kD heat shock protein (hsp10) from Mycobacterium tuberculosis modulates adjuvant arthritis

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(Accepted for publication 28 November 1995)

SUMMARY

The heat shock protein, hsp10, is an abundant protein in Mycobacterium tuberculosis (Mtb), its nucleotide sequence encoding a protein of 99 amino acids with a molecular mass of 10.7 kD. This sequence is phylogenetically conserved, being represented by the GroES homologue of Escherichia coli. Hsp10 and GroES are members of the chaperonin 10 family of molecular chaperones, and GroES is necessary for the optimal activity of GroEL, a member of the chaperonin 60 family and the E. coli homologue of mycobacterial hsp65. Since hsp65 has been implicated in both experimental and human rheumatoid arthritis, we aimed to assess the immunomodulatory effects of its co-chaperonin, hsp10, in experimental arthritis. Our results show that an aqueous solution of a mycobacterial hsp10 delayed the onset and severity of adjuvant-induced arthritis in rodents when administered after disease induction but before joint involvement occurred. This biological activity was specific for the hsp10 of Mtb, since neither GroES nor the rat homologue was effective. Using synthetic hsp10 fragments, the activity was localized to the N-terminal region of the molecule. Assessment of circulating antibody levels to mycobacterial hsp10 and hsp65 indicated that all arthritic rats had increased titres to both hsp10 and hsp65: hsp10-treated rats showed further elevation of this humoral response not only to hsp10 but also to hsp65 when compared with the untreated arthritic control. This is the first report of the immunomodulatory activity of mycobacterial hsp10 in experimental arthritis, and exhibits a potential role for this co-chaperonin in pathophysiological situations.

Keywords experimental arthritis heat shock protein(s) chaperonin 10 immunoregulation early pregnancy factor

INTRODUCTION

Heat shock (stress) proteins (hsp) are ubiquitous throughout nature, essential to life and are induced by a number of stressors, including inflammatory mediators; they are classified into families according to molecular weight [1]. Since stress protein genes are highly conserved across many genera, the ability of immune hosts to recognize their protein products (self) as antigens raises important questions about the regulation of the immune response, autoimmunity occurring due to failure of these discriminatory mechanisms [2]. In particular, a possible link between hsp and rheumatoid arthritis (RA) [3–7] and hss and experimental arthritis [8–10] has been reviewed by many authors.

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Hsp 10-kD (hsp10) is an abundant protein in Mycobacterium tuberculosis (Mtb), its nucleotide sequence encoding a protein of 99 amino acids with a molecular mass of 10.7 kD [11]. Widely conserved among different organisms, sequence homologies have been defined among Mycobacteria [12] and enteric bacteria [13]. More recently, a rat hsp10 was identified [14], and the human homologue has been cloned [15].

Functionally, hsp10 is a chaperonin and co-operates with hsp65 to achieve optimal protein folding and assembly [16]. Since hsp65 is also highly immunogenic, several investigators have addressed the question of immunoreactivity elicited by the 10-kD hsp. Immunization of guinea pigs with whole Mtb sonicates followed by challenge with hsp10 induced DTH [17], and the 10-kD antigen was later shown to be an important component of the cell wall protein–peptidoglycan complex of Mtb, towards which Mtb-specific T cell lines proliferate [18]. Antibodies reacting with this 10-kD antigen have been detected in 35–50% of tuberculosis patients, but not in healthy subjects [19–21]. Recently, Barnes et al. [22] demonstrated that the
response induced by the 10-kD antigen of Mtb in peripheral blood mononuclear cells (PBMC) of healthy bacille Calmette–Guerin (BCG)-positive individuals was similar in magnitude to that elicited by whole Mtb, but greater than that induced by other proteins isolated from an Mtb culture filtrate, suggesting that this antigen is immunodominant. Of particular interest here, the early pregnancy factor (EPF), a known immunomodulatory molecule [23–25], was sequenced and found to share 99% identity with mammalian (rat) hsp10 [26]. This factor has been shown to alter lymphocyte reactivity \textit{in vitro} in the rosette inhibition test and \textit{in vivo} in the adoptive transfer of contact sensitivity [23–25].

Thus, it appears that hsp10 shows immunodominance, elicits T and B cell immune responses, and may have immunomodulatory activity. Previously, using immunohistology, we have demonstrated enhanced expression of hsp10 and hsp65 in human arthritic synovial membrane [27]. Since, \textit{in vitro}, hsp10 functions co-operatively with hsp65 [16], we studied the immunogenicity of a chemically synthesized hspl0 protein from \textit{Mycobacterium tuberculosis} and its effect on adjuvant-induced arthritis (AA) in rats.

**MATERIALS AND METHODS**

**Animals**

Wistar rats were obtained from Charles River (Margate, UK) and Lewis rats from Harlan Olac (Bicester, UK). The rats were fed a standard diet and tap water. Rats weighed ±150 g at the start of the experiment. Young female rats were chosen because they are more susceptible to arthritis induction.

**Antigens**

Recombinant hsp65 (rhs65) of \textit{Mycobacterium leprae} (gift of Dr M. J. Colston, The National Institute for Medical Research, London, UK) [28], \textit{E. coli} GroES (Boehringer Mannheim, Lewes, UK), synthetic p24 [29], synthetic rat hspl0, synthetic hsp10 from Mtb (hspl0) and chemically synthesized fragments spanning residues 1–25, 1–58, 25–99, 51–99, and 75–99 of hspl0, were used. All synthetic proteins/peptides were purified to homogeneity and their chemical integrity was confirmed by amino acid analysis, microsequencing and mass spectrometry [30].

**Protocol for induction of AA**

Heat-killed human strains C, DT and PN of \textit{Mycobacterium tuberculosis} (Central Veterinary Laboratory, Weybridge, UK) were finely ground in a pestle and mortar and suspended in light paraffin oil to a final concentration of 10 mg/ml. Rats were inoculated intradermally at the base of the tail with a total of 100 μl of the suspension [31]. The day of arthritis induction was designated as day 0 and disease severity evaluated using the following standard clinical scoring system [31], adapted from the work of Currey & Ziff [32]: 0, no inflammation; 1, slight redness and swelling of the foot; 2, swelling of the foot such that the tendons are no longer visible; 3, swelling extending to the ankle joint; 4, gross inflammation and deformity of the ankle joint; the tail was scored 0 or 1 according to the absence or presence of cutaneous nodules; ears were scored 0 or 1 according to the absence or presence of subcutaneous haemorrhages. Scores were summed, giving a potential maximum of 18 for each animal. Animals were killed after 28 days (earlier if adverse reactions ensued or protection was minimal) and exsanguinated. Sera were stored at −20°C.

**Treatment with hspl0 after AA induction**

On day 0, AA was induced in Lewis and Wistar rats. On days 4, 5 and 6 groups of five to six animals were injected with hspl0 or related fragments, each receiving 50 μg of protein/peptide in 100 μl PBS intradermally at the base of the tail. A control group received PBS alone on days 4, 5 and 6, while the absolute control was not treated and received Mtb only on day 0. To assess the specificity of the hspl0 response similar experiments were carried out on Lewis rats using rhs65, \textit{E. coli} GroES, or a chemically synthesized rat hspl0 in place of the mycobacterial hspl0.

**Prolonged treatment with synthetic hspl0**

On day 0, AA was induced in Lewis rats. The rats were treated with a dose of 50 μg of hspl0 in 100 μl PBS on days 0, 2, 4, 6, 8, 10, 12 and 14. Control groups received 100 μl PBS only (PBS control) or no injection (absolute control).

**ELISA for antibodies to hspl0 and hspl0**

Serum samples were thawed immediately before use, and circulating antibodies to recombinant \textit{Mycobacterium leprae} hspl0 (rhhsp65 antibodies) and to synthetic mycobacterial hspl0 (rhhsp65 antibodies) were measured by conventional ELISA as previously described [33]. Briefly, microtitre plates were coated with rhp65 or synthetic hspl0 in carbonate buffer pH 9.6. Plates were blocked with 1% bovine serum albumin (BSA) to minimize non-specific reactions and test sera diluted accordingly: 1:25–1:200 for hspl0 antibodies and 1:400–1:51 200 for hspl0 antibodies. Peroxidase-conjugated rabbit anti-rat immunoglobulin (Dako Ltd, High Wycombe, UK) was added and the plates were developed by the addition of ortho-phenylene diamine supplemented with hydrogen peroxide. Absorbance was monitored at a wavelength of 492 nm.

All sera were assayed in duplicate and a reference serum was included in duplicate on each plate. Since OD_{492} readings can vary greatly between assays, the use of a reference serum (OD_{492} control) allows a more accurate comparison. The reference serum was from a pool of eight naive rats, and OD_{492} readings (± s.e.m.) varied as follows: 0.027 (± 0.003); it was also stored in aliquots with the test sera at −20°C. Interassay and intra-assay variabilities were always < 10%.

Results were calculated as the ratio (OD_{492} sample/OD_{492} control) using serum dilutions of 1:50 for rhs65 antibodies and 1:12 800 for hspl0 antibodies. The dilutions were chosen so that the OD_{492} levels lay on the linear portion of the curve.

**Statistical analysis**

Statistical comparisons for clinical scores and antibody levels were made using the Mann–Whitney U-test with Minitab data analysis software (Minitab Inc, Coventry, UK).

**RESULTS**

**Treatment with hspl0 in PBS after AA induction**

Figures 1a,b show the effect of i.d. administration of hspl0 in, respectively, Wistar and Lewis rats on days 4, 5 and 6 after AA induction. In Wistar rats (Fig. 1a), disease severity was
significantly decreased versus both control groups ($P < 0.01$ versus PBS and absolute (Abs) controls on days 9, 10, 12, 25, 28; $P < 0.05$ versus PBS and Abs controls on days 17, 20, 23). Lewis rats (Fig. 1b) experienced a more modest, yet still significant protective effect ($P < 0.01$ versus Abs control and $P < 0.02$ versus PBS control on days 9–14; $P < 0.02$ on days 17 and 18 and $P < 0.05$ on days 7 and 19 versus Abs control).

This experiment was repeated, with analogous results, in six separate experiments using different batches of hsp10, but, due to restrictions in the amount of synthetic compound available in each batch, a full dose response was not ascertained. Therefore, the results presented in Figs 1a,b do not necessarily represent optimal modulation. In Lewis rats, the pattern of response was very reproducible: on day 10, there was $\approx 80\%$ reduction in clinical scores, but this was not sustained. By day 12, only a 60% reduction in severity was observed, and, by days 18, 19 and 20, animals showed only a 15–20% benefit (e.g. in Fig. 1b, on day 20, mean clinical scores for hsp10 treatment were 12-2 while the untreated Abs control group showed a mean score of 16-3; this equates to a 25% improvement).

All further investigations on the characterization of this protective response were carried out using Lewis rats. The reasons for this choice are discussed below.

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**Fig. 1.** Time course of adjuvant arthritis (AA) induced with *Mycobacterium tuberculosis* in oil on day 0 in Wistar rats (a) and Lewis rats (b). The animals were treated intradermally on days 4, 5 and 6 (arrowed) after AA induction with either (a) 50 μg hsp10 in PBS (○; means ± s.e.m.), or (b) PBS (■; means ± s.e.m.), or (c) left untreated (absolute control) (□; means ± s.e.m.). Groups were of six rats. Assessment of severity was made by clinical scoring. (a) Levels of significance compared with the absolute control and PBS control were *$P < 0.01$ and **$P < 0.05$, respectively (Mann–Whitney U-test). (b) Levels of significance compared with the absolute control were *$P < 0.01$; **$P < 0.02$; ***$P < 0.05$. Level of significance compared with the PBS control was †$P < 0.02$.

**Fig. 2.** Circulating antibody levels to hsp10 (a) and rhsp65 (b) in hsp10-treated and untreated Lewis rats with adjuvant arthritis (AA). Sera were obtained from Lewis rats at the end of the experiment (day 22) and the results at serum dilutions of 1:12 800 for hsp10 antibodies (a) and 1:50 for rhsp65 antibodies (b) are presented. Groups were of 9–11 rats. The bar represents the median level. Hsp10-treated rats had significantly elevated titres of hsp10 antibodies ($P < 0.01$ versus the absolute control and $P < 0.02$ versus the PBS control) and of hsp65 antibodies ($P < 0.05$ versus PBS control).

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Antibodies to rhsp65 and hspl0
To assess the humoral immune response in hspl0-protected Lewis rats, we measured the antibody level to rhsp65 and hspl0. Figure 2a shows the antibody titre to hspl0. The hspl0-treated group showed a significantly elevated antibody level (median 31.2) compared with the Abs control (median 2.2, \( P < 0.01 \)) and the PBS control (median 11.67, \( P < 0.02 \)) groups. Similarly (Fig. 2b), the rhsp65 antibody level in the hspl0-treated group was significantly elevated (median 4.3) in comparison with a PBS control (median 2.2, \( P < 0.05 \)) and approached significance towards the absolute control (median 1.97, \( P < 0.08 \)).

Localization of the biological activity to fragment 1–25
Peptide fragments spanning the whole molecule of hspl0 were assessed to localize the biological activity of hspl0; groups of Lewis rats received 50 \( \mu \)g peptide in 100 \( \mu l \) PBS on days 4, 5 and 6, as described. As previously stated, optimal activity was not determined. In Table 1, two separate experiments (a and b) are reported; only fragment 1–25 showed any significant protective effect. There was a significant delay of the onset at day 11 (\( P < 0.02 \) versus the articular control), but later (day 20) a tendency to reach high clinical scores. Figure 3 compares the kinetics of the disease course using peptide fragment 1–25 and the whole synthetic mycobacterial hspl0. By day 19, hspl0 treatment produced a 21% reduction in clinical score, while fragment 1–25 had ceased to be effective.

Fragment 1–58 was similar in effect, showing an early, although statistically non-significant, effect on disease severity, but later clinical scores were similar to the control groups. Fragments 25–99, 51–99 and 75–99 had no effect on disease kinetics when administered on days 4, 5 and 6 after AA induction.

Specificity of the mycobacterial hspl0 modulation
To assess specificity and to control for any artefacts in synthesis, an irrelevant peptide, p24, was synthesized. This peptide corresponds to the 104 amino acids of the C-terminus of HIV virus [29] and is therefore of equivalent length to hspl0 but structurally unrelated. As Table 2a shows, p24 was ineffective when tested under the same experimental conditions. Neither rhsp65 (Table 2b) nor the rat hspl0 (Table 2c) could mimic the biological activity of mycobacterial hspl0. GroES, the E. coli homologue of hspl0 (Table 2d), showed a slight but statistically insignificant activity which was lost immediately following onset of disease.

Table 1. Effect of treatment with hspl0 fragments on days 4, 5 and 6 after adjuvant arthritis (AA) induction

| Treatment group | Day 11 (onset) \( \pm \) s.e.m. | Day 20 \( \pm \) s.e.m. |
|-----------------|---------------------------------|------------------------|
| a. 1–25         | 3.8 \pm 0.4*                    | 17.0 \pm 0.6           |
| 1–58            | 5.8 \pm 1.2                     | 16.0 \pm 0.7           |
| Arthritic control | 9.4 \pm 0.7                    | 17.2 \pm 0.41          |
| b. 25–99        | 11.7 \pm 1.5                    | 17.0 \pm 0.4           |
| 51–99           | 11.8 \pm 2.0                    | 16.0 \pm 0.7           |
| 75–99           | 9.1 \pm 1.7                     | 14.7 \pm 1.6           |
| Arthritic control | 9.2 \pm 1.6                    | 15.4 \pm 1.3           |

Groups of five to six animals were injected with hspl0 fragments (50 \( \mu g/rat \)) in PBS. Clinical scores (mean \( \pm \) s.e.m.) relative to the day of onset (day 11) and at the end of experiment (day 20) are reported.

a. and b. represent two separate experiments.

* \( P < 0.02 \) versus articular control.

Table 2. Effect of treatment with control antigens on days 4, 5 and 6 after adjuvant arthritis (AA) induction

| Treatment group | Day 11 (onset) \( \pm \) s.e.m. | Day 20 \( \pm \) s.e.m. |
|-----------------|---------------------------------|------------------------|
| a. p24          | 5.3 \pm 1.1                     | 14.9 \pm 1.6           |
| Arthritic control | 4.3 \pm 1.1                    | 15.4 \pm 1.3           |
| b. rsps65       | 4.0 \pm 1.4                     | 14.2 \pm 2.1           |
| Arthritic control | 4.6 \pm 2.2                    | 11.4 \pm 2.4           |
| c. Rat hspl0    | 10.2 \pm 1.4                    | 17.6 \pm 0.3           |
| Arthritic control | 9.4 \pm 0.7                    | 17.2 \pm 0.4           |
| d. GroES        | 6.7 \pm 1.0                     | 15.2 \pm 1.0           |
| Arthritic control | 11.3 \pm 1.7                   | 17.0 \pm 0.7           |

Groups of five to six animals were injected with p24, rsps65, rat hspl0 or GroES (50 \( \mu g/rat \)) in PBS. Clinical scores (mean \( \pm \) s.e.m.) relative to the day of onset (day 11) and at the end of experiment (day 20) are reported.

a., b., c. and d. represent four separate experiments.

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erbated arthritis. The mechanism is clearly complex. Since co-operativity between chaperonins has been demonstrated with variable success [35]. The finding that prior injection of 50 μg hsp10 in PBS (C; means ± s.e.m.), or PBS (M; means ± s.e.m.), or left untreated (absolute control) (□; means ± s.e.m.). Groups were of six rats. Assessment of disease severity was made by clinical scoring. Levels of significance compared with the absolute control were *P < 0.01; **P < 0.02. Levels of significance compared with the PBS control were †P < 0.01 (Mann–Whitney U-test).

**DISCUSSION**

AA is recognized as a T cell-mediated disease, since early therapeutic intervention with T cell antisera (e.g. anti-CD4) abrogates disease induction [32,34]; similar immunomodulation of human RA has been attempted using T cell MoAbs, with variable success [35]. The finding that prior injection of mycobacterial hsp65 (chaperonin 60) protected Lewis rats against AA induction [36] fuelled speculation of a cure for arthritis. However, Hogervorst et al. [37] demonstrated that administration of hsp65 following disease induction, exacerbated arthritis. The mechanism is clearly complex. Since co-operativity between chaperonins has been demonstrated [16], we examined the effect of mycobacterial hsp10 (chaperonin 10) on the early response to induction of adjuvant disease.

In this study, we have described a new biological effect of a chemically synthesized chaperonin 10 protein, the mycobacterial 10-kD heat shock protein, hsp10. Although hsp10 appeared to have a more pronounced effect on AA in Wistar rats (Fig. 1a), we chose to examine AA in Lewis rats, as much of the published work with regard to hsp protection uses this model [8–10]. Moreover, the Lewis rat may be more indicative of the clinical situation, since differences in the response of Lewis rats may be attributable to the known impairment in their hypothalamo–pituitary–adrenal axis [38], a condition also noted in patients with RA [39].

Modulation of AA was specific to the mycobacterial form, since GroES (the E. coli homologue) and the rat homologue did not elicit protection in our system. Inactivity of the rat (auto-logous) hsp10 is interesting, as rat hsp10 shows almost 100% homology with human EPF [26], an immunomodulatory molecule [23–25]. Patients with RA often show a transient remission during pregnancy [40], perhaps akin to the effect seen here with hsp10.

As our results show, the specificity of the system is exquisite, since there is no observed response to self hsp10 (rat) or GroES (E. coli) while the mycobacterial protein promotes a suppressive response. This accords well with a recent report by Anderton et al. [41] using chaperonin 60 proteins, which showed that, although mycobacterial hsp65 can protect Lewis rats against AA induction, the corresponding self (rat) protein is ineffective. Moreover, they identified a peptide sequence in the N-terminus (residues 256–270) of hsp65, and immunization with this peptide was able to confer protection against AA in Lewis rats: a homologous epitope occurred in rat chaperonin 60. Using T cell lines derived from rats immunized with this chaperonin 60 peptide 256–270 (self or mycobacterial) they inferred that recognition of cross-reactive epitopes on self and bacterial hsp60, having low MHC affinity, might be used to maintain a protective autoreactive memory T cell population, thereby eliminating the requirement for mycobacterial involvement. In our study, only mycobacterial fragments were tested.

An alternative hypothesis, derived following studies using a murine arthritis model, suggests that autoimmune disease arises through stimulation of immunologically ignorant autoreactive T cells [42]. In this model, although arthritis is induced in the absence of Mtby a non-antigenic paraffin oil (pristane), arthritic mice display T and B cell immunity to both mycobacterial and mammalian chaperonin 60, and mice can be protected against arthritis induction by inoculation with hsp65. Examination of the mechanisms involved demonstrated that arthritic mice showed T cell reactivity to multiple epitopes on hsp65 while protected mice had a limited repertoire. Therefore it is possible that, in our experiments, this ‘epitope spreading’ was limited by early treatment with hsp10.

From the humoral aspect we have demonstrated that in Lewis rats with AA, antibody production to hsp10 is significantly elevated, underlining the importance of hsp10 as a relevant antigen in this experimental disease. Furthermore, rats receiving three consecutive injections of hsp10 in an aqueous solvent (PBS) after adjuvant injection (Freund’s complete adjuvant (FCA)) mounted, in comparison with the arthritic controls, a significantly elevated response to hsp10 itself and, interestingly, also to hsp65. Cross-reactivity between chaperonin antibodies is not surprising since the genes are...
highly conserved; however, the degree of cross-reactivity is highly dependent on the nature and site of antigen presentation [43]. Sequence similarities within the *E. coli* chaperonins GroEL (hsp65 homologue) and GroES (hsp10 homologue) are documented [44] and, using the Daresbury computer database, we showed that hsp10 shares 69% homology with the 65-kD (*Myco. leprae*) antigen [33]. Thus, it is not inconceivable that our results might be explained by a sequence or by a stoichiometric cross-reaction. Moreover, as increased hsp65-specific antibody levels are associated with resistance to AA induction in Wistar rats [45], it is attractive to hypothesize that the 10-kD antigen is acting as a modulator of the immune response towards related heat shock (chaperonin) proteins.

Recently, a 12-kD protein of *Myco. tuberculosis* has been ascribed a protective role in a model of experimental encephalomyelitis [46], and Ferrero *et al.* [47] have evidence that a protein belonging to the GroES class is a protective antigen in mucosal infection in mice. Our results provide further support for the potential use of chaperonin 10 proteins in the therapy of autoimmune diseases and infection.

ACKNOWLEDGMENTS

This work was supported by Italfarmaco Research Centre SpA, Milan, Italy, and the Arthritis and Rheumatism Council (VRW/DRC/CJM; Grant No. B0141). Chesterfield, UK. We are most grateful to Dr M. J. Colston (National Institute for Medical Research, London, UK) for kindly providing the recombinant *Myco. leprae* 65-kD heat shock protein, and we wish to thank Professor W. van Eden (University of Utrecht, The Netherlands) for helpful discussions.

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