Arthritogenic potential of the 65 kDa stress protein – an experimental model

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Abstract

Objectives—To assess the effect of an intra-articular presentation of stress (heat shock) proteins (hsp) on joint inflammation.

Methods—Wistar rats were sensitised with a suspension of heat killed *Mycobacterium tuberculosis* in oil in the scruff of the neck and challenged intra-articularly with stress protein or *M tuberculosis* preparations. Inflammation was assessed by joint swelling and, using immunohistology, cellular infiltration of the synovium and antibody induction by an enzyme-linked immunosorbent method.

Results—It was shown, for the first time, that the intra-articular administration of a recombinant mycobacterial 65 kDa hsp can induce joint inflammation in *M tuberculosis* sensitised recipients; both powdered *M tuberculosis* and the purified protein derivative of tuberculin (PPD) produced a similar response, with T cell infiltration of the synovium and a time course typical of delayed type hypersensitivity. This response was specific to the 65 kDa protein as another immunodominant mycobacterial stress protein of 10 kDa was ineffective. Furthermore, intra-articular injection of the 65 kDa hsp induced an antibody response against both the 65 kDa and 10 kDa proteins and the antibody titres continued to rise when knee swelling had subsided.

Conclusions—These results support the hypothesis that 60 kDa proteins are a relevant arthritogenic stimulus in an *M tuberculosis* background. Moreover, when antigen presentation occurs in the synovium of previously sensitised individuals, circulating antibodies are generated which persist and recognise cross-reactive epitopes on several stress proteins.

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In recent years, considerable attention has been given to the role of stress (heat-shock) proteins (hsp) in arthritis, with studies centring on their putative inflammatory action as well as their unquestionable defence activities. Hsp genes are highly conserved between species while their protein products vary slightly in molecular mass and therefore are defined as 'families' (for example, 60 kDa or 70 kDa family).¹

Stress proteins of the 60 kDa and 70 kDa families are detectable in arthritic human synovia^{2,3} and circulating antibodies are elevated,⁴ suggesting that immune responses to them may be involved in the initiation and/or maintenance of arthritis. Support for this contention comes from the pioneering work of Cohen *et al*⁵ who showed that T cell clones, isolated from Lewis rats with adjuvant-induced arthritis (AA) proliferated in response to the 65 kDa protein (60 kDa family) of *Mycobacterium tuberculosis*, a major constituent of Freund's adjuvant. Moreover, adoptive transfer experiments delineated disease-inducing (A2b) and disease-protective (A2c) sub-clones. Circulating T cells, which proliferated in response to the 65 kDa protein, were identified in patients with rheumatoid arthritis (RA) and 'molecular mimicry' proposed as a mechanism leading to T cell-mediated cartilage destruction.⁶ Conversely, administration of the 65 kDa protein before arthritis induction abrogated disease.⁷

In vivo, the 60 kDa proteins function as molecular chaperones. However, many stress proteins act cooperatively and it has been shown that optimal activity of the 60 kDa protein is dependent on the presence of another stress protein, having a molecular mass of 10 kDa in mycobacteria.⁸ Using immunohistology, we have detected increased expression of a 10 kDa homologue in the synovia of patients with arthritis.² Thus in vivo, ongoing synovitis causing cell destruction may lead to the intra-articular release of these highly immunogenic stress proteins. To assess the effects that the presence of these molecules may have in situ on joint inflammation, we injected them into rat joints and showed that intra-articular presentation of a recombinant 65 kDa protein can induce joint swelling in sensitised individuals.

Materials and methods

Wistar rats were obtained from Charles River (Margate, Kent). Females were chosen to provide a direct comparison with the Lewis rat model used for the previous AA studies,⁷ and weighed 150–170 g at the start of the experiment.

Animals were inoculated in the scruff with 100 µl of a suspension of heat-killed *M tuberculosis* in light paraffin oil (10 mg/ml; strains C, DT and PN; Central Veterinary Laboratory, Weybridge, UK). After seven days, groups of six rats were challenged intra-articularly in one knee with 50 µl of either *M*

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tuberculosis or antigen (listed below) in PBS. Three groups of negative controls were included, an uninjected group, a group injected with the vehicle (PBS) alone and a third group receiving the p24 peptide (a synthetic polypeptide corresponding to the 104 amino acids of the C-terminus of HIV and thus of a length similar to the 10 kDa hsp but structurally unrelated). Initially, 1 µg or 10 µg of antigen was used and this preliminary data has been reported in abstract form;⁹ for subsequent experiments, the dose used was 50 µg/rat knee (1 mg/ml). Knee swelling, used as an index of joint inflammation, was the difference in width, measured with a

micrometer, between the injected and uninjected joints. Measurements were made at two and four hours and days 1, 2, 3, 4, 7 and 8 after injection. At day 3, half of the animals were killed and exsanguinated.

Recombinant mycobacterial 65 kDa protein (r65) from *M leprae* was prepared as originally described.¹⁰ The LPS content was assessed using a *Limulus* assay (E-TOXATE; Sigma, Poole, UK). Additionally, purification of r65 (pur65) was carried out using a Sephacryl S-100 HR (Pharmacia, Uppsala, Sweden) column (370 mm × 16 mm) to remove low molecular weight contaminants and LPS. Using known sequence data, the mycobacterial 10 kDa polypeptide and the 104-mer from HIV p24 were chemically synthesised using a combination of solid phase peptide synthesis and the t-BOC chemistry protocol.¹¹ The synthetic proteins were purified to homogeneity and their chemical integrity confirmed by amino acid analysis, microsequencing and mass spectrometry. Mtb (positive control) was prepared as above and suspended in PBS; PPD (Central Veterinary Laboratory, Weybridge, Surrey; strain 298) and *E coli* LPS (Sigma; serotype 0:55:B5) were also administered in PBS.

Serum samples were coded and stored at -20°C until use. Circulating antibodies were measured using a standard enzyme-linked immunosorbent assay (ELISA) with blocking, essentially as described for human sera¹² and using the same preparation of r65 or 10 kDa protein used for injection. All sera were assayed in duplicate. In addition, a reference serum (pooled normal Wistar rat serum) was included in duplicate or triplicate on each plate. Results were calculated as the ratio [(OD⁴⁹² sample ÷ OD⁴⁹² reference) × 100] using a dilution of 1/100 since this dilution lay on the linear part of the curve. Inter- and intra-assay variability were 10% and 5% respectively.

Using commercially available antibodies (Serotec, Kidlington, Oxford), synovial sections were assessed immunohistologically by a glucose oxidase enhanced technique for the presence of the αβ T cell receptor (R7·3; MCA 453) and resident (ED2; MCA 342) or recruited (ED1; MCA 341) macrophages.

Since the data obtained were parametric, comparison of knee joint differences or antibody levels were made using a paired *t* test.

Figure 1 Effect of an intra-articular presentation of r65 in *M tuberculosis* sensitised animals. Figure 1A shows the time course of joint swelling following intra-articular injection of r65 into *M tuberculosis* sensitised Wistar rats and fig 1B, their serum antibody response. Swelling was induced by 10 µg r65 in PBS (open columns; means with standard deviations) but not PBS alone (filled columns; means only) and was maximal on day 3. Injection of 1 µg r65 produced less swelling over an identical time course. No oedematous response occurred in unsensitised animals or in animals injected with the 10 kDa polypeptide. Levels of significance compared with PBS were **p* < 0.02; ***p* < 0.01; ****p* < 0.001. Antibodies to both r65 and the 10 kDa polypeptide were measured in all groups (fig 1B), 3 days (filled columns) and 8 days (open columns) following intra-articular injection. The antibody response was compared with that in sensitised rats receiving PBS alone (**p* < 0.05; ***p* < 0.025) and the results expressed in ELISA units (see text). Doses of 1 µg and 10 µg of each preparation were used giving four treatment groups; thus 65 (10) and 65 (1) denote groups of rats receiving 10 µg and 1 µg of r65 intra-articularly while 10 (10) and 10 (1) refer to intra-articular injection of 10 µg or 1 µg of the 10 kDa hsp. Increased antibody levels did not occur in unsensitised animals.

Results

EFFECT OF INTRA-ARTICULAR R65 ON JOINT INFLAMMATION AND ANTIBODY INDUCTION

Figure 1A shows that intra-articular injection of 10 µg r65 induced knee swelling (open bars) in sensitised animals which was maximal between 48 hours and 72 hours after injection but returned to normal by day 7. This response was dose dependent; thus 1 µg r65 injected intra-articularly produced less swelling over an identical time course. By contrast, no such response was observed using either the vehicle (PBS) or the 10 kDa polypeptide. Figure 1B shows the serum antibody levels to 10 kDa and 60 kDa proteins measured at day 3 (maximum

Degree of swelling induced following intra-articular injection of microbial proteins

Injected Antigen	n	Degree of Swelling†				
		Day 1	Day 2	Day 3	Day 5	Day 6
PBS	12*	–	–	–	–	–
r65	6	++	+++	+	–	–
pur65	6	+	+++	–	–	–
LPS	6	–	–	–	–	–
PPD	12*	++++	++++	+++	+	–
<i>M tuberculosis</i>	6	++++	++++	+++	+	–
10 kDa	6	–	–	–	–	–
p24	6	–	–	–	–	–

* mean scores of 2 experiments.

† mean joint differences (MJD) and standard deviations (SD) were calculated for all groups on all days and scored relative to the PBS group as follows:–

– MJD test group $\leq$ MJD + 2SD PBS group+ MJD test group $\leq$ MJD + 4SD PBS group++ MJD test group $\leq$ MJD + 6SD PBS group+++ MJD test group $\leq$ MJD + 8SD PBS group++++ MJD test group $>$ MJD + 8SD PBS group

Figure 2 Analysis of cellular recruitment by immunohistology. Synovial sections were obtained from *Mtb*-sensitized Wistar rats three days after intra-articular injection with either r65 (A, B, C) or PBS (D, E). Using r65, cells staining for ED1 (A), ED2 (C) and the $\alpha\beta$ T cell receptor (B) were detected in the synovium. With PBS, no T cell infiltration was apparent (D) but both ED1+ (E) and ED2+ macrophages were present.

swelling) and again at the end of the experiment. At day 3 (filled bars), significantly raised antibodies to the 65 kDa protein were present in animals receiving an intra-articular injection of r65 ($p < 0.025$) but not the 10 kDa polypeptide. At the end of the experiment (day 8), when swelling had subsided, significantly raised antibodies to both 10 kDa ($p < 0.05$) and 65 kDa ($p < 0.025$) hsp were detected only in the animals receiving the higher dose (10 μ g) of r65, administered intra-articularly (open bars) but this should not be overinterpreted as the numbers are small ($n = 3$).

A range of antigens was tested (table 1). In all cases, except LPS, the animals received an intra-articular dose of 50 μ g of antigen. To ascertain whether the inflammatory response observed following intra-articular injection of r65 was attributable merely to contaminating LPS, sensitised rats were injected with LPS in an amount equal to that present in 50 μ g of r65 (that is, 15 EU). LPS, at this concentration, had no significant effect while both r65 and pur65 induced statistically significant joint swelling. *M tuberculosis* and PPD, which contain the 65 kDa protein, were also effective but neither the 10 kDa nor the p24 polypeptides induced oedema. Serum antibody levels were measured six weeks following intra-articular challenge. Raised antibodies to r65 or the 10 kDa polypeptide were again detected only in sensitised animals challenged intra-articularly with the 65 kDa protein preparations (that is, r65, *M tuberculosis* or PPD): Intra-articular challenge with *M tuberculosis* and PPD, however, appeared to favour an anti-10 kDa response (ELISA Units: mean anti-10 kDa = 1.63 for PBS, 12.2 for *M tuberculosis* and 11.37 for PPD; mean anti-65 kDa = 2.10 for PBS, 2.00 for *M tuberculosis* and 2.20 for PPD), but these numbers represent only 2 animals per group. Sensitised animals challenged intra-articularly with LPS or the 10 kDa or p24 polypeptides had normal antibody levels.

EFFECT OF INTRA-ARTICULAR MICROBIAL PREPARATIONS IN NON-IMMUNISED ANIMALS

To determine whether the knee swelling induced by r65 was dependent before sensitisation with *M tuberculosis*, a group of unsensitised rats were injected intra-articularly with either PPD or *M tuberculosis*; no measurable joint swelling occurred and serum stress protein antibody levels were normal.

JOINT ASSESSMENT

Groups of sensitised animals, injected with r65 or the 10 kDa polypeptide, were maintained for six weeks with no subsequent measurable knee swelling. Visual assessment of the joints after sacrifice revealed that animals injected with r65 had an increased fluid volume with synovial proliferation and bone erosions.

Figure 2 shows the immunohistological findings. Intra-articular injection of r65 resulted in recruitment, on day 3, of both ED1 + macrophages (fig 2A) and $\alpha\beta$ + T cells (fig 2B); by day 8, few if any T cells remained

and ED1 staining was considerably reduced. No T cell infiltration was noted on day 3 using PBS (fig 2D), but ED1 + macrophages were present (fig 2E). ED2 + macrophages persisted throughout the experiment in both r65 (fig 2C) and PBS injected animals.

Discussion

Several groups have demonstrated a protective effect for the 65 kDa stress protein in rodent models of arthritis;^{7,13} the route of administration, however, was critically important.^{7,13} Although the 65 kDa protein was shown to exacerbate existing disease,¹³ no arthritogenic potential was demonstrated.⁷ Here we have shown that, in sensitised animals, a recombinant 65 kDa stress protein of *M leprae* can indeed induce joint inflammation in the absence of existing disease, implying a role for this protein in arthritis induction. This result was not attributable to bacterial contaminants as a purified preparation of r65 caused similar swelling and intra-articular administration of LPS itself showed little effect (table 1). Macrophage recruitment occurred following injection with either r65 or PBS but only r65 induced T cell infiltration of the synovium (fig 2).

Antigen-induced arthritis, based on the Glynn/Dumonde rabbit model¹⁴ is well documented. Our model differs in that we sensitised animals in the scruff with *M tuberculosis* in oil but in the absence of soluble antigen. Both the 65 kDa and 10 kDa proteins are immunodominant antigens of *M tuberculosis* but only r65 induced arthritis. Comparison of 'local' (whole protein) primary amino acid sequences using the SERC/Daresbury computer programme revealed a 69% homology between the 65 kDa (*M leprae*) and 10 kDa (*M tuberculosis*) proteins used here, suggesting that the oedematous response obtained is specific to an unshared epitope on the 65 kDa protein. By analogy with the AA model of Cohen,⁵ we speculate that this epitope may induce recruitment of A2b-type arthritogenic T cells. However, it must be noted that the two stress proteins vary considerably in size and, following a series of elegant experiments in a murine model, van Lent *et al*¹⁵ showed that both molecular weight and charge have a striking effect on antigen retention in the joint. Small molecular weight proteins (14 to 47 kDa) were cleared rapidly whereas cationic proteins of high molecular weight (67 to 150 kDa) persisted. Both the stress proteins used here are cationic. This provides an alternative explanation for the lack of swelling using the 10 kDa and p24 polypeptides.

The antibody results require comment since patients with RA have raised serum antibodies to the 65 kDa protein which do not correlate with disease activity.⁴ In the model described here, we have demonstrated that intra-articular injection of r65 into sensitised animals is able to mount an antibody response which persists when the inflammation has settled. Thus these results mimic the human disease situation. The antibody response is clearly hsp-specific as

antibody levels were normal in animals challenged with LPS or p24. Moreover, following intra-articular injection of *M tuberculosis* or PPD, which contain both 60 kDa and 10 kDa stress proteins, antibody levels against the 10 kDa protein predominated, underlining the fact that immune responses vary with the context in which antigen is presented,¹⁶ the 60 kDa protein perhaps acting as a carrier molecule. Furthermore, the generation of 10 kDa antibodies following intra-articular administration of r65 suggests cross-reactivity against a common epitope.

In humans, T cells isolated from healthy individuals show specificity to self-epitopes shared by the human and mycobacterial 65 kDa stress proteins; thus the potential for generating autoimmune disease exists, by breaking tolerance through the ability of T cells to recognise cross-reactive epitopes on infecting organisms.¹⁷ The majority of the Western population are immunised (that is, sensitised) with *M bovis* BCG and all are differentially MHC restricted. The *M bovis* hsp 60 has close amino acid identity with that of *M leprae* (used here), differing by only 28 residues. As previously stated, the route of administration, as well as antigen charge and size, is of major importance to the response obtained. We used immunisation followed by intra-articular challenge. We speculate that this model mimics human arthritis. Taken as an acute model, it could be likened to a reactive arthritis. Extensive investigations have shown that, in these patients, T cells are directed principally to the 60 kDa protein of the infecting organism but show cross-reactive responses.¹⁸ However, hsp60 could be the elusive persistent synovial antigen of RA. Thus in the human arthritic joint, where 60 kDa proteins are highly expressed,^{2,3} ongoing inflammation within the synovium may lead to cell destruction with the concomitant release of these proteins and subsequent recruitment of arthritogenic T cells; this may account for the acute 'flares' seen in RA. Differential MHC restriction, governing susceptibility to such T cells, could account for the more severe disease of HLA DR4 positive patients with RA.

In summary, we have provided evidence in support of the hypothesis that, in *M tuberculosis*-sensitised individuals, stress proteins presented within the synovium, perhaps following cell destruction or microbial infection, can recruit arthritogenic T cells, thereby creating flares of acute inflammation and initiating or prolonging the disease. Further, we have provided an explanation for the occurrence of increased levels of cross-reactive antibodies to stress proteins in patients with RA.

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