

A MYCOBACTERIAL 65-KD HEAT SHOCK PROTEIN
INDUCES ANTIGEN-SPECIFIC SUPPRESSION
OF ADJUVANT ARTHRITIS, BUT
IS NOT ITSELF ARTHRITOGENIC

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A nonapeptide epitope contained in the 65-kD species of heat shock protein of many mycobacterial species shares homology with a peptide on a mammalian cartilage link protein (1), with four of the amino acids identical. Structures containing this epitope, including proteoglycans extracted from human rheumatoid synovial effusions (2), induce proliferation of an arthritogenic, rat T lymphocyte line (3) that has been subdivided into two clones, A2b and A2c, which, respectively, induce adjuvant arthritis or suppress the developing and established phases of this experimental model of autoimmunity (4, 5). Both clones proliferate in the presence of the epitope, as do T lymphocytes from some rheumatoid arthritis patients (6), but it is not established if the epitope plays a role in the pathogenesis of the arthritis. Administration of the recombinant (r) 65-kD shock protein antigen intraperitoneally did not induce arthritis in rats, but rendered such rats resistant to further attempts to induce arthritis with mycobacteria (1) or streptococcal cell walls (7). It is known, however, that adjuvant arthritis development requires the arthritogens to be administered in an oil vehicle by either the intradermal or subcutaneous routes (reviewed in reference 8), and that the peptidoglycan component of the mycobacterial cell wall is essential (8).

Using high levels of the r65-kD protein in an oil vehicle, and even combining the protein with low levels of a synthetic, nonimmunogenic adjuvant, CP20961 (9), to mimic the peptidoglycan portion of mycobacteria, we have been unable to induce arthritis with the r65-kD shock protein, though the induction of resistance was confirmed. At high levels, the synthetic adjuvant CP20961, which is known to be arthritogenic (9), induced arthritis in rats rendered resistant to mycobacterial arthritis by the r65-kD shock protein. Type II collagen-induced arthritis also developed in r65-kD protein-pretreated rats. These results strongly suggest that the r65-kD shock protein produces an antigen-specific resistance to classical, mycobacterial adjuvant arthritis, since it is much less effective at inhibiting the arthritis induced by both CP20961 and type II collagen. Furthermore, the pathogenesis of the actual adjuvant arthritis does not appear to involve the 65-kD protein.

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Materials and Methods

Adjuvant Arthritis. This was induced in female inbred Lewis rats (Olac, Bicester, UK) (140–160 g at the beginning of an experiment) by injection intradermally of 250 μg of *Mycobacterium tuberculosis* (*M.tb.*) (heat-killed human strains C, DT, and PN; Central Veterinary Laboratory, Weybridge, UK) into the tail base. The *M.tb.* had been finely ground in a pestle and mortar and suspended evenly in light paraffin oil at a concentration of 2.5 mg/ml. Development of arthritis was measured by a scoring system assessing the symptoms at eight sites; hind feet, fore feet, ears, eyes, tail, and nose on the basis, 0 = no signs to 4 = severe, so that a total score of 32 was obtainable in each rat. In practice, arthritis development was terminated at a score of 25, but in these experiments this score was rarely reached.

CP20961 Arthritis. This synthetic adjuvant (a lipoidal amine, Avridine; Pfizer, Groton, CT [*N,N*-dioctadecyl-*N',N'*-bis(2-hydroxyethyl)propanediamine]) induces an arthritis essentially identical (9) to classical adjuvant arthritis. 5 mg of CP20961 in light paraffin oil (50 mg/ml) was injected intradermally into the tail base of Lewis rats, and the arthritis development evaluated as described for adjuvant arthritis above.

Type II Collagen-induced Arthritis. This was induced in the Lewis rat (above) by injection into the tail base of 2 mg of native bovine type II collagen. The type II collagen was prepared by standard procedures (10) and was prepared for injection at a concentration of 10 mg/ml in a 1:1 emulsion of 0.1 M acetic acid and IFA (Difco Laboratories Inc., Detroit, MI). Arthritis was measured as for adjuvant arthritis above; however, the arthritis was mainly confined to the hind feet, so that a score of 8 was frequently the maximum obtained.

Adoptive Transfer of Adjuvant Arthritis with T Lymphocytes. The technique used was that described by Taugog et al. (11). Essentially, lymph nodes and spleens were removed from Lewis rats (described above) 10 d after injection of *M.tb.*; these were homogenized by use of a stomacher (Seward Medical, London, UK) while suspended in RPMI 1640 supplemented with 10% FCS and 1% penicillin/streptomycin (Gibco Laboratories, Paisley, Scotland). T lymphocytes were obtained after passage over nylon wool columns, washed in medium, and suspended at 2.5×10^6 ml of medium in 175-cm² flasks (Gibco Laboratories); these were incubated with Con A (Sigma Chemical Co., Poole, UK) at a concentration of 2.5 $\mu\text{g}/\text{ml}$ for 48 h at 37°C in 5% CO₂ in air. After incubation, T lymphocytes were washed in PBS, concentrated to 5×10^8 cells/ml, and immediately injected intravenously into naive, syngeneic female Lewis rats (described above). Arthritis developed within 5–7 d and was assessed by the scoring method above.

Administration of r65-kD Antigen (Heat Shock Protein) to Rats. r65-kD antigen was dissolved in PBS and subsequently mixed with an equal volume of IFA (Difco Laboratories Inc.), and emulsified to give emulsions containing 10–2,500 $\mu\text{g}/\text{ml}$. 0.1 ml of the emulsions was injected intradermally into the tail base.

Expression and Purification of the r65-kD Mycobacterial Antigen. The procedures were essentially those described previously (12). Briefly, the gene encoding the *Mycobacterium leprae* r65-kD antigen was subcloned from a λ gt11 clone into the high copy number plasmid pUC8. *Escherichia coli* (TG2) containing the recombinant plasmid expresses large amounts of the antigen. Cultures of recombinant *E. coli* were grown overnight at 37°C, harvested by centrifugation, and resuspended in citrate-EDTA buffer. The bacteria were lysed by sonication, and the particulate material was dissolved in phosphate-buffered 6 M urea. This material was loaded onto a preparative polyacrylamide gel and subjected to electrophoresis overnight in 6 M urea. The 65-kD band was excised and homogenized in phosphate-buffered urea, filtered, and precipitated by the addition of ammonium sulfate. The protein was dissolved in PBS, dialyzed and filtered, and stored at -70°C before use.

Results and Discussion

Attempts to Induce Arthritis with r65-kD Protein. To determine whether r65-kD mycobacterial antigen alone could induce adjuvant arthritis, rats were immunized with the protein intradermally in IFA at doses of 1, 10, 50, and 250 μg . Control rats, receiving *M.tb.* in oil developed arthritis after 10 d, whereas no arthritis was

seen in any of the rats receiving r65-kD protein (Fig. 1 *a*). Interestingly, descriptions of the histopathology of adjuvant arthritis (see reference 8) do not indicate a direct attack on cartilage, where the shared epitope on the link protein exists. Our Lewis strain of rat develops extensive bone destruction and periostitis, but the cartilage is often left relatively undamaged.

We also attempted to induce arthritis by administering the r65-kD protein in combination with a suboptimal dose of the synthetic adjuvant CP20961, to mimic the peptidoglycan adjuvant component of mycobacteria; this compound alone, at a 5-mg level, induced arthritis similar to that seen with *M.tb.* (Fig. 1 *b*). At the lower dose of 2 mg, a modest degree of arthritis was induced by CP20961 alone; in fact, 5 of the 10 rats so treated developed some degree of arthritis. However, the rats receiving a combination of 2 mg CP20961 plus r65-kD protein failed to develop any symptoms of arthritis.

These results indicate that the r65-kD antigen, even when given by the optimal route of administration (subcutaneously) and at dosages that far exceed the amount of protein that is administered when *M.tb.* is given, was unable to induce arthritis. Furthermore, when added to a suboptimal dose of the arthritis-inducing adjuvant CP20961, the r65-kD protein abrogated the induction of arthritis.

Resistance to Arthritis. Rats that had been immunized with r65-kD protein in IFA, intradermally, were challenged 3–4 wk later either with *M.tb.* (Fig. 2 *a*) or CP20961 (Fig. 2 *b*). Pretreatment with amounts of r65-kD protein as low as 1 μ g significantly protected against arthritis after challenge with *M.tb.*; only a few rats developed mild, transient symptoms (Fig. 2 *a*), confirming earlier results (1). Interestingly, rats pretreated intraperitoneally with 10 μ g r65-kD protein in saline developed full arthritis when rechallenged with *M.tb.*

Rats, however, that were pretreated intradermally with r65-kD protein and subsequently challenged with 5 mg CP20961, all developed symptoms of arthritis, though this was not as severe as that seen in naive rats given the CP20961 (Fig. 2 *b*); in this experiment, rats rechallenged with *M.tb.* were again totally resistant to arthritis. It appears, therefore, that the r65-kD protein produces an antigen-specific resistance

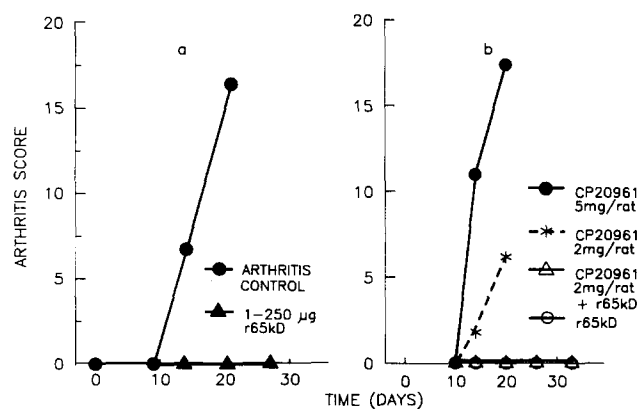


FIGURE 1. (a) Ability of r65-kD protein to induce arthritis. Groups of five rats received either *M.tb.* or r65-kD protein into the tail base. Only those rats receiving *M.tb.* developed arthritis; r65-kD protein at levels much greater than that present in whole mycobacteria failed to induce arthritis. Group means are plotted. (b) Effect of combining r65-kD protein with a synthetic adjuvant on arthritis development. Combining the r65-kD protein with CP20961, to mimic the adjuvant peptidoglycan component of the *M.tb.* cell wall, failed to induce

arthritis. In fact, the r65-kD protein abrogated the arthritogenic activity of the synthetic adjuvant at the 2-mg level. Groups of 10 rats were used, and pretreatment with r65-kD protein was at 50 μ g/rat when used; group means are plotted.

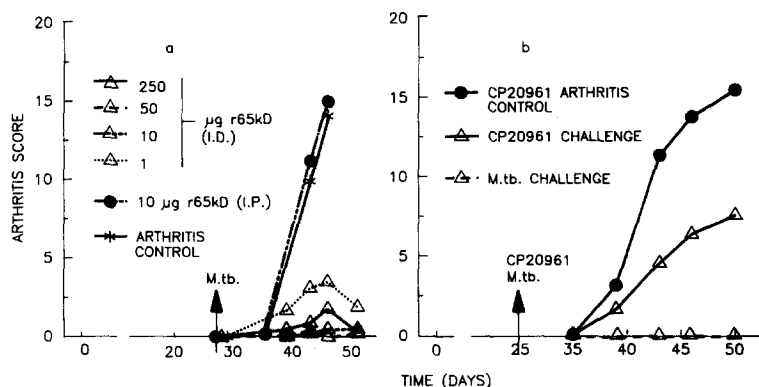


FIGURE 2. (a) Rechallenge of r65-kD protein-pretreated rats with *M.tb.* Rats pretreated with r65-kD protein from 1–250 µg were significantly protected against arthritis in comparison with the control group receiving *M.tb.* Giving the r65-kD protein in saline intraperitoneally, however, was not protective. Groups of five rats were used; group means are plotted. (b) Rechallenge of r65-kD protein protected rats with the synthetic adjuvant, CP20961. A level of 50 µg r65-kD protein protected rats against *M.tb.*-induced arthritis; however, 5 mg of CP20961 induced significant arthritis in similarly pretreated rats, though this was not as severe as in the CP20961 control group. Groups of 10 rats were used; group means are plotted.

to the arthritis induced by *M.tb.*, even at a 1-µg level, but is markedly less effective against CP20961 arthritis. Interestingly, resistance to adjuvant arthritis can be induced by preadministration of very low doses of *M.tb.*, and such resistance is also overridden by the administration of CP20961 (13).

Effect of r65-kD Protein on Type II Collagen-induced Arthritis. Rats were treated with 20 µg r65-kD protein 3–4 wk before challenge with type II collagen. The results are presented in Table I, and show the data for day 23, at the peak of arthritis development. Compared with control rats, fewer r65-kD-pretreated rats developed type II collagen arthritis, and the symptoms tended to be milder, but, as with CP20961 arthritis, the collagen-induced arthritis was mildly reduced, though not prevented, by the r65-kD protein. This contrasts markedly with the virtual total inhibition of mycobacterial adjuvant arthritis, and again emphasizes the antigen-specific nature of the influence of the r65-kD protein on arthritis development. The mild inhibition of the CP20961 and type II collagen arthritis is intriguing, nonetheless, and requires further investigation.

TABLE I
Effect of Pretreatment with r65-kD Protein on the Development of Type II Collagen-induced Arthritis

Treatment	Arthritis score						
	0	1	2	3	4	5	6
Type II collagen control	1	-	3	4	1	1	-
r65-kD pretreated group	4	3	-	2	-	1	-

10 rats were used in each group, and the numbers with a given score are shown over the range, 0 = no symptoms to 8 = severe symptoms in both hind feet. No rats achieved maximum scores in this experiment. r65-kD protein reduced the incidence and severity of arthritis, but did not totally prevent it.

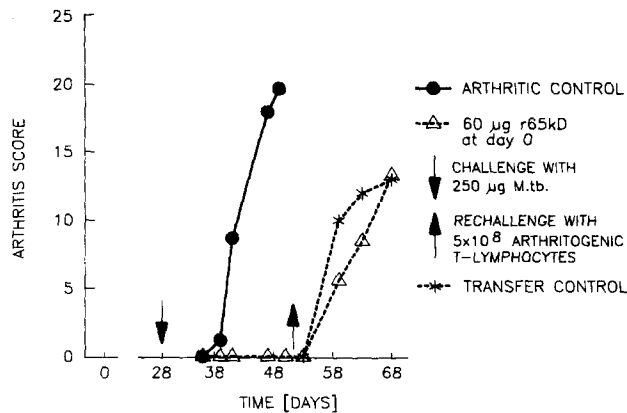


FIGURE 3. Rechallenge of r65-kD protein-pretreated rats with arthritogenic T lymphocytes. Rats receiving the r65-kD protein were protected against arthritis development, when compared with control rats challenged with *M.tb.* However, they developed arthritis when given arthritogenic T lymphocytes, comparable with that in naive, transfer control rats receiving the same cells. Groups of five rats were used; group means are plotted.

Resistance Induced by r65-kD Protein is Overcome by Transfer of Arthritogenic T Cells. A CD4⁺ population of helper T lymphocytes is known to be responsible for the induction of arthritis by *M.tb.* and CP20691, from both adoptive transfer experiments (11, 14) and the use of anti-CD4 mAbs (14). To test whether resistance to adjuvant arthritis was due to suppression of the activity, or inhibition of clonal expansion of these autoreactive CD4⁺ T lymphocytes, we transferred 5×10^8 arthritogenic T lymphocytes to rats pretreated with r65-kD protein (Fig. 3). The pretreated rats developed severe arthritis when given the arthritogenic T lymphocytes, comparable with that seen with age-matched controls receiving similar T lymphocytes, whereas pretreated rats that were rechallenged with *M.tb.* did not develop arthritis. Thus, the prior administration of r65-kD protein does not result in the generation of a population of cells that inhibits the activity of arthritogenic T lymphocytes, but, presumably, prevents them being developed in the first place. van den Broek et al. (7) have, in fact, demonstrated that resistance induced by the r65-kD protein resides within a T lymphocyte population, since such cells transfer resistance to naive rats.

Summary

A recombinant (r)65-kD protein from *Mycobacterium leprae*, at levels far in excess of those present in whole mycobacteria, was unable to induce arthritis. Even when combined with a synthetic adjuvant, CP20961, to mimic the peptidoglycan adjuvant component of the mycobacterial cell wall, the r65-kD protein failed to induce arthritis. Pretreatment with as little as 1 µg r65-kD protein protected rats against arthritis induced by *M. tuberculosis*, but this r65-kD protein was markedly less able to protect against arthritis induced by the synthetic adjuvant, CP20961, or type II collagen. The r65-kD protein appears, therefore, to produce an antigen-specific protection against arthritis induced by bacterial cell walls containing the 65-kD protein. Such protection can be overcome, however, by arthritogenic T lymphocytes, suggesting that protection occurs by preventing clonal proliferation of autoreactive T lymphocytes that are induced by the adjuvant properties of mycobacterial cell walls. How the r65-kD protein abrogates this particular adjuvant activity, and the nature of the arthritogenic self antigen(s), remain to be elucidated.

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