

PROTECTION AGAINST STREPTOCOCCAL
CELL WALL-INDUCED ARTHRITIS BY PRETREATMENT
WITH THE 65-kD MYCOBACTERIAL HEAT SHOCK PROTEIN

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Arguments for an involvement of bacteria in the pathogenesis of chronic joint inflammation (arthritis) are several (1-12). To study the pathogenetic mechanism of bacterium-induced sterile arthritis, different animal models are used, among which, *Mycobacterium tuberculosis*-induced or adjuvant arthritis (AA)¹ (2) and streptococcal cell wall (SCW)-induced arthritis (1) are the best known. In both models, bacterium-specific T lymphocytes have been demonstrated to play a crucial role (13-24, and van den Broek, M. F., M. C. J. van Bruggen, A. J. Severijnen, and W. B. van den Berg, manuscript submitted for publication), and one aspect of this role might be the cross-reactive nature of these T lymphocytes to cartilage-associated components (22, 25).

In AA it is a well known fact that rats that have recovered from arthritis are resistant to a subsequent induction of AA (17, 18), and it has been demonstrated recently that pretreatment of rats with the mycobacterial 65-kD protein resulted in the same resistance to AA when tested 35 d later (13). Since the 65-kD protein is an ubiquitous bacterial common antigen (26), with a homologous molecule present also in streptococci, and probably even associated with cell walls (27), we tested the effects of pretreatment with the mycobacterial 65-kD protein on the development of SCW arthritis.

Here we show that intraperitoneal administration of 50 μ g 65-kD protein to rats 35, 25, 15, or even as short as 5 d before induction of SCW arthritis completely protected the rats against the chronic erosive polyarthritis. The resistance against SCW arthritis is transferrable by spleen T cells to naive recipients. The protection coincides with a suppression of SCW-specific T cell responses, thus displaying features much alike suppression or tolerance leading to resistance in other models for autoimmune diseases (28-31).

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¹ Abbreviations used in this paper: AA, adjuvant arthritis; dm, dutch modification; DTH, delayed-type hypersensitivity; SCW, streptococcal cell wall; ^{99m}Tc, ^{99m}Technetium.

Materials and Methods

Rats. Female Lewis rats were obtained from the Zentral Institut für Versuchstierzucht (Hannover, FRG). The rats were housed in our animal laboratory and were fed a standard diet and tap water ad lib. Rats weighed 100–125 g at the start of the experiments.

SCW. *Streptococcus pyrogenes* T12 organisms were cultured in Todd-Hewitt broth and harvested after 24 h. The bacteria were mechanically disrupted and subsequently treated with various degrading enzymes as described (1). Further isolation was carried out by differential ultracentrifugation steps, as has been described (32), and the resulting 100,000-g pellet was used throughout the experiments. This preparation contained 12.8% muramic acid (33). The SCW were kindly provided by Dr. A. J. Severijnen, Erasmus University, Rotterdam, the Netherlands.

Induction of SCW Arthritis. To induce a chronic polyarthritis, rats were injected intraperitoneally with a sterile aqueous suspension of SCW at a dose of 15 μ g muramic acid per gram rat body weight. The arthritis was scored macroscopically by measuring paw thickness with a caliper, and by histology.

Induction of Adjuvant Arthritis. Lewis rats were injected intracutaneously at the base of the tail with 1 mg heat-killed *M. tuberculosis* organisms (H37Ra; Difco Laboratories, Inc., Detroit, MI) suspended, but not emulsified, in oil (2). Polyarthritis occurred after 14–16 d and lasted ~4 wk.

Induction of Zymosan Arthritis. To induce a nonimmune arthritis, 6 μ l of a sterile suspension of zymosan (10 mg/ml) (Koch-Light Lab., Coinbrook, Bucks, UK) in PBS was injected into the right knee joint (34). The control joint was injected with 6 μ l PBS. Because the resulting arthritis was a unilateral one, it could be quantified by the 99m Tc-uptake method as previously described (35). Briefly, rats were sedated with pentobarbital and injected with 1 MBq 99m Tc. After 20 min, radioactivity in the right and left knee joint was measured by external gamma counting. Arthritis was expressed as the ratio of 99m Tc uptake between right and left joint. All ratios >1.10 were interpreted as inflammation.

Induction of Delayed-type Hypersensitivity (DTH) Reaction. To analyze the effect of pretreatment with 65-kD protein on cellular immune responses, we immunized 65-kD protein-pretreated and IFA-pretreated control rats with OVA or with OVA + SCW emulsified in IFA by injection of 100 μ l (1 mg/ml) of the emulsion into the footpads of the forepaws. A DTH reaction as a measure of cellular immunity was induced by injection of 10 μ g OVA into the right, and saline into the left, ear of rats. The resulting swelling of the ears was measured 6, 24, and 48 h thereafter. The swelling is expressed as right over left ratios.

Pretreatment with Recombinant 65-kD Mycobacterial Protein. The 65-kD protein of various *Mycobacterium* species has recently been cloned and expressed in *Escherichia coli* K12 M1070 (36–38). The recombinant 65-kD protein was prepared from *E. coli* carrying plasmid pRIB1300. Induced cultures were resuspended in 100 mM Tris, 5 mM EDTA, pH 8, and sonicated by eight pulses of 30 s at 80 W. The resulting lysate contained the 65-kD protein up to 30%. The protein was purified from the lysate by ammonium sulphate precipitation followed by DEAE column chromatography as described (39). The isolated protein was checked for purity by SDS-PAGE (Fig. 1).

Lewis rats were treated by intraperitoneal injection of 50 μ g 65-kD protein suspended in IFA. Control rats were injected with IFA alone.

Transfer of 65-kD Protein-induced Protection. To investigate whether the 65-kD protein-induced protection against SCW arthritis was mediated by cellular immunity, transfer experiments were done. For this purpose, we injected Lewis rats intraperitoneally with 50 μ g 65-kD protein/IFA or with IFA alone. After 20 d, we removed the spleen. From spleens a single cell suspension was made. The cells were washed once with RPMI 1640 dutch modification (dm) (Flow Laboratories, Inc., McLean, VA) and the erythrocytes were lysed with 0.16 M $\text{NH}_4\text{Cl}/0.17$ M Tris-HCl, pH 7.2, for 2 min at room temperature. The white cells were washed twice with RPMI 1640 dm. Subsequently, the cells were incubated for adherence to a culture flask in RPMI 1640 dm supplemented with 20 mM glutamine and 2% (vol/vol) SF1 (Costar, Cambridge, MA) as serum substitute for 45 min at 37°C in a CO_2 incubator. The nonadherent cells were collected by aspiration. These cells were pelleted and resuspended

in 1 ml RPMI 1640 dm + glutamine + SF1 and were applied to a nylon wool column for further removal of adherent cells. After 45 min in a CO₂ incubator at 37°C, the nonadherent population was eluted with 25 ml warm RPMI 1640 dm. Of the resulting population, 95% or more of the cells were W3/13⁺.

Naive Lewis rats were injected intravenously with 5×10^8 total spleen cells or with 5×10^7 spleen T cells. Immediately thereafter, SCW were injected intraperitoneally to induce arthritis as described above.

Proliferative Response of Spleen T Cells. Spleens were removed aseptically from rats 4 wk after intraperitoneal SCW injection and a single cell suspension was made. T cell-enriched spleen cells (see above) were incubated in microtiter round-bottomed plates (Costar) with the following stimuli: SCW (6-2-0.6 $\mu\text{g/ml}$ muramic acid), 65 kD (30-10-3 $\mu\text{g/ml}$), and Con A (1.25 $\mu\text{g/ml}$). The final density of cells was $10^5/200 \mu\text{l}$ RPMI 1640 dm + 10 mM glutamin + 2% SF1 + 40 $\mu\text{g/ml}$ gentamycin.

After 3 d, 3.7×10^4 Bq (1 μCi) [³H]thymidine (Amersham, Amsterdam) ($0.7\text{--}1.1 \times 10^8$ MBq/mmol sp act) was added per well. 24 h thereafter, the cells were harvested onto glass fiber filters and the amount of incorporated radioactivity was counted in a liquid scintillation analyzer. All values are expressed as the mean of at least triplicate cultures.

ELISA for Anti-65-kD Protein or Anti-SCW Antibodies. 96-well flat-bottomed microtiter plates were precoated with 150 μl 0.5 mg/ml protamine-HCl (Kabi AB, Stockholm, Sweden) for 2 h in the case of an SCW-specific plate. Precoating was not necessary for 65-kD ELISA. After three washes with PBS + 0.05% (vol/vol) Tween-20, plates were coated with 65-kD protein and native or denatured SCW (10 min, 100°C, 1:1 diluted with 10% glycerol, 5% β -mercaptoethanol, 5% SDS), 5 $\mu\text{g/ml}$, 150 $\mu\text{l/well}$ overnight. The plates were washed three times and postcoated to avoid nonspecific binding with 150 $\mu\text{l/well}$ 1% (wt/vol) BSA for 2 h. The plates were incubated with 100 $\mu\text{l/well}$ of twofold dilutions of pooled rat sera in 1% (wt/vol) BSA for 1 h. The plates were washed five times and then incubated with 100 $\mu\text{l/well}$ horseradish peroxidase-conjugated goat-anti-rat Ig (Nordic, Tilburg, the Netherlands) diluted 500 times in 0.5% (wt/vol) BSA for 1 h. After five washes, 100 μl of the following freshly prepared substrate solution, 0.8 mg/ml 5-aminosalicylic acid dissolved in 50 mM phosphate buffer, pH 6.0, containing 0.8 $\mu\text{l/ml}$ 30% (vol/vol) H₂O₂, was added to each well. After 30 min, the absorbtion at 450 nm was measured in a Titertek Multiskan (Flow Laboratories, Irvine, Scotland). All values were corrected for the signal obtained by omitting the antigen-coating step. All incubations were carried out at room temperature.

Immunological Relationship between SCW and Mycobacterial 65-kD Protein. To investigate whether on 65-kD protein and SCW, similar T cell epitopes were present, rats were immunized in the footpads of the forepaws with 65-kD protein or SCW emulsified in IFA. 9 d thereafter, draining lymph nodes were removed aseptically and a single cell suspension was made. The cells were stimulated in vitro with 65-kD protein (10, 3, and 1 $\mu\text{g/ml}$), SCW (6, 2, and 0.6 $\mu\text{g/ml}$ muramic acid), and mitogen (Con A; 1.3 $\mu\text{g/ml}$), and the resulting proliferative response was measured as described above.

To test serological relationship between the mycobacterial 65-kD protein and SCW, several 65-kD protein-specific mAbs and polyclonal sera were tested for binding to 65-kD protein and SCW in ELISA as described above. In the ELISA system, blocking studies were performed as follows: 1:200 diluted anti-SCW or anti-65-kD protein sera were incubated overnight at 37°C with different concentrations of inhibitor (65 kD protein or SCW). Subsequently, the sera were placed at 4°C for 2 h to favor the formation of complexes and the sera were centrifuged (10 min, 2,000 rpm). The "supernatants" were tested in an ELISA as described above. In addition, antibodies were tested on a Western blot as follows: SCW and 65-kD protein were incubated with a similar volume of denaturing buffer (0.01% bromophenol blue, 10% glycerol, 5% β -mercaptoethanol, 5% SDS for 10 min at 100°C. The samples were separated using a 10% SDS-polyacrylamide gel. The material was electroblotted onto nitrocellulose (4 h, 60 V, 0.2 A) using the following buffer: 200 ml ethanol + 3 g Tris + 14.4 g glycine + 0.3 g SDS/liter. Nitrocellulose (NC) strips were incubated with blocking buffer (PBS + 2% BSA) for 2 h and subsequently with 1:200 diluted sera or mAbs in washing buffer (PBS + 1% Triton X-100 + 0.1% SDS + 0.5% BSA) and the appropriate peroxidase-

conjugated antisera (Nordic, Tilburg, the Netherlands) in a dilution of 1/500 for 1 h. NC strips were washed between each step for 3–5 min. Finally, the strips were incubated with freshly prepared substrate: 0.5 mg/ml 4-chloro-1-naphtol + 0.015% H₂O₂ in water. The reaction was stopped with water.

Histology of Knee Joints. Ankle joints were removed and processed for histology as described (40) with the following alterations: formalin for 2 wk and HCOOH for 2 wk. 7- μ m paraffin sections were made and stained with hematoxylin and eosin. Scoring of inflammation was done by two independent observers on coded slides. A scale of 0–3 was used to quantify the amount of infiltrate and exudate.

Results

Effect of Pretreatment with the Mycobacterial 65-kD Protein on SCW-induced Arthritis

Joint Inflammation. It has been described (13) that pretreatment by intraperitoneal injection of 50 μ g 65-kD protein/IFA (Figure 1) 35 d before challenging with the whole *M. tuberculosis* in oil protected Lewis rats against adjuvant arthritis. To investigate whether this particular protein was similarly protective in another bacterium-induced arthritis model, we tested the same treatment regime in SCW-induced arthritis in Lewis rats. Fig. 2 shows the arthritis in pretreated and control rats as measured by thickness of the hind paws. Complete protection against arthritis was found in the group pretreated with 65-kD protein/IFA, while in the controls, a severe, erosive polyarthritis was seen. Additional experiments were undertaken to evaluate the kinetics of the pretreatment effects (Fig. 2). Intraperitoneal injection of 50 μ g 65-kD protein/IFA 35, 25, 15, or 5 d before induction of SCW arthritis completely protected rats against joint inflammation. The histology of arthritic and protected joints is shown in Fig. 3, A and B. Fig. 3 A shows a severely inflamed joint, while Fig. 3 B, pretreated on day -25, shows only marginal inflammation. Joints from rats that were pretreated 35, 15, or 5 d before SCW arthritis induction show the same histology (data not shown). Besides the massive infiltrate and exudate in the joint space and bone marrow of control rats (Fig. 3 A), also a complete destruction of cartilage, muscle, and bone structures is observed.

In addition, at autopsy it became clear that in successfully protected rats some

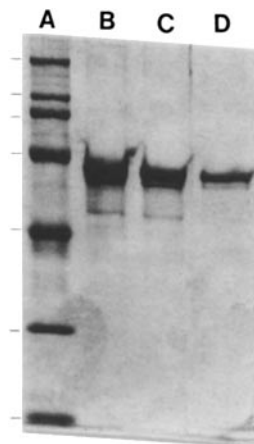


FIGURE 1. SDS-PAGE from the isolated mycobacterial 65-kD heat shock protein that was used throughout the studies. (A) Molecular weight markers: 200, 116, 95.5, 66.2, 45, 31, and 21.5 kD; (B, C, and D) 4, 2, and 1 μ g purified mycobacterial 65-kD protein, respectively.

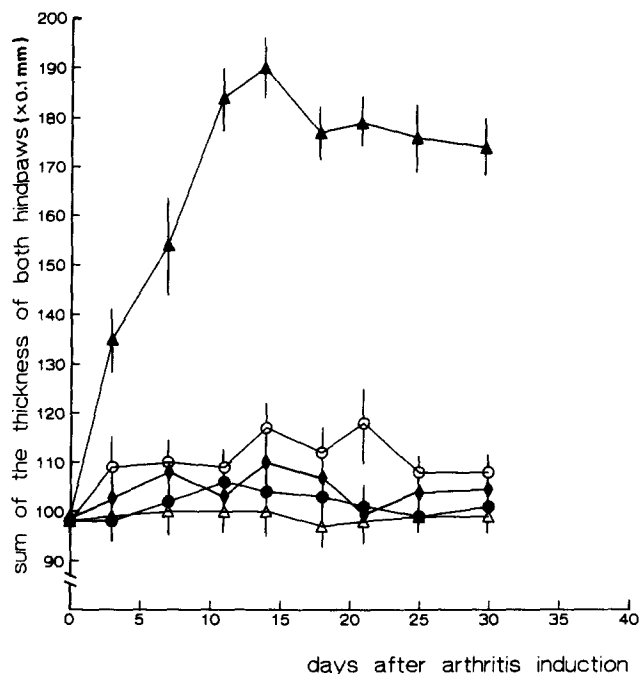


FIGURE 2. Protection of Lewis rats from SDW arthritis by pretreatment with 50 μ g 65-kD protein/IFA. 65-kD protein/IFA was injected intraperitoneally 35 (◆), 25 (△), 15 (●), or 5 d (○) before arthritis induction. The control group received IFA alone at day -25 (▲). All groups consisted of six to seven rats. Arthritis was measured with a caliper and the values represent the sum of the thickness of both hindpaws \times 0.1 mm.

other characteristics of SCW disease, like granulomas in the liver, adhesions of the spleen, and loss of body weight, were absent (data not shown).

Administration of 50 μ g 65-kD protein during arthritis (day 15, 35, or 55 after SCW injection), however, did not lead to an amelioration of arthritis but to a significant visual (redness and swelling) and functional (limping) deterioration instead.

Immune Response to SCW and 65-kD Protein in SCW Arthritis and Protected Rats. Because expression of SCW-induced arthritis is dependent on T cells (14–16), and in all probability on SCW-specific ones (16, van den Broek, M. F., M. C. J. van Bruggen, A. J. Severijnen, and W. B. van den Berg, manuscript submitted for publication), we investigated the relation between arthritis and SCW-specific T cell responses in successfully protected rats and control rats. Table I shows spleen T cell proliferation in response to mitogen, SCW, or 65-kD protein (>95% W3/13⁺, data not shown). Cells were obtained 30 d after intraperitoneal injection of an arthritogenic dose of SCW. SCW-specific and 65-kD protein-specific T cell responses are clearly present in arthritic (IFA-pretreated) rats, as compared with naive control rats.

In protected rats, pretreated with 65-kD protein at day -5 or -15, the responses to SCW, 65-kD protein, and mitogen are significantly lower than those in arthritic rats. Thus, the 65-kD protein-induced protection against SCW arthritis may result from specific T cell regulation. Proliferative responses of T cell-enriched spleen cells obtained from rats pretreated at day -25 or -35 are similar to those shown in Table I for rats pretreated at day -15 or -5 (data not shown).

Pretreatment with 65-kD protein had no effect on antibody levels to SCW, but the antibody levels against 65-kD protein showed a positive correlation with the time

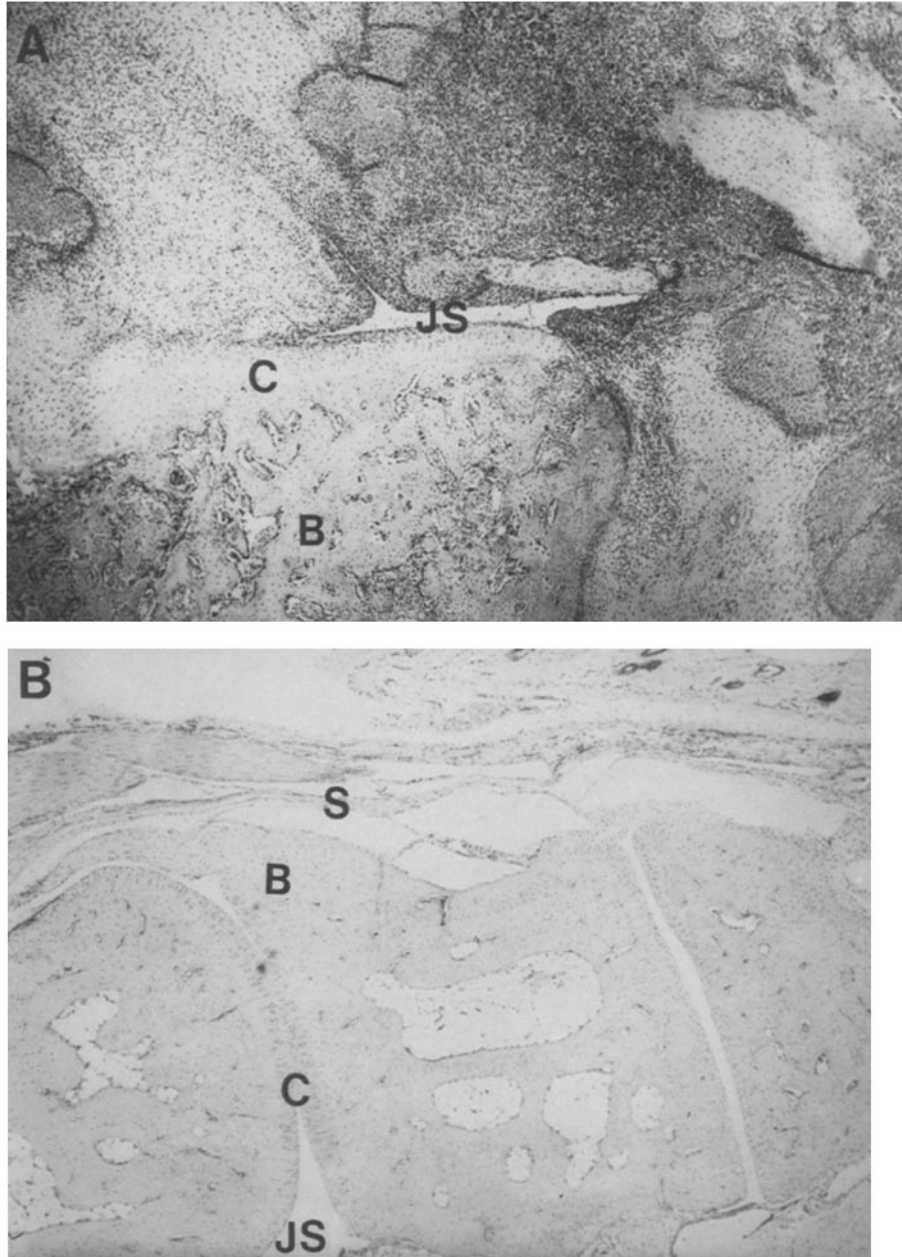


FIGURE 3. Histology of SCW arthritis: frontal sections of rat ankle joint. Staining H&E. JS, joint space; S, synovium; B, bone; C, cartilage. (A) Pretreated with IFA on day -25 ($\times 40$). (B) Pretreated with 65-kD protein/IFA on day -25 ($\times 40$). Note the mass of inflammatory cells in the joint space and bone marrow, leading to a complete joint destruction.

TABLE I
Proliferative Responses of 10⁵ T Cell-enriched Spleen Cells of Rats, Isolated 30 d after Intraperitoneal Injection of an Arthritogenic Dose of SCW or from Naive Rats

Antigen	Dose $\mu\text{g/ml}$	Proliferative responses (SIs) at day 30 of:			
		Rats injected with SCW i (day 0)			Naive rats
		65-kD protein at day - 15	65-kD protein at day - 5	IFA at day - 15	None
SCW	6	4	5	40	2
	2	2	2	26	1
	0.6	1	1	4	1
65-kD protein	10	3	4	26	8
	3	2	2	8	3
	1	1	1	2	1
Con A	1.3	7	6	212	149

Proliferation was measured by [³H]thymidine incorporation during the last 16 h of a 96-h culture with various stimuli. Proliferative responses are expressed as stimulation indices (amount of radioactivity incorporated due to stimulus *X* divided by that due to medium alone). The values represent the mean of at least triplicate cultures and the variation was always <10%. Spleens from each group were pooled.

elapsed between 65-kD protein injection and serum sampling, as expected (data not shown). This indicated that although pretreatment with 65-kD protein suppressed SCW-specific T cell responses and SCW-induced arthritis, it had no effect on anti-SCW antibody levels.

Transfer of Protection by T Cells. Fig. 4 shows the severity of SCW arthritis in Lewis rats that were injected intravenously at the day of arthritis induction (day 0) with 5×10^8 spleen cells (*A*) or with 5×10^7 spleen T cells (*B*) obtained from rats protected by 65-kD protein pretreatment or IFA-pretreated control Lewis rats. It is obvious that both total spleen cells and spleen T cells from protected rats were able to transfer this protection to naive recipient rats, while spleen cells from control rats were not. In the group receiving total spleen cells, the transferred protection was not complete, however, one of six rats developed a SCW arthritis almost as severely as those in the control group, but less chronic. Another rat also showed a substantial arthritis, although significantly less severe than that in control rats. The other four rats had a very mild arthritis with a delayed onset (2 wk) and a short duration. In the group receiving spleen T cells, the protection was complete in all recipients, even at a 10-fold lower transferred cell number. These results suggest that the protection against SCW arthritis induced by pretreatment with 65-kD protein is mediated by T cells.

Effect of Recovery from AA on SCW Arthritis Susceptibility

It is known that rats that have recovered from AA are resistant to a subsequent AA induction (17, 18). To investigate whether SCW-induced arthritis is also prevented

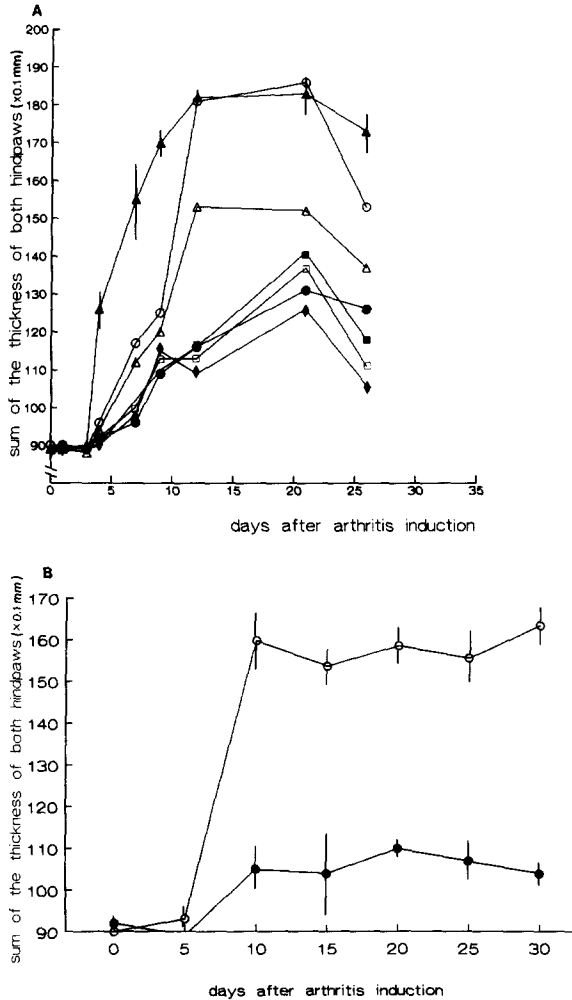


FIGURE 4. Transfer of 65-kD-induced protection against SCW arthritis: donor rats were injected intraperitoneally with 50 μ g 65-kD protein/IFA or with IFA alone at day -20. At day 0 spleen cells were isolated and 5×10^8 cells (A) or 5×10^7 purified spleen T cells (B) were injected intravenously into recipient rats. Immediately thereafter an arthritogenic dose of SCW was injected intraperitoneally. SCW arthritis is measure as the sum of the thickness of both hindlegs $\times 0.1$ mm. (A) Each group consisted of six rats. The mean value \pm SD is shown of rats which received IFA spleen cells (\blacktriangle), and individual values of rats that received 65-kD spleen cells are shown. (B) Each group consisted of four rats. The mean value \pm SD of rats injected with IFA splenic T cells (O) of 65-kD splenic T cells (\bullet) is shown.

by a foregoing AA, we induced SCW arthritis in rats that had recovered from AA (35 d after induction). However, all ex-AA rats died between 6 and 16 h after intraperitoneal SCW administration, showing edematous lungs and hemorrhagic lymph nodes at autopsy, while control rats (injected with IFA alone instead of IFA/*M. tuberculosis*) developed a normal SCW arthritis (Table II).

When rats were injected with whole *Streptococcus pyogenes* organisms suspended in IFA in the adjuvant-like manner (0.1 ml intracutaneously, 10 mg/ml), an adjuvant-like polyarthritis developed within 2 wk, which resolved after ~ 40 d. Injection with an aqueous suspension SCW (arthritogenic dose) after resolution of disease did not result in death of rats recovered from streptococcus arthritis, but led to a reactivation of the joint swelling and redness within several hours instead, thus suggesting an accelerated onset of SCW arthritis. Summarizing, immunization with *M. tuberculosis* (and the resulting arthritis) leads to a lethal hypersensitivity upon challenge

TABLE II
Effect of Pretreatment with M. Tuberculosis on the Expression of SCW-induced Arthritis

Pretreatment		Condition of rats at day 60		
Day 0	Day 35	Dead	Arthritic	Nonarthritic
-	Mt/oil	0	20	2
Mt/oil	Mt/oil	0	0	10
65 kD/oil	Mt/oil	0	0	10
-	SCW	0	20	0
Mt/oil	SCW	13*	1†	0
65 kD/oil	SCW	0	0	14
Sp/oil	SCW	0	10‡	0

Effect of pretreatment with *M. tuberculosis* (Mt) in oil (adjuvant arthritis), its immunodominant 65-kD protein in oil, or *S. pyogenes* (Sp) in oil (streptococcus arthritis) on the expression of SCW-induced arthritis. Rats were pretreated 35 d before intraperitoneal administration of SCW. Arthritis was scored by measuring joint swelling and by histology.

* Rats died within 24 h after challenge.

† One rat did not die, but developed only the T cell-independent acute phase.

‡ All rats developed a severe arthritis with accelerated onset (within 48 h, normal SCW arthritis develops after 11–14 d).

with SCW. This phenomenon is not seen after the immunization with *S. pyogenes* (and the resulting arthritis).

Effect of Pretreatment with the Mycobacterial 65-kD Protein on Zymosan-induced Arthritis

To exclude the possibility that the observed protection against SCW arthritis was a nonspecific antiinflammatory effect of the 65-kD mycobacterial protein, we tested the effect of pretreatment with this protein on a nonimmune joint inflammation, zymosan-induced arthritis (34). Arthritis is quantified by determining the ratio of ^{99m}Tc uptake in the right (arthritic) over the left (control) joint. The amount of ^{99m}Tc is influenced by edema and increased blood flow. In both groups the R/L ratios were comparable: 4 d after zymosan injection the mean ratio \pm SD of the IFA-treated group was 1.33 ± 0.04 ($n = 5$) and that of the 65-kD protein-treated group was 1.29 ± 0.04 ($n = 5$). Also, histologically, no difference between the two groups was observed (data not shown). These results indicate that 65-kD protein is not a nonspecific antiinflammatory agent.

Effect of Pretreatment with the Mycobacterial 65-kD Protein on a DTH Reaction

To exclude that the protection against bacterial arthritis by 65-kD protein is mediated by a nonspecific immunosuppressive capacity of the mycobacterial protein, we analyzed the effect of 65-kD protein pretreatment on the cellular immune response in vivo, a DTH reaction. Rats were pretreated 20 d before immunization with $50 \mu\text{g}$ 65-kD protein/IFA or with IFA alone. Table III shows the ear swelling in the four groups as a result from challenge with OVA. In 65-kD protein-pretreated and control OVA-primed groups, a comparable swelling is observed, thus implying that the effect of 65-kD protein on bacterial arthritis is not a nonspecific immunosuppressive one.

To test whether 65-kD protein primes for suppression that can be activated by

TABLE III
Effect of Pretreatment with 65-kD Protein/IFA or IFA Alone on a DTH Reaction as Measure for a Cellular Immune Response In Vivo

Pretreatment at day -34	R/L ratio of ear swelling of rats immunized at day -14 with:			
	OVA/IFA		OVA + SCW/IFA	
	24 h	48 h	24 h	48 h
65-kD protein/IFA	2.18 ± 0.08	1.71 ± 0.11	1.32 ± 0.03*	1.21 ± 0.05
IFA	2.11 ± 0.11	1.64 ± 0.07	1.96 ± 0.08	1.69 ± 0.07

DTH was induced by injection of 10 µg OVA into the pinna of the right and saline into the pinna of the left ear of rats, which were treated as follows: day -34, pretreatment with 65-kD protein/IFA ($n = 6$) or IFA ($n = 6$) intraperitoneally; day -14, immunization with OVA/IFA or OVA + SCW/IFA in forepaws; day 0, intrapinnal challenge with OVA; day 1 and 2, measurement of DTH reaction.

* $P < 0.001$ compared with its IFA-pretreated control group as determined by the two-tailed Mann Whitney U test.

rechallenge with a 65-kD protein-containing antigen, we immunized 65-kD-protein-pretreated and control rats with OVA/IFA supplemented with SCW to activate this suppression and induced a DTH with OVA subsequently. Table III depicts that the OVA-elicited DTH reactions in OVA/IFA- and OVA + SCW/IFA-primed control rats are comparable to each other and to that in 65-kD protein-pretreated, OVA/IFA-primed rats, but that the DTH reaction in 65-kD protein-pretreated, OVA + SCW/IFA-primed rats is significantly decreased.

Immunological Relationship between SCW and the Mycobacterial 65-kD Protein

To see whether SCW and 65-kD protein are immunologically interrelated, we searched for crossreactivity at both the humoral and the cellular level. Recently, a 65-kD protein has been demonstrated in *S. pyogenes* by mAbs (26), but no data are available for the presence of 65-kD proteins in isolated cell walls of *S. pyogenes*. For Coxiella, however, it has been described that isolation of cell walls resulted in enrichment for the 65-kD heat shock protein (27).

The results of our protection experiments already suggest a similarity with respect to T cell epitopes and Table IV shows further evidence: SCW-primed T cells proliferate in vitro in response to 65-kD protein, and 65-kD protein-primed T cells proliferate in response to SCW. In addition, Table I shows that administration of SCW in saline intraperitoneally in an arthritogenic dose induces 65-kD protein-specific T cell responses.

Also at the level of antibody recognition, SCW and 65-kD protein are related; anti-65 kD sera react with SCW on immunoblots and vice versa (data not shown), and of six anti-65-kD monoclonals, each recognizing different epitopes in the molecule (41-46), monoclonal E423 and 67-2 recognized SCW. A control monoclonal (WT31, directed against human T3, [42]) was negative. Table V shows the ELISA results: the binding of antiserum to its own antigen and to the crossreactive antigen could be blocked by preincubation of the serum with either antigen, albeit not in all combinations to the same extent.

TABLE IV
*Immunological Relationship at the T Cell Level between the
Mycobacterial 65-kD Protein and SCW*

Antigen	Dose $\mu\text{g/ml}$	Proliferative response 9 d after immunization with:		
		65-kD protein/IFA	SCW/IFA	IFA
SCW	6	12.1	48.1	1.6
	2	6.5	20.6	1.1
	0.6	4.1	8.3	1.2
65-kD protein	10	20.0	25.4	7.9
	3	10.5	11.1	2.7
	1	4.6	6.4	1.1
Con A	1.3	143.0	163.0	149.2

Rats were immunized with 65 kD protein or SCW in IFA and the proliferative response of cells isolated from draining lymph nodes was measured 9 d later. Proliferation was measured by [^3H]thymidine incorporation during the last 16 h of a 96-h culture with 65-kD protein, SCW, or mitogen as stimulus. Proliferative responses are expressed as stimulation indices (amount of radioactivity incorporated due to stimulus X divided by that due to medium alone). The values represent the mean of quadruplicate cultures of pooled lymph nodes from three rats and the variation was always <10%.

* Muramic acid equivalents.

TABLE V
Serological Relationship between SCW and the Mycobacterial 65-kD Protein

Serum specificity	Coated Ag	Inhibiting Ag	Percent inhibition at inhibitor ($\mu\text{g/ml}$)		
			12.5	25	50
SCW	SCW	SCW	25	36	64
		65-kD protein	0	19	30
	65-kD	SCW	0	6	20
		65-kD protein	8	26	51
65-kD protein	SCW	SCW	34	60	58
		65-kD protein	10	21	32
	65-kD	SCW	12	15	27
		65-kD protein	22	56	71

The reactivity of polyclonal rat sera with a specificity for SCW or 65-kD protein were tested by ELISA and blocking studies for reactivity against 65-kD protein and SCW. Sera and monoclonals were diluted 1:200. Inhibitor was added to final concentrations ranging from 0 to 200 $\mu\text{g/ml}$. Sera were incubated for 16 h at 37°C and a subsequent 2 h at 4°C, and were centrifuged. Sups were tested in an ELISA. All signals were corrected for nonspecific binding (binding of sera +/− inhibitor to microtiterplates prepared as described but with omission of the antigen-coating step).

Discussion

Both clinical and experimental data provide evidence that bacteria play a role in the induction or maintenance of arthritis (1–12). Two animal models used to study this are AA (2) and SCW-induced arthritis (1). Both models are inducible in Lewis rats, but differ in the way of induction: AA is induced by subcutaneous injection

of a suspension of killed mycobacteria (1 mg) in oil, and SCW arthritis is induced by an intraperitoneal injection of an aqueous suspension of SCW (10–15 mg). In AA, a polyarthritis develops 2–3 wk after induction, which wanes after another 4 wk, whereafter rats are resistant to a subsequent AA induction. Immediately after injection of SCW, however, an acute systemic illness is observed that lasts for 3–5 d, followed by a chronic polyarthritis on week 2–3 with a duration ranging from 2 to 6 mo.

Also with respect to the pathogenetic mechanism, the models are considered to be different. In AA it is obvious that mycobacterium-specific T lymphocytes, of which some crossreact with cartilage, can be responsible for the disease (17–22), while in SCW arthritis the most important role was thought to be played by antigen persisting in the joint in concert with macrophages trying to degrade the material (23, 47, 48). In addition, AA is transferable to naive rats by *M. tuberculosis*-specific lymph node or spleen cells or by a T cell clone (17–22), whereas similar transfers in SCW arthritis have not been described yet. More recent studies, however, suggested also in SCW arthritis an important if not determining role for (SCW-specific) T lymphocytes (15, 16, 24, 49, and van den Broek, M. F., M. C. J. van Bruggen, A. J. Severijnin, and W. B. van den Berg, manuscript submitted for publication).

A recent study in the AA model (13) demonstrated that administration of a small amount of an immunodominant mycobacterial protein, the 65-kD protein, in oil to rats 35 d before arthritis induction, made those rats completely resistant to AA. This 65-kD protein is immunologically related to a similarly sized ubiquitous bacterial common antigen (26) and shows homology with prokaryotic and eukaryotic heat shock proteins (50). Because of the homology with proteins of other bacteria and the clear relationship between bacterial infections of different kind and joint inflammation, we tested this mycobacterial 65-kD protein with respect to its capacity to vaccinate against arthritis induced by group A streptococci, bacteria that are unrelated to mycobacteria.

Here we present, in concordance with the findings in AA, that SCW-induced arthritis in Lewis rats can be prevented by pretreatment of the rats with the mycobacterial 65-kD protein. Also, we show that protection by 65-kD protein is equally successful when rats are injected with 65-kD protein 25, 15, or even as short as 5 d before arthritis induction. This suggests that the protection takes at the most 5 d to develop completely and lasts for 35 d or maybe even longer.

An obvious question arising from these observations is which mechanism could be responsible for this. To address this question we analyzed the cellular and humoral immune response to SCW in protected and control rats. Regarding the humoral immune response, no difference in anti-SCW antibodies could be detected between the protected or control rats, suggesting that anti-SCW antibodies are of minor, if of any, importance with respect to expression of SCW arthritis. This is in agreement with earlier studies in the SCW arthritis model (16, 51). A different picture is obtained when the cellular immune response is analyzed. T cells from successfully protected rats showed almost no proliferative response to SCW in vitro, while T cells from control arthritis rats displayed a vigorous SCW-specific response. This confirms the involvement of SCW-specific T lymphocytes in chronic SCW arthritis (16, 24, 49, and van den Broek, M. F., M. C. J. van Bruggen, A. J. Severijnin, and W. B. van den Berg, manuscript submitted for publication). And because pretreatment with 65-kD protein had no effect at all on zymosan-induced arthritis (nonspecific

inflammatory reaction) or on a DTH reaction to a protein antigen (cellular immune response), the possibility that the 65-kD protein activity is a nonspecific immunosuppressive or antiinflammatory one could be excluded.

A peculiar observation is the suppression of the proliferative response to mitogen in 65-kD protein-pretreated, SCW-injected rats. The explanation could be that by intraperitoneal injection of the mycobacterial 65-kD heat shock protein, the rat is primed for suppression or tolerance to arthritogenic bacterial epitopes. After an SCW injection in 65-kD protein-pretreated rats, suppression of SCW-induced arthritis is therefore elicited, and, as immunosuppression often has nonspecific aspects in its effector phase, suppression of the mitogen-driven proliferation is also seen. This explanation is supported by the data shown in Table III: the OVA-specific DTH reaction is only suppressed in 65-kD protein-pretreated rats when these rats are "challenged" with SCW.

The reason why pretreatment with whole mycobacteria instead of the isolated immunodominant protein does not induce protection against SCW-induced arthritis, but induces an anaphylactic kind of hypersensitivity, is not clear yet. This phenomenon obviously does not occur in the same way for all bacterial stimuli, because pretreatment with whole *S. pyogenes* does not induce this lethal hypersensitivity to SCW, but rather primes. This lethal hypersensitivity reaction also indicates the immunological relationship between some antigens present in both mycobacteria and streptococci.

Also, in other animal models for autoimmune diseases the induction of tolerance to certain disease-inducing epitopes has been demonstrated. In experimental allergic encephalomyelitis (28, 29) and collagen-induced arthritis (30, 31), pretreatment with fragments of the inductive protein (but not the inducing agent itself) or with the intact protein when administered orally completely protected animals against the disease, or at least delayed the onset and diminished the severity. Like in the study presented here, in experimental allergic encephalomyelitis the myelin basic protein-specific T cell responses were severely suppressed in protected Lewis rats while T cell responses to irrelevant antigens were normal.

Compatible with these findings seems to be the observation of Kohashi et al. (52), who showed that AA-resistant F344 rats (conventional, CV, or specific pathogen-free) became susceptible when kept at germ-free conditions. Upon recolonization with live bacteria, the susceptibility to AA decreased again. One might conclude from these data that certain antigens present on endogenous bacteria may somehow induce tolerance to bacterial arthritogenic epitopes. In addition, conventional F344 rats are resistant to SCW-induced arthritis and also, this resistance might be due to active suppression of SCW-specific and thus of crossreactive anticartilage T cell responses (16, 25). Also, the latter observation supports the view of tolerance as a mechanism for resistance.

Resistance to SCW-induced arthritis can be transferred into naive rats by spleen cells from 65-kD protein-treated rats (Fig. 4), leaving a role for whether T cells (antigen-specific), macrophages (nonspecific), or both are responsible for the protection. The fact, however, that isolated T cells from spleens from 65-kD protein-protected rats were even more capable than unpurified spleen cells to transfer protection, strongly suggests a role for T cells alone.

Experiments in other models suggest a role for antigen-specific suppressor T lymphocytes (53–55). If a lack of tolerance is the reason for arthritis susceptibility, this

then suggests in the mean time that the Lewis rat is defective in building tolerance to certain bacterial epitopes or to antigens in general. The fact that most models for autoimmune disease are inducible in the Lewis rat and often in the Lewis rat only is at least not contradictory to this statement.

Our attempts to interfere with SCW-induced arthritis in a sense of curing were not successful. Instead of a decreased joint swelling, we observed an acute exacerbation of the arthritis, which manifested itself as redness, swelling, and a complete inability to walk, while before injection of 65-kD protein (50 μ g i.p.), an inferior kind of walking was possible. The exacerbation occurred 4 d after 65-kD protein administration and waned within 10–14 d. We observed this feature in an SCW-induced arthritis that had just come to expression (15 d) and in a fully established chronic phase (35 or 55 d). This observation was not completely unexpected because of our (56, 57, and van den Broek, M. F., M. C. J. van Bruggen, A. J. Severijnen, and W. B. van den Berg, manuscript submitted for publication) experience and that of others (58, 59) with reactivations of joint inflammation in different arthritis models. Exacerbations of arthritis occur after systemic challenge with small amounts of the relevant antigen and the expression thereof is dependent on antigen-specific T lymphocytes.

Tolerance induction to SCW by 65-kD protein demands immunological similarity between 65-kD protein and SCW. This similarity is present both at the T cell and at the humoral level. Looking at epitopes with the use of anti-65-kD monoclonals, we found that (at least) two distinct 65-kD protein-specific epitopes were present in SCW preparations. At the moment studies are undertaken to see whether the minimal epitope as defined in AA (amino acid 180–188, [13]) also plays a similar role in SCW-induced arthritis.

Summary

We report that streptococcal cell wall (SCW)-induced arthritis in rats, a T cell-dependent chronic, erosive polyarthritis, can be prevented by pretreatment of the rats with the mycobacterial 65-kD heat shock protein. This 65-kD protein shows extensive amino acid homology with prokaryotic and eukaryotic 65-kD heat shock proteins and is a ubiquitous bacterial common antigen.

Both the clinical and histopathologic manifestations of the arthritis were prevented completely when rats were pretreated with 50 μ g of 65-kD protein intraperitoneally at 35, 25, 15, or 5 d before administration of SCW. In such protected rats, SCW-specific T cell responses were suppressed, as compared with responses in arthritic rats. Pretreatment with 65-kD protein had no effect on the production of antibodies against SCW, on a nonspecific inflammatory reaction (zymosan-induced arthritis), or on general cellular immunity *in vivo* (delayed type hypersensitivity reaction to a nonrelated protein antigen). Furthermore, the protection against SCW arthritis was transferable by splenic T cells to naive recipients.

Our data show that pretreatment with the 65-kD mycobacterial heat shock protein protects rats against a subsequent bacterium-induced arthritis. This protection is immunologically specific and resides in the lymphoid cell population.

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