Activation of T Cells Recognizing Self 60-kD Heat Shock Protein Can Protect against Experimental Arthritis

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Summary

Lewis rats are susceptible to several forms of experimental arthritis-induced using heat-killed *Mycobacterium tuberculosis* (adjuvant arthritis, or AA), streptococcal cell walls, collagen type II, and the lipoidal amine CP20961. Prior immunization with the mycobacterial 65-kD heat shock protein (hsp65) was reported to protect against AA, and other athritis models not using *M. tuberculosis*, via a T cell–mediated mechanism. Hsp65 shares 48% amino acid identity with mammalian hsp60, which is expressed at elevated levels in inflamed synovia. Several studies have reported cross-reactive T cell recognition of mycobacterial hsp65 and self hsp60 in arthritic and normal individuals. We previously described nine major histocompatibility complex class II–restricted epitopes in mycobacterial hsp65 recognized by Lewis rat T cells. Of these only one, covering the 256-270 sequence, primed for cross-reactive T cell responses to the corresponding region of rat hsp60. Here we have tested each hsp65 epitope for protective activity by immunizing rats with synthetic peptides. A peptide containing the 256-270 epitope, which induced cross-reactive T cells, was the only one able to confer protection against AA. Similarly, administration of a T cell line specific for this epitope protected against AA. Preimmunization with the 256-270 epitope induced T cells that responded to heat-shocked syngeneic antigen-presenting cells, and also protected against CP20961-induced arthritis, indicating that activation of T cells recognizing an epitope in self hsp60 can protect against arthritis induced without mycobacteria. Therefore, in contrast to the accepted concept that cross-reactive T cell recognition of foreign and self antigens might induce aggressive autoimmune disease, we propose that cross-reactivity between bacterial and self hsp60 might also be used to maintain a protective self-reactive T cell population. This discovery might have important implications for understanding T cell–mediated regulation of inflammation.

As the pathogenic mechanisms underlying rheumatoid arthritis (RA) remain unclear, extensive use is made of experimental rodent arthritis models. Lewis rats are susceptible to arthritis after administration of various arthritogenic preparations including heat-killed *Mycobacterium tuberculosis* (Mt)[1] suspended in IFA (adjuvant arthritis, or AA) (1) streptococcal cell walls (SCW-arthritis) (2), collagen type II (3), and the lipoidal amine CP20961 (4).

The antigenic epitope recognized by the arthritogenic T cell clone A2b (generated from a rat with AA) was identified as residues 180-188 of the 65-kD mycobacterial heat shock protein (hsp65) (5). After this discovery, attempts to induce AA by immunization with hsp65 alone failed, but did induce a state of resistance to AA induction using Mt (5, 6) which was transferable to naive rats using splenic T cells (7). Preimmunization with hsp65 has subsequently been reported to confer protection against other forms of experimental arthritis induced with SCW (8), collagen type II (6), or CP20961 (6) in Lewis rats and pristane in mice (9).

Mycobacterial hsp65 belongs to the hsp60 family of heat shock proteins that is highly conserved throughout evolution, and shares 48% amino acid identity with the mammalian homologue, P1 or hsp60 (10). Expression of mammalian hsp60 is known to be upregulated as a physiological response to various stressful stimuli, and has been shown to be elevated in inflamed synovia of arthritis patients (11, 12) and rats with AA (13). There have been numerous reports of cross-reactive T cell recognition of mycobacterial hsp65 and self hsp60 (14–17). This led to the suggestion that T cells specific for self hsp60 might in some way play a role

[1] *Abbreviations used in this paper:* AA, adjuvant arthritis; DDA, dimethyl dioctadecyl ammonium bromide; hsp65, mycobacterial 65-kD heat shock protein; hsp60, mammalian 60-kD heat shock protein; M.256-270, synthetic peptide of mycobacterial hsp65 256-270 sequence; Mt, heat-killed *Mycobacterium tuberculosis*; PLNC, primed lymph node cell; R.256-270, synthetic peptide of rat hsp60 256-270 sequence.

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in the regulation of the inflammatory response, and that the protective effect of preimmunization with hsp65 involved cross-reactive T cells recognizing self hsp60 (16). Alternatively, induction of T cell responses to mycobacterial-unique epitopes after hsp65 preimmunization might mediate the protective mechanism.

To test these hypotheses, we are undertaking a detailed analysis of Lewis rat T cell responses to epitopes in mycobacterial hsp65 and rat hsp60. Previously we reported MHC class II ($RT1.B^l$)–restricted T cell responses to nine hsp65 epitopes, and that the pattern of responsiveness differs after immunization with hsp65 versus whole Mt (18). Here we report that immunization with individual synthetic peptides primes for T cell responses to eight of these epitopes. To identify the T cell epitopes involved in the protective effects of preimmunization with hsp65, the effects of preimmunization with peptides were studied. A peptide corresponding to hsp65(256-270) induced effective protection against the development of AA. Peptides containing other epitopes had no effect. Administration of a T cell line specific for the 256-270 epitope at the time of AA induction with Mt also protected against AA. Preimmunization with 256-270 also protected against CP20961-induced arthritis. The 256-270 epitope activated T cells that also recognized the 256-270 sequence of rat hsp60. Thus activation of T cells capable of responding to self hsp60 has a protective effect in arthritis. These findings may have important implications for vaccine development in human arthritic diseases.

Materials and Methods

Animals. Male inbred Lewis rats ($RT1^l$ MHC haplotype) were obtained from the University of Limburg (Maastricht, The Netherlands). Rats were 5–8-wk-old at the start of each experiment.

Antigens and Adjuvants. Mt strain H37Ra was obtained from Difco (Detroit, MI). Purified recombinant hsp65 of *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) (which is identical to *M. tuberculosis* hsp65) was kindly provided by Dr. J.D.A. van Embden (National Institute of Public Health and Environmental Hygiene, Bilthoven, The Netherlands). IFA (Difco) and dimethyl dioctadecyl ammonium bromide (DDA; Eastman Kodak, Rochester, NY) (19) were used as adjuvants. DDA was prepared as a 20-mg/ml suspension in PBS and sonicated to produce a gel which was mixed 1:1 with Ag solution before immunization. The synthetic adjuvant *N,N*-dioctadecyl-N^1,N^1-bis[2-hydroxyethyl]propanediamine (CP20961, or avridine) (4) was obtained from Pfizer (Sandwich, Kent, UK).

Peptides were prepared by automated simultaneous multiple peptide synthesis (SMPS). The SMPS set-up was developed using a standard autosampler (model 221; Gilson Medical Elec. Inc., Middleton, WI) as described previously (20). Briefly, for the concurrent synthesis of peptides, standard Fmoc chemistry was employed using Pfp-activated amino acids (Dhbt for serine and threonine) in a sixfold molar excess and Hobt as catalyst. Peptides were obtained as COOH-terminal amides from 6 mg resin/peptide (0.33 meq/g PAL™ resin; Millipore Corp., Bedford, MA). 15-mer peptides containing individual hsp65 epitopes described previously (18) were used. Sequences of these peptides are shown in Table 1. Extensive use was made of the hsp65 peptide 256-270 (M.256-270) and the homologous peptide of rat hsp60 (R.256-270)

Immunizations and Primed Lymph Node Populations. Rats were immunized with 50 μg of synthetic peptide in PBS/DDA in each hind footpad (i.e., 100 μg/rat). 10 d later, draining popliteal lymph nodes were removed, disaggregated, washed three times, and used as a source of primed lymph node cells (PLNC). In some experiments, PLNC were derived as pooled inguinal and popliteal lymph nodes from AA rats 35–42 d after Mt immunization. In control experiments, splenocytes and lymph nodes from unimmunized rats and PLNC from rats immunized with PBS/DDA alone were used.

Tissue Culture Reagents. IMDM supplemented with 5% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all from GIBCO BRL, Gaithersburg, MD) and 5×10^{-5} M 2-ME was used as culture medium. Cell populations were washed in IMDM without supplements.

T Cell Proliferation Assays. PLNC were cultured in triplicate in 200 μl flat bottom microtiter wells (Costar Corp., Cambridge, MA) at 2×10^5 cells per well with or without antigen. PLNC were tested for responsiveness to individual peptides and hsp65 at 20 μg/ml. Con A (2 μg/ml) was used as a positive control for T cell proliferation. Cultures were incubated for 96 h at 37°C in a humidified atmosphere of 5% CO_2. Cultures were pulsed for the final 16 h with [^{3}H]TdR (Amersham International, Amersham, Bucks, UK, 1 μCi/well) and TdR uptake measured using a liquid scintillation β counter. Assays using T cell lines were performed as above using 2×10^4 line cells/well with irradiated (30 Gy) syngeneic accessory cells (2×10^5 splenocytes/well).

Results are expressed as mean counts per minute of triplicate cultures. In experiments where responses to Ag were low, responses were considered significant if the stimulation index (mean counts per minute with Ag divided by mean counts per minute without Ag) was greater than 2.5 and Student's *t* tests gave $p < 0.01$.

T Cell Lines. The generation of $RT1.B^l$-restricted T cell lines with specificity for individual mycobacterial hsp65 epitopes from hsp65 immunized rats has been described previously (18). Short-term epitope-specific T cell lines were also generated from rats immunized with synthetic peptides. Peptide-PLNC were cultured at 5×10^6/ml in culture medium supplemented with 2% normal rat serum (NRS) in the presence of 10 μg/ml peptide. After 3 d, viable cells were harvested using a Ficoll-Isopaque gradient and cultured for a further 4 d in culture medium plus 5% FCS and 5% T cell growth factor (TCGF) (Con A–activated rat spleen supernatant). 7 d after initial stimulation, lines were restimulated with irradiated spleen accessory cells and 10 μg/ml peptide in culture medium plus NRS. Lines were maintained in this 7-d restimulation cycle.

Induction and Clinical Assessment of Experimental Arthritis. Arthritis was induced by a single intradermal injection in the base of the tail. AA was induced with 0.5 mg Mt suspended in 100 μl IFA. CP20961 arthritis was induced with 2 mg CP20961 in 100 μl light mineral oil (Sigma Chemical Co., St. Louis, MO). Rats were examined daily for clinical signs of arthritis. Severity of arthritis was assessed by scoring each paw from zero to four based on degree of swelling, erythema, and deformity of the joints. Thus the maximum possible arthritis score was 16 (3).

Modulation of Arthritis with hsp65 Peptides and Epitope-specific T Cell Lines. Synthetic peptides corresponding to eight T cell epitopes present in mycobacterial hsp65 were tested for protective effects on arthritis development. Rats were immunized, as described above for PLNC generation, with 100 μg of individual peptide 7 d before arthritis induction. Control rats received PBS/DDA.

Epitope-specific T cell lines were also tested for protective activity by intravenous administration of lines at the time of arthritis induction. T cell lines were restimulated in vitro with irradiated spleen APC and specific peptide. 3 d later, T cell blasts were har-

vested by Ficoll gradient, washed, and applied to a second Ficoll gradient to remove any contaminating APC. T cells were washed twice in wash medium and twice in PBS and finally suspended at 2.5 × 10^7/ml in PBS. Immediately before injection of Mt, 200 μl (i.e., 5 × 10^6) T cells were injected intravenously in the tail vein.

mAbs. Anti-MHC mAbs were added to proliferation assays to determine the MHC restriction of T cell line responses to peptides. OX6 (anti-RT1.B, class II), OX17 (anti-RT1.D, class II), OX18 (anti-RT1.A, class I), and UD15 (antichloramphenicol control antibody) were used. All antibodies were mouse IgG1 and were added at concentrations of 5–40 μg/ml.

FACS® Analysis. FACS® analysis was used to phenotype T cell lines. Cells were incubated with either R73 (anti-α/β TCR), W3/25 (anti-CD4) or OX8 (anti-CD8), all mouse IgG1 antibodies. Second-step staining was with FITC-conjugated goat anti-mouse Ig (Becton Dickinson & Co., Mountain View, CA). Cells were analyzed using a FACScan® analyzer (Becton Dickinson & Co.).

Results

Immunization with hsp65 Peptides Primes for Epitope-specific T Cell Responses. To determine whether immunization of rats with synthetic peptides could prime for T cell reactivity to previously described hsp65 epitopes (18), we examined in vitro proliferative responses after immunization with nine peptides, each containing individual T cell epitopes. PLNC isolated 10 d after immunization were tested for responsiveness to the immunizing peptide, overlapping peptides, and to hsp65 (Table 1). PLNC responses were observed after immunization with seven of the peptides. Responses were not seen after immunization with peptides 386-400 and 511-525.

Bulk stimulation generated T cell lines to eight of the nine peptides tested (attempts to generate a line against peptide 386-400 were unsuccessful). These eight lines were tested for specificity after four in vitro restimulations (Table 1). All responded to the immunizing peptide and, to some extent, to hsp65. Also, the lines were tested for responsiveness to overlapping peptides and, in all but one case, showed identical response patterns to T cell lines generated previously against the same epitopes after immunization with whole hsp65. These findings indicate that T cells activated in vivo by immunization with hsp65 or synthetic peptides recognize the same core epitopes.

The one exception to this was the T cell line generated from rats immunized with the 86-100 peptide. A T cell line specific for this peptide generated from rats immunized with hsp65 (18) responded to peptides 86-100 and 91-105 (i.e., recognizing a core epitope of 91-100). The T cell line generated after immunization with the 86-100 peptide also responded to peptides 81-95 and 91-105. This suggested the presence of two T cell populations, one recognizing residues 86-95, the other recognizing residues 91-100. Interestingly, the sequences of mycobacterial hsp65 and rat hsp60 covering residues 86-95 are identical. Accordingly, the line responded to peptide 81-95 of rat hsp60. Thus, immunization with the mycobacterial hsp65 86-100 peptide, but not intact hsp65, could prime for responsiveness to this epitope in self hsp60.

Previously we demonstrated that line H.52 (generated from hsp65-immunized rats and recognizing epitope 256-265) also

Table 1. *Immunization with hsp65 Peptides Primes for T Cell Responses*

Immunizing peptide	In vitro peptide	PLNC	T cell line	Response after hsp65 immunization*
		cpm		
86-100	0	2,518	1,127	
	81-95	15,296	15,785	–
	86-100	57,925	49,422	+
	91-105	1,999	18,444	+
	Rat 86-100	6,196	25,535	–
176-190	0	1,784	1,255	
	171-185	3,082	1,640	–
	176-190	57,707	31,400	+
	181-195	1,916	1,653	–
	180-188	7,859	23,275	+
211-225	0	986	1,479	
	206-220	645	988	–
	211-225	39,765	121,978	+
	216-230	11,117	102,341	+
226-240	0	1,286	882	
	221-235	7,381	100,306	+
	226-240	22,761	152,071	+
	231-245	1,655	862	–
256-270	0	2,448	762	
	251-265	6,391	66,063	+
	256-270	16,423	69,037	+
	261-275	2,553	646	–
	Rat 256-270	4,152	14,987	+
396-410	0	3,399	2,095	
	391-405	2,280	39,335	+
	396-410	8,477	88,916	+
	401-415	3,211	2,011	–
446-460	0	1,928	1,028	
	441-455	4,715	17,805	+
	446-460	22,918	38,324	+
	451-465	2,130	1,283	–
511-525	0	2,804	537	
	506-520	2,754	61,293	+
	511-525	3,164	129,373	+
	516-530	2,821	1,231	–

Rats were immunized with synthetic peptides containing individual hsp65 epitopes (100 μg peptide/DDA per rat). 10 d later, PLNC were isolated and tested for responses to overlapping peptides (20 μg/ml shown here). Peptide-specific T cell lines were generated by bulk in vitro stimulation of PLNC with immunizing peptide. Lines were tested for responses to overlapping peptides (10 μg/ml). All PLNC (except 511-525-PLNC) and T cell lines showed signficant responses to 20 μg/ml hsp65 (data not shown). Results are expressed as mean cpm of triplicate cultures. All SEM were <20%. Significant responses are underlined.

* As determined previously using T cell lines generated from hsp65-immunized rats (17).

responded to the highly identical R.256-270 peptide (18). Accordingly the M.256-270 specific T cell line derived from peptide immunized rats also responded to R.256-270 (Table 1). This line also showed an increased "autoreactive" response to syngeneic APC that had been heat-shocked (1 h at 42°C before irradiation) in comparison with control APC cultured at 37°C (Table 2). This suggested that increased expression of endogenous hsp60 by APC results in presentation of this cross-reactive epitope in association with MHC for T cell recognition.

Analysis of the Ability of hsp65 Peptides to Vaccinate against Arthritis. To determine the epitope(s) in hsp65 responsible for the T cell–mediated protective effect of preimmunization with hsp65, we analyzed the effects of preimmunization with synthetic peptides containing individual epitopes on the development of AA (Fig. 1). The eight peptides which primed for epitope-specific T cells were tested. Rats were immunized with 100 μg of peptide 7 d before AA-induction with Mt.

Preimmunization with M.256-270 resulted in clear protective effects against AA development. The mean maximum arthritis score after M.256-270 preimmunization was 2.4 (24 rats in five separate experiments) compared with a mean maximum score of 11.5 for control rats preimmunized with PBS. Of the 24 rats preimmunized with M.256-270, 12 did develop clinical signs of arthritis, which were milder than those developed by control rats. Also, whereas control rats suffered permanent joint deformities that persisted after the initial arthritis had subsided, the M.256-270–preimmunized rats which

Table 2. *Heat-shocked APC Stimulate hsp65(256-270)-specific T Cells*

T cell line:	P.M52	H.46
Specificity:	256-265	226-235
	cpm	
T cells (no APC)	44	22
37°C APC (no antigen)	470	33
Plus mycobacterial 256-270	120,744	nt
Plus rat 256-270	18,061	nt
Plus mycobacterial 226-240	nt	162,785
Control peptide	28	34
42°C APC (no antigen)	15,960	37
Plus mycobacterial 256-270	115,626	nt
Plus rat 256-270	25,842	nt
Plus mycobacterial 226-240	nt	150,887
Control peptide	12,643	41

T cell lines (2 × 10^4/well) were cultured with APC (2 × 10^5/well) that had been cultured for 1 h at either 37°C or 42°C before irradiation. Cells were cultured with or without specific peptide as Ag (10 μg/ml). Line H.46, specific for the noncross-reactive, mycobacterial hsp65-unique, epitope 226-235 was used as a control. Each line was also tested against a control peptide (211-225 of hsp65) as an irrelevent epitope. Results are expressed as mean cpm of triplicate cultures. All SEM were <20%. *nt*, not tested.

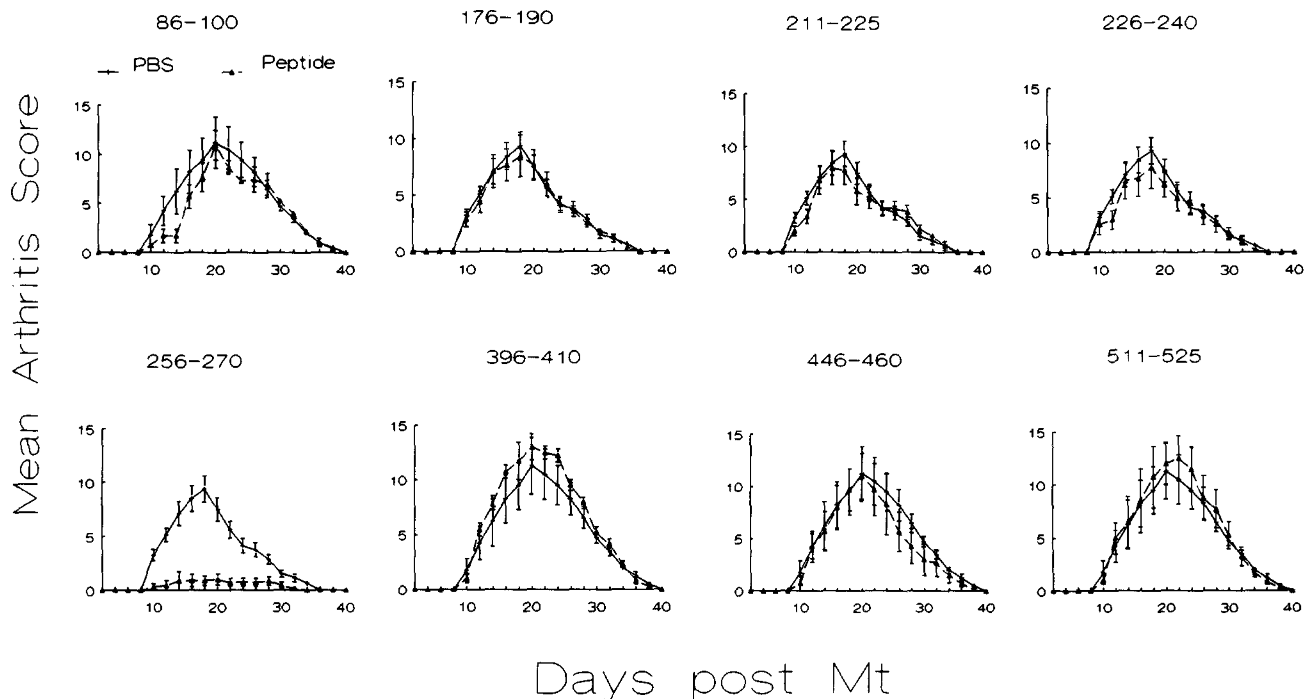

Figure 1. Modulation of AA development by preimmunization with hsp65 peptides. Rats were immunized in the hind footpads with 100 μg of individual synthetic peptides or PBS in DDA 7 d before AA induction using 0.5 mg Mt in 100 μl IFA injected intradermally at the base of the tail. Five rats were used in each preimmunization group. Arthritis scores were assessed daily from 8 d after Mt injection. Results of two separate experiments are shown (experiment 1 tested 176-190, 211-225, 226-240, and 256-270; experiment 2 tested 86-100, 396-410, 446-460, and 511-525).

developed mild AA were remarkably free of permanent deformities. Preimmunization with the other seven peptides containing hsp65 T cell epitopes had no effect on AA.

PLNC from preimmunized rats were tested for responsiveness to hsp65 epitopes 42 d after Mt administration (Fig. 2). PLNC from rats preimmunized with PBS/DDA alone showed significant responses to peptide 176-190 (containing the AA-associated 180-188 epitope) but not to any other of the defined hsp65 epitopes. PLNC from rats preimmunized with individual hsp65 peptides all showed responses to the immunizing peptide, although the level of proliferation varied. Thus, whereas preimmunization with peptides 211-225 and 446-460 induced strong responses, preimmunization with peptide M.256-270 (which protected against AA) induced marginal PLNC responses. Responses to peptide 176-190 were present in all PLNC populations (and enhanced after 176-190 preimmunization) with one important exception. PLNC from rats protected from AA by preimmunization with peptide M.256-270 showed a lack of response to 176-190. This suggested that M.256-270–specific T cells might have an inhibitory effect on potentially arthritogenic 180-188–specific T cells. As 176-190 is a codominant epitope recognized after immunization with hsp65 (18), we tested whether preimmunization with M.256-270 could inhibit responses to 176-190 in hsp65-PLNC. Preimmunization with M.256-270 7 d before immunization with hsp65 showed no significant effect on the response of PLNC to 176-190 compared with control PLNC preimmunized with PBS (data not shown). This finding suggests that the lack of responsiveness to 176-190 in PLNC from M.256-270–protected rats was not the result of a T cell–T cell suppressive activity. Rather, we favor the idea that this was due to the lack of arthritis in the protected rats.

A T Cell Line Specific for the Cross-reactive 256-265 Epitope Protects Against AA. Preimmunization with M.256-270 protected against arthritis. To determine whether this activity was the result of activation of T cells specific for this epitope, we used epitope-specific T cell lines generated previously from hsp65-immunized rats (18). T cell lines were administered to rats at the same time as Mt for AA induction. In two experiments, administration of line H.52 recognizing the 256-265 epitope clearly reduced the severity of AA compared with control animals receiving PBS (Fig. 3). The T cell lines H.36, H.43, and H.46 (specific for epitopes 180-188, 216-225, and 226-235, respectively) were also tested and had no effect on AA development. To ensure that the protective effect of line H.52 was not the result of administration of residual M.256-270 peptide carried over from the in vitro restimulation of the line, line H.46 was restimulated with specific peptide (226-240) in the presence of 10 μg/ml M.256-270. Administration of the resulting 226-235–specific T cell blasts failed to protect against AA.

Preimmunization with Peptide 256-270 also Vaccinates against CP20961-induced Arthritis. The only hsp65 peptide that protected against AA was found to be M.256-270, which induced T cell reactivity against R.256-270. This finding suggested that the hypothesis involving self-reactive T cells to account for hsp65-induced protection against arthritis might be correct. If so this mechanism would be expected to protect against arthritis induced by other compounds and not to be dependent on the use of mycobacteria. To investigate this, we tested the capacity of M.256-270 to induce protection in the CP20961-induced model. As CP20961 is a lipoidal amine, there was no possibility of this arthritogenic preparation containing an antigenic component with potential cross-reactivity with M.256-270. Preimmunization with M.256-

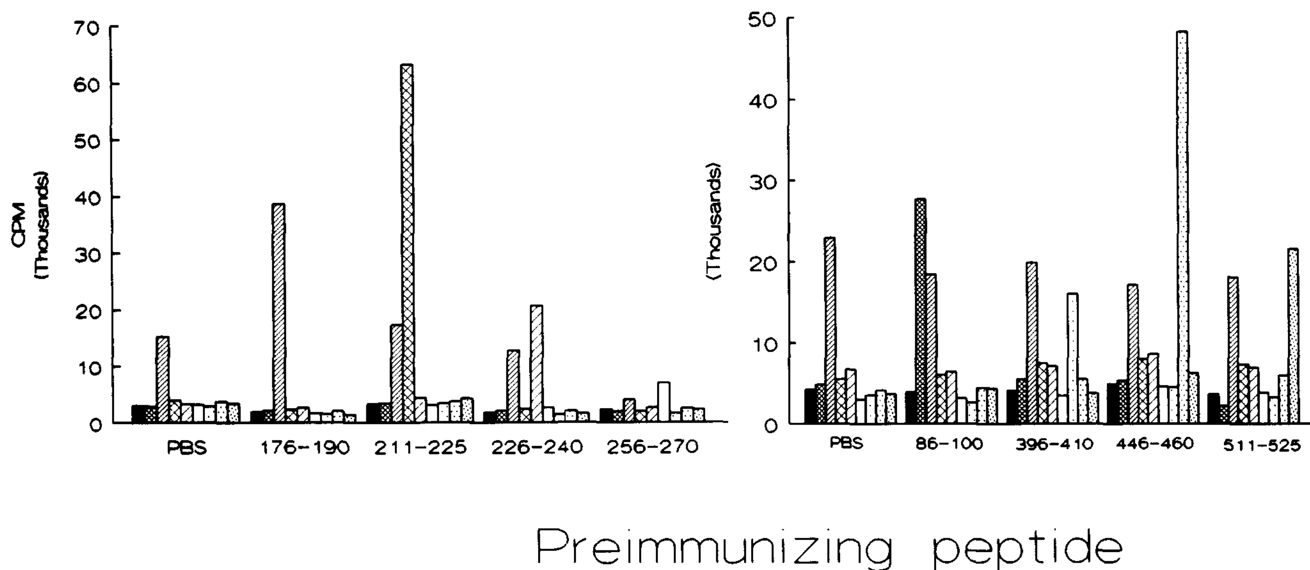

Figure 2. PLNC responses of peptide-preimmunized AA rats. Rats were preimmunized and Mt-immunized as described in Fig. 1. PLNC (pooled inguinal and popliteal lymph node cells from rats used in Fig. 1) were isolated 42 d after Mt immunization and tested for responses to hsp65 peptides containing defined T cell epitopes (20 μg/ml shown here). All PLNC responded to hsp65 and Mt (responses were all >50,000 cpm). All SEM were <20%. (■) Medium; (▩) 86–100; (▨) 176–190; (▧) 211–225; (▨) 226–240; (□) 256–270; (□) 396–410; (□) 446–460; (□) 511–525.

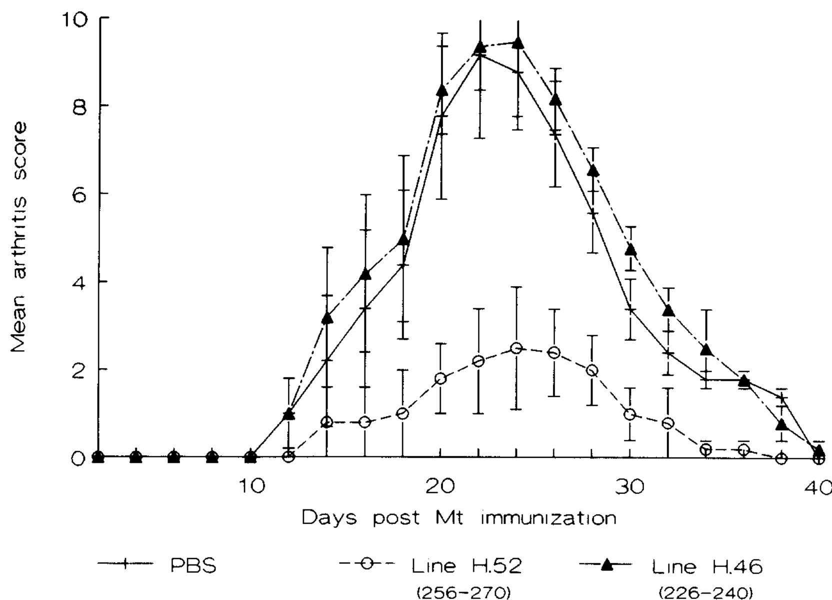

Figure 3. Modulation of AA using epitope-specific T cell lines. Rats were administered with 5 × 10^6 T cells intravenously in PBS or PBS alone at the time of AA induction (0.5 mg Mt in 100 μl IFA injected intradermally at the base of the tail). Five rats were used in each group. Arthritis scores were assessed daily from 8 d after Mt injection. Results using lines H.46 (specific for 226-235) and H.52 (specific for 256-265) are shown. Injection of lines H.36 and H.43 (specific for epitopes 180-188 and 216-225, respectively) had no effect on AA (data not shown).

270, but not a control peptide (hsp65 211-225) strongly protected rats against CP20961-induced arthritis (Fig. 4). Thus, preimmunization with M.256-270 could protect against AA and CP20961-induced arthritis, presumably via a mechanism that is not dependent on the administration of mycobacteria as a component of the arthritogenic inoculation.

Rat hsp60(256-270) Fails to Vaccinate against Arthritis. Preimmunization with M.256-270 provided protection against AA. As T cells specific for this peptide also responded to the homologous rat peptide R.256-270, we tested R.256-270 for similar protective effects (Fig. 5). In two separate experiments protection was observed after preimmunization with M.256-270, but not with R.256-270. An explanation for this discrepancy might be that immunization with R.256-270 does not prime for T cell responses. To investigate this we generated a peptide-specific T cell line from R.256-270 immunized PLNC and compared responses of this line with those of a line from M.256-270–immunized PLNC (Fig. 6). The line generated against M.256-270 responded to peptides representing M.256-270, M.251-265, and a shorter "core" peptide M.256-265, and cross-reacted with R.256-270 and (weakly) R.256-265. In contrast, the line generated against R.256-270 responded to R.256-270, R.261-275, and R.261-270, but not to any mycobacterial peptide.

Thus immunization with M.256-270 primed T cells recognizing the cross-reactive 256-265 core epitope, whereas immunization with R.256-270 primed for responses to the rat-unique 261-270 core epitope (Fig. 6 *B*). The R.256-270 specific line failed to respond to a peptide representing R.260-269 (data not shown) indicating that all five COOH-terminal

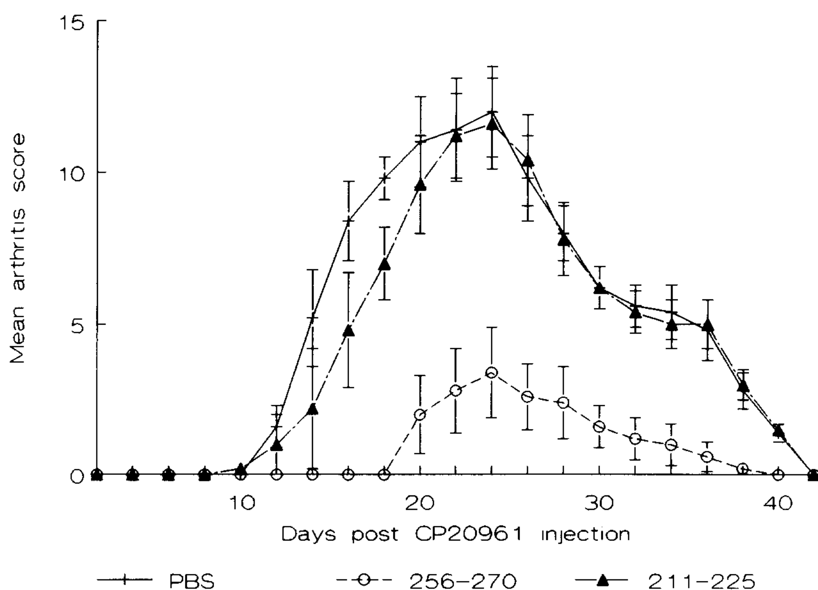

Figure 4. Modulation of CP20961-induced arthritis by preimmunization with hsp65 peptides. Rats were immunized in the hind footpads with 100 μg of individual synthetic peptides (211-225 or 256-270) or PBS in DDA 7 d before arthritis induction (2 mg CP20961 in 100 μl mineral oil injected intradermally at the base of the tail). Five rats were used in each preimmunization group. Arthritis scores were assessed daily from 8 d after CP20961 injection.

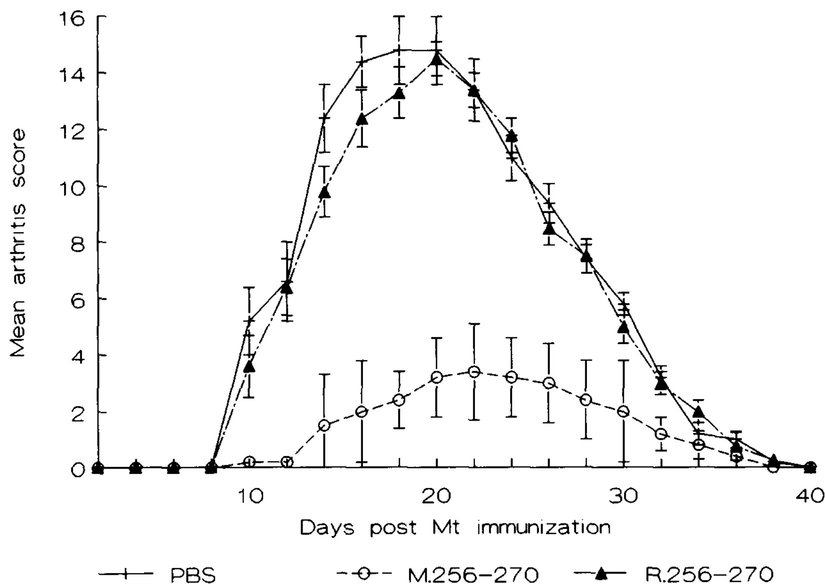

Figure 5. Preimmunization with rat hsp60(256-270) fails to protect against AA. Rats were immunized in the hind footpads with 100 μg of individual synthetic peptides or PBS in DDA 7 d before AA induction (0.5 mg Mt in 100 μl IFA injected intradermally at the base of the tail). Five rats were used in each preimmunization group. Arthritis scores were assessed daily from 8 d after Mt injection. Preimmunization with a control peptide (hsp65 211-225) did not influence AA development (data not shown).

residues of R.256-270 were essential for stimulation of this line. None of these five residues share identity with the mycobacterial sequence (Fig. 6 *B*), presumably accounting for the lack of responsiveness to M.256-270.

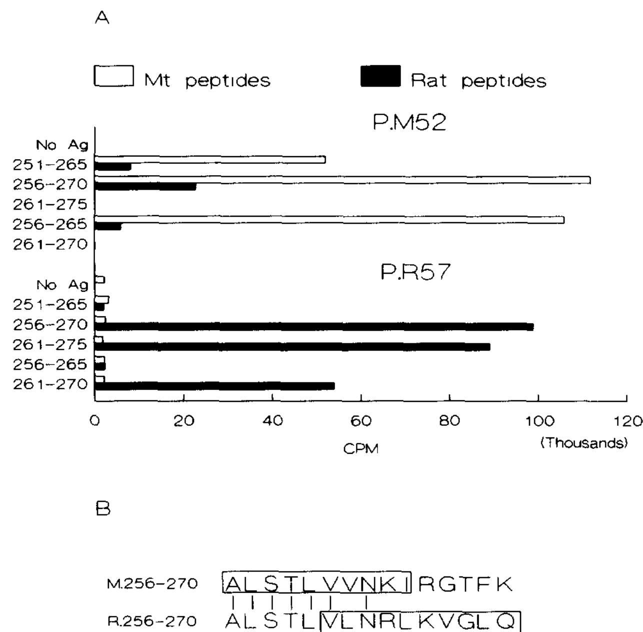

Figure 6. Immunization with hsp65(256-270) and rat hsp60(256-270) primes for T cell responses to distinct epitopes. (*A*) Rats were immunized in the hind footpads with either M.256-270 or R.256-270 in DDA. 7 d later, PLNC were isolated and restimulated in vitro with the immunizing peptide. The resulting T cell lines (line P.M52 recognizing M.256-270, and line P.R57 recognizing R.256-270) were tested for responses to mycobacterial and rat peptides in the presence of irradiated syngeneic spleen APC. All SEM were <20%. (*B*) Comparison of mycobacterial and rat 256-270 sequences. The distinct epitopes recognized by lines P.M52 and P.R57 are boxed. (10).

Furthermore, the T cell lines generated against M.256-270 and R.256-270 were found to have different MHC restrictions. Peptide-induced proliferation of the M.256-270–specific line was inhibited by the OX6 anti-RT1.B mAb, whereas the R.256-270–specific line was inhibited by the OX17 anti-RT1.D mAb (data not shown). FACS® analysis revealed that both lines were α/β-TCR^+CD4^+ (data not shown).

Discussion

Vaccination with mycobacterial hsp65 protects Lewis rats against not only AA (5, 6), but also arthritis induced with SCW (8) collagen type II (6) or the lipoidal amine CP20961 (6). T cell reactivity against hsp65 is believed to be involved in the protective mechanism(s) (7). Previously we characterized the T cell response to hsp65 in Lewis rats and identified nine epitopes recognized in association with the $RT1.B^l$ MHC class II molecule (18). In this study we have analyzed the ability of individual T cell epitopes to influence the development of AA. Rats were preimmunized with synthetic peptides containing individual epitopes 7 d before AA induction with Mt. This approach led to a striking protective effect in rats preimmunized with peptide M.256-270. None of the other peptides tested showed any influence on AA development. The T cell line H.52, originally generated from hsp65-immunized rats and specific for epitope 256-265 also showed a protective effect on AA development when injected intravenously at the time of Mt administration.

Our finding that preimmunization with peptide 176-190 (containing the AA-associated 180-188 epitope) had no effect on AA onset is in contrast with previous reports of protection after preimmunization with peptide 180-188 (21, 22). This difference might be due to the different preimmunization regimens adopted. We immunized once with peptide in the footpads 7 d before Mt immunization using DDA as adjuvant, whereas the previous studies immunized intraperi-

toneally with peptide in IFA three times on days −35, −20, and −5. Lymph node responses to the 176-190 epitope were absent in rats protected by preimmunization with M.256-270. As preimmunization with M.256-270 did not influence lymph node cell responses to 176-190 in rats immunized with hsp65, we conclude that a direct suppressive activity of M.256-270–specific T cells on 176-190 specific (arthritogenic) T cells did not account for this effect. Previously we showed that lymph node cells from rats immunized with the AA-inducing protocol (Mt/IFA) did not respond to 176-190 before the development of clinical AA at ∼14 d (18). Therefore, we suggest that significant responses to 176-190 in polyclonal lymphoid populations after Mt/IFA immunization correlate with development of overt AA.

Line H.52 recognizes the 256-270 sequence of rat hsp60 (18). Similarly, T cell lines derived from rats immunized with M.256-270 responded to R.256-270. These lines also responded to heat-shocked APC, indicating presentation of the endogenous self hsp60 epitope in association with MHC class II for T cell recognition as a result of increased hsp60 expression. These findings support the hypothesis that hsp65 preimmunization protects against arthritis by activation of T cells recognizing a shared epitope in rat hsp60. This hypothesis does not require the "protective" T cells to recognize a mycobacterial component in the joint and therefore provides an attractive single mechanism for explaining hsp65-induced resistance to models not employing bacterial-derived arthritogens. The most notable of these models in the Lewis rat is induced with CP20961, a lipoidal amine with no possible antigenic cross-reactivity with hsp65 (4). It is encouraging that immunization with M.256-270 also protected against CP20961-induced arthritis.

Thus, immunization with M.256-270 activated T cells responsive to R.256-270 and protected against two forms of arthritis induced by different arthritogens and (presumably) having different pathogenic mechanisms. These findings suggest strongly that the mechanism underlying the protective effect involves T cell recognition of the self hsp60 epitope expressed by APC in the inflamed joint. Raised expression in the joint due to cell stress during inflammation (11–13) would make hsp60 a reliable target antigen for regulatory T cell recognition controlling inflammatory responses. The exact nature of the regulatory mechanism activated by the hsp60 "alarm signal" remains to be elucidated. If the self hsp60-specific T cells display a T_H2 pattern of cytokine secretion, they might be expected to have antiinflammatory effects via inhibitory effects of IL-10 on macrophage production of IL-12, and IL-4 on IFN-γ production by "auto-aggressive" T_H1 cells recognizing self antigens in the joint (23). Alternatively, production of TGF-β by the regulatory cells might have an inhibitory effect on the autoaggressive T cell population (24). A further possibility is that regulatory T cells have cytotoxic activities on APC expressing increased hsp60 and MHC class II levels. $CD4^+$ T cells capable of MHC class II–restricted lysis of macrophages pulsed with self hsp60 peptides have been isolated from healthy human donors (14) and RA patients (17). Experiments to determine cytotoxic activity and cytokine profiles of M.256-270–specific T cells are in progress.

Interestingly, preimmunization with rat 256-270 failed to protect against AA. T cell lines derived from rats immunized with M.256-270 and R.256-270 recognized two distinct epitopes. Immunization with M.256-270 induced $RT1.B^l$-restricted T cells specific for the cross-reactive core epitope 256-265. Immunization with R.256-270 induced $RT1.D^l$-restricted T cells specific for the rat-unique core epitope 261-270. Thus the protective effect observed here required T cell recognition of the 256-265 epitope in association with $RT1.B^l$. The relatively weak responses to R.256-270 after M.256-270 immunization suggest the trimolecular interaction of R.256-270, $RT1.B^l$, and TCR was of relatively low affinity. The strong proliferative responses of the R.256-270-specific T cell line suggest that the R.256-270, $RT1.D^l$, TCR interaction might be of high affinity. The rat-unique R.261-270 epitope might be cryptic (i.e., not expressed with MHC class II after processing of endogenous hsp60 by APC and therefore not available for recognition by potentially protective T cells). If so, T cells with high affinity for this epitope would not be negatively selected in the thymus and would dominate the T cell response after immunization with R.256-270, preventing development of an effective response to the protective 256-265 epitope. If differing affinities of the two interactions result from $RT1.D^l$ having a higher binding affinity than $RT1.B^l$ for R.256-270, a form of "determinant capture" (25) could account for this effect. Analysis of the binding affinities of both MHC class II molecules for R.256-270 and M.256-270 will clarify this point. Experiments testing whether immunization with the R.256-265 core epitope induces protective T cells are in progress. Thus, therapeutic applications may require the use of bacterial hsp60 epitopes that induce relatively low affinity cross-reactive responses to endogenously processed self hsp60. Any self hsp60 epitopes that prove strongly immunogenic when administered as synthetic peptides may well be cryptic as high affinity responses to epitopes of naturally processed endogenous hsp60 would be controlled by normal mechanisms of thymic negative selection and peripheral tolerance.

The aim of this study was to define the T cell epitope(s) involved in protection induced by whole hsp65. Our results provide evidence that T cells recognizing an appropriate self hsp60 epitope have a beneficial influence on arthritis development. The conventional view is that T cells with cross-reactivity for foreign Ag and structurally similar self Ags may play a role in autoimmune pathogenesis (26). In addition, we now suggest that T cells with low affinity for self hsp60 may be used as a normal Ag-specific mechanism of regulating inflammation. These T cells would be maintained in the periphery through stimulation by shared epitopes in hsp60 cognates of commensal bacteria. On this point it is noteworthy that Fisher rats (normally arthritis resistant) are susceptible to AA when raised in germ-free conditions (27), and resistance can be re-established by infection with *Escherichia coli* (28). Also, it has been reported that BCG treatment has beneficial effects in diabetes (29) and that vaccination with a peptide corresponding to mouse hsp60 (437-460) can protect against diabetes in NOD mice (30). Recent evidence from studies in arthritis patients lend support to the idea of T cell

recognition of self hsp60 as a regulatory mechanism. Two T cell lines generated from a patient with a short history of RA responded to epitopes in the highly conserved 243-265 region of hsp65, and one cross-reacted with the homologous human hsp60 peptide (17). Also, juvenile chronic arthritis patients with mild oligoarticular disease of favorable prognosis show T cell responses to recombinant human hsp60 (31), whereas patients with severe disease do not (Prakken, A. B. J., manuscript in preparation). If these findings can be extended to show that patients with severe disease lack appropriate responses to self hsp60, immunotherapy by vaccination for T cell responsiveness to self hsp60 would be an attractive possibility.

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