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*J Immunol* 1998; 160:4628-4634; 
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An Immunodominant Epitope from Mycobacterial 65-kDa Heat Shock Protein Protects Against Pristane-Induced Arthritis

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Previous studies showed that mice with pristane-induced arthritis (PIA) and those protected from the disease by preimmunization with mycobacterial 65-kDa heat shock protein (hsp65) possess raised immune responses to hsp65. Additionally, T cells from hsp65-protected mice, but not from pristane-injected or normal mice, produced the Th2-associated cytokines IL-4, IL-5, and IL-10 in response to stimulation with hsp65. Here we demonstrate that the specificity of the immune response to hsp65 and related heat shock protein (hsp) differs between protected and PIA mice. T cells from hsp65-protected mice respond to the bacterial hsps tested but not to the mammalian homologue, hsp58. Similarly, they exhibit high serum titers of anti-hsp65 Abs, yet they have virtually undetectable levels of anti-hsp58 IgG. By contrast, both cellular and humoral immune responses are detectable to bacterial and mammalian hsps in mice with PIA. An immunodominant T cell epitope has been identified in hsp65-protected mice corresponding to amino acids 261–271 from hsp65. Immunization of mice, either before or after the induction of arthritis, with this bacterial peptide, but not its mammalian homologue, protects mice from the development of PIA, and protection is associated with the production of Th2-type cytokines. Other experiments revealed that T cells primed with bacterial 261–271 or the mammalian homologue do not cross-react at the proliferative or cytokine level. These results demonstrate that an hsp65 peptide-specific Th2 response confers protection from PIA but do not support the idea that protection is mediated by a cross-reaction with self hsp58 in the joints. The Journal of Immunology, 1998, 160: 4628–4634.

A proportion of mice injected i.p. with the paraffin oil pristane (2,6,10,14-tetramethylpentadecane) develop a chronic inflammatory arthritis in the peripheral joints between 60 and 200 days later depending on the strain of mice (1–3). The time course of pristane-induced arthritis (PIA) distinguishes it from other established animal models resembling rheumatoid arthritis (RA) such as adjuvant arthritis, streptococcal cell wall arthritis, and collagen-induced arthritis. Nevertheless, the histopathologic changes in the affected joints of mice with PIA are similar to those in RA with polymorphonuclear cell infiltration and synoviocyte hyperplasia with cartilage erosions and the formation of pannus (1, 2). Moreover, PIA is immunologically driven. Irradiated mice fail to develop the disease unless they are reconstituted with lymphoid cells containing CD4 T cells (2, 4). Similarly, nude mice are resistant to the development of PIA, and treatment of susceptible strains with anti-CD4 Ab ameliorates disease (3, 5).

How could an oil injected at one site induce arthritis at another site via an immune mechanism? We have previously suggested that pristane promotes an autoimmune response induced by sensitization to microbes in the normal environment (6, 7). A number of observations lend credence to this suggestion. For example, specific-pathogen-free mice maintained under sterile conditions in an isolator are resistant to the development of PIA while the return of such mice to the conventional environment restores their susceptibility to the disease (6). The possibility that the 60-kDa family of heat shock proteins (hsp) are stimulatory and target Ags arose from the discovery that mice immunized with mycobacterial hsp65 in IFA before pristane challenge are protected from PIA (7–9). This protective effect is specific to hsp65 and is not induced by the Escherichia coli equivalent 60-kDa hsp (GroEl) or other unrelated Ags incorporated in IFA (7–9) and cannot be attributed to antigenic competition (9). A further argument follows from the fact that this family of hsps are dominant Ags in the immune response to microorganisms, despite their extraordinarily high sequence conservation throughout the eukaryotic and prokaryotic kingdoms (10). Thus, mycobacterial hsp65 is studded with self epitopes for any animal with an immune system, and hsp58, its mammalian equivalent, is a normal constituent of all mammalian cells, although its synthesis is increased by many different forms of cellular stress. Since hsp58 has been detected in the joints of patients with RA (11) and PIA (12) and T cells from mice with PIA react with joint extracts (7, 13), it seems reasonable to postulate that hsp58 could be a target Ag in the joints of mice developing PIA. This hypothesis may explain the paradox that both mice with PIA and animals protected from the development of arthritis by hsp65 preimmunization exhibit elevated immune responses to the 65-kDa...
mycobacterial heat shock protein as compared with age-matched normal mice (7).

We have recently demonstrated that the qualities of the anti-hsp65 response of T cells derived from mice with pristane-induced arthritis and hsp65-protected mice differ. Use of a sensitive CεELISA to measure Ag-driven lymphokine production revealed that spleen cells from hsp65-protected mice, but not from pristane-injected or normal mice, produced the Th2-associated cytokines IL-4, IL-5, and IL-10 in response to stimulation with hsp65. By contrast, the Th1-associated cytokines IL-2 and IFN-γ were produced by spleen cells from mice of all groups in response to hsp65 (14). As far as specificity of the response is concerned, it may be predicted that only mice with PIA develop autoimmune responses to the 60-kDa family of hsp, whereas the response of mice pre-immunized with hsp65 could be restricted to microbial specific determinants. In other words, the specificity of the response elicited by immunization with hsp65 in IFA differs from that induced in mice with PIA. The prime purpose of this work was thus to determine whether the specificity of the hsp65-reactive T cells from normal, hsp65-protected, and PIA mice differed. This was achieved by comparing the ability of these T cells to proliferate in vitro in response to bacterial hsp65 and mammalian hsp58 and to synthetic overlapping peptides corresponding to the sequence of hsp65. An immunodominant T cell epitope was identified in hsp65-protected mice corresponding to amino acids 261–271 from hsp65. The quality and specificity of the response elicited in mice immunized with this peptide were characterized along with the ability of the peptides to protect mice from the development of PIA.

Materials and Methods

Animals

Age-matched male CBA/Igα (H2-k) mice were used in each experiment (originally a kind gift from Professor H. S. Micklem, Department of Zoology, University of Edinburgh, Edinburgh, U.K.). These mice were conventionally housed and fed ad libitum.

Induction and assessment of arthritis

Two 0.5-ml injections of pristane were administered i.p., 50 days apart (Fluka, Gillingham, U.K.). The animals were examined for the incidence of arthritis in the ankle joints at various times from day 80 onward, with the final incidence assessed 200 days post-pristane injection. Ankle joints were measured with a micrometer as previously described (7). The swollen joints ranged from 3.0 to 4.6 mm, compared with normal joints which had a range from 2.5 to 2.8 mm. This difference could easily be distinguished, and in most experiments the joints were assessed visually, arthritis being scored as present or absent.

Immunization of animals

Some groups of mice were immunized i.p. 10 days before pristane challenge with 50 μg of mycobacterial hsp65 (WHO Antigen Bank, Braunschweig, Germany) administered as an emulsion in IFA (Sigma Chemical, Poole, U.K.). This regimen has been previously reported to specifically block arthritis induction, whereas immunization with BSA, GroEl (the E. coli homologue of hsp65), or human Ig in IFA had no effect on the incidence of disease (7, 9). In addition, other groups were immunized with 100 μg of synthetic peptides derived from the sequences of hsp65 and hsp58 or saline, all as an emulsion in IFA.

Stimulation of T cells

Spleens were aseptically removed and macerated individually in petri dishes containing α modification of Eagle’s medium (α-MEM; Life Technologies, Paisley, U.K.). Large debris was removed by decanting followed by washing twice at α-MEM. T cells were enriched before culture. The enrichment of T cells was performed according to the panning method of Englemann et al. (15). Briefly, 10-cm-diameter petri dishes (Sterilin, Hounslow, U.K.) were coated with 5 ml of 0.5 mg/ml mouse γ-globulin in HBSS at room temperature for 60 min. After one washing with α-MEM, petri dishes were incubated with 7 ml of an appropriate dilution of rabbit anti-mouse Ig serum (as defined by Ouchterlony) at 4°C overnight. After a washing, the spleen cell suspensions (~7.5 × 10⁷ cells) were poured into the Ig-rabbit anti-mouse Ig-coated petri dishes and incubated at room temperature for 60 min. The nonadherent cells were then gently aspirated followed by washing with medium. These cells were then used as the T cell-enriched fraction. A purity of >90% was achieved as assessed by anti-Thy-1.2 staining using flow cytometry (FACScan, Becton Dickinson, Oxford, U.K.). Unfractionated autologous whole spleen cells were irradiated (1000 rads from a cesium source; Gradamot Industries, Gosport, U.K.) and used as APCs in enriched T cell cultures.

The medium used in all tissue culture was α-MEM supplemented with 4 mM L-glutamine (Life Technologies), 100 U/ml benzyl penicillin (Sigma), 100 μg/ml streptomycin sulfate (Sigma), 5 × 10⁻⁴ M 2 ME (Sigma), 20 mM HEPES (Sigma), and 0.5% fresh normal mouse serum. The cultures consisted of 1.2 × 10⁶ purified T cells plus 0.8 × 10⁶ APC per ml in volumes of 1 or 2 ml in 48- or 24-well plates, respectively (ICN Biomedicals), in the presence or absence of Ag. All cultures were incubated at 37°C in a humidified atmosphere of 3% CO₂ and 95% air.

Antigens

The following Ags were used in cultures at concentrations of 1 to 20 μg/ml: hsp65, GroEl, and BSA as an irrelevant Ag. The mitogen Con A (Sigma) was used at 2 μg/ml.

Synthetic peptides

A library consisting of 106 overlapping peptides, between 15 and 19 amino acids long, corresponding to the complete sequence of hsp65, was synthesized by a simultaneous multiple-peptide solid-phase synthetic method using a polyamide resin and FOMC chemistry. Completed peptides were extracted from the resin using trifluoroacetic acid and suitable scavengers, and isolated by solvent evaporation and precipitation with methanol and diethyl ether. Purity was checked by amino acid analysis and by HPLC. Additional peptides corresponding to the sequence of mycobacterial hsp65 or mammalian hsp58 were also synthesized.

Assay for proliferation

At different time points, triplicate 100-μl samples from cultures were transferred to 96-well, round-bottom culture plates (ICN Biomedicals) and pulsed with 0.5 μCi per well of [3H]Tdr (sp. act. 25 Ci/mmol; Amersham International, Amersham, U.K.) in 16 μl of culture medium and incubated for 6 h. The cells were harvested onto glass fiber filter mats (LKB-Wallac, Turku, Finland) using a Mach III Harvester 96 (Tomtec, Orange, NJ) and [3H]Tdr incorporation measured using a scintillation counter (Microbeta +, LKB-Wallac).

Assays for secreted cytokine

Cytokine production by unstimulated and Ag-driven cultures were measured by CeELISA as described in detail elsewhere (16). Briefly, 100-μl samples were taken from cultures on the indicated day and incubated for a further 24 h in the wells of microtiter plates that had been previously coated with monoclonal anti-cytokine Abs. After washing, bound cytokines were detected by adding biotinylated anti-cytokine monoclonals directed to non-overlapping epitopes on the cytokine (IL-2: capture Ab (clone) JES6-1A12, detecting Ab biotin-labeled JES6-5H4; IL-4: capture Ab 11B11, detecting Ab biotin-labeled BVD6-24G2; IL-5: capture Ab TRFK5, detecting Ab biotin-labeled TRFK4; IFN-γ: capture Ab R4-6A2, detecting Ab biotin-labeled XMG1,2, all from PharMingen, San Diego, CA). The levels of each cytokine in the test samples were determined by regression analysis from a standard curve constructed using recombinant cytokine (IL-4: Genzyme, Boston, MA; IL-2, IL-5, and IFN-γ: PharMingen).

Assays for serum Abs

Serum levels of Abs specific for hsp65 or hsp58 were determined by ELISA using anti-mouse Ig Abs known to react with all murine IgG isotypes. Alkaline phosphatase-conjugated rat mAbs were affinity purified F(ab′)₂ fragments specific for mouse IgG (Sigma). Briefly, Maxisorp Immunoplates were coated for 1 h at 37°C with 50 μl/well of mycobacterial hsp65 or mammalian hsp58 diluted to 5 μg/ml in carbonate/bicarbonate buffer (pH 9.6). After two washings in PBS/0.1% Tween 20, plates were blocked at 37°C for 1 h with 200 μl/well of a PBS/1% BSA solution. After two washes, sera to be tested were then added at 50 μl/well in dilutions ranging from 1/10 to 1/500 and incubated at 37°C for 1 h. Pooled sera from hsp65- or hsp58-immunized mice were used as positive standards. After a further three washes in PBS/Tween 20, conjugate was added at an optimal (1/500) dilution in PBS/BSA and incubated at 37°C for 30 min. After further washes, the amount of enzyme bound to the wells was assessed.
using p-nitrophenyl phosphate as the substrate (1 mg/ml in bicarbonate buffer) for 20 min at 37°C. The plates were then read at OD \textsubscript{405 nm} on a multiscan plate reader (Labsystems, Basingstoke, U.K.).

Statistical analysis

Student’s t test and the \chi^2 test were used for the statistical comparisons, with \( p < 0.05 \) considered significant.

Results

Proliferative responses to the hsp60 gene family

The in vitro proliferative responses of purified splenic T cells obtained from mice with PIA, animals protected from the development of pristane-induced arthritis by preimmunization with mycobacterial hsp65 and normal age-matched controls to bacterial hsp65 and GroEl and mammalian hsp58 were measured. Figure 1 summarizes the results of a representative experiment. It can be seen that T cells from PIA mice respond not only to the bacterial hsps (hsp65 and GroEl) but also to the mammalian form (hsp58). By contrast, T cells from hsp65-protected mice proliferate only on stimulation with the bacterial hsps. The proliferative response of T cells from PIA mice to hsp58 was significantly higher than those from hsp65-protected mice (mean stimulation index, 9.5 \pm 2.6 vs 1.2 \pm 0.3, respectively; \( p < 0.01 \)). Normal age-matched mice demonstrated significant responses to both bacterial and mammalian hsp, albeit at a lower magnitude. These differences were confirmed in subsequent experiments. T cell proliferative responses to an irrelevant Ag, i.e., BSA, were not enhanced or impaired in any of the animal groups tested. Con A responses were not significantly different between the groups tested (stimulation index for PIA, 297 \pm 72 vs hsp65 protected 312 \pm 92 vs normal 316 \pm 142).

Serum anti-hsp65 and hsp58 Ab levels

The anti-hsp65 and anti-hsp58 Ab levels in the serum of mice with PIA, hsp65-protected and age-matched normal mice were measured and compared. Figure 2 shows that in agreement with previous work (7), mice with PIA have significantly higher serum anti-hsp65 IgG than those of normal age-matched control animals (\( p = 0.01 \)), although not surprisingly, protected animals preimmunized with hsp65 exhibited the highest anti-hsp65 titers. It is striking that these mice had virtually undetectable levels of anti-hsp58 IgG, whereas the titers in mice with PIA were elevated as compared with those of normal mice, although this difference did not attain statistical significance.

Mapping of the epitopes within hsp65 recognized by murine T cells

A panel of 106 overlapping peptides between 15 and 19 amino acids long, corresponding to the complete sequence of hsp65, was generated and tested for their ability to induce proliferative responses in PIA and hsp65-protected T cells. Figure 3 shows that T cells from PIA mice recognized a number of peptides, including those corresponding to amino acids 120-135, 136-151, and 152-167. By contrast, T cells from hsp65-protected mice responded only to peptides corresponding to amino acids 120-135 and 136-151.

FIGURE 1. Comparison of proliferation to hsp65 and to the E. coli and mammalian homologues (GroEl and hsp58, respectively) of T cells derived from mice (\( n = 4 \) group) protected from the development of pristane-induced arthritis by preimmunization with hsp65 before pristane challenge, arthritic mice (PIA), and normal mice. BSA was also tested as a control Ag. In all cases the background cpm was <1500. S.I., stimulation index.

FIGURE 2. Comparison between the serum levels of anti-hsp65 and anti-hsp58 IgG Abs in samples obtained from normal animals (\( n = 5 \)), mice with pristane-induced arthritis (PIA, \( n = 7 \)), and animals protected from the development of PIA by preimmunization with hsp65 before pristane challenge (\( n = 9 \)).
tested for the ability to stimulate splenic T cells from PIA, hsp65-protected, and normal mice. The peptides were divided into 11 pools for initial screening. Pool 1 contained peptides 1–10, pool 2 contained peptides 11–20, and so on. Each pool therefore encodes for 60 amino acids. Figure 3 illustrates the peak proliferative responses obtained from purified splenic T cells derived from individual animals (n = 6/group). T cells from PIA mice displayed varied responses to all 11 peptide pools (Fig. 3b). By contrast, T cells from hsp65-protected mice (Fig. 3a) gave a more restricted pattern of response. T cells from most of the six mice did not respond to the first five peptide pools or to pools 7 and 11, although T cells from five of six mice proliferated vigorously to peptide pool 6 (aa 251–312). T cells from normal, age-matched mice gave minimal responses to all of the peptide pools (Fig. 3c).

T cells from both PIA and hsp65-protected mice responded to pool 6. Experiments were therefore set up to determine whether the T cells from the two groups of animals responded to the same or different peptides. The responses in normal mice were also examined for comparison. Figure 4 illustrates the peak proliferative responses obtained from purified splenic T cells derived from individual animals (n = 7–12/group). T cells from normal mice predominantly recognized the peptide aa 291–306, whereas T cells from mice immunized with hsp65 were additionally stimulated by the peptide aa 261–276. Similar to the results with the 11 peptide pools, the individual peptides within region 6 elicited variable responses from the T cells derived from animals with PIA.

To investigate the minimal amino acid sequence or core epitope within peptide 261–276, N-terminal single-amino acid-frame-shift peptides and C-terminal truncated peptides were synthesized and used in in vitro T cell proliferative assays. The T cells utilized were purified from the spleens of hsp65-immunized mice (Fig. 5). Firstly, using the frame-shift peptides, it was noted that loss of the N-terminal valine at position 261 led to a significant reduction in T cell stimulation compared with the parent molecule or peptides 260–271 and 261–272 (p = 0.02); loss of the neighboring valine at position 262 completely abolished the recall response of this peptide. Secondly, using the C-terminal truncations, it was noted that the recall response was not affected significantly until serine 271 was removed (hsp65 or 261–276 or 261–271 vs 261–270, p = 0.024). Further truncations completely abolished the ability of the peptides to stimulate T cell proliferation. From these experiments, it was clear that the peptide of amino acid sequence 261–271 was the minimal peptide capable of stimulating vigorous hsp65-primed T cell-proliferative responses.
Effects of peptide immunization on the incidence of PIA

We have previously shown that mice immunized with hsp65 are protected from the development of PIA (7, 9, 14). The facts that mammalian hsp58 is expressed in the joints of mice with PIA (12) and that Figure 1 demonstrates that such mice harbor autoreactive T cells directed against this Ag led us to compare the ability of bacterial peptide 261–271 to protect against PIA with that of its mammalian homologue. The sequences of the two peptides are: VVKNIRGTFFKS, peptide 261–271 from hsp65; and LLNLR-LKVGLQV, peptide homologue from mammalian hsp58. Note that there are only two residues conserved between the peptides (shown in bold and underlined).

Groups of mice were immunized i.p. with 100 μg of peptide emulsified in IFA 10 days before pristane challenge or at the time of onset of symptoms 60 days after the initial pristane injection. The final incidence of arthritis was assessed at day 200. Figure 6a confirms our previous finding that immunization with hsp65 10 days before pristane challenge protects mice from the development of PIA. Likewise immunization with the bacterial peptide 261–271, but not the mammalian homologue, significantly reduces the incidence of PIA as compared with the pristane only group (pristane only 8 of 27 vs bacterial 261–271 immunization 1 of 30, p = 0.025 χ² test). Immunization at day 60 (around the time of disease onset) with hsp65 reduced the incidence of PIA from 10 of 28 in the control group to 5 of 21, although this did not attain statistical significance (Fig. 6b). Again, immunization at this time point with the bacterial peptide, but not the mammalian homologue, significantly reduced the final incidence of PIA (pristane only 10 of 28 vs bacterial peptide 1 of 42, p < 0.01 χ² test).

Cytokine production following immunization with hsp65 or bacterial peptide

We have previously demonstrated that hsp65-preimmunized mice are resistant to PIA induction and characteristically produce Th2-associated cytokines (IL4, IL-5, and IL-10) in vitro in response to this Ag (14, 16). This led us to compare the Ag-driven cytokine production in cultures derived from mice immunized with the bacterial peptide 261–271 or with the parent molecule hsp65. Figure 7 illustrates the Ag-driven Th1 (IFN-γ and IL-2) and Th2 (IL-4 and IL-5) cytokine production from hsp65 and peptide 261–271-primed cells elicited on stimulation with the priming Ag. There is no significant difference in the levels of IL-2, IL-4, or IL-5 produced between the two groups. However, cells primed with peptide and stimulated with peptide secreted less IFN-γ than hsp65-primed T cells stimulated with hsp65, although this difference did not reach statistical significance.

**FIGURE 5.** Comparison of proliferation to hsp65, to peptide 261–276, and to single-amino acid-frame-shift overlapping and truncation peptides of T cells derived from hsp65-immunized mice (n = 3).

**FIGURE 6.** Effect of preimmunization (10 days before pristane challenge) (a) or postimmunization (60 days after the initial pristane injection) with hsp65, bacterial peptide 261–271, or its mammalian homologue (b) on the incidence of pristane-induced arthritis. The incidence of arthritis was measured at 200 days.

**FIGURE 7.** Comparison of peak Ag-driven IFN-γ, IL-2, IL-4, and IL-5 from T cells derived from hsp65-immunized mice stimulated in culture with hsp65 and T cells derived from peptide 261–271-immunized mice restimulated in culture with peptide (n = 5/group). T cells were isolated 10 days postimmunization.
**Table I. Assessment of T cells for in vitro proliferation and cytokine production in response to stimulation with bacterial and mammalian peptides**

<table>
<thead>
<tr>
<th>Immunization (100 μg in IFA)</th>
<th>Peptide Stimulant (10 μg/ml)</th>
<th>Proliferation (Δcpm ± SD)</th>
<th>IFN-γ (ng/ml)</th>
<th>IL-4 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterial peptide</strong></td>
<td>Bacterial</td>
<td>43,954 ± 1,036</td>
<td>43.6</td>
<td>262</td>
</tr>
<tr>
<td></td>
<td>Mammalian</td>
<td>1,484 ± 450</td>
<td>0.15</td>
<td>20</td>
</tr>
<tr>
<td><strong>Mammalian peptide</strong></td>
<td>Bacterial</td>
<td>26,710 ± 805</td>
<td>4.82</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Mammalian</td>
<td>12,473 ± 650</td>
<td>0.26</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>22,748 ± 1,203</td>
<td>2.84</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Mammalian</td>
<td>654 ± 210</td>
<td>0.19</td>
<td>12</td>
</tr>
</tbody>
</table>

*Mice (n = 5/group) were immunized s.c. with 100 μg of bacterial 261–271 peptide or the mammalian homologue peptide given as an emulsion in IFA. After 14 days, the draining lymph node T cells were isolated and stimulated in culture with both peptides. The peak proliferative response (where the background cpm was always <2000) and the corresponding peak Ag-driven cytokines are recorded in the table.

**T cell cross-reactivity**

Heat shock proteins are conserved during evolution, and although the bacterial T cell epitope under investigation here has only two identical residues with its mammalian homologue, it also has several conserved amino acid replacements. It was important therefore to ascertain whether T cells primed with the bacterial epitope could be stimulated with the mammalian homologue. Accordingly, groups of mice were immunized with 100 μg of bacterial 261–271 or the mammalian peptide in IFA and their T cells were assessed for in vitro proliferation and cytokine production in response to stimulation with both peptides (Table I). First, it is evident that T cells derived from mice immunized with the bacterial peptide proliferate vigorously in vitro to this peptide and produce high concentrations of both IFN-γ and IL-4 in response. By contrast, bacterial primed T cells do not respond, at either the proliferative or the cytokine level, to stimulation with the mammalian peptide. Secondly, T cells derived from mice immunized with the mammalian peptide proliferate to a modest degree in vitro to this peptide and also produce low concentrations of IFN-γ and IL-4 in response. Interestingly, these T cells appear to proliferate and produce high levels of IFN-γ, but not IL-4, when stimulated with the bacterial peptide. However, T cells derived from naive mice respond in a similar manner on stimulation with the bacterial peptide. Only T cells from mice immunized with the bacterial peptide are capable of producing high concentrations of IL-4.

**Discussion**

These results show that splenic T cells from arthritic mice proliferate against both mycobacterial hsp65 and mammalian hsp58 (Fig. 1). Likewise, the serum of arthritic mice contains elevated levels of both anti-hsp65 and anti-hsp58 Abs as compared with normal controls (Fig. 2). In short, autoimmune responses against the 60-kDa family of heat shock proteins are induced in arthritic mice. We have previously demonstrated that there are a wide range of joint Ags under immunologic attack in mice with PIA (13). Whether hsp58 is the primary autoantigen in the pathogenesis of PIA or merely the consequence of the inflammatory process stimulating B and T cells to respond to numerous and irrelevant Ags is open to debate. However, the fact that T cells derived from mice with PIA respond to many peptides throughout the sequence of hsp65 as compared with normal mice or hsp65-protected animals (Figs. 3 and 4) is reminiscent of the observation in murine experimental allergic encephalomyelitis (EAE), where immunization with peptide 1–11 from guinea pig myelin basic protein leads both to the induction of EAE and to epitope spreading, as judged by the recall proliferative responses by splenic T cells to three other endogenous peptides (17, 18). Similarly, in nonobese diabetic mice, the T cell proliferative response to glutamic acid decarboxylase spreads to numerous peptides as the disease develops (19). In contrast to the situation in arthritic mice, splenic T cells from hsp65-protected mice respond only to the bacterial hsp65 (Fig. 1). Similarly, they exhibit high serum titers of anti-hsp65 Abs, yet they have virtually undetectable levels of anti-hsp58 IgG, surprisingly even lower than that observed in normal mice (Fig. 2). These results are in accordance with those of Barrios et al. (20), who demonstrated that Abs elicited by immunization with mycobacterial hsp65 cross-react with the *E. coli* equivalent, GroEl, but not mammalian hsp58. Perhaps the Th2 cytokine environment elicited by hsp65 immunization (14) focuses both the T cell and Ab response toward hsp65 by restricting the “natural” processing and presentation of self hsp58 epitopes observed in normal mice.

Strikingly, as illustrated in Figure 3, T cells from protected mice respond only to a limited number of hsp65 peptide pools, the majority of the reactivity being directed against peptides present in pool 6. Since mice with PIA also demonstrated T cell reactivity against this pool, we next asked whether there was any difference in specificity of the responses between arthritic and protected mice toward individual peptides within pool 6. The results presented in Figure 4 confirm that T cells derived from mice with PIA do not exhibit a restricted or focused response, but individual mice can respond to all of the peptides tested. By contrast, T cells from protected mice predominantly proliferated against two peptides namely aa 261–276 and aa 291–306. We have previously put forward the hypothesis that it is the balance between the Th1 and Th2 cytokine environment of APCs that determines epitope spreading or T cell “repertoire limitation” (for full discussion, see Ref. 21). T cells from normal mice are also capable of responding vigorously to peptide aa 291–306. This may be due to “environmental priming,” i.e., the animals’ immune system being exposed to bacterial heat shock proteins associated with nonpathogenic microorganisms in our conventional animal husbandry facilities. In support of this contention, T cells from normal specific-pathogen-free mice maintained under sterile conditions, in an isolator, do not proliferate in response to the bacterial heat shock proteins hsp65 or GroEl, but the response is restored if the mice are returned to the conventional environment for 1 week (our unpublished observations). Since normal mice respond to peptide 291–306 and are susceptible to the development of PIA, it is evident that i.p. immunization with hsp65 in IFA elicits T cell responses predominantly toward the peptide 261–276 and that this response appears to be associated with protection from the development of disease. Subsequent experiments aimed at mapping the core epitope, recognized by hsp65-primed T cells, within peptide 261–276 revealed that the minimal epitope that elicited vigorous proliferation was peptide aa 261–271 (Fig. 5).

To investigate the hypothesis that immune responses directed toward the T cell epitope aa 261–271 are protective, experiments were set up to induce such responses both before the induction of PIA and around the time of onset of arthritic symptoms. The results illustrated in Figure 6 reveal a number of interesting points. Firstly, mice immunized with the bacterial peptide 261–271 are protected from the development of PIA even when the peptide was administered 60 days after disease induction. The control peptide, mammalian 261–271, did not afford such protection. Secondly, the bacterial peptide was more efficacious at preventing disease, administered at day 60, than was the parent molecule (hsp65). This may be due to the fact that the molar concentration of the relevant “protective epitope” is much higher in peptide administration regimen than in hsp65 immunization.
We have previously reported that CD4+ Th2 cells specific for hsp65 mediate protection from PIA. Protection requires both Ag specificity and Ag-driven Th2-associated cytokines, given that other immunogens known to elicit Th2 cytokines do not protect against PIA and deviation of the hsp65 response toward Th1, by coadministration of IL-12, breaks the protection afforded by hsp65 immunization (14). Since T cells from normal and hsp65-immunized mice produce the Th1-associated cytokines IL-2 and IFN-γ in response to stimulation with hsp65 (14), it follows, therefore, that it is the balance between hsp65-driven Th1 and Th2 cytokines that governs disease outcome. Results presented in Figure 7 show that immunization with the hsp65-derived peptide 261–271 was equally as good as the parent molecule at eliciting the production of Th2 cytokines (IL-4 and IL-5) in vitro restimulation assays. By contrast, the production of IFN-γ by peptide-immunized mice was approximately one-half that produced by hsp65-injected mice. These cytokine profiles represent a two- to threefold greater shift toward a “Th2 environment” in peptide-immunized mice than that exhibited in hsp65-immunized animals. This more strongly biased Th2 response may also help to explain why peptide administration at the time of disease onset, when inflammation is ongoing, was more effective at preventing disease than the parent molecule. These findings are in accordance with other Th1-mediated models of autoimmunity where Th2-type responses are also seen to protect (22–25).

How can an hsp65 peptide-specific Th2 response confer protection from PIA? One possibility is that the response induced by peptide preimmunization cross-reacts with the autologous homologue of hsp65, hsp58, in the joint, resulting in an antinflammatory Th2 response. This hypothesis is suggested by analogy with the protective effect of hsp65 preimmunization on rat adjuvant-induced arthritis. Anderton et al. (26) demonstrated that T cells from protected rats responded to a hsp65 peptide (256–265), conserved between the bacterial and self homologues, and that these T cells reacted with rat hsp58. Additional work showed that preimmunization with the partially conserved hsp65 peptide 256–270, but not other peptides, protected rats against adjuvant arthritis. However, work reported here reveals that T cells from mice protected against PIA by preimmunization with hsp65 rarely respond to the conserved hsp65 peptide 251–266 but mount a dominant response to the nonconserved bacterial-specific peptide 261–271 eliciting a Th2 cytokine profile. Preimmunization of mice with this nonconserved peptide, but not the mammalian homologue, protects mice against PIA. Furthermore, T cells isolated from either bacterial or mammalian peptide-immunized mice do not cross-react (Table I). Thus, to date our results do not favor the above idea. A second possibility is that Th2 cells exert their protective effects in the joints of pristane-injected animals where they may cross-react with a joint Ag other than self hsp58. Preliminary immunohistochemical analysis has revealed the presence of IL-4-secreting cells in the joint space of hsp65-immunized mice exclusively and that hsp65-reactive T cells have the ability to cross-react with an as yet undefined joint Ag (7, 12). This idea predicts that hsp65-reactive T cells specifically home to the joint where they are stimulated and exert a protective antinflammatory effect.

In conclusion, the protection and therapy against PIA afforded by the hsp65 derived peptide 261–271 are mediated by peptide-specific Th2 cell cytokine production. Ultimately, it is the specific control mechanisms behind the production of these cytokines after peptide stimulation and their subsequent effects on their target cells that must be established before the mechanisms of protection can be defined. The elucidation of such mechanisms in PIA may reveal new avenues to the development of specific peptide immunotherapy in human disease.

Acknowledgments
We thank A. G. Lamont and T. Johnson (Peptide Therapeutics) for the provision of some of the peptides utilized in this study.

References