DNA Fragments of the Human 60-kDa Heat Shock Protein (HSP60) Vaccinate Against Adjuvant Arthritis: Identification of a Regulatory HSP60 Peptide[1]

Francisco J. Quintana, Pnina Carmi, Felix Mor, and Irun R. Cohen[2]

Adjuvant arthritis (AA) is induced by immunizing Lewis rats with *Mycobacterium tuberculosis* suspended in adjuvant. The mycobacterial 65-kDa heat shock protein (HSP65) contains at least one epitope associated with the pathogenesis of AA: T cell clones that recognize an epitope formed by aa 180–188 of HSP65 react with self-cartilage and can adoptively transfer AA. Nevertheless, vaccination with HSP65 or some of its T cell epitopes can prevent AA by a mechanism that seems to involve cross-reactivity with the self-60-kDa HSP60. We recently demonstrated that DNA vaccination with the human *hsp60* gene can inhibit AA. In the present work, we searched for regulatory epitopes using DNA vaccination with HSP60 gene fragments. We now report that specific HSP60 DNA fragments can serve as effective vaccines. Using overlapping HSP60 peptides, we identified a regulatory peptide (Hu3) that was specifically recognized by the T cells of DNA-vaccinated rats. Vaccination with Hu3, or transfer of splenocytes from Hu3-vaccinated rats, inhibited the development of AA. Vaccination with the mycobacterial homologue of Hu3 had no effect. Effective DNA or peptide vaccination was associated with enhanced T cell proliferation to a variety of disease-associated Ags, along with a Th2/3-like shift (down-regulation of IFN-γ secretion and enhanced secretion of IL-10 and/or tumor growth factor β1) in response to peptide Mt176–190 (the 180–188 epitope of HSP65). The regulatory response to HSP60 or its Hu3 epitope included both Th1 (IFN-γ) and Th2/3 (IL-10/tumor growth factor β1) secretors. These results show that regulatory mechanisms can be activated by immunization with relevant self-HSP60 epitopes. ***The Journal of Immunology*****, 2003, 171: 3533–3541.**

Adjuvant arthritis (AA)[3] is an experimental autoimmune disease that models several features of human rheumatoid arthritis (1). AA is induced in Lewis rats by immunization with heat-killed *Mycobacterium tuberculosis* (Mt) suspended in IFA (1). T cell reactivity against the mycobacterial 65-kDa heat shock protein (HSP65) is involved in the progression of AA. HSP65-specific T cells directed against an epitope formed by aa 180–188 (2) cross-react with a self-Ag present in cartilage (3) and can adoptively transfer AA (4, 5). However, vaccination with HSP65 or HSP65 peptides can also prevent the development of AA (6–11). The regulatory properties of HSP65 in AA are thought to involve the activation of T cells cross-reactive with the endogenous 60-kDa HSP60 (12). This hypothesis is supported by the finding that immunization with a recombinant vaccinia virus encoding human HSP60 (~95% homologous to rat HSP60) prevents (13) or treats (14) AA. We have recently reported that DNA vaccination with human HSP60 inhibits AA (15). Protection from AA was associated with the activation of T cells responding to HSP60 (15). However, the identity of HSP60 epitopes involved in prevention of AA by DNA vaccination remained to be uncovered. The aim of this work was to identify regulatory HSP60 T cell epitopes using an approach that combined DNA vaccination with HSP60 fragments and the study of T cell responses directed to overlapping peptides of HSP60.

Materials and Methods

Animals

Female Lewis rats were raised and maintained under pathogen-free conditions in the Animal Breeding Center (The Weizmann Institute of Science, Rehovot, Israel). One- to 2-mo-old rats were used for DNA vaccination and peptide-vaccination experiments. The experiments were performed under the supervision and guidelines of the Animal Welfare Committee.

Ags and adjuvants

Peptides were synthesized as previously described (15). The HSP60 peptides used in these studies are listed in Table I. Two HSP65 peptides were also used: Mt176–190, EESNTFGLQLELTEG (16), and Mt3, AYDEE ARRGLERGLNALADA. Purified recombinant HSP65 was generously provided by Prof. R. van der Zee (Institute of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht, The Netherlands). Recombinant HSP60 was prepared in our laboratory as described (17). Mt strain H37Ra and IFA were purchased from Difco (Detroit, MI). Tuberculin purified protein derivative (PPD) was provided by the Statens Seruminstitut (Copenhagen, Denmark). OVA and Con A were purchased from Sigma-Aldrich (Rehovot, Israel).

DNA plasmids

The vector containing the human *hsp60* gene (DNA vaccine encoding HSP60 (pHSP60)) has been described (17). The construct encoding *Mycobacterium leprae* HSP65 (DNA vaccine encoding HSP65 (pHSP65)) was kindly provided by Dr. D. Lowrie (Medical Research Council, London, U.K.). Both vectors have been shown to be effective in inhibiting AA (8, 15). Five fragments of the human *hsp60* gene were amplified by PCR from *hsp60* cDNA in pGEM (Promega, Madison, WI) using specific oligonucleotides containing restriction sites for the enzymes *Bam*HI (oligonucleotide 5′) or *Hin*dIII (oligonucleotide 3′), and cloned into the pcDNA3 vector (Invitrogen, Leek, The Netherlands) using standard molecular biology techniques (Table II). The 5′ oligonucleotide also included an ATG

Department of Immunology, The Weizmann Institute of Science, Rehovot, Israel

Received for publication February 28, 2003. Accepted for publication July 29, 2003.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] These studies were funded by a grant from the Center for the Study of Emerging Diseases and by the European Community, through the Fifth Framework of the Quality of Life Program. I.R.C. is the incumbent of the Mauerberger Chair in Immunology.

[2] Address correspondence and reprint requests to Dr. Irun R. Cohen, Department of Immunology, The Weizmann Institute of Science, Rehovot, 76100, Israel. E-mail address: irun.cohen@weizmann.ac.il

[3] Abbreviations used in this paper: AA, adjuvant arthritis; Mt, *M. tuberculosis*; HSP, heat shock protein; PPD, purified protein derivative; LNC, lymph node cell; SI, stimulation index; EAE, experimental autoimmune encephalomyelitis.

0022-1767/03/$02.00

Table I. *Overlapping peptides of human HSP60, region 1–275*

Peptide	Position	Sequence
Hu1	1–20	MLRLPTVFRQMRPVSRVLAP
Hu2	16–35	RVLAPHLTRAYAKDVKFGAD
Hu3	31–50	KFGADARALMLQGVDLLADA
Hu4	46–65	LLADAVAVTMGKGRTVIIE
Hu5	61–80	TVIIEQSWGSPKVTKDGVTV
Hu6	76–95	DGVTVAKSIDLKDKYKNIGA
Hu7	91–110	KNIGAKLVQDVANNTNEEAG
Hu8	106–125	NEEAGKGTTTATVLARSIAK
Hu9	121–140	RSIAKEGFEKISKGANPVEI
Hu10	136–155	NPVEIRRGVMLAVDAVIAEL
Hu11	151–170	VIAELKKQSKPVTTPEEIAQ
Hu12	166–185	EEIAQVATISANGDKEIGNI
Hu13	181–199	EIGNIISDAMKKVGRKGVI
Hu14	195–214	RKGVITVKDGKTLNDELEII
Hu15	210–229	ELEIIEGMKFDRGYISPYFI
Hu16	225–244	SPYFINTSKGQKCEFQDAYV
Hu17	240–259	QDAYVLLSEKKISSIQSIVP
Hu18	255–275	QSIVPALEIANAHRKPLVIIA

sequence necessary for protein translation. The plasmids were sequenced to confirm correct insertion of the cDNA and transcribed in vitro to check that they were functional (data not shown).

Plasmid DNA was prepared in large scale and injected after pretreatment with cardiotoxin (Sigma-Aldrich) as previously described (15). Briefly, rats were vaccinated in the quadriceps three times (on days −40, −26, −12 relative to AA induction) with 150 μg of pcDNA3, DNA vaccine encoding aa 1–140 of human HSP60 (pI), DNA vaccine encoding aa 130–260 of human HSP60 (pII), DNA vaccine encoding aa 250−410 of human HSP60 (pIII), DNA vaccine encoding aa 400−470 of human HSP60 (pIV), or DNA vaccine encoding aa 460–540 of human HSP60 (pV). Endotoxin levels were checked by *Limulus* amoebocyte lysate and found always to be under acceptable levels for in vivo use (<0.02 endotoxin U/μg DNA). AA was induced 12 days after the last injection of DNA. The empty vector pcDNA3 was used as a DNA vaccination control.

AA induction and assessment

AA was induced as described (15), using 1 mg per rat of heat-killed Mt strain H37Ra (Difco). Each experimental and control group contained at least eight rats. The day of AA induction was designated as day 0, and disease severity was assessed by direct observation of all four limbs in each animal. A relative score between 0 and 4 was assigned to each limb, based on the degree of joint inflammation, redness, and deformity; thus the maximum possible score for an individual animal was 16 (15). The mean AA score (±SEM) is shown for each experimental group. Arthritis was also quantified by measuring hind limb diameter with a caliper. Measurements were taken on the day of the induction of AA and 26 days later (at the peak of AA); the results are presented as the mean ± SEM of the difference between the two values for all the animals in each group. The person who scored the disease was blinded to the identity of the groups. Experiments were repeated at least three times and produced similar results.

T cell proliferation

Unless otherwise stated, T cell proliferation assays were performed at day 26 after the induction of AA, when the disease is at its peak, as previously described (15). Briefly, popliteal and inguinal lymph node cells (LNC), were cultured in quadruplicates in 200-μl round-bottom microtiter wells (Costar, Cambridge, MA) at 2×10^5 cells per well with or without Ag. The T cell mitogen Con A 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. T cell responses were detected by the incorporation of [*methyl*-^{3}H]thymidine (1 μCi/well; Amersham, Buckinghamshire, U.K.), which was added to the wells for the last 18 h. The stimulation index (SI) was computed as the ratio of the mean cpm of Ag- or mitogen-containing wells to control wells cultured with medium alone. The results of T cell proliferation experiments are shown as SI ± SEM, T cell responses with SI < 2 were considered not significant.

Table II. *Plasmids constructed containing overlapping fragments of the hsp60 gene*

Plasmid	Position[a]	Peptides Contained[b]
pI	1–140	Hu1-Hu9
pII	130–260	Hu10-Hu18
pIII	250–410	ns
pIV	400–470	ns
pIV	460–540	ns

[a] The position is expressed as aa number.
[b] ns, not synthesized.

Peptide vaccination

Female Lewis rats were immunized i.p. with a single dose of 100 μg of peptide emulsified in IFA.

Transfer of cells

Spleen cells were prepared from peptide-vaccinated rats, either 7 days after peptide vaccination or 26 days after AA was induced following peptide vaccination. The splenocytes (10^7 cells per ml) were activated with 2.5 μg/ml Con A for 48 h at 37°C in a humidified atmosphere of 5% CO_2. The cells were washed with sterile PBS and injected i.v. into naive rats (5×10^7 cells per rat). Three days after the transfer of the splenocytes, AA was induced.

Cytokine assays

Supernatants were collected after 72 h of stimulation with each of the Ags tested. Rat IL-10 and IFN-γ were quantitated in culture supernatants by ELISA using an OPTEIA kit (BD PharMingen, San Diego, CA) as described (15). Rat TGFβ1 was quantified using the TGFβ1 E_{max} Immuno-Assay System (Promega) according to the manufacturer's instructions. Cytokine levels are expressed as picograms per milliliter based on calibration curves constructed using recombinant cytokines as standards. The lower limits of detection for the experiments described in this paper were 15 pg/ml for TGFβ1, IL-10, and IFN-γ.

Statistical significance

The InStat 2.01 program (Graph Pad Software, San Diego, CA) was used for statistical analysis. The Student *t* test and the Mann-Whitney *U* test were conducted to assay significant differences between the different experimental groups.

Results

HSP60 DNA fragments inhibit AA

We previously demonstrated that DNA vaccination with human HSP60 inhibited the development of AA (15). To identify regions of HSP60 involved in the inhibition of AA by DNA vaccination, the cDNA corresponding to the human *hsp60* gene was divided into five fragments, each with a 30-bp overlap, and each fragment was cloned into the pcDNA3 vector (Table II). In this way, we generated five constructs corresponding to HSP60-derived fragments overlapping by 10 aa: pI, aa 1–140; pII, aa 130–260; pIII, aa 250−410, pIV, aa 400−470, and pV, aa 460–540 (Table II and data not shown). Lewis rats were vaccinated with one of the HSP60 fragment constructs or with pcDNA3 as a control, and AA was induced. Although all of the rats vaccinated with pI or pII developed AA, Fig. 1*A* shows that such vaccination induced significantly decreased arthritis compared with rats vaccinatedwith constructs pIII, pIV, or pV, or with control pcDNA3. The protective effect of vaccination with constructs pI and pII was also reflected by a significant reduction in ankle swelling (Fig. 1*B*).

A regulatory HSP60 epitope

To learn whether the suppression of AA by DNA vaccination with pHSP60 (15), pI, or pII (Fig. 1) was associated with T cell reactivity to a specific HSP60 peptide epitope, we studied the proliferation of LNC isolated from pHSP60-vaccinated rats 26 days after the induction of AA in response to a panel of overlapping peptides spanning the region of human HSP60 encoded by pI and pII (aa 1- 275; Table I). Controls were LNC prepared from rats

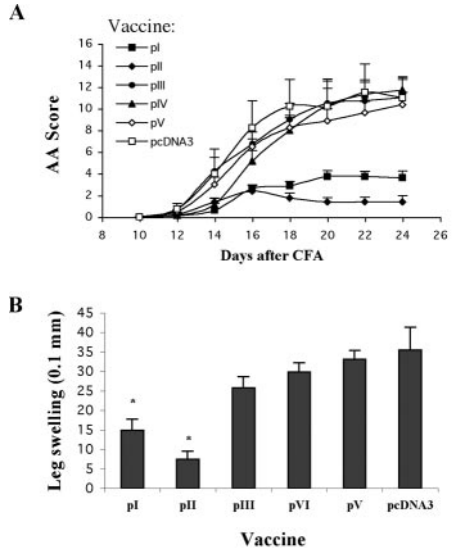

FIGURE 1. Inhibition of AA by vaccination with pI and pII. *A*, Time course of AA. Rats were vaccinated in the quadriceps three times (on days −40, −26 −12 relative to AA induction) with 150 μg of pcDNA3, pI, pII, pIII, pIV, or pV. On day 0, AA was induced by injecting 1 mg of Mt suspended in 100 μl of IFA, and arthritis scores were assessed every 2 or 3 days starting at day 10. Bars show the mean ± SEM assessment of disease severity for each experimental group. Statistically significant differences ($p < 0.05$) were obtained at every determination from days 14 to 24 when pI-vaccinated rats and pII-vaccinated rats were compared with pcDNA3-vaccinated rats. *B*, Leg swelling measured at day 26 after AA induction. The results are presented as the mean ± SEM of the difference between the values for hind limb diameter taken on days 0 and 26 for each experimental group. ∗, $p < 0.001$.

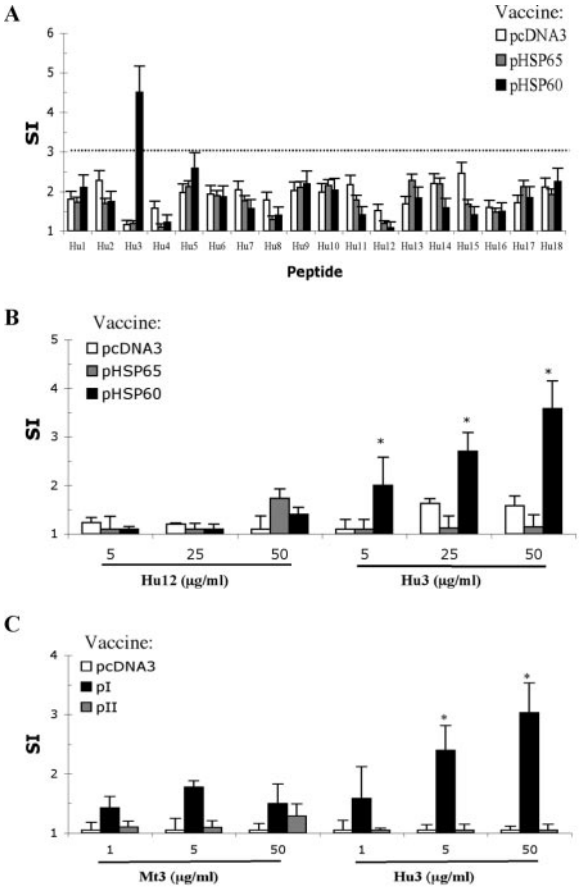

FIGURE 2. pHSP60 and pI-vaccination activate T cell responses to Hu3. *A*, Proliferative responses to overlapping peptides corresponding to the first 260 aa of HSP60. Rats vaccinated according to the protocol described in Fig. 1 were killed and LNC were collected on day 26 after induction of AA. Bars show the mean ± SEM SI of quadruplicate cultures in the presence of 25 μg/ml of each peptide. *B*, Dose-response to Hu3 of pHSP60, pHSP65, and pcDNA3-vaccinated rats. Rats vaccinated according to the protocol described in Fig. 1 were killed and LNC were collected on day 26 after induction of AA. The results are expressed as the mean ± SEM cpm of quadruplicate cultures in the presence of different concentrations of Hu3 or the control peptide Hu12. *C*, Dose-response to Hu3 of pI-, pII-, and pcDNA3-vaccinated rats. Rats vaccinated according to the protocol described in Fig. 1 were killed and LNC were collected on day 26 after induction of AA. The results are expressed as the mean ± SEM cpm of quadruplicate cultures in the presence of different concentrations of Hu3 or the control peptide Hu12. ∗, $p < 0.005 + p < 0.02$.

vaccinated with pcDNA3 or pHSP65 and challenged with Mt suspended in IFA to induce AA. Fig. 2*A* shows that only peptide Hu3 (aa 31–50 of human HSP60) induced a significant response in LNC taken from pHSP60-vaccinated rats; cells prepared from pHSP65 or pcDNA-vaccinated rats did not respond to Hu3. Note that in the 31–50 region rat and human HSP60 have the same amino acid sequence; thus Hu3 is a self-peptide (Table III). We were unable to identify an HSP60 peptide that was specifically recognized by LNC taken from pII-immunized rats. Fig. 2*B* shows a dose-dependent proliferative response to Hu3 using LNC isolated from pHSP60-vaccinated rats. No significant responses to the control peptide Hu12 (aa 166–185 of human HSP60) were detected.

To confirm these results, T cell proliferative responses were studied in LNC taken 26 days after the induction of AA from rats vaccinated with pI, pII, or pcDNA3. Fig. 2*C* shows that Hu3 triggered a significant proliferation of LNC taken from pI-immunized rats, but not of LNC from rats vaccinated with pII or pcDNA3. LNC from pI-vaccinated rats also showed some degree of T cell reactivity directed against the mycobacterial homologue of Hu3, termed Mt3. However, this Mt3-specific T cell response always had an SI < 2, and was therefore considered not significant. In summary, these results show that pHSP60- and pI-vaccinated rats manifested up-regulated T cell responses to the Hu3 peptide of HSP60.

Peptide Hu3 inhibits AA

We studied the immune effects of vaccination with Hu3 or its mycobacterial counterpart Mt3 (Table III). Female Lewis rats were immunized with a single i.p. dose of 100 μg of peptide in IFA, and 7 days later their LNC were prepared and the T cell responses to HSP65, Mt3, HSP60, and Hu3 were assessed. Fig. 3*A* shows that each peptide induced specific proliferative responses to itself. However, vaccination with Hu3 also induced significant proliferative responses to HSP60 and HSP65. The analysis of IFN-γ and IL-10 secretion (Fig. 3, *B* and *C*) revealed that vaccination with Hu3 induced Hu3-specific T cells that secreted IL-10 and traces of IFN-γ, but also T cells that secreted relatively larger amounts of IFN-γ and IL-10 in response to HSP60 or HSP65. Moreover, Hu3 vaccination also induced IFN-γ-secreting T cells that cross-reacted with Mt3. In contrast, vaccination with Mt3 induced T cells that secreted relatively small amounts of IL-10 upon stimulation with Mt3 and small amounts of IFN-γ upon stimulation with HSP65 or HSP60.

To establish a link between the immune response to Hu3 and the inhibition of AA, rats were vaccinated with Hu3, or with Mt3

Table III. *Comparison of human HSP60, rat HSP60, and mycobacterial HSP65 in the region corresponding to the Hu3 sequence*[a]

Homo sapiens	31	**KFGADARALMLQGVDLLADA**	50
Rattus norvergicus	31	**KFGADARALMLQGVDLLADA**	50
M. tuberculosis	5	A*Y*DE*E***AR**RG*L*ER**G***L*N**ALADA**	24

[a] Residues sharing identity with the corresponding sequence of human HSP60 are shown in bold, and conserved substitutions are italicized.

(Table III) or Hu12 (Table I) as controls. Each rat received a single i.p. dose of 100 μg of peptide in IFA 7 days (day -7) before the induction of AA (day 0). Fig. 4*A* shows that although all the Hu3-vaccinated rats manifested signs of AA, the severity of the disease was significantly decreased compared with nonimmunized rats or rats that had been vaccinated with PBS, Hu12, or Mt3. This reduction in AA was also reflected by a significant reduction in ankle swelling ($p = 0.02$ compared with PBS-vaccinated rats; Fig. 4*B*). Immunization with Hu3 had no effect on the induction of experimental autoimmune encephalomyelitis (EAE) (18) in control groups of rats: the EAE maximal score $\pm$ SD was 2.4 $\pm$ 0.2 in nontreated rats, compared with 2.4 $\pm$ 0.3 in Hu3-vaccinated rats ($p > 0.05$). Therefore, vaccination with Hu3 could specifically inhibit AA.

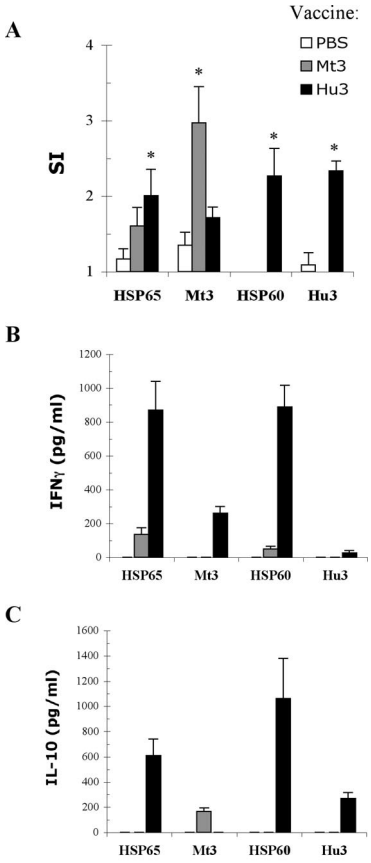

FIGURE 3. Immune response to Hu3. Rats were vaccinated once with 100 μg of Hu3, Mt3, or PBS in 100 μl of IFA; 7 days later LNC were collected and stimulated in vitro with Mt3, Hu3, HSP65, and HSP60 for 72 h. The proliferative responses (*A*) and the amounts of secreted IFN-γ (*B*) or IL-10 (*C*) were determined. The proliferation results are expressed as the mean $\pm$ SEM SI of quadruplicate cultures, and the cytokine data are shown as the mean $\pm$ SD of triplicates. $*$, $p < 0.05$.

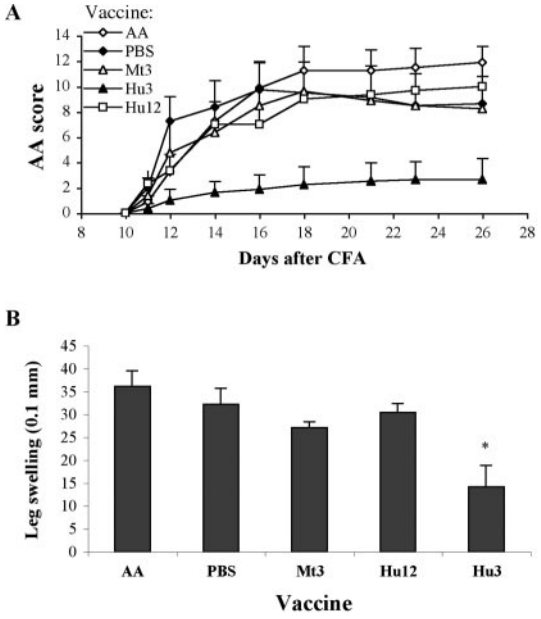

FIGURE 4. Inhibition of AA by vaccination with Hu3. *A*, Time course of AA. Rats were vaccinated once (on day -7 relative to AA induction) with 100 μg of Hu3, Hu12, Mt3, or PBS in 100 μl of IFA, or left unvaccinated (AA) and AA was induced on day 0 and arthritis scores were assessed. Bars show the mean $\pm$ SEM assessment of disease severity for each experimental group. Statistically significant differences ($p < 0.05$) were obtained at every determination from days 14 to 26 when Hu3-vaccinated rats were compared with the other vaccinated rats. *B*, Leg swelling measured on day 26 after AA induction. The results are presented as the mean $\pm$ SEM of the difference between the values for hind limb diameter taken on days 0 and 26 for each experimental group. $*$, $p = 0.02$.

Adoptive transfer of peptide-induced regulation

To learn whether the inhibition of AA triggered by Hu3 vaccination could be adoptively transferred by activated T cells, we prepared splenocytes from rats that had been vaccinated 7 days earlier with Hu3. Control splenocytes were taken from Mt3-vaccinated rats. The T cells were stimulated in vitro for 2 days with the T cell mitogen Con A, washed, and injected i.v. into naive rats (5×10^7 cells per rat). Three days later, AA was induced and arthritis was scored. Although all the rats developed AA, the recipients of cells taken from the Hu3-vaccinated group developed a significantly milder disease (Fig. 5, *A* and *B*). Adoptive transfer of these T cells had no effect on the induction of EAE (18) in control groups of rats: the EAE maximal score $\pm$ SD was 2 $\pm$ 0.7 in nontreated rats, compared with 1.9 $\pm$ 0.8 in Hu3-vaccinated rats ($p > 0.05$). No protection was seen in rats that had received Con A-activated cells from Mt3-vaccinated rats. Thus, vaccination with Hu3 induced T cells that specifically protected rats from AA.

To learn whether the induction of AA might affect the adoptive transfer of protection, we vaccinated rats with Hu3 or Mt3, induced AA 3 days later, and obtained splenocytes for adoptive transfer 26 days after the induction of AA. The splenocytes were activated in vitro with Con A and transferred into naive rats, and the rats were challenged 3 days later with Mt and followed for the development of AA. Fig. 5, *C* and *D*, shows that the degree of protection achieved after AA induction in the donors was even stronger than that observed by donors without the induction of AA (compare Fig. 5, *A* and *B*, with *C* and *D*). Thus, inhibition of AA by vaccination with Hu3 could be adoptively transferred by activated T cells, and the regulatory T cell population appeared to be enhanced by the induction of AA.

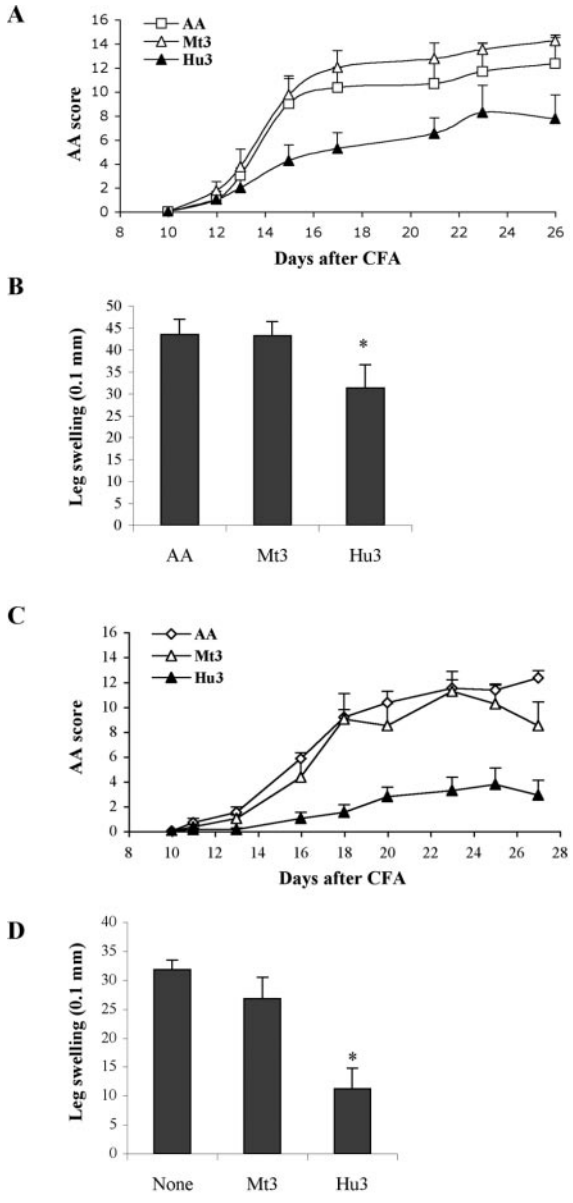

FIGURE 5. Inhibition of AA by transfer of Con A-activated splenocytes from Hu3-vaccinated rats. *A*, Time course of AA. Rats were vaccinated once with 100 μg of Hu3 or Mt3 in 100 μl of IFA, and 7 days later splenocytes were collected, activated for 48 h with Con A, and transferred i.v. (5×10^7 per rat) to naive rats. Three days later, AA was induced in the recipients and arthritis scores were assessed. Bars show the mean ± SEM assessment of disease severity for each experimental group. Statistically significant differences ($p < 0.05$) were obtained at every determination from days 15 to 26 when Hu3-vaccinated rats were compared with PBS- or Mt3-vaccinated rats. *B*, Leg swelling measured on day 26 after AA induction. The results are presented as the mean ± SEM of the difference between the values for hind limb diameter taken on days 0 and 26 for each experimental group. ∗, $p < 0.05$. *C*, Time course of AA. Rats were vaccinated once (on day −7 relative to AA induction) with 100 μg of Hu3 or Mt3 in 100 μl of IFA and AA was induced on day 0. Splenocytes were collected on day 26 after induction of AA, activated for 48 h with Con A, and transferred iv (5×10^7 per rat) to naive rats. Three days later, AA was induced in the recipients and arthritis scores were assessed. Bars show the mean ± SEM assessment of disease severity for each experimental group. Statistically significant differences ($p < 0.05$) were obtained at every determination from days 16 to 27 when Hu3-vaccinated rats were compared with PBS- or Mt3-vaccinated rats. *D*, Leg swelling measured on day 26 after AA induction. The results are presented as the mean ± SEM of the difference between the values for hind limb diameter taken on days 0 and 26 for each experimental group. ∗, $p < 0.01$.

Immune responses of rats protected from AA by pI or pII vaccination

To study the immune responses associated with the inhibition of AA by DNA vaccination with pI or pII, we analyzed the T cell responses of immunized rats 26 days after the induction of AA. We stimulated the LNC in vitro with a collective of mycobacterial Ags known to be associated with AA: HSP65, PPD, and Mt176–190. The Mt176–190 peptide contains the 180–188 epitope of HSP65 described by van Eden et al. (2) which is recognized by HSP65-specific T cell clones that can transfer AA. We also studied the immune response directed to mammalian HSP60, HSP60-peptide Hu3, and control HSP60-peptide Hu12. OVA was included as a control Ag. None of the experimental groups showed significant responses to OVA or to Hu12, and they did not differ in their responses to Con A (data not shown). Nevertheless, inhibition of AA by DNA vaccination with the pI or pII constructs was associated with the up-regulation of the T cell proliferative responses to the panel of mycobacterial Ags (PPD, HSP65, and Mt176–190; Fig. 6*A*). Fig. 6*B* depicts the proliferative responses to HSP60 and its Hu3 peptide. It can be seen that both pI and pII vaccination induced significant T cell responses to HSP60, however, only LNC from pI-vaccinated rats manifested reactivity to Hu3.

Our previous findings indicated that prevention of AA by whole HSP60 DNA vaccination was associated with a Th2/3-like shift in the cytokine responses induced by the mycobacterial Ags PPD, HSP65, and Mt176–190 (15). Therefore, we studied cytokine secretion by LNC taken from rats that had been vaccinated with pI, pII, or pcDNA3. Inhibition of AA by DNA vaccination with pI was associated with a decrease in IFN-γ secretion (Fig. 7*A*), and an increase in IL-10 and TGFβ1 secretion upon stimulation with PPD, HSP65, or Mt176–190 (Fig. 7, *B* and *C*).

LNC from pII-vaccinated rats also showed a decrease in IFN-γ secretion upon stimulation with PPD, HSP65, or Mt176–190 (Fig. 7*A*); however, IL-10 secretion was only detected after activation with HSP65 while TGFβ1 secretion was only detected following activation with Mt176–190 or PPD (Fig. 7, *B* and *C*). Note that cells from pI- or pII-vaccinated rats secreted detectable amounts of TGFβ1 upon activation with Mt3. Thus, protection from AA by DNA vaccination with pI or pII was associated with decreased IFN-γ secretion and a concomitant increase in IL-10 and/or TGFβ1 secretion upon stimulation with the mycobacterial Ags PPD, HSP65, or Mt176–190 (Fig. 7).

In addition to the responses to mycobacterial Ags, we studied the effects of DNA vaccination with HSP60 fragments on the responses to HSP60 and Hu3. IFN-γ was not secreted in response to HSP60 or Hu3 by the LNC of either the pI- or pII-vaccinated rats (Fig. 7*A*). LNC taken from pI-vaccinated rats secreted both IL-10 and TGFβ1 in response to HSP60 or Hu3 (Fig. 7, *B* and *C*). LNC taken from pII-vaccinated rats, in contrast, secreted TGFβ1 upon activation with HSP60, but IL-10 was not detected. Therefore, both pI or pII vaccination induced the secretion of TGFβ1 in response to HSP60. However, only pI appeared to trigger the secretion of IL-10.

Immune responses in Hu3-vaccinated rats

To compare the effects of peptide vaccination to DNA vaccination, we studied the T cell responses after induction of AA in rats that had been vaccinated with peptides Hu3, Hu12, or Mt3. All three peptides were immunogenic; significant and specific T cell responses could be detected in the immunized rats to each peptide (Fig. 8*A*). However, only the LNC taken from Hu3-vaccinated rats

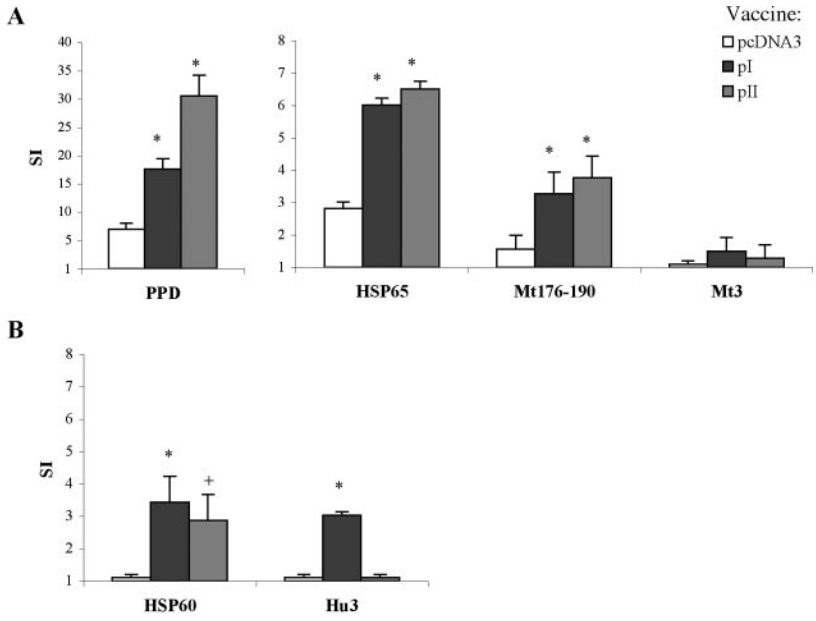

FIGURE 6. T cell responses after DNA vaccination. Lewis rats were vaccinated with pI, pII, or pcDNA3 as described in Fig. 1 and AA was induced. Twenty-six days later, LNC were collected, and the proliferative responses to (*A*) PPD, HSP65, Mt176–190, Mt3, (*B*) HSP60, and Hu3 were studied. The results are expressed as the mean ± SEM SI of quadruplicate cultures. Three independent experiments produced similar results. *, $p < 0.005$ + $p < 0.02$.

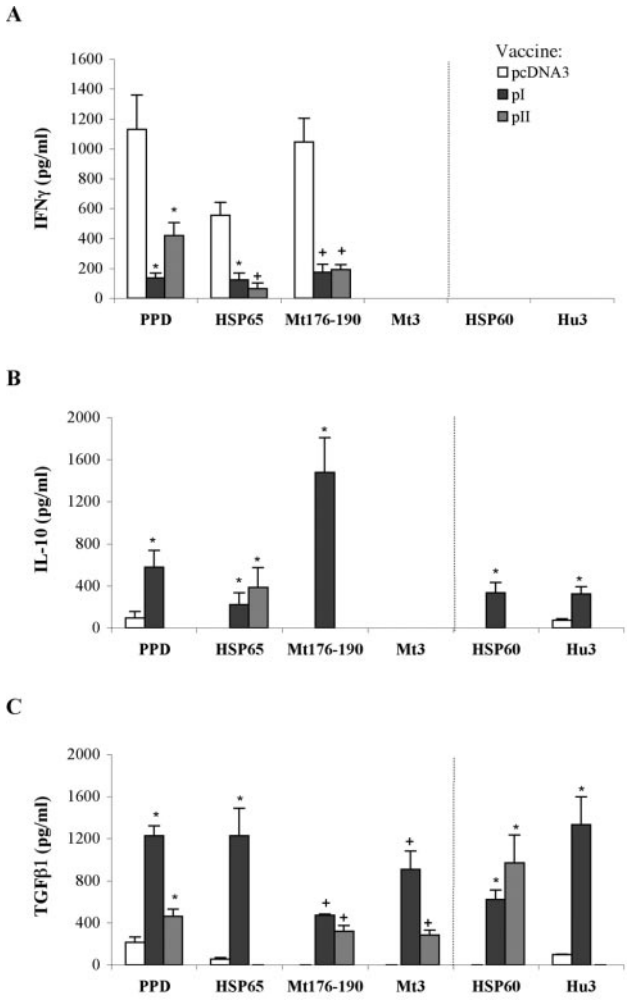

FIGURE 7. Cytokine secretion after DNA vaccination. Lewis rats were vaccinated with pI, pII, or pcDNA3 as described in Fig. 1 and AA was induced. Twenty-six days later, LNC were collected, stimulated in vitro with PPD, HSP65, Mt176–190, Mt3, HSP60, and Hu3 and the supernatants were tested after 72 h for the amounts of secreted (*A*) IFN-γ, (*B*) IL-10, or (*C*) TGFβ1. The data are shown as the mean ± SD of triplicates. Three independent experiments produced similar results. *, $p < 0.05$ + $p < 0.02$.

showed up-regulated T cell proliferative responses to the mycobacterial Ags PPD, HSP65, and Mt176–190 (Fig. 8*B*). Furthermore, vaccination with Hu3 led to the induction of a specific response to HSP60 (Fig. 8*B*). None of the experimental groups showed significant responses to OVA, and they did not differ in their response to Con A (data not shown).

With regard to cytokine secretion, vaccination with Hu3 led to a reduction in IFN-γ secretion (Fig. 9*A*), and to a concomitant increase in the secretion of IL-10 (Fig. 9*B*) and TGFβ1 (Fig. 9*C*) upon stimulation with PPD, HSP65, or Mt176–190. Hu3-vaccination also led to the induction of T cells that secreted IFN-γ, IL-10, and TGFβ1 in response to Hu3. The response to whole HSP60 was predominantly TGFβ1. Thus, the effects of Hu3 vaccination were similar to those observed following DNA vaccination with whole pHSP60, or with the pI or pII fragments.

Discussion

In these studies, we mapped regions of HSP60 that can regulate AA following DNA vaccination with pHSP60. We found that DNA vaccination with HSP60 fragments pI (1–140) or pII (130–260) inhibited AA (Fig. 1). To detect regulatory epitopes within the 1–260 region of HSP60, we studied the T cell responses of pHSP60-vaccinated rats against a panel of overlapping HSP60 peptides and detected reactivity to Hu3 (Fig. 2). Vaccination with Hu3 or pHSP60 induced low but significant T cell responses to Hu3, HSP60, and HSP65 (Fig. 3 and data not shown). Similarly, van Tienhoven et al. (19) reported that weak T cell proliferative responses could be detected following vaccination with a plasmid encoding the immunogenic 180–188 T cell epitope of HSP65. However, the T cell responses could be amplified by the Mt immunization used to induce AA (Figs. 8 and 9 and Ref. 15). For this reason, we studied the effect of vaccination on immune responses on day 26 after the induction of AA. Hu3-vaccinated rats were protected from AA (Fig. 4); protection could be adoptively transferred with Con A-activated splenic T cells (Fig. 5, *A* and *B*), but it was more effective when the cells were taken after Hu3-vaccinated rats had been challenged with Mt (Fig. 5, *C* and *D*). These results suggest that the induction of AA enhanced the expansion and/or activation of the regulatory cells induced by vaccination with Hu3. Indeed, AA itself is known to induce regulatory cells.

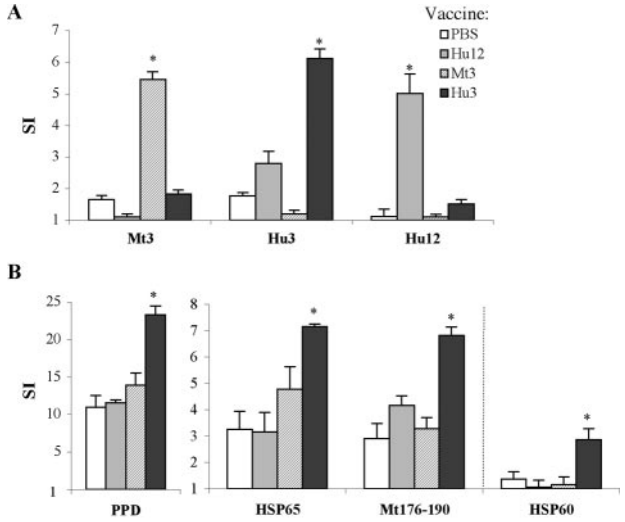

FIGURE 8. T cell responses after peptide vaccination. Lewis rats were vaccinated with Hu3, Mt3, Hu12, or PBS as described in Fig. 3 and AA was induced. Twenty-six days later, LNC were collected, and the proliferative responses to (*A*) Mt3, Hu3, Hu12, (*B*) PPD, HSP65, Mt176–190, and HSP60 were studied. The results are expressed as the mean ± SEM SI of quadruplicate cultures. Three independent experiments produced similar results. *, $p < 0.005$.

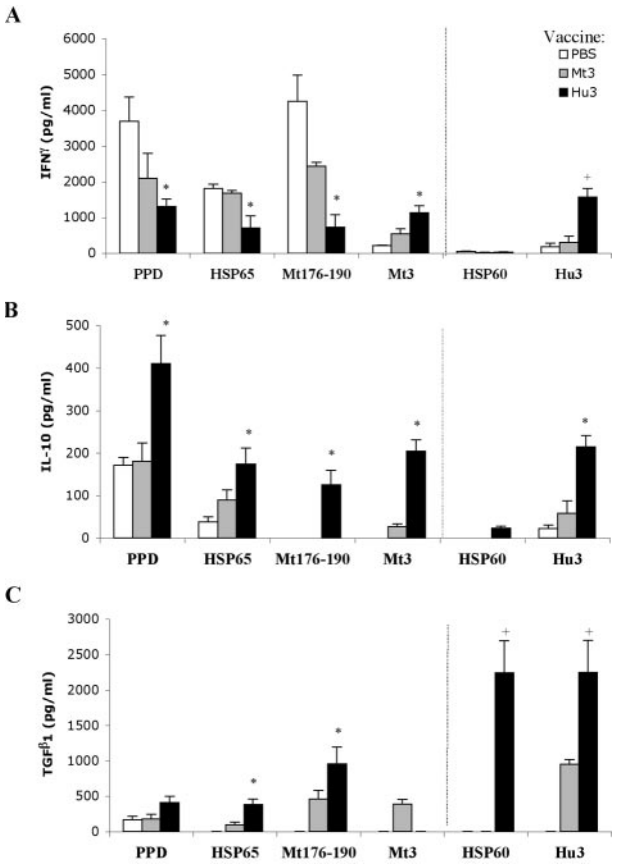

FIGURE 9. Cytokine secretion after peptide vaccination. Lewis rats were vaccinated with Hu3, Mt3, Hu12, or PBS as described in Fig. 3, and AA was induced. Twenty-six days later, LNC were collected, stimulated in vitro with PPD, HSP65, Mt176–190, Mt3, HSP60, and Hu3 and the supernatants were tested after 72 h for the amounts of secreted (*A*) IFN-γ, (*B*) IL-10, or (*C*) TGFβ1. The data are shown as the mean ± SD of triplicates. Three independent experiments produced similar results. *, $p < 0.05$ + $p < 0.02$.

Rats recovered from AA acquire resistance to further attempts to induce the disease (1).

We now report that modulation of AA by treatment with pI or Hu3 was associated with three observations: 1) a decrease in the secretion of IFN-γ (Figs. 7*A* and 9*A*) and a concomitant increase in IL-10 and TGFβ1 secretion (Figs. 7, *B* and *C*, and 9, *B* and *C*) upon stimulation with mycobacterial Ags; 2) the induction of IL-10 and TGFβ1 secretion in response to HSP60 or Hu3 (Figs. 7, *B* and *C*, and 9, *B* and *C*); and 3) the induction of IFN-γ in response to HSP60 or Hu3.

IFN-γ secreted by effector T cells has been implicated in the progression of AA (20). Therefore, the cytokine shift to Th2/3 in response to the mycobacterial peptide epitope cross-reactive with cartilage (3), might reflect the control of T cell clones involved in the progression of AA. It is conceivable that the HSP60-specific T cells induced after vaccination with pI, pII, or Hu3 supported this cytokine shift by secreting IL-10 and TGFβ1. IL-10 and TGFβ1 are immunomodulatory cytokines capable of inhibiting experimental arthritis (21–23). Indeed, T cells reactive with self-HSP60 have been previously shown to control AA (24). Inflammation up-regulates HSP60 expression in lymphocytes (25), macrophages (26), and synovial cells (27, 28). Moreover, Ag-specific T cells induced by DNA vaccination with constructs coding for joint Ags home to the joints (29). Therefore, we propose that HSP60-specific T cells induced by vaccination with HSP60-derived vaccines are recruited to the inflamed joints, where they are stimulated to secrete IL-10 and TGFβ1 that finally control the pathogenic T cell clones that drive AA. However, the induction of IFN-γ in response to Hu3 (Fig. 8) indicates that regulatory cells need not be exclusively Th2/3. Indeed, regulatory T cells in AA (24), EAE (30), and insulin-dependent diabetes mellitus (31) have been reported to secrete IFN-γ. The role of IFN-γ secretion in the regulation of AA needs clarification.

Note that Hu3 is not the only regulatory epitope involved in the control of AA, and the induction of an Hu3-specific T cell response is probably not the only regulatory mechanism triggered by DNA vaccination with HSP60. In fact, we have shown here that pII (which does not contain the Hu3 region) could also confer protection against AA (Fig. 1). Moreover, Abs directed against peptide 61–80 from HSP60 can also inhibit AA (32); and several regulatory epitopes of HSP60 cross-reactive with mycobacterial HSP65 have been described. Paul et al. (24) described HSP65 peptide epitope 256–270, which corresponds to 283–297 in reference to HSP60. Moudgil et al. (9) reported HSP65 peptide epitopes 417–431, 441–455, 465–479, 513–527, and 521–535, which correspond to 441–458, 469–483, 493–507, 541–555, and 549–563 in reference to HSP60. These epitopes are probably not relevant to the inhibition of AA triggered by pHSP60 vaccination, because the DNA fragments of HSP60 that contain these epitopes (pIII, and pIV and pV) did not inhibit the disease (Fig. 1). So we can conclude that the induction of a T cell response against Hu3 is only one of several mechanisms that can lead to protection against AA following vaccination with pHSP60.

Vaccination with pII (aa 130–260) also inhibited AA and induced HSP60-specific T cell responses, but we could not detect significant T cell responses specific for any peptide in the 130–260 region of HSP60, neither in pHSP60 nor in pII-vaccinated rats (Fig. 2*C* and data not shown). Thus, protection from AA triggered by vaccination with pII might involve the recognition of an epitope that was not represented in our peptide library (perhaps such epitope was split between two consecutive peptides), was below the detection level of the technique used in our assay, or simply

involved a different mechanism. A pII-specific mechanism of protection is supported by the different patterns of cytokine secretion observed when LNC taken from pI- or pII-vaccinated rats were stimulated with mycobacterial HSP65 or HSP60 epitopes (Fig. 7). Similar to what we observed in pI-vaccinated rats, LNC from pII-vaccinated rats showed a decrease in IFN-γ secretion upon stimulation with PPD, HSP65, and Mt176–190 (Fig. 7*A*). However, IL-10 and TGFβ1 secretion by LNC from pII-vaccinated rats was restricted to activation with HSP65 or Mt176–190 and PPD, respectively (Fig. 7, *B* and *C*). Future studies are needed to elucidate the regulatory mechanisms triggered by pII vaccination.

The present findings may be relevant for human rheumatoid arthritis and juvenile chronic arthritis, where T cell responses to HSP60 and HSP65 have been described (33–37). Human anti-HSP60 T cells secrete regulatory cytokines and their presence is associated with a milder disease in rheumatoid arthritis (36, 37) and in juvenile chronic arthritis (35, 38). This suggests that reactivity to HSP60 is involved in the control of human arthritis as well as in rat AA. Boosting the HSP60-regulatory response might therefore constitute an approach to managing human arthritis. For that purpose, it would be desirable to use defined regulatory epitopes like Hu3, and not whole proteins that harbor multiple epitopes, some of which might trigger adverse immunopathological reactions. At present, a peptide derived from HSP60 is undergoing clinical trials for type I diabetes mellitus and preliminary results have been encouraging (39). These observations highlight the importance of HSP60 reactivity, a form of regulatory autoimmunity, in the control of inflammation and body homeostasis (40, 41).

Acknowledgments

We thank Danielle Sabah-Israel for excellent secretarial assistance. We are grateful to Prof. Doug Lowrie for providing the pHSP65 construct, and to Prof. Ruurd van der Zee for the HSP65 used in our studies.

References

1. Wauben, M. H. M., J. P. A. Wagenaar-Hilbers, and W. van Eden. 1994. Adjuvant arthritis. In *Autoimmune Disease Models.* I. R. Cohen and A. Miller, eds. Academic Press, San Diego.
2. van Eden, W., J. E. Thole, R. van der Zee, A. Noordzij, J. D. van Embden, E. J. Hensen, and I. R. Cohen. 1988. Cloning of the mycobacterial epitope recognized by T lymphocytes in adjuvant arthritis. *Nature 331:171.*
3. van Eden, W., J. Holoshitz, Z. Nevo, A. Frenkel, A. Klajman, and I. R. Cohen. 1985. Arthritis induced by a T-lymphocyte clone that responds to Mycobacterium tuberculosis and to cartilage proteoglycans. *Proc. Natl. Acad. Sci. USA 82:5117.*
4. Holoshitz, J., A. Matitiau, and I. R. Cohen. 1984. Arthritis induced in rats by cloned T lymphocytes responsive to mycobacteria but not to collagen type II. *J. Clin. Invest. 73:211.*
5. Holoshitz, J., Y. Naparstek, A. Ben-Nun, and I. R. Cohen. 1983. Lines of T lymphocytes induce or vaccinate against autoimmune arthritis. *Science 219:56.*
6. Billingham, M. E., S. Carney, R. Butler, and M. J. Colston. 1990. A mycobacterial 65-kD heat shock protein induces antigen-specific suppression of adjuvant arthritis, but is not itself arthritogenic. *J. Exp. Med. 171:339.*
7. Hogervorst, E. J., L. Schouls, J. P. Wagenaar, C. J. Boog, W. J. Spaan, J. D. van Embden, and W. van Eden. 1991. Modulation of experimental autoimmunity: treatment of adjuvant arthritis by immunization with a recombinant vaccinia virus. *Infect. Immun. 59:2029.*
8. Ragno, S., M. J. Colston, D. B. Lowrie, V. R. Winrow, D. R. Blake, and R. Tascon. 1997. Protection of rats from adjuvant arthritis by immunization with naked DNA encoding for mycobacterial heat shock protein 65. *Arthritis Rheum. 40:277.*
9. Moudgil, K. D., T. T. Chang, H. Eradat, A. M. Chen, R. S. Gupta, E. Brahn, and E. E. Sercarz. 1997. Diversification of T cell responses to carboxy-terminal determinants within the 65-kD heat-shock protein is involved in regulation of autoimmune arthritis. *J. Exp. Med. 185:1307.*
10. Anderton, S. M., R. van der Zee, B. Prakken, A. Noordzij, and W. van Eden. 1995. Activation of T cells recognizing self 60-kD heat shock protein can protect against experimental arthritis. *J. Exp. Med. 181:943.*
11. Yang, X. D., J. Gasser, and U. Feige. 1992. Prevention of adjuvant arthritis in rats by a nonapeptide from the 65-kD mycobacterial heat shock protein: specificity and mechanism. *Clin. Exp. Immunol. 87:99.*
12. van Eden, W., U. Wendling, L. Paul, B. Prakken, P. van Kooten, and R. van der Zee. 2000. Arthritis protective regulatory potential of self-heat shock protein cross-reactive T cells. *Cell Stress Chaperones 5:452.*
13. Lopez-Guerrero, J. A., J. P. Lopez-Bote, M. A. Ortiz, R. S. Gupta, E. Paez, and C. Bernabeu. 1993. Modulation of adjuvant arthritis in Lewis rats by recombinant vaccinia virus expressing the human 60-kilodalton heat shock protein. *Infect. Immun. 61:4225.*
14. Lopez-Guerrero, J. A., M. A. Ortiz, E. Paez, C. Bernabeu, and J. P. Lopez-Bote. 1994. Therapeutic effect of recombinant vaccinia virus expressing the 60-kd heat-shock protein on adjuvant arthritis. *Arthritis Rheum. 37:1462.*
15. Quintana, F. J., P. Carmi, F. Mor, and I. R. Cohen. 2002. Inhibition of adjuvant arthritis by a DNA vaccine encoding human heat shock protein 60. *J. Immunol. 169:3422.*
16. Anderton, S. M., R. van der Zee, A. Noordzij, and W. van Eden. 1994. Differential mycobacterial 65-kDa heat shock protein T cell epitope recognition after adjuvant arthritis-inducing or protective immunization protocols. *J. Immunol. 152:3656.*
17. Quintana, F. J., A. Rotem, P. Carmi, and I. R. Cohen. 2000. Vaccination with empty plasmid DNA or CpG oligonucleotide inhibits diabetes in nonobese diabetic mice: modulation of spontaneous 60-kDa heat shock protein autoimmunity. *J. Immunol. 165:6148.*
18. Steinman, L. 1999. Assessment of animal models for MS and demyelinating disease in the design of rational therapy. *Neuron 24:511.*
19. van Tienhoven, E. A., C. T. ten Brink, J. van Bergen, F. Koning, W. van Eden, and C. P. Broeren. 2001. Induction of antigen specific $CD4^+$ T cell responses by invariant chain based DNA vaccines. *Vaccine 19:1515.*
20. Schmidt-Weber, C. B., D. Pohlers, A. Siegling, H. Schadlich, E. Buchner, H. D. Volk, E. Palombo-Kinne, F. Emmrich, and R. W. Kinne. 1999. Cytokine gene activation in synovial membrane, regional lymph nodes, and spleen during the course of rat adjuvant arthritis. *Cell. Immunol. 195:53.*
21. Garcia, G., Y. Komagata, A. J. Slavin, R. Maron, and H. L. Weiner. 1999. Suppression of collagen-induced arthritis by oral or nasal administration of type II collagen. *J. Autoimmun. 13:315.*
22. Song, X. Y., M. Gu, W. W. Jin, D. M. Klinman, and S. M. Wahl. 1998. Plasmid DNA encoding transforming growth factor-β1 suppresses chronic disease in a streptococcal cell wall-induced arthritis model. *J. Clin. Invest. 101:2615.*
23. Prakken, B. J., S. Roord, P. J. van Kooten, J. P. Wagenaar, W. van Eden, S. Albani, and M. H. Wauben. 2002. Inhibition of adjuvant-induced arthritis by interleukin-10-driven regulatory cells induced via nasal administration of a peptide analog of an arthritis-related heat-shock protein 60 T cell epitope. *Arthritis Rheum. 46:1937.*
24. Paul, A. G., P. J. van Kooten, W. van Eden, and R. van der Zee. 2000. Highly autoproliferative T cells specific for 60-kDa heat shock protein produce IL-4/IL-10 and IFN-γ and are protective in adjuvant arthritis. *J. Immunol. 165:7270.*
25. Sato, H., M. Miyata, and R. Kasukawa. 1996. Expression of heat shock protein on lymphocytes in peripheral blood and synovial fluid from patients with rheumatoid arthritis. *J. Rheumatol. 23:2027.*
26. Ferm, M. T., K. Soderstrom, S. Jindal, A. Gronberg, J. Ivanyi, R. Young, and R. Kiessling. 1992. Induction of human hsp60 expression in monocytic cell lines. *Int. Immunol. 4:305.*
27. Miyata, M., H. Sato, Y. Sato, E. Kodama, and R. Kasukawa. 1999. Significance of endogenous heat shock protein in adjuvant arthritis. *J. Rheumatol. 26:2210.*
28. Schett, G., M. Tohidast-Akrad, G. Steiner, and J. Smolen. 2001. The stressed synovium. *Arthritis Res. 3:80.*
29. Nakajima, A., C. M. Seroogy, M. R. Sandora, I. H. Tarner, G. L. Costa, C. Taylor-Edwards, M. H. Bachmann, C. H. Contag, and C. G. Fathman. 2001. Antigen-specific T cell-mediated gene therapy in collagen-induced arthritis. *J. Clin. Invest. 107:1293.*
30. Waisman, A., P. J. Ruiz, D. L. Hirschberg, A. Gelman, J. R. Oksenberg, S. Brocke, F. Mor, I. R. Cohen, and L. Steinman. 1996. Suppressive vaccination with DNA encoding a variable region gene of the T-cell receptor prevents autoimmune encephalomyelitis and activates Th2 immunity. *Nat. Med. 2:899.*
31. Quinn, A., B. McInerney, E. P. Reich, O. Kim, K. P. Jensen, and E. E. Sercarz. 2001. Regulatory and effector CD4 T cells in nonobese diabetic mice recognize overlapping determinants on glutamic acid decarboxylase and use distinct Vβ genes. *J. Immunol. 166:2982.*
32. Ulmansky, R., C. J. Cohen, F. Szafer, E. Moallem, Z. G. Fridlender, Y. Kashi, and Y. Naparstek. 2002. Resistance to adjuvant arthritis is due to protective antibodies against heat shock protein surface epitopes and the induction of IL-10 secretion. *J. Immunol. 168:6463.*

33. Celis, L., C. Vandevyver, P. Geusens, J. Dequeker, J. Raus, and J. Zhang. 1997. Clonal expansion of mycobacterial heat-shock protein-reactive T lymphocytes in the synovial fluid and blood of rheumatoid arthritis patients. *Arthritis Rheum. 40:510*.
34. De Graeff-Meeder, E. R., R. van der Zee, G. T. Rijkers, H. J. Schuurman, W. Kuis, J. W. Bijlsma, B. J. Zegers, and W. van Eden. 1991. Recognition of human 60 kD heat shock protein by mononuclear cells from patients with juvenile chronic arthritis. *Lancet 337:1368*.
35. de Graeff-Meeder, E. R., W. van Eden, G. T. Rijkers, B. J. Prakken, W. Kuis, M. M. Voorhorst-Ogink, R. van der Zee, H. J. Schuurman, P. J. Helders, and B. J. Zegers. 1995. Juvenile chronic arthritis: T cell reactivity to human HSP60 in patients with a favorable course of arthritis. *J. Clin. Invest. 95:934*.
36. MacHt, L. M., C. J. Elson, J. R. Kirwan, J. S. Gaston, A. G. Lamont, J. M. Thompson, and S. J. Thompson. 2000. Relationship between disease severity and responses by blood mononuclear cells from patients with rheumatoid arthritis to human heat-shock protein 60. *Immunology 99:208*.
37. van Roon, J. A., W. van Eden, J. L. van Roy, F. J. Lafeber, and J. W. Bijlsma. 1997. Stimulation of suppressive T cell responses by human but not bacterial 60-kD heat-shock protein in synovial fluid of patients with rheumatoid arthritis. *J. Clin. Invest. 100:459*.
38. Prakken, A. B., W. van Eden, G. T. Rijkers, W. Kuis, E. A. Toebes, E. R. de Graeff-Meeder, R. van der Zee, and B. J. Zegers. 1996. Autoreactivity to human heat-shock protein 60 predicts disease remission in oligoarticular juvenile rheumatoid arthritis. *Arthritis Rheum. 39:1826*.
39. Raz, I., D. Elias, A. Avron, M. Tamir, M. Metzger, and I. R. Cohen. 2001. β-Cell function in new-onset type 1 diabetes and immunomodulation with a heat-shock protein peptide (DiaPep277): a randomised, double-blind, phase II trial. *Lancet 358:1749*.
40. Schwartz, M., and I. R. Cohen. 2000. Autoimmunity can benefit self-maintenance. *Immunol. Today 21:265*.
41. Cohen, I. R. 2000. *Tending Adam's Garden: Evolving the Cognitive Immune Self*. Academic Press, London.