Inhibition of Adjuvant Arthritis by a DNA Vaccine Encoding Human Heat Shock Protein 60[1]

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

Adjuvant arthritis (AA) is an autoimmune disease inducible in rats involving T cell reactivity to the mycobacterial 65-kDa heat shock protein (HSP65). HSP65-specific T cells cross-reactive with the mammalian 60-kDa heat shock protein (HSP60) are thought to participate in the modulation of AA. In this work we studied the effects on AA of DNA vaccination using constructs coding for HSP65 (pHSP65) or human HSP60 (pHSP60). We found that both constructs could inhibit AA, but that pHSP60 was more effective than pHSP65. The immune effects associated with specific DNA-induced suppression of AA were complex and included enhanced T cell proliferation to a variety of disease-associated Ags. Effective vaccination with HSP60 or HSP65 DNA led paradoxically to up-regulation of IFN-γ secretion to HSP60 and, concomitantly, to down-regulation of IFN-γ secretion to the P180-188 epitope of HSP65. There were also variable changes in the profiles of IL-10 secretion to different Ags. However, vaccination with pHSP60 or pHSP65 enhanced the production of TGFβ1 to both HSP60 and HSP65 epitopes. Our results support a regulatory role for HSP60 autoreactivity in AA and demonstrate that this control mechanism can be activated by DNA vaccination with both HSP60 or HSP65. *The Journal of Immunology,* 2002, 169: 3422–3428.

Adjuvant arthritis (AA),[3] inducible in susceptible rats by immunization with heat-killed *Mycobacterium tuberculosis* (Mt), is characterized by peripheral joint lesions that share many features with human rheumatoid arthritis (RA), although certainly not all features (1). Mycobacterial 65-kDa heat shock protein (HSP65) is a target of pathogenic T cells in AA; a T cell clone (A2b) specific for the epitope contained between amino acids 180 and 188 of HSP65 could adoptively transfer AA (2). The A2b T cell clone was also found to react to an epitope of cartilage proteoglycan, suggesting that targeting of inflammation to the joints might be due to cross-reactivity between P180-188 and a self-component in cartilage (3). However, HSP65 or some of its T cell epitopes can also induce resistance to AA when administered i.p., orally, or injected, expressed in a recombinant vaccinia virus or as a DNA vaccine (4–7). Inhibition of AA by treatment with HSP65 is thought to be mediated by regulatory T cells cross-reactive with the self-60 kDa heat shock protein (HSP60) (8, 9). Indeed, T cell reactivity to self-HSP60 was reported to be associated with a favorable prognosis in human RA; thus, HSP60 autoimmunity is thought to contribute to the control of RA and oligoarticular juvenile chronic arthritis (10, 11). Hence, mycobacterial HSP65 appears to provide epitopes with different immune func-

tions in AA: the cross-reaction of P180-188 with cartilage may be involved in the pathogenic effector mechanism, and a cross-reactivity between HSP65 and self-HSP60 might be involved in regulation of the disease.

In this work we studied the effect on AA of DNA vaccination using vaccines encoding for human HSP60 (pHSP60) or mycobacterial HSP65 (pHSP65). Human HSP60 is 97% identical to rat HSP60 at the amino acid level; thus, pHSP60 is rich in self-epitopes. We found that both DNA vaccines inhibited AA. We then studied both T cell proliferation and cytokine production to several Ags known to be associated with the disease. Our strategy was to document the immune effects of DNA vaccination on the immune reactions to the Ags that develop at the peak of AA.

Materials and Methods

Rats

Female Lewis rats were raised and maintained under pathogen-free conditions in the Animal Breeding Center of this institute. Experiments were conducted under the supervision and guidelines of the Animal Welfare Committee. The rats were 1 mo old at the start of the experiments.

Ags, peptides, and adjuvants

Mt strain H37Ra was obtained from Difco (Detroit, MI). Purified recombinant HSP65 of Mt was kindly provided by Dr. R. van der Zee (Institute of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht, The Netherlands). Recombinant HSP60 was prepared as described (12). The HSP65-derived peptide P176-190 used in this work was EESNT FGLQLELTEG (13); this peptide includes the P180-188 epitope. Peptides were synthesized by a standard F-moc procedure. The peptides were purified by reversed-phase HPLC and their compositions were confirmed by amino acid analysis. Con A and OVA were purchased from Sigma (Rehovot, Israel). IFA was purchased from Difco. The purified protein derivative (PPD) of Mt was provided by the Statens Seruminstitut (Copenhagen, Denmark).

DNA plasmids

The vector containing the human HSP60 gene (pHSP60) has been described (14). A construct coding for the 14.5-kDa isoform of murine myelin basic protein (pMBP) was kindly provided by Dr. L. Steinman (Department of Neurological Sciences, Stanford University, Stanford, CA). The construct encoding *Mycobacterium leprae* HSP65 (pHSP65) was kindly provided by Dr. D. Lowrie (Medical Research Council, London,

[3] Abbreviations used in this paper: AA, adjuvant arthritis; HSP65, 65-kDa heat shock protein; HSP60, 60-kDa heat shock protein; pHSP65, DNA vaccine encoding HSP65; pHSP60, DNA vaccine encoding HSP60; Mt, *Mycobacterium tuberculosis*; RA, rheumatoid arthritis; PPD, purified protein derivative; pMBP, DNA vaccine encoding murine myelin basic protein; DLN, draining lymph node; SI, stimulation index; RT, room temperature.

U.K.) and has been shown to be effective in controlling AA (7). The empty vector pcDNA3 (14) was used as a DNA vaccination control.

Plasmid DNA was prepared in large scale using the alkaline lysis method of Qiagen Plasmid Mega Prep (Qiagen, Santa Clarina, CA). Plasmid DNA was ethanol precipitated and resuspended in sterile PBS. Spectrophotometric analysis revealed 260/280 nm ratios ≤1.80. Purity of DNA preparations was confirmed on a 1% agarose gel. Endotoxin levels were checked by *Limulus* amebocyte lysate and were found always to be under acceptable levels for in vivo use (<0.02 EU/μg DNA).

Groups of rats were injected i.m. with 200 μl of 10 mM cardiotoxin (Sigma) into the quadriceps using a sterile 27-gauge syringe, witted with a plastic collar to limit needle penetration to 2 mm. The test DNA vaccine (150 μl of 1 mg/ml) was administered i.m. three times: 5, 19, and 33 days after the cardiotoxin. AA was induced 12 days after the last injection of DNA.

Detection of HSP60

Blood samples were collected at day 12 after the last injection of DNA, and serum was prepared as previously described (15) and kept at −20°C until used. HSP60 was quantified by a capture ELISA as previously reported by Rea et al. (16). ELISA plates (Maxisorp; Nunc, Roskilde, Denmark) were coated overnight at 4°C with the murine mAb LK1, specific for human HSP60 (17). Nonspecific binding was blocked by incubation with 1% BSA for 3 h at room temperature (RT), and serum samples or recombinant HSP60 were added and incubated overnight at 4°C. After washing the plates, bound HSP60 was detected using purified anti-human HSP60 rabbit polyclonal Abs (provided by Dr. G. Nussbaum, Department of Immunology, Weizmann Institute of Science, Rehovot, Israel) followed by an anti-rabbit murine mAb conjugated to alkaline phosphatase (Sigma). The plates were washed, alkaline phosphatase substrate (Sigma) was added, and samples were read at 405 nm. HSP60 levels in serum are expressed as micrograms per milliliter based on a calibration curve constructed using recombinant human HSP60 as a standard; the lower limit of detection was 20 ng/ml.

AA induction and assessment

Heat-killed Mt strain H37Ra (Difco) was finely ground using a pestle and mortar, and was suspended to a final concentration of 10 mg/ml in IFA. Test rats were injected at the base of the tail with a total of 100 μl of the Mt suspension. The day of AA induction was designated as day 0. 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. 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 ± SE of the difference between the values for hind limb diameter taken on days 0 and 26.

Joint histology

The rats were killed at day 26, the peak of AA, and their hind paws were removed and fixed in formol saline for 48 h, decalcified in 5% formic acid with formol saline, and stained with H&E (7). Sixteen individual joints were analyzed in each group. The stained sections were scored using a semiquantitative scale (18, 19) that separately measures synovial inflammation (0–4) and bone and cartilage erosions (0–4). The results are shown as the mean score ± SE each for synovial inflammation and for bone and cartilage erosion.

T cell proliferation

T cell proliferation was measured at day 26, the peak of AA. Draining lymph node (DLN) cells (prepared from inguinal and popliteal lymph nodes) were cultured in quadruplicate in round-bottom microtiter wells (Costar, Cambridge, MA) in 200 μl of stimulation medium at 2×10^5 cells per well with or without Ag. Stimulation medium was made up of DMEM (Life Technologies, Paisley, U.K.) supplemented with $5 \ 10^{-5}$ M 2-ME (Sigma), 2 mM L-glutamine (Biological Industries, Kibbutz Beit Haemek, Israel), 1 mM sodium pyruvate (Sigma), 100 U/ml penicillin (Biological Industries), 100 μg/ml streptomycin (Biological Industries), 1% v/v non-essential amino acids (Bio Lab, Jerusalem, Israel), and 1% v/v autologous serum. The T cell mitogen Con A was used at a concentration of 1.25 μg/ml as a positive control for T cell proliferation. Cultures were incubated for 96 h at 37°C in a humidified atmosphere of 7.5% CO_2. T cell responses were detected by the incorporation of 1 μCi/well [methyl-^{3}H]thymidine (Amersham Pharmacia Biotech, Little Chalfont, U.K.) added to the wells for the last 18 h. The stimulation index (SI) was computed as the ratio of
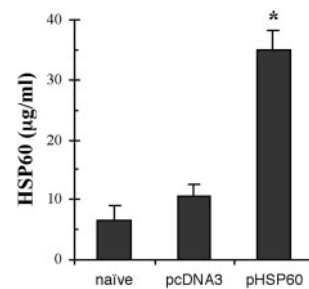

FIGURE 1. pHSP60 vaccination induces increased serum HSP60. The concentration of HSP60 in serum was measured 12 days after the last injection of DNA by a sandwich ELISA. The bars represent the mean values ± SE for each group of eight rats. ∗, $p < 0.0001$ compared with the pcDNA-treated or naive rats.

the mean cpm of Ag- or mitogen-containing wells to control wells cultured with medium alone. In the case of DLN cells taken from pHSP60- or pHSP65-treated rats, the results are also expressed as the percentage of increase in the proliferation compared with the proliferation seen in DLN cells isolated from pcDNA3-treated rats. The percentage of increase in the proliferation for each Ag was calculated as $100 \times$ (SI in the pHSP60 or pHSP65 group − SI in the pcDNA3 group)/SI in the pcDNA3 group.

Cytokine assays

Supernatants were collected after 72 h of stimulation with test Ags. Rat IL-10 and IFN-γ were quantified in the culture supernatants by ELISA using the OPTEIA kit (BD PharMingen, San Diego, CA) with some modifications. Briefly, ELISA plates (Maxisorp; Nunc) were coated overnight at 4°C with anti-rat cytokine monoclonal capture Abs. Nonspecific binding
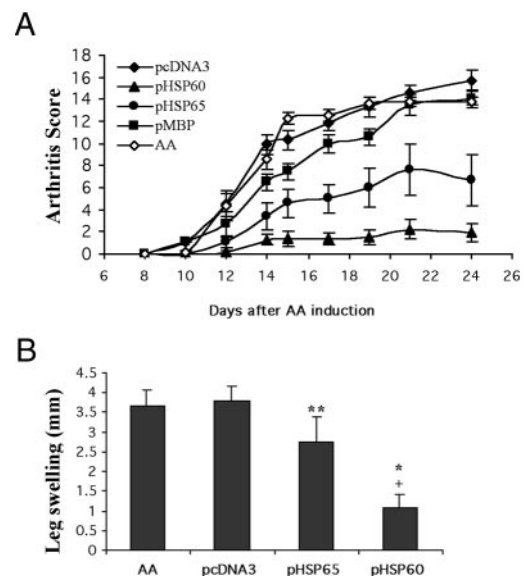

FIGURE 2. Inhibition of AA by DNA vaccination with pHSP60 or pHSP65. *A*, Time course of AA. Rats, 11 in each group, were not vaccinated (AA) or were vaccinated with control pcDNA3 or pMBP, or with pHSP60 or pHSP65. On day 0, AA was induced and arthritis scores were assessed every 2 or 3 days starting at day 8. Bars show the mean ± SEM disease score. The differences between the unvaccinated or control vaccinated rats and the rats vaccinated with pHSP60 or pHSP65 were significant ($p < 0.05$) at every determination at days 14–24. Vaccination with pHSP60 was more effective than vaccination with pHSP65 starting from day 15 ($p < 0.05$). *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. The difference between pHSP60- and pHSP65-treated rats is significant ($p < 0.05$).

was blocked by incubation with 1% BSA for 1 h at RT, and culture supernatants or recombinant cytokines were added for 2 h at RT. After washing the plates, biotinylated detection Abs were added for 1 h at RT, then extensively washed and incubated with streptavidin conjugated to alkaline phosphatase (Jackson ImmunoResearch Laboratories, West Grove, PA) for 30 min at RT. The plates were washed, alkaline phosphatase substrate (Sigma) was added, and samples were read at 405 nm after 30 min of incubation at RT. Rat TGFβ1 was quantified using the TGFβ1 E$_{max}$ ImmunoAssay System (Promega, Madison, WI) according to the manufacturer's instructions. The determinations were made at day 26, the peak of AA. Cytokine levels in supernatants 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. In the case of DLN cells taken from pHSP60- or pHSP65-treated rats, the results are also expressed as the percentage of change in cytokine secretion compared with the secretion detected in DLN isolated from pcDNA3-treated rats. The percentage of change was calculated as $100 \times$ (pg/ml of cytokine in the pHSP60 or pHSP65 group $-$ pg/ml in the pcDNA3 group)/pg/ml in the pcDNA3 group. When no cytokine secretion was detected in DLN cells from pcDNA3-vaccinated rats, 15 pg/ml (the lower detection limit) was used for the calculation of the change in cytokine secretion.

Statistical significance

The InStat 2.01 program was used for statistical analysis. Student's *t* test and the Mann-Whitney test were conducted to assay significant differences between the different experimental groups.

Results

DNA vaccination with pHSP60 induces increased levels of serum HSP60

The features of the pHSP65 construct used in these studies have been previously described (7). To determine whether the injection of pHSP60 could lead in vivo to the production of HSP60, eight rats were vaccinated three times (5, 19, and 33 days after the pretreatment with cardiotoxin) with pHSP60 or with the empty vector pcDNA3. A group of naive rats was also included as a control. Twelve days after the last injection, serum samples were collected and HSP60 was quantified using a specific ELISA. Fig. 1 shows that pHSP60-vaccinated rats manifested significantly higher levels of HSP60 in serum ($p < 0.0001$) when compared with pcDNA3-vaccinated or naive rats. These results are in accordance with pre-

vious reports that describe the detection of the Ag encoded by DNA vaccines in the serum of injected animals (20, 21). Furthermore, they demonstrate that vaccination with pHSP60 leads to a significant increase in HSP60 serum levels.

DNA vaccination: human pHPS60 is more effective than is mycobacterial pHSP65

We investigated the effects on AA of vaccination with DNA encoding human pHSP60 compared with mycobacterial HSP65. We used a construct encoding the full-length human HSP60 (pHSP60) and a construct encoding the full-length HSP65 of *M. leprae*
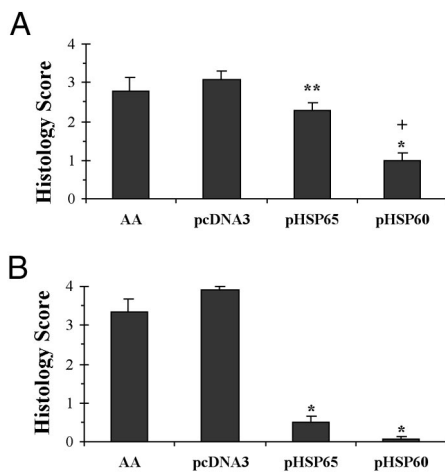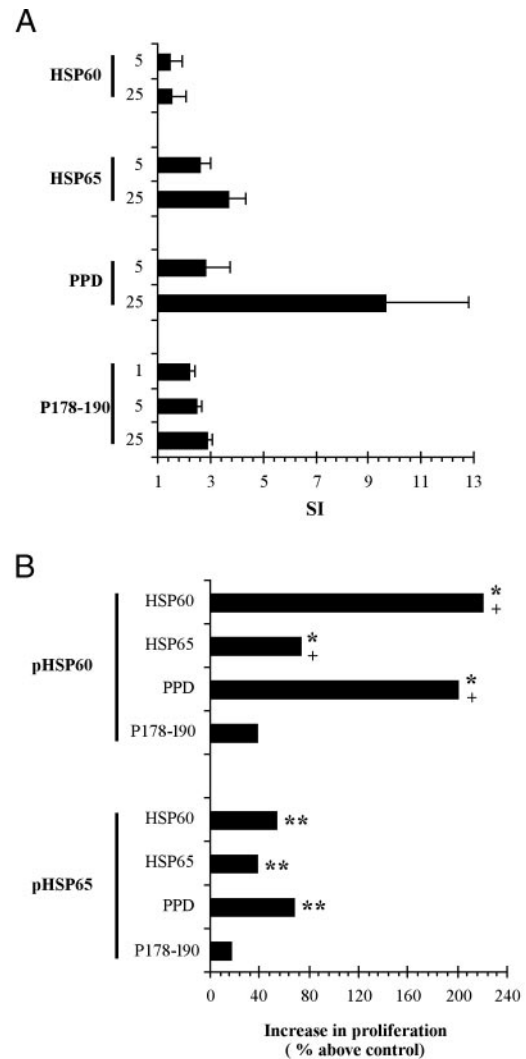

FIGURE 4. Effect of DNA vaccination on T cell proliferation in AA. *A*, T cell responses in pcDNA3-vaccinated rats. Rats were vaccinated with the empty vector pcDNA3, and their DLN T cell responses were measured on day 26 after induction of AA. The simulating Ags, at 1, 5, or 25 μg/ml, were HSP60, HSP65, PPD, and P178-190 peptide of HSP65. The proliferative responses are expressed as the mean SI $\pm$ SEM of quadruplicate cultures. *B*, Changes in T cell responses in pHSP60- and pHSP65-vaccinated rats. Rats were vaccinated with pHSP60 or pHSP65, and on day 26 after induction of AA the T cell responses of their DLN cells were studied. The results are presented as the mean of three independent experiments and are expressed as the percentage of increase in proliferation relative to the responses of control vaccinated rats for the same Ag (see *A*). Only the results corresponding to the optimum concentration of the Ag are shown. The SE was <15% of the mean between different experiments. $*$, $p < 0.005$ compared with the pcDNA3 group; $**$, $p < 0.04$ compared with the pcDNA3 group; $+$, $p < 0.05$ compared with the pHSP65 group.

FIGURE 3. Histological examination of AA joints following DNA vaccination. A semiquantitative histological analysis was performed on joints (16 per group) obtained from DNA-vaccinated rats 26 days after the induction of AA. *A*, Mean histological scores $\pm$ SE for synovial inflammation (maximal score, 4). *B*, Mean histological scores $\pm$ SE for bone and cartilage erosion (maximal score, 4). $*$, $p < 0.0001$ compared with the control groups; $**$, $p < 0.05$ compared with the control groups; $+$, $p = 0.005$ compared with the pHSP65 group.

Table I. *Proliferation and cytokine secretion by DLN cells of DNA-treated rats upon in vitro Ag stimulation[a]*

| | Treatment | | | | | | | | | | | |
| | pcDNA3 | | | | pHSP65 | | | | pHSP60 | | | |
Ag	SI	IFN-γ	IL-10	TGFβ1	SI	IFN-γ	IL-10	TGFβ1	SI	IFN-γ	IL-10	TGFβ1
None	1	BD	BD	BD	1	BD	BD	BD	1	BD	BD	BD
HSP60	1.5 ± 0.5	62 ± 13	BD	BD	2.3 ± 0.3	649 ± 76	83 ± 13	92 ± 26	4.8 ± 1.7	566 ± 63	128 ± 15	264 ± 33
HSP65	3.7 ± 0.7	1440 ± 87	BD	BD	5.1 ± 0.5	1713 ± 196	31 ± 5	708 ± 154	6.4 ± 0.5	1658 ± 235	72 ± 11	339 ± 37
PPD	9.6 ± 3.2	2084 ± 127	128 ± 31	37 ± 3	16.1 ± 2.2	1486 ± 524	292 ± 35	985 ± 101	28.9 ± 1.3	1981 ± 273	654 ± 85	855 ± 169
P178–190	2.9 ± 0.2	2152 ± 91	BD	BD	3.4 ± 0.4	821 ± 54	BD	664 ± 54	4 ± 0.4	1232 ± 154	76 ± 12	1277 ± 345

[a] Rats (eight per group) were vaccinated with DNA preparations and AA was induced. The DLN cells were pooled on day 26, the peak of AA, and proliferation and cytokine secretion were measured. Similar results were obtained in each of three independent experiments. Only the results corresponding to the optimal concentration of the Ag are shown. Proliferation is shown as SI. Cytokine secretion into the culture medium is shown as picograms per milliliter. The sensitivity for IFN-γ, IL-10, and TGFβ1 was 15 pg/ml. BD, Below detection.

(pHSP65), shown in the past to modulate AA (7). Two control constructs were used: an empty vector (pcDNA3) and pMBP. Fig. 2*A* shows that vaccination with pcDNA3 or pMBP did not have any effect on AA. In contrast, rats immunized with pHSP60 or pHSP65 manifested a significantly milder arthritis. Inhibition of AA was also reflected as a diminished swelling of the ankle, as shown in Fig. 2*B*. Histological examination of the joints revealed that pHSP60- and pHSP65-vaccinated rats manifested reduced bone and cartilage erosion and synovial inflammation (Fig. 3). The degree of protection achieved by immunization with pHSP65 reproduces that which has been previously reported by others (7). However, it can be seen that pHSP60 was more effective than pHSP65 in modulating the autoimmune process. The difference between pHSP60 and pHSP65 was statistically significant with regard to the maximal AA score (2.3 ± 0.7 vs 7.7 ± 1.8; $p = 0.02$), the degree of leg swelling (10.6 ± 3.4 vs 27.5 ± 6.4; $p = 0.03$), and the synovial inflammation score (2 ± 0.2 vs 1 ± 0.2; $p = 0.005$).

AA immune response in vaccinated rats: effects on T cell proliferation

To provide a background for the immunological effects of DNA vaccination, we first established the T cell response profile of AA in rats with unmodified disease. Twenty-six days after the induction of AA, DLN cells were prepared from untreated rats or from rats that had been control-treated with pcDNA3. We stimulated the DLN cells in vitro using a collective of Ags previously known to be targeted or associated with AA: HSP60, HSP65, PPD, and peptide P176-190 (which contains the 180-188 epitope of HSP65) (4). OVA was included as a control Ag. We found that the results were essentially the same whether the AA was induced in untreated rats or in rats pretreated with control injections of pcDNA3 (data not shown). Fig. 4*A* depicts the results obtained with DLN cells isolated from pcDNA3-treated animals, showing the proliferative responses at the peak of clinical AA: day 26. The rats showed a strong proliferative response to PPD and low but significant responses to HSP65 and P176-190; no significant proliferation was detected in response to stimulation with HSP60.

Fig. 4*B* shows the T cell proliferative responses measured 26 days after the induction of AA in DLN cells isolated from rats vaccinated with pHSP60 or pHSP65. The results presented in Fig. 4*B* are the mean ± SE of three independent experiments and are expressed as the percentage of increase in reactivity relative to the proliferation obtained using cells from pcDNA3-treated rats. In addition, Table I shows the SI values of a representative experiment. The pHSP60- or pHSP65-vaccinated rats, with reduced AA (see Fig. 2), showed markedly increased proliferative responses to the mycobacterial Ags PPD and HSP65 and to human HSP60. The

response to P178-190 peptide was only slightly increased relative to the other Ags. The increase in the proliferation to HSP60, HSP65, and PPD was stronger in pHSP60-treated rats than in those treated with pHSP65. Thus, vaccination with pHSP60, which inhibits AA, can actually augment T cell proliferation to HSP65 as well as to HSP60 itself; likewise, HSP65 vaccination augments T cell proliferation to HSP60 and to itself.

None of the experimental groups showed significant T cell responses to OVA, and they did not differ in their responses to Con A (data not shown). Thus, inhibition of AA by vaccination with either pHSP60 or pHSP65 is accompanied by the up-regulation of T cell proliferative responses to HSP60 and to some mycobacterial Ags.

AA immune response in vaccinated rats: effects on cytokine secretion

Cytokine release upon in vitro stimulation was studied to further characterize the T cell responses of DNA-treated rats 26 days after AA induction. DLN cells isolated from untreated rats (data not shown) or from rats pretreated with control injections of pcDNA3 gave essentially the same results (shown in Table I). Note the cytokine profile in unmodified AA: although the proliferative response to P176-190 was quite low, this peptide induced the release of IFN-γ to at least the same levels as those achieved by stimulation with PPD. IFN-γ was secreted to a lower extent in response to HSP65; very low secretion was detected upon stimulation with HSP60. IL-10 and TGFβ1 were detected only upon activation with PPD. Thus, induction of AA up-regulates IFN-γ secretion in response to mycobacterial Ags, but the T cells in these animals do not appear to respond to HSP60. The question was how the cytokine profile might be modified by effective DNA vaccination with pHSP60 or pHSP65.

The mean values of cytokine secretion obtained in three independent experiments done using cells isolated from rats treated with pHSP60 or pHSP65 are presented in Fig. 5, as the percentage of change relative to the amount of cytokines secreted from cells taken from control-treated rats. In addition, Table I shows the results corresponding to a representative experiment, expressed as picograms per milliliter. The effect of DNA vaccination on IFN-γ secretion depended on the stimulating Ag. DLN cells from pHSP60- and pHSP65-vaccinated rats secreted significantly lower amounts of IFN-γ upon stimulation with P176-190 than did rats with unmodified AA; secretion was reduced by half. Remarkably, IFN-γ secretion was relatively increased in response to HSP60 after vaccination with pHSP60 or pHSP65. Thus, inhibition of AA was associated with a reduction in IFN-γ secretion to P176-190 and with an increase in IFN-γ secretion to HSP60.
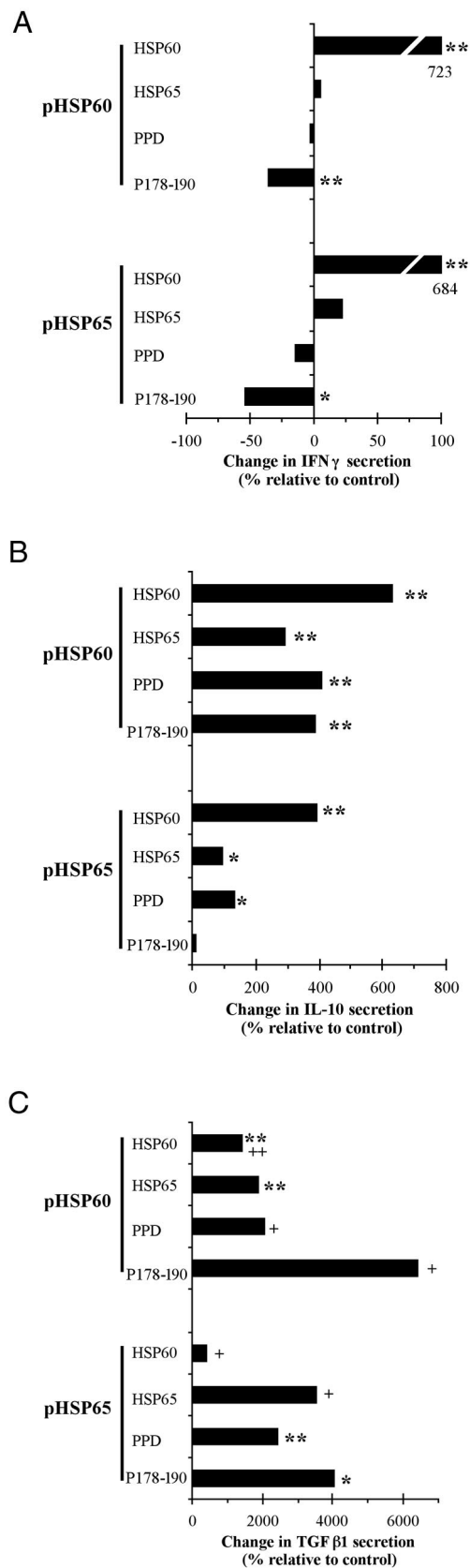
FIGURE 5. Effect of DNA vaccination on cytokine secretion in AA. Rats were vaccinated with pHSP60 or pHSP65, control vaccinated with the empty vector pcDNA3, or unvaccinated, and AA was induced. On day 26 after induction of AA, the cytokine secretion of their DLN cells was studied. The cells were stimulated for 72 h in the presence of various Ags, and the secretion of IFN-γ (A), IL-10 (B), or TGFβ1 (C) was measured. The results are presented as the mean change in cytokine secretion obtained in

Regarding IL-10, PLN cells from pHSP60- and pHSP65-vaccinated rats secreted IL-10 in response to stimulation with PPD or HSP65, but only those cells taken from pHSP60-vaccinated rats secreted IL-10 upon activation with P176-190. Cells taken from both pHSP60- and pHSP65-vaccinated animals released significant IL-10 upon stimulation with HSP60 (Fig. 5B).

In contrast to the Ag-dependent changes in IFN-γ and IL-10 secretion, markedly increased amounts of TGFβ1 were produced by the cells of vaccinated rats in response to stimulation with all the Ags tested: mycobacterial Ags HSP65, PPD, P176-190, and HSP60 (Fig. 5C).

In summary, inhibition of AA by vaccination with specific DNA vaccines was associated with three observations: first, decreased secretion of IFN-γ upon stimulation with the HSP65 peptide P178-190. Second, increased secretion of IFN-γ in response to stimulation with HSP60. Third, augmentation of IL-10 and TGFβ1 secretion in response to both mycobacterial Ags and HSP60.

Discussion

In this work we compared the effects of DNA vaccination with human HSP60 and mycobacterial HSP65 on the course of AA. HSP60 DNA vaccination led to increased serum levels of the encoded Ag (Fig. 1); thus, the vaccine was functional. Although both constructs inhibited AA, vaccination with pHSP60, which is much closer to rat self-HSP60 than is HSP65, had a significantly stronger effect (Fig. 2). Protection from AA with both DNA constructs was associated with increased proliferative responses to mycobacterial Ags and to HSP60 (Fig. 4B). Up-regulation of the proliferation to mycobacterial Ags has also been found when AA was inhibited using a recombinant vaccinia virus coding for HSP60 or HSP65, and by immunization with mycobacterial HSP65 as a recombinant protein or a DNA vaccine (7, 13, 15, 16). Our finding of increased T cell proliferation to human HSP60 in suppressed AA is compatible with the observation that increased T cell proliferative reactivity to HSP65 and HSP60 is associated with milder forms of RA and oligoarticular juvenile chronic arthritis (10, 11).

The immune effects of DNA vaccination on AA were considerable. Significant differences were found between control and pHSP60-treated animals, in decreased disease both clinically (Fig. 2) and histologically (Fig. 3). Immunologically, effective vaccination was associated with increased T cell proliferation to specific Ags (Fig. 4B). However, how can increased T cell reactivity to disease-associated Ags correlate with protection from AA?

Th cells have been assigned to different categories according to the cytokines they secrete upon stimulation with their target Ags (17). Among these categories, self-reactive Th1 cells (defined by IFN-γ secretion) have been classically associated with autoimmune pathology, while "regulatory" Th2/3 cells (secreting IL-10 and/or TGFβ1) were proposed to protect against autoimmune disease (22–24). In our experiments, protection by DNA vaccination

three independent experiments and are expressed as a percentage of the responses of pcDNA3-vaccinated rats for the same Ag. The SE was <15% of the mean between different experiments, except for the response to PPD (25%). Only the results corresponding to the optimum concentration of the Ag are shown. When cytokine release was not detected in the controls upon stimulation with a certain Ag, the value corresponding to the lower level of sensitivity was used to estimate the percentage of change in cytokine secretion. The sensitivity was 15 pg/ml for IFN-γ, IL-10, and TGFβ1. *, $p < 0.005$ compared with the pcDNA3 group; **, $p < 0.02$ compared with the pcDNA3 group; +, $p < 0.05$ compared with the pcDNA3 group; ++, $p < 0.05$ compared with the pHSP65 group.

was associated with two different effects in the same rats: down-regulation of IFN-γ secretion in response to P176-190 and up-regulation of IFN-γ secretion in response to HSP60, along with up-regulation of IL-10 and TGFβ1 (Table I and Fig. 5). Regarding P176-190 the picture is clear: this peptide contains an epitope from HSP65 that has been linked to the autoimmune process involved in AA. A T cell clone specific to this epitope, which cross-reacts with cartilage, could transfer arthritis to irradiated rats (2–4). Thus, the decrease in IFN-γ release and increase in IL-10 and TGFβ1 upon in vitro stimulation with P176-190 might reflect the effective control of pathogenic T cell clones (3) in the DNA-vaccinated rats involving a switch from Th1 to Th2/3.

However, the induction of increased IFN-γ secretion in response to "self" HSP60 does not seem to fit a simple cytokine shift hypothesis. Indeed, other investigations suggest that IFN-γ secretion might mark some regulatory T cells. A recent study by Paul et al. (25) reported that HSP65-specific rat T cells able to confer protection to AA cross-react with mammalian HSP60 and secrete IL-10 and IFN-γ. Similar IFN-γ-associated regulatory mechanisms have been described to operate controlling self-reactivity after T cell vaccination in humans (26, 27), or vaccination with TCR-derived peptides (28) or DNA vaccines (29, 30) in experimental models of autoimmunity. In these examples, vaccination induced a response rich in IFN-γ against the protective vaccine, but this response correlated with a shift toward Th2 in the pathogenic response to the target Ags of the pathogenic T cells. Finally, the Tr1 regulatory cells (22, 23) first described by Groux et al. (31) are characterized by the secretion of IL-10, TGFβ1, and IFN-γ, but no IL-4, in response to Ag stimulation, and have been shown to control Th1 (31) and Th2 (32) immune responses. Hence, based on these results we might propose that DNA vaccination with pHSP60 or pHSP65 induces a T cell response to HSP60 that modulates the T cell response to P176-190 and other target Ags. The HSP60-specific T cells secrete both the Th1 cytokine IFN-γ and the Th2/3 cytokines IL-10 and TGFβ1. By yet-unknown mechanisms, the regulatory HSP60 specific T cells induce a more polarized shift to the Th2/3 cytokine profile of the autoimmune clones responding to the P176-190 target Ag.

In our experiments, the immune response toward the P176-190 target peptide was enriched in IL-10 and/or TGFβ1 and decreased in IFN-γ secretors. Administration of recombinant murine IL-10 or of T cells engineered to secrete rat IL-10 constitutively was shown to down-regulate autoimmune arthritis (33, 34). In addition, IL-10 can render activated T cells susceptible to the action of TGFβ1 (35). TGFβ1 is a suppressor cytokine with broad spectrum of action (36). Although its role in the control of experimental arthritis is just starting to be understood (37, 38), secretion of TGFβ1 by DLN cells taken from pHSP60- and pHSP65-vaccinated rats might reflect the induction of regulatory cells. Thus, the HSP60-specific regulatory T cells might exert their effects through the secretion of IL-10 and TGFβ1.

But what could be the origin of the HSP60-specific regulatory T cells in pHSP65-treated animals and how might this explain why pHSP65 is less effective than HSP60 in vaccinating against AA? HSP65 and HSP60 are members of a family of highly conserved proteins, and immunization with HSP65 activates T cells cross-reactive with self-HSP60 (39). The results presented in this work indicate that HSP60-specific T cells are indeed activated in pHSP65-vaccinated animals upon induction of AA (Fig. 4B). Nevertheless, vaccination with pHSP60 is apparently more effective than pHSP65 in activating an anti-HSP60 regulatory response. Vaccination with pHSP60 induced stronger proliferative responses to HSP60 (Fig. 4B), accompanied by the secretion of higher amounts of the regulatory cytokines IL-10 and TGFβ1 upon acti-

vation with mycobacterial Ags (Fig. 5, B and C). Thus, although pHSP65 vaccination induced HSP60-specific T cells, the response to HSP60 was not as strong as that induced by vaccination with pHSP60 itself, leading to the generation of less IL-10 and/or TGFβ1 secretors and to a weaker control of AA by pHSP65.

In this work we demonstrate that it is feasible to control AA with a DNA vaccine aimed to up-regulate the specific immune response directed to self-HSP60. Our results are in agreement with the need for controlled autoreactivity for the proper functioning of the immune system and body homeostasis (40–42). Hence, therapies aimed at activating built-in regulatory networks might serve as effective tools for the management of autoimmune diseases. Indeed, Raz et al. (43) have recently demonstrated that autoimmune regulation can be activated in humans by vaccination with an HSP60 peptide leading to arrest of β cell destruction in new-onset type 1 diabetes.

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, San Diego, CA, p. 201.
2. 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.*
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. 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.*
5. Haque, M. A., S. Yoshino, S. Inada, H. Nomaguchi, O. Tokunaga, and O. Kohashi. 1996. Suppression of adjuvant arthritis in rats by induction of oral tolerance to mycobacterial 65-kDa heat shock protein. *Eur. J. Immunol. 26:2650.*
6. 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.*
7. 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.*
8. van Eden, W., R. van der Zee, A. G. Paul, B. J. Prakken, U. Wendling, S. M. Anderton, and M. H. Wauben. 1998. Do heat shock proteins control the balance of T-cell regulation in inflammatory diseases? *Immunol. Today 19:303.*
9. van der Zee, R., S. M. Anderton, A. B. Prakken, A. G. Liesbeth Paul, and W. van Eden. 1998. T cell responses to conserved bacterial heat-shock-protein epitopes induce resistance in experimental autoimmunity. *Semin. Immunol. 10: 35.*
10. Prakken, A. B., M. J. van Hoeij, W. Kuis, A. Kavelaars, C. J. Heynen, E. Scholtens, I. M. de Kleer, G. T. Rijkers, and W. van Eden. 1997. T-cell reactivity to human HSP60 in oligo-articular juvenile chronic arthritis is associated with a favorable prognosis and the generation of regulatory cytokines in the inflamed joint. *Immunol. Lett. 57:139.*
11. 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.*
12. Elias, D., T. Reshef, O. S. Birk, R. van der Zee, M. D. Walker, and I. R. Cohen. 1991. Vaccination against autoimmune mouse diabetes with a T-cell epitope of the human 65-kDa heat shock protein. *Proc. Natl. Acad. Sci. USA 88:3088.*
13. 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.*
14. 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.*
15. Quintana, F. J., and I. R. Cohen. 2001. Autoantibody patterns in diabetes-prone NOD mice and in standard C57BL/6 mice. *J. Autoimmun. 17:191.*
16. Rea, I. M., S. McNerlan, and A. G. Pockley. 2001. Serum heat shock protein and anti-heat shock protein antibody levels in aging. *Exp. Gerontol. 36:341.*

17. Boog, C. J., E. R. de Graeff-Meeder, M. A. Lucassen, R. van der Zee, M. M. Voorhorst-Ogink, P. J. van Kooten, H. J. Geuze, and W. van Eden. 1992. Two monoclonal antibodies generated against human hsp60 show reactivity with synovial membranes of patients with juvenile chronic arthritis. *J. Exp. Med. 175: 1805.*

18. Ronaghy, A., B. J. Prakken, K. Takabayashi, G. S. Firestein, D. Boyle, N. J. Zvailfler, S. T. Roord, S. Albani, D. A. Carson, and E. Raz. 2002. Immunostimulatory DNA sequences influence the course of adjuvant arthritis. *J. Immunol. 168:51.*

19. Carol, M., C. Pelegri, C. Castellote, A. Franch, and M. Castell. 2000. Immunohistochemical study of lymphoid tissues in adjuvant arthritis (AA) by image analysis: relationship with synovial lesions. *Clin. Exp. Immunol. 120:200.*

20. Chastain, M., A. J. Simon, K. A. Soper, D. J. Holder, D. L. Montgomery, S. L. Sagar, D. R. Casimiro, and C. R. Middaugh. 2001. Antigen levels and antibody titers after DNA vaccination. *J. Pharmacol. Sci. 90:474.*

21. Tripathy, S. K., E. C. Svensson, H. B. Black, E. Goldwasser, M. Margalith, P. M. Hobart, and J. M. Leiden. 1996. Long-term expression of erythropoietin in the systemic circulation of mice after intramuscular injection of a plasmid DNA vector. *Proc. Natl. Acad. Sci. USA 93:10876.*

22. Groux, H., and F. Powrie. 1999. Regulatory T cells and inflammatory bowel disease. *Immunol. Today 20:442.*

23. Groux, H. 2001. An overview of regulatory T cells. *Microbes Infect. 3:883.*

24. O'Garra, A., L. Steinman, and K. Gijbels. 1997. CD4$^+$ T-cell subsets in autoimmunity. *Curr. Opin. Immunol. 9:872.*

25. 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.*

26. Hermans, G., R. Medaer, J. Raus, and P. Stinissen. 2000. Myelin reactive T cells after T cell vaccination in multiple sclerosis: cytokine profile and depletion by additional immunizations. *J. Neuroimmunol. 102:79.*

27. Hermans, G., U. Denzer, A. Lohse, J. Raus, and P. Stinissen. 1999. Cellular and humoral immune responses against autoreactive T cells in multiple sclerosis patients after T cell vaccination. *J. Autoimmun. 13:233.*

28. Elias, D., Y. Tikochinski, G. Frankel, and I. R. Cohen. 1999. Regulation of NOD mouse autoimmune diabetes by T cells that recognize a TCR CDR3 peptide. *Int. Immunol. 11:957.*

29. Kumar, V., J. Maglione, J. Thatte, B. Pederson, E. Sercarz, and E. S. Ward. 2001. Induction of a type 1 regulatory CD4 T cell response following Vβ8.2 DNA vaccination results in immune deviation and protection from experimental autoimmune encephalomyelitis. *Int. Immunol. 13:835.*

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. Groux, H., A. O'Garra, M. Bigler, M. Rouleau, S. Antonenko, J. E. de Vries, and M. G. Roncarolo. 1997. A CD4$^+$ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature 389:737.*

32. Cottrez, F., S. D. Hurst, R. L. Coffman, and H. Groux. 2000. T regulatory cells 1 inhibit a Th2-specific response in vivo. *J. Immunol. 165:4848.*

33. Quattrocchi, E., M. J. Dallman, A. P. Dhillon, A. Quaglia, G. Bagnato, and M. Feldmann. 2001. Murine IL-10 gene transfer inhibits established collagen-induced arthritis and reduces adenovirus-mediated inflammatory responses in mouse liver. *J. Immunol. 166:5970.*

34. Setoguchi, K., Y. Misaki, Y. Araki, K. Fujio, K. Kawahata, T. Kitamura, and K. Yamamoto. 2000. Antigen-specific T cells transduced with IL-10 ameliorate experimentally induced arthritis without impairing the systemic immune response to the antigen. *J. Immunol. 165:5980.*

35. Cottrez, F., and H. Groux. 2001. Regulation of TGF-β response during T cell activation is modulated by IL-10. *J. Immunol. 167:773.*

36. Prud'homme, G. J., and C. A. Piccirillo. 2000. The inhibitory effects of transforming growth factor-β-1 (TGF-β1) in autoimmune diseases. *J. Autoimmun. 14:23.*

37. Ditzian-Kadanoff, R. 1999. Testicular-associated immune deviation and prevention of adjuvant-induced arthritis by three tolerization methods. *Scand. J. Immunol. 50:150.*

38. Chernajovsky, Y., G. Adams, K. Triantaphyllopoulos, M. F. Ledda, and O. L. Podhajcer. 1997. Pathogenic lymphoid cells engineered to express TGFβ1 ameliorate disease in a collagen-induced arthritis model. *Gene Ther. 4:553.*

39. 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.*

40. Cohen, I. R. 2000. *Tending Adam's Garden: Evolving the Cognitive Immune Self.* Academic, London.

41. Cohen, I. R. 2000. Discrimination and dialogue in the immune system. *Semin. Immunol. 12:215.*

42. Schwartz, M., and I. R. Cohen. 2000. Autoimmunity can benefit self-maintenance. *Immunol. Today 21:265.*

43. 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 randomized, double-blind, phase II trial. *Lancet 358:1749.*