

Brief Report

Immunomodulation and Protection Induced by DNA-hsp65 Vaccination in an Animal Model of Arthritis

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ABSTRACT

We described a prophylactic and therapeutic effect of a DNA vaccine encoding the *Mycobacterium leprae* 65-kDa heat shock protein (DNA-hsp65) in experimental murine tuberculosis. However, high homology of the vaccine to the corresponding mammalian hsp60, together with the CpG motifs in the plasmidial vector, could trigger or exacerbate an autoimmune disease. In the present study, we evaluate the potential of DNA-hsp65 vaccination to induce or modulate arthritis in mice genetically selected for acute inflammatory reaction (AIR), either maximal (AIRmax) or minimal (AIRmin). Mice immunized with DNA-hsp65 or injected with the corresponding DNA vector (DNA_v) developed no arthritis, whereas pristane injection resulted in arthritis in 62% of AIRmax mice and 7.3% of AIRmin mice. Administered after pristane, DNA-hsp65 downregulated arthritis induction in AIRmax animals. Levels of interleukin (IL)-12 were significantly lower in mice receiving pristane plus DNA-hsp65 or DNA_v than in mice receiving pristane alone. However, when mice previously injected with pristane were inoculated with DNA-hsp65 or DNA_v, the protective effect was significantly correlated with lower IL-6 and IL-12 levels and higher IL-10 levels. Our results strongly suggest that DNA-hsp65 has no arthritogenic potential and is actually protective against experimentally induced arthritis in mice.

INTRODUCTION

DNA VACCINES have presented a radically new approach to disease immunoprophylaxis. It has been shown that, after intramuscular injection of a naked expression vector, plasmid DNA is taken up by muscle cells and maintained episomally, allowing expression of the encoded antigens (Wolff *et al.*, 1992). Therefore, after single or repeated injections of DNA, cellular and humoral immune responses to the protein are elicited, and long-lived memory lymphocytes are induced (Hassett *et al.*, 2000). Ulmer *et al.* were the first to report that DNA vaccination could protect mice against influenza infection, providing remarkable evidence of how this vaccination strategy could mediate protective immunity (Ulmer *et al.*, 1998). In addition, DNA vaccination has been used in cancer treatment

(Stevenson *et al.*, 1999) and in modulation of autoimmune processes (Waisman *et al.*, 1996). Our group has previously shown that a DNA plasmid encoding the *Mycobacterium leprae* 65-kDa heat shock protein (DNA-hsp65) displayed both a prophylactic (Bonato *et al.*, 1998) and therapeutic effect in a murine model of tuberculosis (Lowrie *et al.*, 1999; Bonato *et al.*, 2004). The protection was attributed to the induction of a cellular immune response dominated by hsp65 of *M. leprae*-specific T lymphocytes that produce interferon- γ (IFN- γ) and are cytotoxic (Silva *et al.*, 1996; Silva and Lowrie, 2000).

The main argument against taking DNA-hsp65 into clinical trial is that it could trigger an autoimmune response because *M. leprae* hsp65 protein has been shown to be 55% homologous to the equivalent mammalian protein (Feige and van Eden, 1996). In support of this argument, other studies have shown

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humoral and cellular immune responses against bacterial hsp65 in diabetes (Elias *et al.*, 1991; Gupta *et al.*, 1991; Bras and Aguas, 1996; Kallmann *et al.*, 1999), atherosclerosis (Afek *et al.*, 2000; Keren *et al.*, 2000), and arthritis (van Eden, 1991; Danieli *et al.*, 1992; Thompson *et al.*, 1998; Tanaka *et al.*, 1999). However, the *Mycobacterium* hsp65 antigen has been implicated not only in the immunopathology of rheumatoid arthritis (RA) (Kaufmann, 1990; Winfield and Jarjour, 1991) but also in protection against induced experimental disease. Specifically, protection against arthritis has been demonstrated in Lewis rats immunized with recombinant vaccinia virus expressing the *Mycobacterium* hsp65 (Hogervorst *et al.*, 1991) or human hsp60 (Lopez-Guerrero *et al.*, 1993) gene.

Pristane-induced arthritis (PIA) has proven to be a useful experimental model of RA. In susceptible inbred lines of mice such as BALB/c (Portter and Wax, 1981) and CBA *Igh^b* (Thompson *et al.*, 1993), 15 to 25% developed inflammation of the ankle and wrist joints approximately 200 days after pristane injection (Thompson and Elson, 1993). Susceptibility to PIA has been shown to be associated with elevated levels of agalactosyl IgG caused by unregulated interleukin (IL)-6 production (Rook *et al.*, 1991) and with major histocompatibility complex genes (the *H-2^q*, *H-2^d*, and *H-2^r* alleles are susceptible) (Wooley *et al.*, 1989). Such susceptibility also seems to be CD4⁺ T (helper T cell type 1 [Th1] or Th2) cell dependent (Stasiuk *et al.*, 1997). Interestingly, PIA incidence has been associated with elevated humoral and cellular responses to *M. tuberculosis* hsp65 (Thompson *et al.*, 1990). In addition, it has been suggested that protection against PIA is mediated by Th2 cytokines produced after immunization with recombinant hsp65 (Beech *et al.*, 1997).

The role of inflammatory and specific immune response in PIA was also investigated with heterogeneous mouse lines produced by bidirectional selective breeding for acute inflammatory reaction (AIR)—either maximal or minimal (AIRmax and AIRmin, respectively)—by comparing the outcome of PIA to the hsp65-induced humoral and cellular response (Vigar *et al.*, 2000). These lines originated from a highly polymorphic population (F₀) obtained by the intercross of eight inbred mouse strains (A, DBA2, P, SWR, CBA, SJL, BALB/c, and C57BL/6) (Stifell *et al.*, 1990). The AIR phenotype was defined by the local leukocyte influx and plasma protein exudation measured 24 hr after the subcutaneous injection of polyacrylamide beads, a nonantigenic, insoluble, and chemically inert substance (Ibanez *et al.*, 1992). The progressive divergence of the AIRmax and AIRmin lines in the successive generations of selective breeding reached 10- and 2.5-fold differences in leukocyte and protein exudate infiltration, respectively. These differences resulted from the accumulation of alleles that have opposite effects on the inflammatory response (Ibanez *et al.*, 1992). Genetic analysis of this selection indicated that AIR regulation involves at least 11 quantitative trait loci (QTL) (Biozzi *et al.*, 1998). This selective process has been demonstrated to be appropriate for the study of the mechanisms of acute inflammation and their interactions with the specific immune response in infectious (Araujo *et al.*, 1998), tumorous (Biozzi *et al.*, 1998), and autoimmune (Vigar *et al.*, 2000) diseases. The impact of genetic control of the inflammatory reaction was demonstrated in these mice during the induction of experimental arthritis, as evidenced by chronic inflammation, which is the

main pathogenic factor of the disease. In this way, it was observed that AIRmax mice were highly susceptible to PIA and presented higher numbers of spleen cells secreting IL-6, IL-4, and tumor necrosis factor (TNF)- α than did resistant AIRmin mice, which, in contrast, presented greater numbers of IFN- γ -secreting cells. Differences in isotype production were also noted (Vigar *et al.*, 2000). Within this context, our study was designed to determine the ability of DNA-hsp65 vaccination to directly induce arthritis in the AIRmin and AIRmax mouse phenotypes. In addition, we investigated the ability of this vaccine to up- or downmodulate PIA in these animals.

MATERIALS AND METHODS

Animals

Lines of mice endowed with the AIRmax or AIRmin phenotype were produced and maintained at the Instituto Butantan animal facilities in the city of São Paulo, Brazil. Experiments were carried out with 2- to 4-month-old male and female mice.

Induction and assessment of arthritis

Groups comprising similar numbers of male and female AIRmax and AIRmin mice received two intraperitoneal injections of pristane (0.5 ml; Sigma, St. Louis, MO) administered 60 days apart, and signs of arthritis were assessed over a 150- to 180-day period. Ankle joints were measured with a micrometer, and mice were considered positive for arthritis when the swollen joints ranged from 3.0 to 4.0 mm in diameter.

Plasmid purification and immunization protocol

The DNA-hsp65 construct was derived from its corresponding vector, pcDNA3 (DNAv) (Invitrogen, Carlsbad, CA), previously digested with *Bam*HI and *Not*I (GIBCO-BRL; Invitrogen, Gaithersburg, MD) by inserting a 3.3-kb fragment corresponding to the *M. leprae* hsp65 gene and the cytomegalovirus intron A. Vector without the hsp65 gene was used as a control. We cultured DH5- α *Escherichia coli* transformed with pcDNA3 plasmid alone or with pcDNA3 plasmid carrying the hsp65 gene in Luria-Bertani liquid medium (GIBCO-BRL) containing ampicillin (100 μ g/ml). The plasmids were purified with a Concert High Purity Maxiprep system (GIBCO-BRL). Plasmid concentration was determined by spectrophotometry at $\lambda = 260$ nm and at $\lambda = 280$ nm, using a GeneQuant II apparatus (Amersham Biosciences/GE Healthcare, Buckinghamshire, UK). Mice were immunized with plasmidial DNA encoding the hsp65 gene from *M. leprae*. Each animal received three 100- μ g intramuscular injections of DNA (50 μ g of DNA in each quadriceps muscle) at 2-week intervals. Control animals were injected with DNAv.

Assessment of DNA effect on development of arthritis

Initially, we used a protocol designed to investigate the ability of DNA (DNA-hsp65 or DNAv) to induce arthritis or to modulate PIA in AIRmin and AIRmax mice. In this protocol, arthritis induction and the immunization procedure began concomitantly on day 0. The second protocol used was designed to evaluate a possible immunomodulatory effect of the DNA vaccine, using AIRmax mice, which develop an intense in-

flammatory response. In this case, the immunization procedure began 45 days after the first pristane injection.

Cytokine evaluation

After arthritis assessment, the mice were killed and their splenocytes ($5 \times 10^6/\text{ml}$) were cultured in RPMI 1640 medium (Invitrogen, Grand Island, NY) and stimulated *in vitro* with recombinant hsp65 (rhsp65, 20 $\mu\text{g}/\text{ml}$). Cytokine levels in culture supernatants were evaluated 48 hr later by enzyme-linked immunosorbent assay (ELISA) in accordance with the manufacturer's instructions. The detection limit was 15 pg/ml. The following capture and biotinylated monoclonal antibodies were used, respectively, for IL-12, IL-10, and IL-6: C15.6 and C17.8; JES5-2A5 and SXC-1; and MP5-20F3 and MP5-32C11. Antibodies and recombinant cytokines were purchased from BD Biosciences Pharmingen (San Diego, CA).

Histopathological assessment

After sacrifice, ankle joints of AIRmax mice were dissected out, fixed in neutral-buffered formalin, and decalcified. Longitudinal sections were prepared and stained with hematoxylin and eosin.

Statistical analysis

Results represent means \pm SD. The statistical significance of the data was determined by Student *t* test or by Fisher exact test with the Yates correction. Computer-assisted evaluation of the results was used to calculate *p* values. The level of statistical significance adopted was $p < 0.05$.

RESULTS AND DISCUSSION

Incidence of arthritis in AIRmax mice injected with pristane was decreased by concomitant administration of DNA-hsp65

In this study, we evaluated the potential of DNA-hsp65 to induce arthritis in AIR mice. In our first protocol, AIRmax and AIRmin mice received pristane simultaneously with the first dose of DNA-hsp65 or DNAv. The AIRmax group receiving pristane alone presented a 50% incidence of arthritis on day 120 (data not shown) and a 62% incidence on day 180, when the final assessment was made, at which time the AIRmin mice receiving pristane alone presented only a 7.3% incidence of arthritis (Fig. 1A). These data are consistent with the findings of Vigar *et al.*, who showed that pristane injection induced a higher incidence of arthritis in AIRmax mice than in AIRmin mice (Vigar *et al.*, 2000). Therefore, using only the AIRmax model, we obtained an arthritis incidence of 62% after pristane injection, confirming that this is a good model for our analyses. However, we found that AIRmax and AIRmin mice injected with DNA-hsp65 alone or DNAv alone showed no signs of arthritis during the 180-day follow-up period. We believe that, within the context of vaccine safety, this is a significant finding because it indicates that neither DNA-hsp65 nor DNAv per se induced arthritis in the AIRmax or AIRmin mouse model. It is notable that the combination of pristane and DNA-hsp65 in

AIRmax mice, which typically present a high incidence of PIA, did not increase the incidence of the disease. In fact, these mice presented significantly less arthritis incidence than did those receiving pristane alone (Fig. 1A). We believe that the immunomodulatory effect triggered by DNA-hsp65 was specific for hsp65 because the lower arthritis incidence was much more pronounced in DNA-hsp65-vaccinated mice (57% lower than in mice receiving pristane alone) than in the group injected with DNAv (24.6% lower) (Fig. 1A).

These results were confirmed by radiological evaluation and histological analysis. One hundred and eighty days after pristane injection or DNA immunization, or both, radiographic images of the hind paws of AIRmax mice injected with pristane alone (Fig. 1B) showed joint displacement and reduced latency due to demineralization and areas of recalcification, indicative of new bone formation. These alterations were not seen in the radiographic images of AIRmax mice receiving DNA-hsp65 alone (Fig. 1C). Histological analysis of AIRmax mice injected with pristane alone (Fig. 1D), DNA-hsp65 plus pristane (Fig. 1E), or DNAv plus pristane (Fig. 1F) showed that only those injected with pristane alone presented alterations compatible with arthritis, presenting articular cartilage degeneration, synovial hyperplasia, and pannus formation indicative of severe inflammation (arrow, Fig. 1D). The PIA mice presented polyarticular arthritis, although no elastic cartilage damage was observed. The DNA-hsp65-vaccinated AIRmax mice presented no inflammatory infiltrate such as that observed in those injected with pristane alone.

DNA-hsp65 decreased the incidence of arthritis in AIRmax mice pretreated with pristane

To evaluate the immunomodulatory effect of DNA-hsp65 vaccination in this model of arthritis, the first dose of DNA-hsp65 or DNAv was administered 45 days after the first pristane injection, followed at 15-day intervals by the second and third DNA doses. This schedule was chosen on the basis of the knowledge that the immunological alterations leading to the development of arthritis in the pristane model are settled at about 60 days after pristane injection (Bedwell *et al.*, 1987). However, macroscopic alterations are not yet evident at that time.

After the first pristane injection, we assessed arthritis in AIRmax mice receiving pristane alone and found the incidence to be 30% on day 90, 40% on day 120, and 52% on day 150 (the end point of the study). In contrast, a clear and pronounced modulatory effect was observed in AIRmax mice receiving pristane followed by DNA-hsp65 vaccination. Therefore, it seems clear that DNA-hsp65 vaccination was protective against arthritis in AIRmax mice, as evidenced by the absence of arthritis incidence during the 150-day follow-up period (Fig. 2). This effect was not observed in mice receiving pristane followed by DNAv administration, although the incidence of arthritis was in these mice was 16%, which was 36% lower than that seen in mice receiving pristane alone (Fig. 2).

Despite the fact that the lower incidence of arthritis seems dependent on the hsp65-specific immune response induced by DNA-hsp65 immunization, we cannot rule out the possibility that DNAv plays a nonspecific role, likely associated with an inflammatory response. The immunostimulatory effect of DNAv is attributed to the CpG motifs present in its backbone,

A)

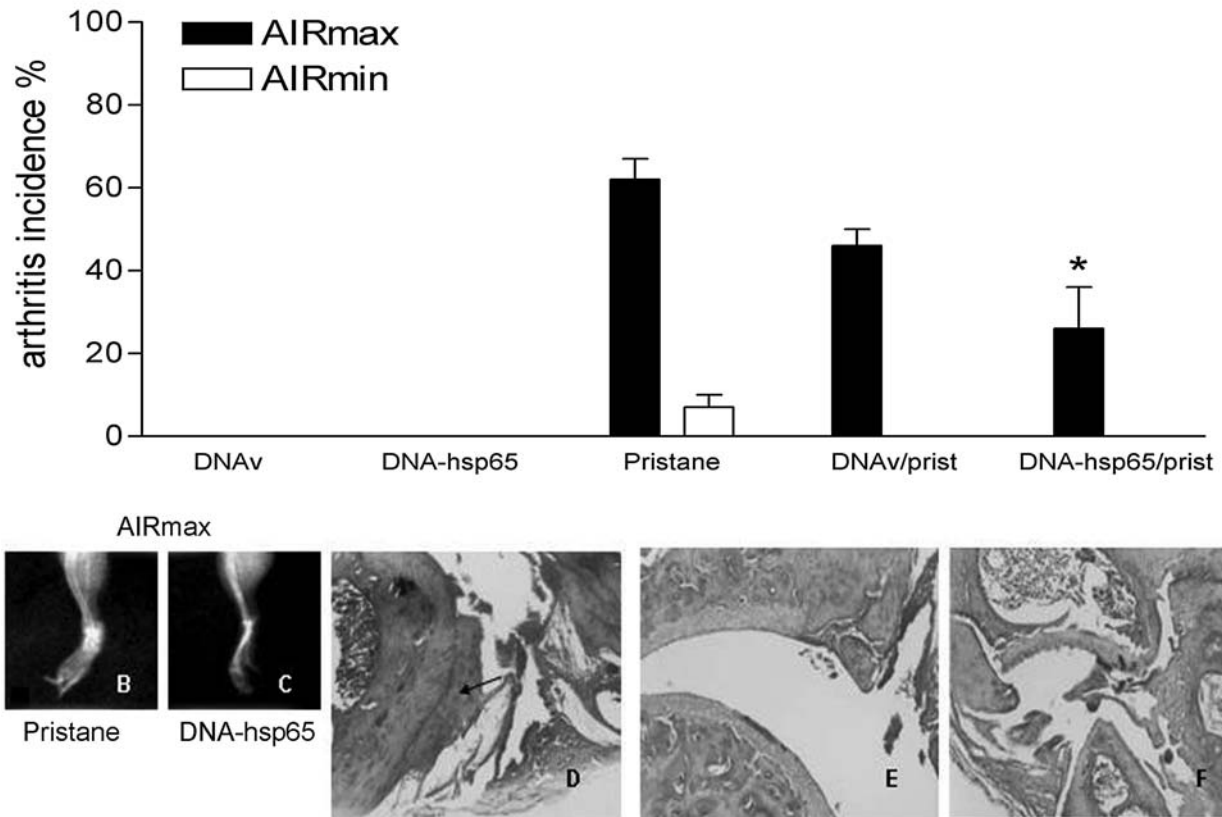


FIG. 1. DNA-hsp65 did not induce arthritis in AIR mice. (A) PIA incidence at 180 days (study end point) in AIRmax and AIRmin mice receiving DNA-hsp65 immunization, vector, and pristane simultaneously. (B and C) Radiographic image of hind paws of AIRmax mice receiving pristane alone (B) and DNA-hsp65 alone (C). (D–F) Histological analysis of ankle joints from AIRmax mice that received pristane alone (D), pristane plus DNA-hsp65 (E), and pristane plus DNAv (F). Arrow in (D) indicates alteration compatible with arthritis. On day 0, mice were injected intraperitoneally with pristane and intramuscularly with DNA as described in Materials and Methods. Arthritis development was assessed over a 180-day follow-up period by measuring the ankle joints with a micrometer. After 180 days, these animals were killed for radiographic and histological analysis. Each experimental group was composed of 10–15 animals. These results represent the means of two experiments. * $p < 0.05$, compared with the pristane-only group.

which stimulate the secretion of IL-12 and IL-6 by monocytes and dendritic cells (Krieg, 2001). However, a downregulatory effect of CpG motifs has previously been described in experimental models of autoimmunity. Quintana *et al.*, using the nonobese diabetic (NOD) mouse model, showed that a DNA construct encoding human hsp60 and the empty vector thereof, as well as an oligonucleotide sequence containing CpG motifs, all modulated spontaneous hsp60 autoimmunity and inhibited NOD diabetes (Quintana *et al.*, 2000). The authors demonstrated that this modulation was associated with high levels of IL-10 production by spleen cells in response to the oligonucleotide (Quintana *et al.*, 2000).

Administration of DNA-hsp65 simultaneously with or after pristane injection modulated cytokine production differentially. The mechanism underlying the higher or lower incidence of arthritis has been correlated with up- or downregulation of cytokine production (Mauri *et al.*, 1996; Beech *et al.*, 1997; Ragno *et al.*, 1997; Thompson *et al.*, 1998). Consequently, modulation of arthritis development after DNA vaccination might also be

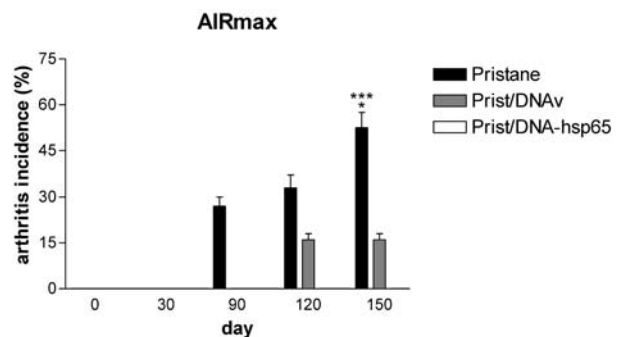


FIG. 2. Immunomodulatory effect of DNA on arthritis incidence. AIRmax mice received two intraperitoneal injections of pristane at 60-day intervals. Mice received the first dose of DNA-hsp65 or DNAv 45 days after the first pristane injection. Arthritis development was assessed over a 150-day period by measuring the ankle joints with a micrometer. Each experimental group comprised 15 animals. * $p < 0.05$ and *** $p < 0.001$, compared with the pristane-only group.

associated with changes in the pattern of cytokine production. In an attempt to understand the immunomodulatory effect of DNA-hsp65 vaccination on arthritis induction, we evaluated secretion of inflammatory (IL-6 and IL-12) and antiinflammatory (IL-10) cytokines by spleen cells of AIRmax mice at study end point (when the arthritis was well established). By 180 days after the first pristane injection, spleen cells from AIRmax mice displayed distinct profiles in terms of proinflammatory and antiinflammatory cytokine production after *in vitro* stimulation with rhsp65. Spleen cells from AIRmax mice injected with pristane alone produced higher levels of IL-6 and IL-12 when stimulated *in vitro* with rhsp65 (Fig. 3A and B). Numerous reports indicate that IL-6 may play an important role in RA (Field *et al.*, 1991; Helle *et al.*, 1991; Kobayashi *et al.*, 2002). It has also been shown that IL-6 is a direct causative agent in the activation of monocytes/macrophages by synovial fluid in RA patients. This role of IL-6 has been clearly demonstrated by the clinical improvement of RA patients who undergo anti-IL-6 treatment

(Wendling *et al.*, 1993) and by the resistance of IL-6 knockout mice to antigen-induced experimental arthritis (Boe *et al.*, 1999). Moreover, susceptibility to PIA has been shown to be associated with elevated levels of agalactosyl IgG resulting from unregulated IL-6 production (Rook *et al.*, 1991). In agreement with these data, our results showed high levels of IL-6 production when arthritis was well established in AIRmax mice injected with pristane (Fig. 3A). The IL-6 levels were also increased when DNA-hsp65 and pristane were injected concomitantly but not when DNA-hsp65 alone was injected (Fig. 3A). We also evaluated IL-12 production after hsp65 antigen stimulation *in vitro*. Levels of IL-12 production in AIRmax mice injected with DNA-hsp65 were comparable to those observed in pristane-injected mice (Fig. 3B). Interestingly, when AIRmax mice were injected simultaneously with DNA-hsp65 and pristane, a significant decrease was observed in IL-12 levels (Fig. 3B). The same was observed when these mice were injected with DNAv and pristane concomitantly (Fig. 3B). Because there is consid-

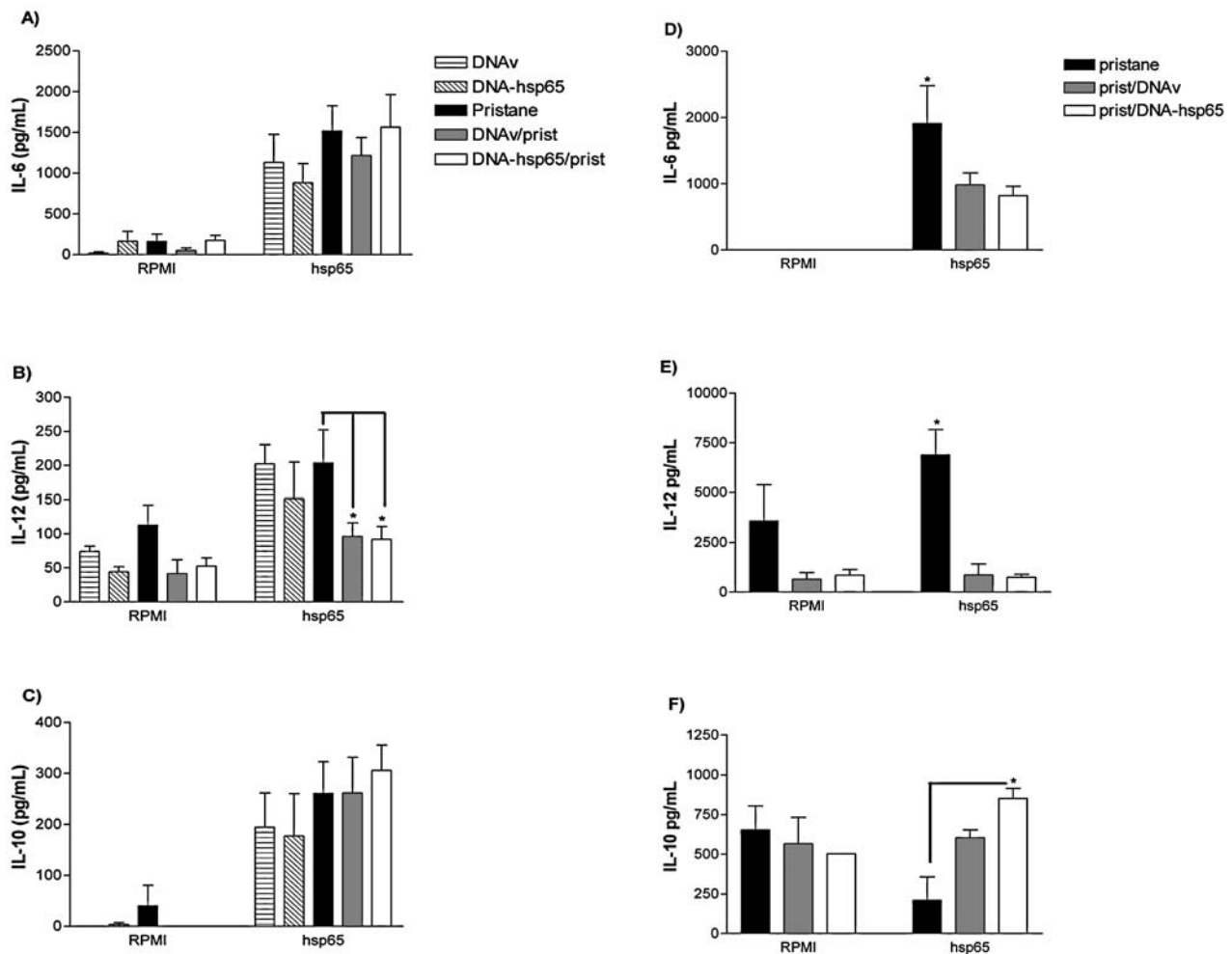


FIG. 3. Cytokine production by DNA-hsp65 or DNAv in AIRmax mice injected simultaneously or after pristane injection. The animals received two intraperitoneal injections of pristane at 60-day intervals. (A–C) Arthritis induction and the immunization procedure initiated concomitantly on day 0. (D–F) Administration of DNA-hsp65 or DNAv began 45 days after the first pristane injection. Levels of IL-6 (A and D), IL-12 (B and E), and IL-10 (C and F) were assessed by ELISA in spleen cell cultures stimulated with recombinant hsp65 (rhsp65) or unstimulated in RPMI. The spleen cultures were performed during the final assessment in each experimental protocol. Results are expressed as the mean of six animals per group. * $p < 0.05$, compared with the pristane group.

erable evidence to implicate the Th1 response in the pathogenesis of collagen-induced arthritis (CIA) (Mauri *et al.*, 1996), the participation of IL-12 in the onset of this disease is expected and has, indeed, been amply demonstrated. The incidence and severity of CIA are reduced in IL-12-deficient mice (McIntyre *et al.*, 1996). Neutralizing anti-IL-12 antibodies delay the onset and slow the progression of disease (Joosten *et al.*, 1997; Malfait *et al.*, 1998; Butler *et al.*, 1999).

In contrast to the deleterious effect that IL-12 and IL-6 in arthritis, it has been reported that IL-10 plays a beneficial role (Ulmansky *et al.*, 2002). This regulatory mechanism has been attributed to local inhibition of both antigen-presenting cell (APC) activity and IL-12 production by macrophages (Mottonen *et al.*, 1998). Our prediction that injecting AIRmax mice with pristane and concomitantly with DNA-hsp65 or DNA_v could control arthritis by increasing IL-10 production was not observed (Fig. 3C), despite the significant downmodulation of IL-12 secretion (Fig. 3B). In AIRmax mice injected with pristane and concomitantly with DNA-hsp65 or DNA_v, IL-10 production was similar to that seen in those injected with pristane alone (Fig. 3C). However, other cytokines, such as TGF- β , could be involved in the inhibition of APCs and effector T cells.

Otherwise, the immunomodulatory effect of DNA (DNA-hs65 or DNA_v) was more evident in mice receiving DNA after pristane injection. In this case, AIRmax mice received the first dose of DNA-hsp65 or DNA_v 45 days after the first pristane injection. In these mice, secretion of IL-6 and IL-12 was significantly downregulated in comparison with mice injected only with pristane (Fig. 3D and E). However, only DNA-hsp65-vaccinated mice produced significantly higher levels of IL-10 than those seen in pristane-injected AIRmax mice (Fig. 3F). In this context, increased levels of IL-10 can have an inhibitory effect on IL-12 secretion by APCs, consistent with the observations of Mottonen *et al.* (1998). In addition, DNA_v administration also induced a similar inhibitory mechanism involving the lowering of IL-6 and IL-12 levels (Fig. 3D and E). Levels of IL-10 were increased in relation to those seen in the pristane-injected group, although the difference was less than significant (Fig. 3F). These data could explain the differential effect of DNA-hsp65 and DNA_v, the former preventing and the latter delaying the onset of arthritis. Furthermore, the incidence of arthritis in mice injected with DNA_v did not exceed approximately 16%.

Among these results, two aspects merit special attention. The first is the high efficacy of DNA-hsp65 in preventing arthritis in the pristane model. The other issue of special interest is the protective effect of DNA_v, which, although less efficient than DNA-hsp65, regulated the inflammatory immune response in mice previously injected with pristane.

Modulation of autoimmune conditions has received a great deal of attention. Gilkeson and coworkers demonstrated that immunization with bacterial DNA can play a regulatory role in several diseases seen in autoimmune NZB/NZW mice, whereas calf thymus DNA was ineffective (Gilkeson *et al.*, 1996). Boccaccio and coworkers also reported that noncoding plasmid DNA can inhibit experimental encephalomyelitis (Boccaccio *et al.*, 1999).

In summary, we have demonstrated that DNA-hsp65, when administered in isolation, does not induce arthritis in mice highly prone to developing the disease. In addition, this vaccine proved to have a protective effect that was more pronounced when it was administered after the onset of PIA.

Downregulation of IL-6 and IL-12, as well as upregulation of IL-10, seem to be at least some of the mechanisms involved in this protective effect. Given that DNA-hsp65 has previously shown potential as a vaccine against tuberculosis, our results suggest that it may have similar potential in the prevention and treatment of arthritis. Further studies are warranted to validate and expand on these findings.

ACKNOWLEDGMENTS

The authors are grateful to Mrs. Izaíra T Brandão for technical assistance. This study was supported by grants from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, Foundation for the Support of Research in the State of São Paulo), the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, National Council of Scientific and Technological Development), and the Rede Brasileira de Pesquisa em TB (REDE-TB, Brazilian Tuberculosis Research Network).

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Received for publication March 30, 2005; accepted after revision August 12, 2005.

Published online: September 29, 2005.