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Effect of hsp65 DNA Vaccination Carrying Immunostimulatory DNA Sequences (CpG Motifs) Against *Mycobacterium leprae* Multiplication in Mice[1]

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One of the important objectives of leprosy control is to reduce the occurrence of new cases, since approximately 690,000 new cases were still detected in 1997 ([24]). Therefore, the development of an effective anti-leprosy vaccine is needed immediately. The efficiency of *Mycobacterium bovis* BCG as a vaccinating agent has been evaluated in several clinical trials and was found to be as high as 50% ([29, 30]). Various attempts have been made to search for entities which are effective in suppressing the replication of *M. leprae,* but none have reached the level of potential clinical usage. As one of the attempts, we recently found that recombinant BCG over-producing an A component of the antigen 85 complex suppressed the multiplication of *M. leprae in vivo* and its suppressing effect was superior to parental BCG ([18]). In addition to the system using live bacteria, DNA vaccinations are considered as another strategy for controlling leprosy infection. Intramuscular or intradermal administration of plasmid DNA (pDNA) expression vectors can expect to initiate intracellular synthesis of the encoded proteins and to induce long-lasting cellular and humoral antigen (Ag)-specific immune responses ([23]). It has been reported that palindromic, single stranded immunostimulatory DNA sequences (ISS) induce production of IFN-α, IFN-β and IFN-γ by murine spleen cells and by human peripheral blood lymphocytes, which are closely associated with activation of natural killer cells ([7, 25]). These ISS include the palindromic CpG-containing hexamers: 5′-GACGTC-3′, 5′-AGCGCT-3′, and 5′-AACGTT-3′. Recently, Sato, *et al.,* reported that a pDNA expression vector (pACB) containing two repeats of 5′-AACGTT-3′ in the *ampR* gene is highly immunogenic, because it elicits strong Ag-specific immunity ([19]).

Heat-shock proteins (hsps) are highly conserved and are produced in response to cellular stress in both prokaryotic and human cells ([4, 13]). The amplitude of T-cell responses to *M. leprae*-derived hsp correlates well with the clinical spectrum of leprosy; i.e., a cell-mediated immune response (CMIR) to hsp is observed in tuberculoid type leprosy patients while little or no CMIR is detected in lepromatous type patients ([1, 10]). There are at least two major CD4+ T cell subsets as categorized by cytokine profile, and the type 1 CD4+ T cells, preferentially producing IL-2 and IFN-γ, play a major role in the protection against mycobacterial infection. The Ag-specific responses of CD4+ T cells to exogenous Ags is generally controlled by MHC class II genes. Therefore, it has been reported that *M. tuberculosis*-derived hsp65 has three DQ8-restricted T cell epitopes ([5]), of which MHC class II Ag restriction was confirmed by using HLA-transgenic mice, murine class II-knock out mice ([5]). Also, the intramuscular immunization of pDNA expressing my-

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cobacterial hsp65 protected mice from disease development by virulent *M. tuberculosis* H37Rv ([2]). Therefore, hsp is likely to have an immunodominant Ag capable of preventing mycobacterial infection in an MCH class II restricted manner. However, as far as we know, there are no reports indicating the potentiality of hsp65 derived from *M. leprae* using animal models. In this study, we test the usefulness of hsp65 as a candidate for a new DNA vaccine against leprosy using a pDNA vector containing two repeats of the palindromic hexamer.

MATERIALS AND METHODS

Mice. Female BALB/cA mice, 6–7 weeks of age, were obtained from CLEA, Shizuoka, Japan. They were housed in the animal care facility of our laboratory after a DNA vaccination and *M. leprae* inoculation, and were kept under specific pathogen-free conditions.

Construction of the DNA vaccine. For DNA vaccination, a 1.9-kb *Xmn*-I—*BssH*II fragment of the *M. leprae* genome carrying hsp65 gene was excised from pUC-N5 ([12, 14]), and inserted, after blunting, into the *Eco*RV site downstream of the cytomegalovirus (CMV) promoter/enhancer region in plasmid pACB ([19]). The orientation of the inserted DNA was determined by comparing the length of the DNA fragment flanked with *Xba*1. The obtained recombinant DNA containing CMV promoter/enhancer region along with the CMV immediate early intron, hsp65 cDNA and bovine growth hormone polyadenylate signal, is termed pACB/hsp65.

pACB and pACB/hsp65 were propagated in *E. coli* DH5, purified using QIAGEN Maxi-Prep, Endo-Free Kit (Qiagen, Chatsworth, California, U.S.A.) and stored at –20°C in TE buffer, pH 8.0, until used.

pACB and pACB/hsp65 were transfected into COS-7 cells using the calcium phosphate precipitation method as described previously ([12]), and the expression of hsp65 in the transformed cells was revealed by Western blotting using a monoclonal antibody (mAb) to bacterial hsp65; MAb-3A ([15]).

***M. leprae* and preparation of hsp65.** *M. leprae* (Thai-53) were maintained in footpads of BALB/cA-*nu/nu* mice, and were isolated according to Nakamura's methods ([11]). The lysate of *M. leprae* was produced as previously described ([14]). The hsp65 protein was purified from cultured *E. coli* JM109 carrying pUC8/N5 by affinity chromatography using MAb-3A ([12]). The absence of contamination of bacterial LPS in the hsp65 preparation was confirmed by tests using polymixin B ([17]).

Immunization. pACB or pACB/hsp65, 50 μg in 25 μl of saline in the presence or absence of lipofectin (Invitrogen, Gaithersburg, Maryland, U.S.A.), was injected intradermally into the base of the tail as reported by Sato, *et al.* ([19]). We used intradermal, not intra-muscular injections, for reasons of safety, because Manickan, E., *et al.,* reported that intradermal gene vaccination with naked pDNA induced a type 1 response to the gene products ([9]). The injection was carried out 3 times every 10 days, and partial bleeding was done at 12, 15, and 18 weeks after initial immunization.

Measurement of Ab to hsp65 in immunized mice. Immunized or control mice (3 mice each) were sacrificed 15 weeks after initial immunization, and the concentration of anti-hsp65 Ab in sera was determined by ELISA assay as described elsewhere ([22]). Briefly, the purified *M. leprae* hsp65 was coated on polystyrene microtiter plates at a concentration of 2 μg per ml, and serum samples, diluted 100 or 1000 fold, were added in duplicate. The level of anti-hsp65 Ab was determined by calculating the difference in optical density (OD) between hsp65-coated and uncoated wells.

Measurement of cytokine concentrations. The spleens were aseptically removed 12 or 18 weeks after the initial immunization from two mice, and were pooled for subsequent analysis. The 1.0×10^6 of spleen cells per well was cultured in triplicate in medium containing 5% heat-inactivated fetal bovine serum. In the secondary stimulation of spleen cells, the cell suspension was cultured with 5 μg/ml of either hsp65 or *M. leprae* lysate in the presence or absence of 20 μg/ml of anti-IL-10 mAb (Pierce-Endogen, Rockford, Illinois, U.S.A.). Supernatants were collected after 72 hours incubation and the concentrations of IFN-γ, IL-10 (Pierce-Endogen, Rockford, Illinois, U.S.A.) and IL-12 (BIOSource, Camarillo, California, U.S.A.) in the supernatants were determined by using commercially available ELISA kits. The measurement of

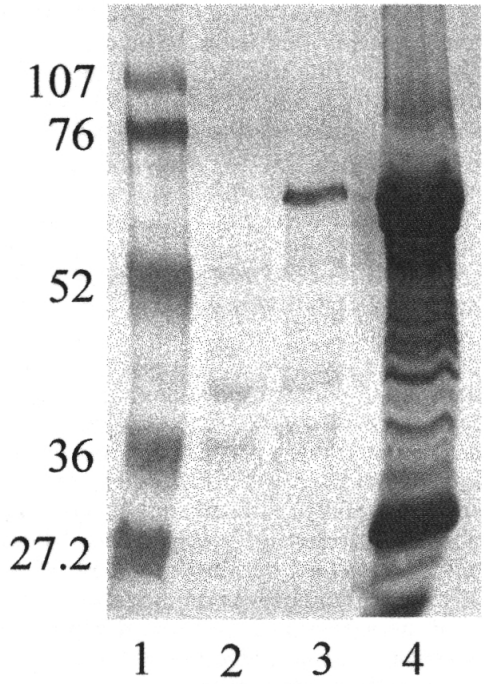

FIG. 1. Western blotting analysis of cells transfected with the gene using mAb to bacterial hsp65 (MAb-3A). Lane 1 = molecular weight standard, lane 2 = COS-7 cells transfected with pACB, lane 3 = COS-7 cells transfected with pACB/hsp65, lane 4 = crude extract of hsp65 from *E. coli* carrying pUC/N5.

nitric oxide (NO) was carried out using the Griess Reagent ([21]).

Protective immunity against *M. leprae*. Four weeks after the initial immunization, the BALB/cA mice were challenged with 5000 live *M. leprae* bacilli in the right-hind footpad. At 30 weeks of *M. leprae* challenge, the mice were killed and the acid-fast bacilli in the footpads were enumerated according to Shepard's method ([20]). The statistical differences were revealed by post-hoc test; Bonferroni/Dunn. A p value less than 0.05 indicates a statistical significant difference.

RESULTS

Effect of hsp65 immunization. pACB and pACB/hsp65 were transfected into COS-7 cells, and the expression of hsp65 was confirmed by Western blotting analysis. Only the cells transfected with pACB/hsp65 produced the protein (Fig. 1). Lipofectin is a liposome formulation of cationic lipid and dioleoyl phosphotidylethanolamine in water, and the reagent interacts spontaneously with DNA to form a lipid-DNA complex. The fusion of the complex with tissue culture cells results in the efficient uptake and expression of the DNA

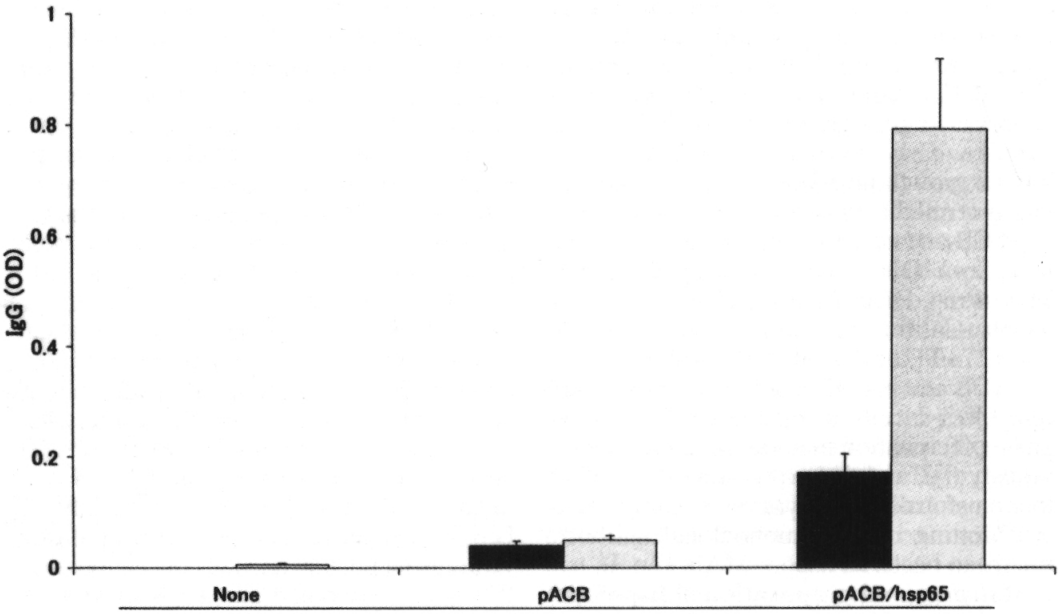

FIG. 2. A production of IgG anti-hsp65 Ab in serum. Female BALB/cA mice were immunized with pACB and pACB/hsp65 in the absence (■) or presence (▨) of lipofectin and were sacrificed at 15 weeks after the initial immunization for assessment of anti-hsp65 Ab concentration. The serum was diluted at 1:100. A representative of three independent experiments is shown. Mean ± SD of three mice are exhibited.

TABLE 1. *Effect of mAb antagonistic to IL-10 on the cytokine production by spleen cells from mice immunized with pACB/hsp65.*[a]

Immunization to mice	Secondary stimulate	IFN-γ (ng/ml)	IL-12 (pg/ml)
pACB	hsp65	0	14.9 ± 0.8
	anti-IL-10 mAb	0	21.5 ± 1.8
	anti-IL-10 mAb + hsp65	1.5 ± 0.1	68.9 ± 4.3
pACB/hsp65	hsp65	0	0
	anti-IL-10 mAb	0	17.7 ± 0.8
	anti-IL-10 mAb + hsp65	67.4 ± 0.4	43.2 ± 2.1

[a] BALB/cA mice were immunized with either pACB or pACB/hsp65. At 18 weeks post-immunization, spleen cells were re-stimulated *in vitro* as indicated. Hsp65 (5 μg/ml) and anti-IL-10 mAb (20 μg/ml) were used for stimulation. Supernatant was obtained from the cells cultured for 3 days. Results are expressed as the mean ± S.D. for triplicate cultures.

in vitro ([26]). However, the efficiency of the lipofectin for transfection of genes *in vivo* is not fully clarified. Therefore, to analyze the efficiency of lipofectin for transfection of genes *in vivo,* the IgG anti-hsp65 Ab level in serum from mice immunized with pACB/hsp65 in the presence or absence of lipofectin was measured (Fig. 2). A significant increase in the level of IgG Ab to hsp65 was observed at both dilutions of sera, 1:100 and 1:1000, and the Ab production was only detected in mice immunized with pACB/hsp65. However, the production of IFN-γ and IL-12 by spleen cells from mice immunized with pACB/hsp65 was not enhanced by *in vivo* treatment with lipofectin (data not shown).

Production of various cytokines and NO *in vitro* by secondary stimulation. IFN-γ, IL-12, IL-10 and NO production was examined to analyze the role of hsp65 by the secondary *in vitro* stimulation of cells from mice immunized with pACB/hsp65 (Fig. 3). The spleen cells from the immunized mice produced IFN-γ without the secondary stimuli and further up-regulated its production by the stimuli with *M. leprae* lysate or hsp65. The IFN-γ production was observed solely in mice immunized for 12 weeks, but not 18 weeks (Fig. 3A). The production of IL-12 was observed again predominantly in mice 12 weeks post-immunization. The level of cytokine produced by spleen cells in the absence of secondary stimulation was higher in mice immunized with pACB/hsp65 than in those receiving pACB alone. However, curiously, the IL-12 production was suppressed by *in vitro* secondary stimulation with *M. leprae* lysate or hsp65. This suppression was dependent on the dose used for *in vitro* stimulation and was confirmed by the lack of IL-12 mRNA (not shown). On the contrary, IL-10 was produced only in mice 18 weeks after immunization (Fig. 3C). However, there seemed to be little difference in IL-10 production between mice immunized with pACB and pACB/hsp65. The mice immunized with mock-transfected *E. coli* products did not affect IL-10 production (not shown). A higher production of IFN-γ and IL-12 was achieved in mice 12 weeks post-immunization and was subsequently down-regulated. The relationship between IL-10 production at 18 weeks post-immunization and the reduction of IFN-γ and IL-12 production was examined by using a mAb antagonistic to IL-10 (Table 1). The production of both IFN-γ and IL-12 upon the secondary stimulation was recovered by an addition of the exogenous mAb antagonistic to IL-10. In contrast to IFN-γ production, IL-12 production was enhanced in both pACB- and pACB/hsp65-immunized mice. However, when *M. leprae* lysate was used as a secondary stimulant, no significant recovery of cytokine production was obtained even in the presence of the anti-IL-10 mAb (data not shown). This lack of recovery may be due to a lower concentration of hsp65 present in *M. leprae* lysate. These results suggest that hsp65-induced IL-10 production at 18 weeks post-immunization, which, in turn, resulted in the suppression of IFN-γ and IL-12 production.

A production of NO by spleen cells from mice immunized with either pACB or pACB/hsp65 was observed at 12 weeks (Fig. 3D). However, no consistent enhancement of the NO production was observed by the secondary stimuli. At 18 weeks after immunization, no apparent production of NO was observed.

Protective effect of pACB and pACB/hsp65 on *M. leprae* multiplication *in vivo*. Mice immunized with either pACB or pACB/hsp65 were challenged by injecting 5000 live bacilli of *M. leprae* into the

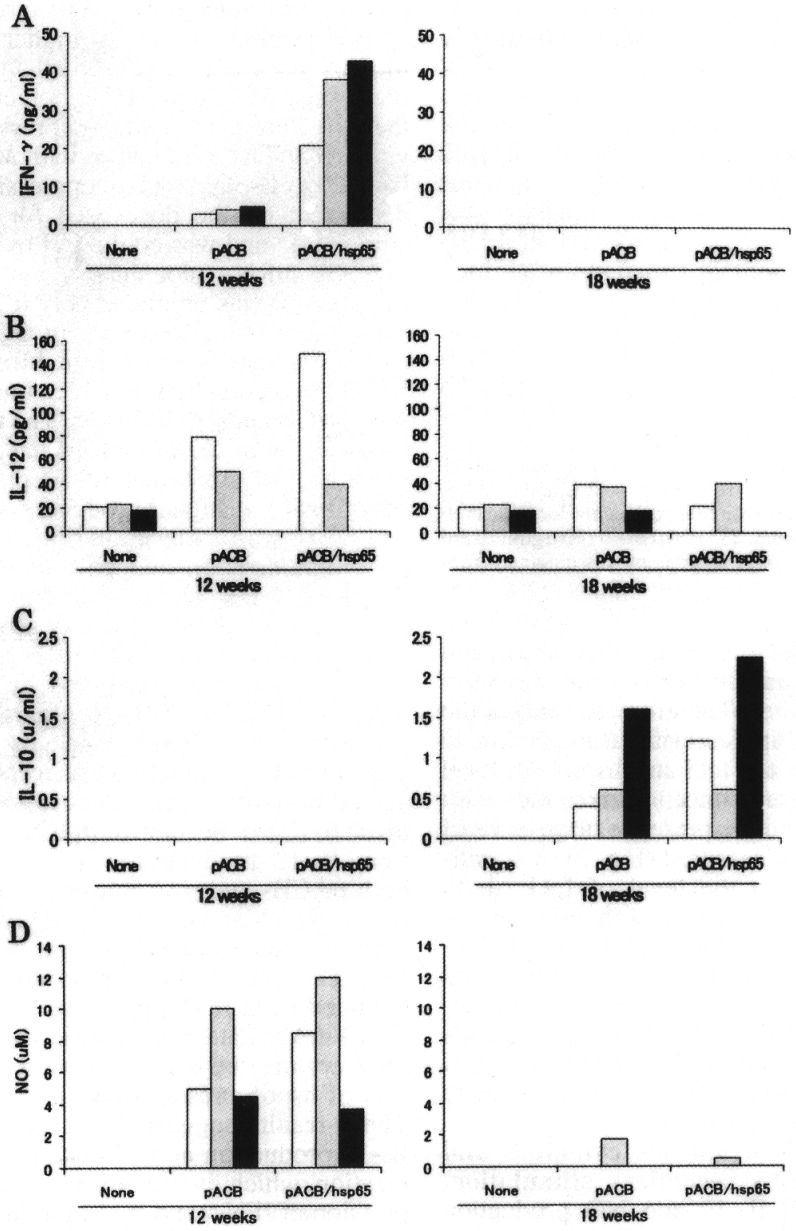

FIG. 3. Production of various cytokines and NO by secondary stimulation of spleen cells from hsp65-immunized mice. Spleen cells were obtained from mice immunized with pACB or pACB/hsp65 or unprimed mice, and were re-stimulated with *M. leprae* lysate or hsp65 *in vitro*. Spleen cells from 2 mice in each group were pooled and used. The *in vitro* stimulation was done in triplicate by using the following stimulator; □: None, ■: *M. leprae* lysate, ▨: hsp65. The mean ± S.D. was calculated. The S.D. was less than 10% of the mean. A representative of two separate experiments is shown. **A:** IFN-γ, **B:** IL-12, **C:** IL-10 and **D**: NO.

footpad (Table 2). The multiplication of *M. leprae* in the footpad at 30 weeks post-challenge was significantly inhibited by immunization with pACB/hsp65. The number of bacilli recovered from pACB/hsp65-immunized mice were significantly lower than that from mice unimmunized or immunized with sole vector (pACB).

DISCUSSION

The goal of our studies is to establish vaccinating agents capable of preventing

TABLE 2. *Effect of pACB/hsp65 DNA vaccine against* M. leprae *multiplication in BALB/c mice.*[a]

Immunization	No. of bacilli recovered[b] (log_{10}/mouse)
PBS	5.37 ± 0.21 (n = 6) [c]
pACB	5.34 ± 0.36 (n = 6) [c]
pACB/hsp65	4.16 ± 0.39 (n = 5)

[a] BALB/cA mice were immunized with indicated DNA and challenged with 5×10^3 *M. leprae* at 4 weeks after immunization. At 30 weeks after *M. leprae* infection, the mice were sacrificed and the number of acid-fast bacilli in the footpad were enumerated. A representative of two separate experiments is shown.

[b] Parenthesis indicates the number of mice examined.

[c] Statistical difference: $p < 0.05$.

leprosy. In this study, we examined the effect of *M. leprae* derived hsp65 and ISS (CpG motif) on the *in vivo* multiplication of *M. leprae.* A DNA plasmid vector (pACB/hsp65), having ISS sequence (CpG motif) and the *M. leprae* hsp65 gene under the control of the CMV promoter, was constructed. *In vitro* experiments using COS-7 cells, proved that hsp65 protein was successfully overproduced in animal cells (Fig. 1). When we vaccinated mice with pACB/hsp65, the serum level of IgG specific to hsp65 was significantly high, indicating that the hsp65 protein was produced *in vivo* (Fig. 2). However the efficiency of lipofectin was not confirmed, because we did not evaluate IL-4 production. The repeated injection of the pACB/hsp65 into mice induced the cytokine production from the spleen cells, which was further enhanced by secondary *in vitro* stimulation (Fig. 3). The up-regulation of humoral and cellular immune responses to hsp65 in immunized mice might partially depend on ISS which is introduced into the vector pACB, because ISS is known to have an adjuvant effect ([19]). This point is closely associated with the report by Elkins, K. L., *et al.,* that bacterial DNA containing ISS (CpG motifs) induced protective immunity against *Listeria monocytogenes* ([3]). Therefore, in mice immunized with pACB/hsp65, both hsp65-specific and ISS-mediated immune responses might be raised. At 12 weeks post-immunization, the hsp65-immunized mice produced cytokines, including IFN-γ and IL-12, that are associated with type 1 CD4+ T cell responses. However, their production was down-regulated thereafter. This reduction was apparently induced by enhanced production of IL-10, because *in vitro* experiments using an mAb antagonistic to IL-10 did reverse the down-regulated production of IFN-γ and IL-12. Therefore, it should be important to control IL-10 production when DNA encoding hsp65 is introduced as a vaccine. Curiously, the IL-12 production was suppressed by secondary stimulation with hsp65 protein. We have no idea how to exactly explain this phenomenon. However, it has been reported that plasmids expressing either hsp65 or IL-12 are effective in controlling *M. tuberculosis* infection, but co-delivery of plasmids expressing both hsp65 and IL-12 showed an antagonistic effect on each other and reduced the therapeutic efficacy ([8]). We have also reported that IL-12 production was consistently observed in the NOD diabetes model mice, but upon further stimulation of the spleen cells with hsp65 up-regulated Il-10 production, which in turn inhibited the production of IL-12 ([16]). A similar mechanism might be involved in our system. This is a subject for further investigation.

IFN-γ plays an essential role in the protection against various infectious agents including mycobacterial infection ([27]). The chief action of IFN-γ is reported to activate macrophages to kill intracellular mycobacteria ([6]). From the fact that increased levels of both IL-12 and IFN-γ were observed after 12 weeks post-immunization, we can envisage that mice immunized with pACB/hsp65 might achieve a cytokine milieu which can control mycobacterial infection. In addition to IFN-γ, NO is also an important mediator controlling mycobacterium infections in mice ([28]). A significant amount of NO was synthesized in mice immunized with pACB/hsp65. Based on these observations, we evaluated the protective effect of the immunization against *M. leprae.* Examination of the number of bacilli revealed that, at 30 weeks after inoculation of live bacilli, mice immunized with pACB/hsp65 significantly inhibited the multiplication of *M. leprae,* as expected. Although a detailed analysis enrolling several time-points and an analysis of the effect of pACB expressing irrelevant protein on the multiplication

of *M. leprae* remain to be conducted, our observations are consistent with the reports showing the protective and therapeutic efficacy of DNA expressing mycobacterial hsp65 in mice infected with virulent *M. tuberculosis* ([2, 8]).

In conclusion, we showed that a DNA containing hsp65 gene in the vector carrying ISS motif was effective in eliciting humoral and cellular immune responses that lead to the prevention of the multiplication of *M. leprae* in the mouse footpad model. Therefore, DNA having an hsp65 encoding gene with an ISS fragment might be a potent vaccine candidate that should be further pursued.

SUMMARY

A DNA expressing hsp65 of *Mycobacterium leprae* (pACB/hsp65) was constructed by using a vector containing immunostimulatory DNA sequences (pACB). At 12 weeks post-immunization, spleen cells from BALB/cA mice immunized with pACB/hsp65, produced a significantly higher amount of IFN-γ than mice immunized with pACB in the absence of any *in vitro* stimulation, and further enhanced its production upon secondary *in vitro* stimulation with *M. leprae* lysate and hsp65. On the other hand, while production of IL-12 was observed in mice immunized with pACB/hsp65 12 weeks before, the cytokine production was inhibited by *in vitro* secondary stimulation with *M. leprae* or hsp65. At 18 weeks post-immunization, the production of both IFN-γ and IL-12 was apparently down-regulated, but that of IL-10 was up-regulated. IL-10 seemed to suppress the IFN-γ and IL-12 productions, because their production was recovered by neutralization of IL-10 with anti-IL-10 mAb. Furthermore, when the efficiency of pACB/hsp65 as a vaccine against *M. leprae* was evaluated *in vivo,* the mice immunized with pACB/hsp65 suppressed the multiplication of subsequently challenged *M. leprae.* These results suggest that a DNA containing *M. leprae*-derived hsp65 and immunostimulatory sequences might be a potent vaccine candidate against *M. leprae* infection.

RESUMEN

Se construyó un DNA conteniendo el gene para la proteína hsp65 de *Mycobacterium leprae* (pACB/hsp65) usando un vector portador de secuencias de DNA relacionadas con inmunoestimulación (pACB). Se encontró que 12 semanas después de la estimulación, los esplenocitos de los ratones BALB/cA inmunizados con la construcción pACB/hsp65 produjeron más interferón que los esplenocitos de los ratones inmunizados sólo con pACB, en ausencia de estimulación *in vitro,* y que estas células produjeron todavía más IFN-γ cuando se estimularon *in vitro* con un lisado de *M. leprae* o con la proteína hsp65. Por otro lado, mientras que la producción de IL-12 se observó aumentada en los ratones inmunizados 12 semanas antes con pACB/hsp65, la producción de esta citocina se inhibió por la estimulación secundaria *in vitro* con *M. leprae* o con hsp65. A las 18 semanas postinmunización, la producción de IFN-γ y de IL-12 apareció suprimida pero la producción de IL-10 se observó aumentada. La IL-10 pareció inducir la supresión de la producción de IFN-γ y de IL-12 ya que la producción de estas citocinas se recuperó cuando la IL-10 se neutralizó con un anticuerpo monoclonal contra la IL-10. Además, cuando se evaluó la eficacia *in vivo* de pACB/hsp como vacuna contra *M. leprae,* los ratones inmunizados con pACB/hsp65 suprimieron la multiplicación del bacilo aplicado como reto. Estos resultados sugieren que el DNA conteniendo la secuencia para hsp65 y secuencias inmunoestimulantes, puede ser un candidato potencial para una vacuna contra la lepra.

RÉSUMÉ

Un système d'expression du gène hsp65 de *Mycobacterium leprae* (pACB/hsp65) fut construit avec un vecteur contenant des séquences d'ADN immunostimulantes (pACB). Douze semaines après immunisation, les splénocytes provenant de souris BALB/cA immunisées avec pACB/hsp65 ont produit une quantité significativement plus importante d'IFN-γ que des souris immunisées avec pACB seul en l'absence de stimulation *in vitro.* Cette production était augmentée lors d'une stimulation secondaire *in vitro* avec un lysat de *M. leprae* et hsp65. Par contre, si une production d'IL-12 était observée chez les souris immunisées 12 semaines auparavant par pACB/hsp65, la production de cette cytokine était inhibée par la stimulation secondaire *in vitro* avec *M. leprae* ou hsp65. A 18 semaines postimmunisation, la production d'IFN-γ et d'IL-12 était apparemment diminuée tandis que la production d'IL-10 était augmentée. IL-10 semblait exercer un feed-back négatif sur les productions d'IFN-γ et d'IL-12, puisqu'elles étaient rétablies par une neutralisation de l'IL-10 par des anticorps monoclonaux anti-IL-10. De plus, lorsque l'efficacité de pACB/hsp65 fut évaluée *in vivo* comme vaccin contre *M. leprae,* les souris immunisées contre pACB/hsp65 ne montrèrent pas de multiplication des *M. leprae* inoculées. Ces résultats suggèrent qu'un ADN contenant la séquence codant pour l'hsp65 dérivée de *M. leprae* pourrait être un candidat vaccinal puissant contre l'infection par *M. leprae.*

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