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Direct Immunization of Malaria DNA Vaccine into the Liver by Gene Gun Protects against Lethal Challenge of *Plasmodium berghei* Sporozoite

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The liver is the first target organ for malaria parasites immediately after the bite of an infected mosquito. We studied local immunization of malaria DNA vaccines at the site of the liver using a gene gun as a useful tool for *in vivo* transfection of foreign genes. A malaria DNA vaccine consisting of the *Plasmodium berghei* circumsporozoite protein (PbCSP) gene plus the mouse IL-12 gene was bombarded directly by a gene gun into mouse liver once or into the skin twice. A marked protective effect was induced by gene bombardment into the liver (more than 71%) compared with that into the skin (less than 33%). A Th1-type immune response and high production of iNOS were observed in the hepatic lymphocytes from mice bombarded into the liver, resulting in more effective protection compared with those bombarded into the skin. These results provide an important implication on the development of efficient malaria vaccine strategies.

Key Words: **malaria; *Plasmodium berghei;* DNA vaccine; liver; CSP; cytokine; IL-12; nitric oxide.**

Malaria is one of the worst health problems in the world. Approximately 300 to 500 million people are infected with malaria annually, and 1.5 to 2.7 million lives are lost in each year. The main hope to control this disease may reside in the development of safe and effective vaccines. Immunization with irradiated *Plasmodium* spp. sporozoites induce sterile protective immunity against malaria in humans, monkeys, mice, and chickens (reviewed in Ref. 1). Protective immunity is directed against the pre-erythrocytic stage of the parasite (i.e., sporozoites and liver stages). In particular, IFN-γ, provided by malaria-specific $CD8^+$ T cells, stimulates infected cells to produce nitric oxide (NO) which destroys infected hepatocytes or parasites within these cells (2–4). However, the irradiated sporozoite vaccine is not practical for large scale human use. Recent studies support the importance of pursuing a DNA vaccine development for malaria control (5–8). A new DNA vaccine should aim to protect immunized subjects against malaria comparable to that induced by the irradiated sporozoite vaccine.

Our primary strategy is to deliver DNA vaccines directly into the liver, where malaria parasites develop into exoerythrocytic forms and multiply by schizogony. Introduction of a target DNA into the liver may result in direct transfection of professional antigen presenting cells like Kupffer cells and/or the presentation of T-cell epitopes encoded by the DNA vaccines in association with MHC-I molecules on the surface of hepatocytes. Among several methods of such liver-directed gene transfer reported for gene therapy (reviewed in Ref. 9), we used a gene-gun mediated gene transfer into the live on the basis of our and Williams *et al.*'s results showing that gene-gun delivery of DNA into the liver is an efficient method of foreign gene introduction (10, 11). In addition to transfection efficacy, multiple gene constructs can be simultaneously delivered in desirable molar ratios, and accurately *in situ* into target organs within only 10 s.

We chose the *Plasmodium berghei* circumsporozoite protein (PbCSP) and the mouse IL-12 genes as a DNA

Abbreviations used: PbCSP, *P. berghei* circumsporozoite protein; NO, nitric oxide; iNOS, inducible nitric oxide synthase; GPI, glycosylphosphatidylinositol; CD, dendritic cells; HVJ, hemagglutinating virus of Japan.

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vaccine candidate for malaria. CSP is present in two developmental stages namely sporozoites and infected hepatocytes (1, 12) and is one of the most extensively studied malaria vaccine candidate antigens (13). Immunization with a DNA vaccine encoding CSP by intramuscular injection conferred 68% protection against sporozoite challenge in the *P. yoelii* model, and the protection was dependent on $CD8^+$ T cells (8). To achieve protective immunity against malaria parasites at the site of the liver, the induction of IFN-γ by $CD8^+$ T cells and subsequent NO production have been shown to be important for the destruction of infected hepatocytes or the parasite within these cells (2, 3, 6, 14–16). IL-12 has been reported to be an important inducer and enhancer of Th-1 type responses including IFN-γ production and cytotoxic activities (reviewed in Ref. 17). Coadministration of DNA vaccine with an IL-12 expression plasmid by intramuscular immunization significantly enhanced cell-mediated immunity by Th-1 cell activation (18–20). Moreover, a single subcutaneous injection of recombinant human IL-12 two days before challenge with *P. cynomolgi* sporozoites protected 100% of rhesus monkeys, suggesting that recombinant human IL-12 protects monkeys through the IFN-γ-dependent elimination of *P. cynomolgi*-infected hepatocytes (21).

In the present study, we demonstrate for the first time that direct immunization with malaria DNA vaccine into the liver effectively protected mice against the challenge of *P. berghei*-infected mosquito bites. These data clearly indicate that optimal vaccines designed to induce protective immune responses in infected hepatocytes should be considered to improve current malaria vaccine strategies.

MATERIALS AND METHODS

Mice and parasite. Female 6- to 8-week-old BALB/c mice (SEASCO, Saitama, Japan) were used in all experiments. *Plasmodium berghei* ANKA strain was maintained by cyclical passage through BALB/c mice and *Anopheles stephensi* (SDA 500 stain).

DNA vaccine. The PbCSP gene was amplified from genomic DNA of *P. berghei* ANKA strain by PCR using a sense primer (5′-GGAGGGCTAGCATGGAGACAGACACACTCCTGCTATGGGTACTGCTGCTCTGGGTTCCAGGTTCCACTGGTGACGCGGATCCACTGCAGGACTACAAGGACGACGTAGACAAGGGATATGGACAAAATAAAAGCATCCAAGCCC-3′: a newly created *Nhe*I site is broken-underlined, the mouse Igκ secretion signal sequence (22) is underlined and the FLAG encoding sequence (23) is double-underlined) and an antisense primer (5′-GGAGGGCGGCCGCATCCCGGGTTTTCTTATTTGAACCTTTTCGTTTTCTAACTCTTATACCAGAACC-3′: a newly created *Not*I site is broken-underlined). The PCR was performed with *Pfu* DNA polymerase (Stratagene GmbH, Heidelberg, Germany) under the following conditions for 30 cycles (denaturation at 94°C for 30 s, annealing at 55°C for 1 min, elongation at 72°C for 2 min). The PCR product encodes PbCSP fused to the mouse Igκ secretion signal sequence in the place of its native signal sequence and without its C-terminal glycosylphosphatidylinositol (GPI) anchor. After purification the PCR product was digested with *Nhe*I and *Not*I, and ligated into *Nhe*I–*Not*I site in pcDNA3.1 (Invitrogen, San Diego, CA). The resulting plasmid was designated pcDNA-CS87 (Fig. 1, A_1).

The mouse IL-12 expression plasmid, designated pcDNA-mIL12p35p40, was constructed as follows. Both mouse IL-12 p35 and p40 cDNAs (kindly provided by Dr. S. Wolf, Genetic Institute, Cambridge, MA) were modified to clone into pcDNA3.1. The primers for IL-12 p35 were 5′-GAGGAGGATCCATGTGTCAATCACGCTACCTCCTCTTTTTGG-3′ (a newly created *Bam*HI site is broken-underlined) and 5′-GGGGAGGGCCCTCAGGCGGAGCTCAGATAGCCCATCAC-3′ (a newly created *Apa*I site is broken-underlined); the primers for IL-12 p40 were 5′-GAGGAGGATCCATGTGTCCTCAGAAGCTAACCATCTCC-3′ (a newly created *Bam*HI site is broken-underlined) and 5′-GGGGAGGGCCCCTAGGATCGGACCCTGCAGGGAACACATGC-3′ (a newly created *Apa*I site is broken-underlined). After purification the PCR product encoding IL-12 p35 was digested with *Bam*HI and *Apa*I, and ligated into *Bam*HI–*Apa*I site in pcDNA3.1. The resulting plasmid was designated pcDNA-mIL12p35. Similarly, the PCR product encoding IL-12 p40 was digested with *Bam*HI and *Apa*I, and ligated into *Bam*HI–*Apa*I site in pcDNA3.1. The resulting plasmid was designated pcDNA-mIL12p40. A 1.8-kb *Mun*I–*Nae*I fragment from pcDNA-mIL12p35 was ligated into *Mun*I–*Nru*I site in pcDNA-mIL12p40 to generate pcDNA-mIL12p35p40. The luciferase gene was removed from pT3/T7 luc (CLONTECH, Palo Alto, CA) by digestion with *Bam*HI and *Not*I and inserted into *Bam*HI–*Not*I site in pcDNA3.1 to generate pcDNA-Luc. The sequences of the PbCSP and the IL-12 genes were verified by DNA sequencing. The sequence of the PbCSP gene obtained from PCR was identical with that reported for ANKA strain PbCSP gene (24), except for a GAA insertion into the amino acid position between 81 and 82. Plasmid DNAs were produced in bacteria and purified using Qiagen Maxi Prep kits (Qiagen, Santa Clara, CA).

Transient transfection in vitro. Proteins expression from pcDNA-CS87 and pcDNA-mIL12p35p40 were analyzed by immunofluorescent staining following transfection into HepG2 cells (American Type Cell Culture, Rockville, MD) using Qiagen lipofectin (Qiagen) according to the manufacturer's protocol. Briefly, 2 days after transfection, cells growing in cover slips were fixed with acetone/methanol (3:2 v/v) and blocked with 3% BSA in PBS. The pcDNA-CS87- transfected cells were incubated with mAb 3D11 (kindly provided by Dr. R. Nussenzweig, New York University School of Medicine) followed with a goat anti-mouse IgG-FITC conjugate. The pcDNA-mIL12p35p40-transfected cells were incubated with rat anti-mouse IL-12 p40 (kindly provided by Dr. S. Wolf, Genetic Institute, Cambridge, MA) followed with a goat anti-rat IgG-FITC conjugate. Fluorescence was observed using a Zeiss fluorescence microscope.

Gene immunization. Gold particles coated with plasmid DNAs and their cartridges were prepared as described previously (10). Eight-week-old BALB/c female mice were anesthetized by injection with a mixture of Rompun-Vetalar (1:2 ratio; Bayer and Park-Davis Veterinary, respectively). For liver bombardment, the abdomen was shaved and the liver surface was exposed by a transverse skin incision. DNA-coated gold particles (1 μg plasmid DNA per shot) were delivered into the left lateral lobe of the liver using a Helios Gene Gun (Nippon Bio-Rad Laboratory, Tokyo, Japan) at a helium discharge pressure of 200 psi. After bombardment the lobe was repositioned, and the incision sutured. For skin bombardment, the abdomen of the skin was shaved and DNA-coated gold particles (1 μg plasmid DNA per shot) were delivered twice (one at time 0 and the 2 weeks later) into the abdomen with the gene gun at a helium discharge pressure of 300 psi.

Protection against challenge. For Experiment 1, two weeks after the second immunization by skin bombardment with pcDNA-Luc alone or pcDNA-CS87 plus pcDNA-mIL12p35p40, or 4 weeks after the immunization by liver bombardment with pcDNA-CS87 plus pcDNA-mIL12p35p40, 3 groups of 10 mice were challenged by the bites of *P. berghei*-infected Anopheles stephensi. Sedated mice were placed on a nylon-mesh screened container containing infected mos-

quitoes. Each mouse was removed after a minimum of five observed mosquito feedings over 5 min. We chose the bites of *P. berghei*-infected mosquitoes for the challenge to emulate the natural route of malaria infection. Mice were considered protected if malaria Giemsa-stained thin blood films taken from day 2 until 14 after challenge were negative by microscopy. One mouse from the group bombarded with pcDNA-CS87 plus pcDNA-mIL12p35p40 into the liver died 2 days after challenge. No malaria infection was found in this mouse. To address the immune responses which conferred protection against sporozoite challenge, protected mice were rechallenged by more than 20 bites of *P. berghei*-infected mosquitoes and examined T cell subsets in the liver.

For Experiment 2, the same procedure as in Experiment 1 was repeated except that 5 groups including non-treated control mice and mice bombarded with pcDNA-Luc into the liver were examined. Each group consisted of 10 mice except for 8 nontreated control mice. Forty-two hours after sporozoite challenge, 3 mice from each group were sacrificed to examine cytokine production in the liver and spleen. Protected mice were rechallenged by more than 20 bites of *P. berghei*-infected mosquitoes to examine the induction of inducible nitric oxide synthase (iNOS) mRNA.

Serum samples from both experiments were collected by tail vein bleeding immediately prior to each DNA bombardment and immediately prior to challenge, and assayed for the presence of anti-PbCSP Abs.

Flow cytometric analysis of hepatic lymphocytes. Hepatic lymphocytes from the protected mice in Experiment 1 were isolated from perfused livers 42 h after sporozoite rechallenge according to the method described by Goossens *et al.* (25). The surface phenotype of cells was analyzed using mAbs specific for FITC-conjugated CD4 and PE-conjugated CD8 (PharMingene, San Diego, CA) in conjunction with a two-color immunofluorescence test and analyzed with a FACScalibur flow cytometer using CellQuest software (Becton–Dickinson, San Jose, CA).

Cytokine production assay. For the *in vitro* cytokine production assay, single-cell suspensions of splenic and hepatic lymphocytes from mice bombarded in Experiment 2 at 42 h after sporozoite challenge were prepared by routine methods (25, 26). Cell populations were plated at 3×10^6 cells per well in 24-well plates (Nunc, Roskilde, Denmark) and cultured in the presence of medium alone or immobilized anti-CD3 mAb (PharMingen) at a final concentration of 2 μg/ml. All cell cultures were incubated at 37°C in an atmosphere of 5% CO_2 in air. Cell-free supernatant fluids were harvested from these cell cultures at 6 h and assayed for IFN-γ or IL-4 activities. IFN-γ levels were measured by standard sandwich ELISA as described previously (27). IL-4 levels were determined by proliferation of IL-4 dependent CT4S cell bioassay as described previously (28). In the case of IL-4, hepatic lymphocytes were stimulated by anti-CD3 mAb because no IL-4 production was detected without the stimulation in any groups. IFN-γ or IL-4 levels were calculated from standard curves constructed by using recombinant mouse IFN-γ or IL-4.

Ab reagents for in vivo depletion and neutralization. Six of 10 protected mice with no parasitemia in Experiment 2 were rechallenged to detect iNOS mRNA in the livers. In experiments assessing the importance of $CD4^+$, $CD8^+$ T-cells and IFN-γ on expression of iNOS mRNA, another 4 protected mice were given the following treatment individually before rechallenge. For depletion of $CD4^+$ T-cells, one of the 4 mice was treated ip on days -6 and -3 and the day of sporozoite rechallenge with 200 μg of the rat anti-mouse CD4 mAb, GK1.5 (29). For depletion of $CD8^+$ T-cells, one of the 4 mice were treated iv on days -6 and -3 and the day of sporozoite rechallenge with 200 μg of the rat anti-mouse CD8 mAb, lyt2.2 (the kind gift of Dr. T. Aji, Okayama University Medical School). For neutralization of IFN-γ, one of the 4 mice was treated iv on days -6 and -3 and the day of sporozoite rechallenge with 200 μg of the rat anti-mouse IFN-γ mAb. For an appropriate control mAb, one of the 4 mice was treated iv on days -6 and -3 and the day of sporozoite rechallenge with 200 μg of the mouse anti-PfMSP-1 mAb (5.2 mAb: American Type Culture Collection, Rockville, MD). Mice were sacrificed 24 h after sporozoite rechallenge and the livers were removed for iNOS mRNA analysis. Meanwhile, spleen cells were prepared from each mice and the efficacy of $CD4^+$ and $CD8^+$ T cell depletion was assessed by flow cytometric analysis (>95%).

Detection of inducible nitric oxide synthase mRNA by RT-PCR. Livers removed from mice were frozen in liquid nitrogen and the total liver RNA was extracted as described previously (2). Primer sequences used for mouse iNOS detection were 5′-CCCTTCCG-AAGTTTCTGGCAGCAGC-3′ forward and 5′-GGCTGTCAGAG-CCTCGTGGCTTTGG-3′ reverse. For mouse β-actin detection, the primer pair was 5′-TGGAATCCTGTGGCATCCATGAAAC-3′ forward and 5′-AACGCAGCTCAGTAACAGTCCGCCTA-3′ reverse. Cycle parameters were 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The identity of the specific PCR products was verified by Southern blot hybridization. The cDNAs encoding iNOS and β-actin were labeled with enzyme horseradish peroxidase using an ECL direct nucleic acid labeling kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). A series of the PCR products was run on a 1.5% agarose gel. After transfer to a nylon membrane, the PCR products were hybridized to the probes. High stringency conditions were used in the hybridization and washing. Positive signals were detected by an ECL detection system (Amersham Pharmacia Biotech) followed by exposure using IR LAS-1000 Lite V1.1 (FUJIFILM, Tokyo, Japan). The amounts of the bands on autoradiographs were quantified by densitometric scanning using Image Gauge V3.3 (FUJIFILM).

Statistical analysis. The statistical significances of differences in survival between groups of mice were analyzed by the χ^2 test and Kaplan–Meier test.

RESULTS

Construction and expression of PbCSP and IL-12 in vitro. Plasmid maps for pcDNA-CS87 and pcDNA-mIL12p35p40 are shown in Fig. 1. pcDNA-CS87 (Fig. 1, A_1) contains the PbCSP gene lacking 5′-signal sequence and 3′-GPI anchor sequence, which corresponds to the amino acid coding region 21 to 299. This gene fragment is fused to the mouse Igκ signal sequence. Transcription initiates from the CMV promoter, continues through the mouse Igκ signal sequence, the FLAG encoding sequence, the mature PbCSP protein coding sequence and terminating at a bovine growth hormone polyadenylation sequence. pcDNA-mIL12p35p40 contains the sequences for the p35 and p40 subunits of mouse IL-12, linked tandemly in the same direction and each driven by CMV promoter, and a bovine growth hormone polyadenylation sequence (Fig. 1, B_1).

To verify the construction of pcDNA-CS87 and pcDNA-mIL12p35p40, HepG2 cells were transfected with one or other. Positive immunofluorescence was observed in cells transfected with pcDNA-CS87 and pcDNA-mIL12p35p40 by the indirect fluorescent Ab test (Fig. 1, A_2 and B_2). A *Bam*HII–*Not*I 900-kb fragment form pcDNA-CS87 was inserted into *Bam*HII–*Not*I site in a baculovirus expression vector pBACgus-13.1scFv (30). Proteins produced in the recombinant baculovirus-infected cells were secreted into medium and recognized with 3D7 mAb in Western blots (data

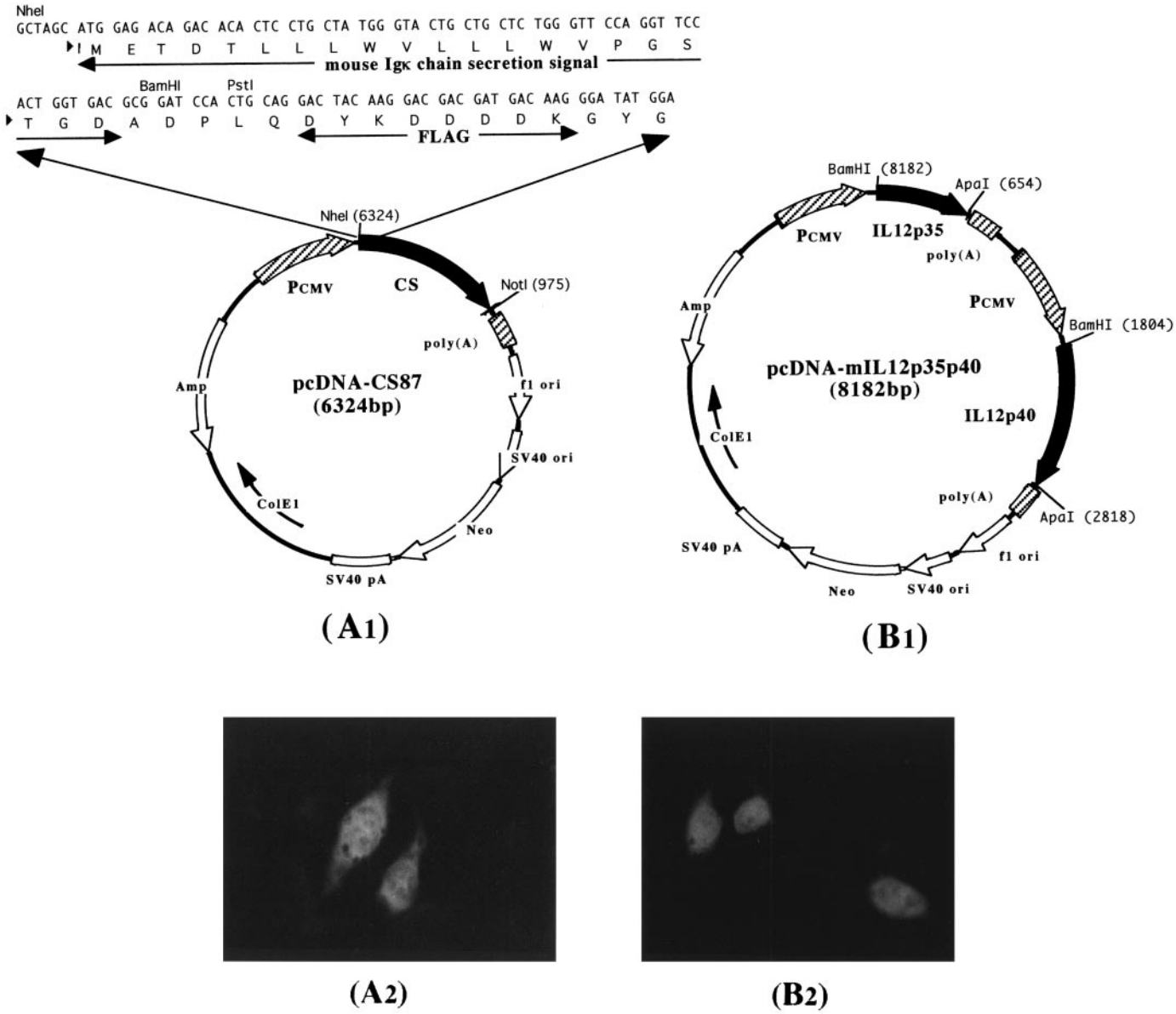

FIG. 1. Transient Expression of the PbCSP gene and the mouse IL-12 gene in HepG2 cells using expression vectors pcDNA-CS87 and pcDNA-mIL12p35p40, respectively. A schematic representation of the pcDNA-CS87 plasmid containing a CMV promoter, the mouse Igk secretion signal, a portion of the PbCSP gene (corresponding amino acid position 21–299) and an SV40 polyadenylation signal is shown in (A_1). The nucleotide sequence present at the junction between the CMV promoter and the PbCSP gene is illustrated above the plasmid map. The mouse Igk secretion signal and FLAG sequences are underlined. A schematic representation of the pcDNA-mIL12p35p40 plasmid containing the mouse IL12p35 gene under the control of a CMV promoter and the mouse IL12p40 gene under the control of a CMV promoter is shown in (B_1). Indirect immunofluorescence assay of acetone-fixed HepG2 cells transfected either with pcDNA-CS87 or pcDNA-mIL12p35p40. HepG2 cells were transfected either with pcDNA-CS87 (A_2) or pcDNA-mIL12p35p40 (B_2), and stained with anti-PbCSP mAb and anti-mouse IL-12p40 mAb, respectively.

not shown). Purified recombinant PbCSP was therefore used as an ELISA antigen to detect antibodies obtained from mice immunized with plasmid DNA.

Protection after immunization. We specifically chose the IL-12 gene as a "genetic adjuvant" for malaria DNA vaccine because the coadministration of DNA vaccines with IL-12 expression plasmids preferentially activates Th1-type cells (18–20), and Th1 type immune responses can play a critical role in protection against intrahepatic parasites (31, 32). We therefore focused on the coadministration of DNA vaccine with the IL-12 expression plasmid into the liver and did not address the contribution of PbCSP alone or IL-12 alone to protection. The liver surface of BALB/c mice was exposed surgically and bom-

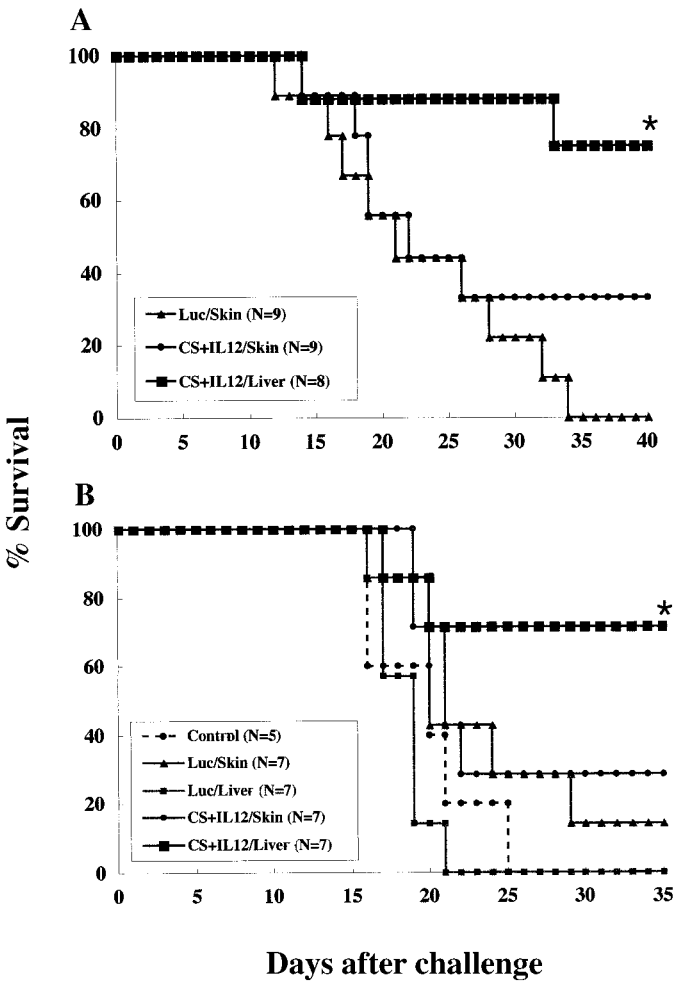

FIG. 2. Survival of mice bombarded with different regimes using a gene gun after lethal sporozoite challenge. Two experiments were performed. (A) Experiment 1: Mice were bombarded twice with pcDNA-Luc into the skin ($N = 9$), twice with pcDNA-CS87 plus pcDNA-mIL12p35p40 into the skin ($N = 9$) or once with pcDNA-CS87 plus pcDNA-mIL12p35p40 into the liver ($N = 8$). (B) Experiment 2: Mice were bombarded twice with pcDNA-Luc into the skin ($N = 7$), twice with pcDNA-CS87 plus pcDNA-IL12p35p40 into the skin ($N = 7$), once with pcDNA-Luc into the liver ($N = 7$), once with pcDNA-CS87 plus pcDNA-mIL12p35p40 into the liver ($N = 7$) or non-immunized mice as a control ($N = 5$). Mice were challenged by the bites of *P. berghei*-infected mosquitoes 2 weeks (mice bombarded into the skin) or 4 weeks (mice bombarded into the liver) after the last immunization. Malaria-infected mice were monitored daily for survival up to 35 or 40 days after challenge. Asterisks indicate that groups of mice immunized with pcDNA-CS87 plus pcDNA-IL12p35p40 into the liver (CS + IL12/Liver) have significantly higher protection rates than groups of mice bombarded with the control plasmid pcDNA-Luc into the skin (Luc/Skin) at $P < 0.05$, using the χ^2 test.

barded with the DNA vaccine by gene gun. Another group was similarly bombarded twice with the same DNA vaccine into the skin. Mice were challenged by the bites of *P. berghei*-infected mosquitoes 4 weeks after liver bombardment or 2 weeks after the second skin bombardment (Fig. 2A). The same experiment was repeated with inclusion of a group of mice bombarded with pcDNA-Luc into the liver (Fig. 2B). We have previously shown that 2 days after bombardment with the *lac*Z gene into the rat liver, β-galactosidase activity was detected to a depth of 2 mm below the liver surface (10). In the two experiments, mice bombarded with the DNA vaccine into the liver had statistically significant protection (73%) compared with those into the skin (31%). All nonimmunized mice and mice bombarded with pcDNA-Luc into the liver died. Although we checked parasitemia daily from 3 to 14 days after challenge in Experiment 1, those animals which became infected showed no significant delay in parasitemia in any group (Fig. 3). It is of interest to note that although all mice once showing parasitemia died of infection, the period of life varied from 13 to 34 days after challenge in Experiment 1 (Fig. 2A) or 16 to 25 days in Experiment 2 (Fig. 2B), even in the control groups.

We assessed the relationship between humoral responses to PbCSP and protection in mice. Serum samples were collected immediately prior to challenge and 2 weeks after challenge. No detectable ELISA antibody responses to recombinant PbCSP were induced in any immunized mice, whereas immunization with the PbCSP plasmid alone by intramuscular injection induced relatively high antibody to PbCSP (data not shown). This is consistent with the low-level Ab response induced by coadministration of HIV gag gene with IL-12 gene (20).

Taken together, these data show that direct immunization into the liver resulted in more effective protection compared to immunization into the skin. In addition, the lack of association of protection with Ab response suggested that the immunity was dependent on cell-mediated responses to PbCSP.

Increase in the number of CD8$^+$ T cells in the liver of protected mice after rechallenge. To test whether sporozoite rechallenge affected the relative proportion of infiltrating T cell subsets, splenic and hepatic lymphocytes from the protected mice of Experiment 1 were isolated 42 h after rechallenge and analyzed by flow cytometry. Two-color staining for CD8 and CD4 showed that CD8 single-positive cells in the liver were substantially increased both in mice bombarded into the liver and skin following rechallenge compared with those in the control mice (Fig. 4). Neither significant change in CD3/IL-2R β/TCR-$\gamma\delta$ subsets nor change in CD8/CD4 subsets was observed in the spleens (data not shown).

Induction of IFN-γ in the hepatic lymphocytes from immunized mice after challenge. Splenic and hepatic lymphocytes from mice bombarded in Experiment 2 at 42 h after challenge were cultured in the presence of medium alone or cross-linked anti-CD3 antibody. Supernatant fluids were assayed for IFN-γ or IL-4 pro-

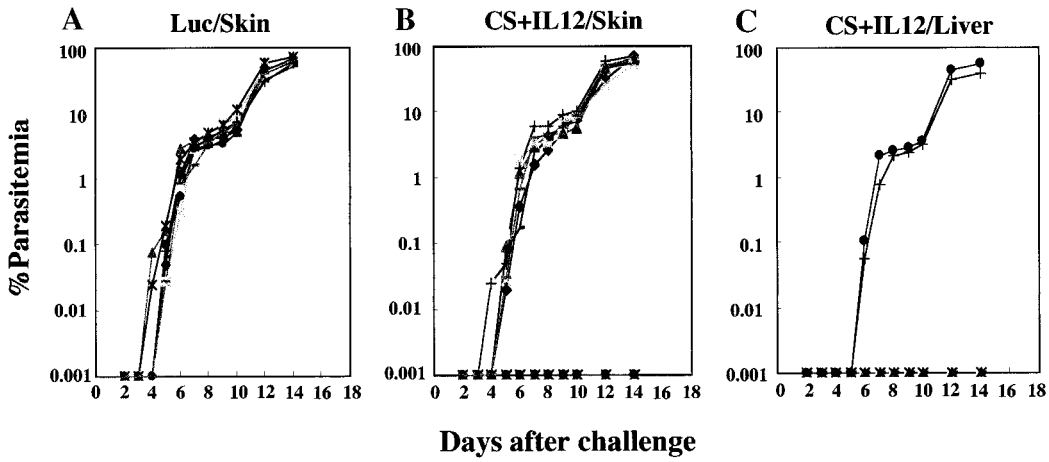

FIG. 3. Parasitemia profiles in groups of mice bombarded with different regimes using a gene gun after lethal sporozoite challenge in Experiment 1. Mice were bombarded with pcDNA-Luc into the skin (A) (N = 9), pcDNA-CS87 plus pcDNA-mIL12p35p40 into the skin (B) (N = 9) or pcDNA-CS87 plus pcDNA-mIL12p35p40 into the liver (C) (N = 9), and challenged as described in the legend to Fig. 2A. Parasitemia percentage was monitored on Giemsa-stained blood films for individual animals from 3 to 14 days after challenge.

duction. Hepatic lymphocytes from mice bombarded into the liver produced without stimulation almost 5-fold greater IFN-γ production than did cells obtained from mice bombarded into the skin (Fig. 5). Control mice, in contrast, produced no detectable IFN-γ. In addition, no IFN-γ was detectable in any culture supernatant fluid from spleen cells of any group (data not shown).

All culture supernatant fluids collected were also assayed for IL-4. There was no detectable IL-4 in any group without stimulation. Curiously, hepatic lymphocytes from mice bombarded into the liver produced much less IL-4 than did cells obtained from another group in response to anti-CD3 antibody stimulation. Thus, the cytokine assays suggested that coadministration of the PbCSP gene with the IL-12 gene induce a Th1-type immune response in the liver.

Detection of iNOS mRNA by RT-PCR. Previous works had demonstrated that in addition to $CD8^+$ T cells and IFN-γ, nitric oxide (NO) is required for the protection induced by immunization with irradiated *P. berghei* sporozoites in mice (2). To determine whether the protected liver-bombarded mice induce iNOS in the liver after sporozoite invasion, RNA was isolated from the livers of protected mice bombarded into the liver in Experiment 2 at 24 h after sporozoite rechallenge and analyzed for the presence of iNOS mRNA by RT-PCR (Fig. 6). iNOS was not detectable in the livers of either normal mouse or mouse bombarded into the liver (lanes 1 and 3), whereas iNOS was induced in the livers of mice bombarded into the liver 24 h after rechallenge (lane 4). A faint level of iNOS mRNA was detected in the liver of normal mouse 24 h after challenge (lane 2).

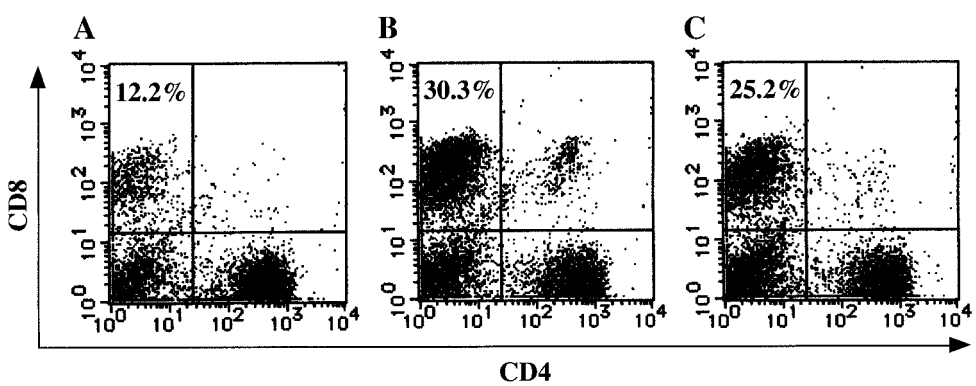

FIG. 4. Flow cytometry analysis of hepatic lymphocytes isolated from mice 42 h after challenge by the bites of *P. berghei*-infected mosquitoes. Mice bombarded into the liver or skin protected challenge as described in the legend to Fig. 2B. The protected liver-bombarded (B) and skin-bombarded mice (C) were rechallenged by the bites of *P. berghei*-infected mosquitoes, and hepatic lymphocytes were isolated from three mice per group 42 h after rechallenge. Naive mice (A) were challenged and the hepatic lymphocytes were prepared as (B) and (C). Cells were stained with PE-coupled anti-CD4 mAb and FITC-coupled anti-CD8 mAb. Two-color immunofluorescence analysis dot blots for the hepatic lymphocytes were generated after exclusion of dead cells by using propidium iodide.

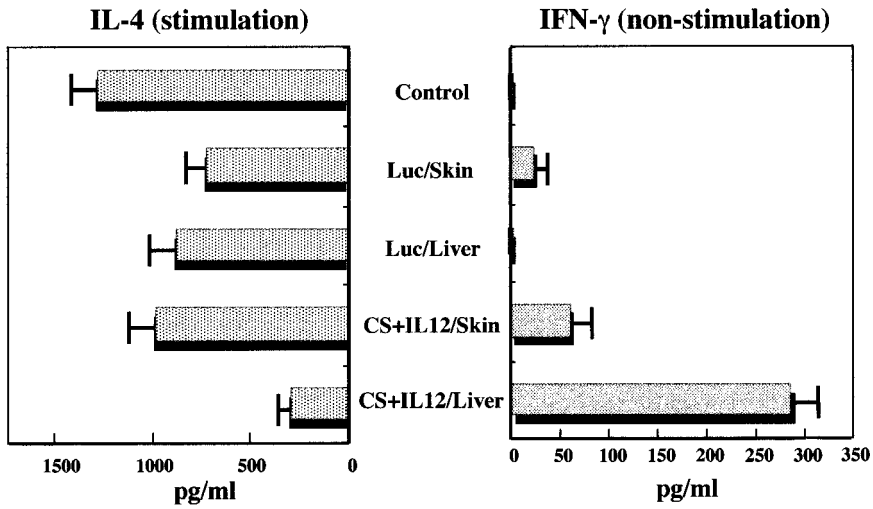

FIG. 5. Comparison of IFN-γ and IL-4 production levels from hepatic lymphocytes of mice bombarded with DNA vaccine followed by challenge of *P. berghei*-infected mosquito bites. Groups of three mice were bombarded and challenged as described in the legend to Fig. 2B. Forty-two hours after challenge, hepatic lymphocytes from three mice were collected and cultured with or without anti-CD3 Ab. IFN-γ (right) and IL-4 (left) productions were determined in the supernatant at 12 h by ELISA and bioassay, respectively. Five groups consisting of naive mice (Control), mice skin-immunized with control plasmid (Luc/Skin), mice liver-bombarded with control plasmid (Luc/Liver), mice skin-bombarded with the DNA vaccine (CS + IL12/Skin) and mice liver-bombarded with the DNA vaccine (CS + IL12/Liver) are indicated at the middle of the panel. Data represent means ± standard deviations and are representative of two replicative experiments.

Since increases in IFN-γ and $CD8^+$ T cells in the livers of mice bombarded into the liver were observed, we assessed the relationship between $CD8^+$ T cells, IFN-γ and liver iNOS. The protected liver-bombarded mice were rechallenged following treatment with either anti-IFN-γ, anti-CD8, or anti-CD4 mAb. iNOS mRNA was detected in the livers of mice administrated both with control mAb and anti-CD4 mAb (lanes 7 and 8). In contrast, we found a significant decrease in the ability of the protected mice to express iNOS in response to sporozoite challenge by administration of anti-CD8 antibody and anti-IFN-γ antibody (lanes 5 and 6). Therefore, induction of iNOS mRNA was dependent on $CD8^+$ T cells and IFN-γ.

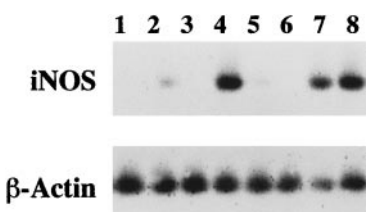

FIG. 6. Detection of iNOS mRNA expression. Mice bombarded with pcDNA-CS87 plus pcDNA-mIL12p35p40 into the liver were protected against challenge as described in the legend to Fig. 2B. The protected mice were inoculated with different mAbs and then rechallenged by the bites of *P. berghei*-infected mosquitoes. RNA was extracted from the livers of a control mouse (lane 1), a protected mouse (lane 3), a control mouse 24 h after challenge (lane 2) and a protected mouse 24 h after rechallenge (lane 4). Other protected mice were rechallenged following the treatment with anti- IFN-γ mAb (lane 5), anti-CD8 mAb (lane 6), anti-CD4 mAb (lane 7) and control mAb (lane 8). RNA was extracted from livers 24 h after rechallenge. Southern hybridization was performed to detect the expression of iNOS and β-actin mRNA from livers following amplification by RT-PCR as described under Materials and Methods. Each lane represents data from an individual mouse.

DISCUSSION

Protective immunity against intrahepatic malaria parasites is induced by immunization with irradiated sporozoites, thereby preventing blood stage malaria. In an attempt to pursue development of vaccines that induce immune responses comparable to those elicited by immunization with irradiated sporozoites, genetic immunization with naked DNA has been shown to induce humoral as well as cellular immune responses with high efficiency, emphasizing the enormous potential of this strategy for vaccination purposes (5–8). We hypothesized that induction of local immunity at the site of the liver could be important in preventing the development of exoerythrocytic stages and subsequent blood infection. In this study, we introduced malaria DNA vaccine into the liver using a gene gun-mediated gene delivery system and examined the protective effects. Protection obtained by gene gun delivery into the liver once (73%) was significantly higher than that by the material into the skin twice (31%), suggesting that direct gene transfer of DNA vaccine to the liver surface is potentially a more effective approach of generating protective immunity against malaria parasites with the CSP immunogen.

One obvious advantage of immunization with a DNA vaccine into the liver, over immunization with the same

DNA vaccine into the skin is that malaria antigens expressed on the cell surface from DNA constructs can be correctly presented to the immune system in a native state at the appropriate site. Thus, immunization with genes encoding malaria liver stage antigens, such as CSP (8), TRAP (33), LSA-1 (34), and HEP17 (6), can in theory elicit a whole range of immune responses, closely resembling the process of natural sporozoite infection. In addition, the success of DNA vaccination depends upon transfection and activation of dendritic cells (DC) followed by the initiation of T cell response (35). Gene gun bombardment into the skin results in direct transfection of bone marrow-derived Langerhans cells as DC, which are only 5% of the cells in the epidermis (36). These DC are deeply involved in priming native T cells. In contrast to the skin, higher frequencies of Kupffer cells as resident DC in the liver to support the recruitment and activation of antigen presenting cells may account for the liver playing a critical role in DNA immunization. Although it is unlikely that this approach will ultimately be used for DNA vaccination in humans, this information may potentially influence the choice of clinical trials of DNA vaccination against not only malaria, but hepatic diseases.

The local cytokine environment of the liver may affect the type of immune responses generated at this site. Manipulation of the cytokine environment by coadministration with cytokine-encoding plasmids has potential to allow the tailoring of a DNA vaccine to ensure that protective immune response is initiated. Analysis of cell-mediated immunity revealed that $CD8^+$ T cells, IFN-γ and iNOS were induced in the livers of the protected mice immediately after rechallenge. In addition, the induction of iNOS in the liver requires $CD8^+$ T cells and IFN-γ. Thus, our results strongly support the notion that coadministration of the PbCS gene with the IL-12 gene may induce Th1-type immune responses into the hepatic lymphocytes, resulting in local secretion of IFN-γ and stimulation of liver cells to produce NO for the destruction of infected hepatocytes or the parasite within these cells.

We demonstrate here for the first time the efficacy of local immunization into the liver with malaria DNA vaccines by gene gun directly in eliciting protective immune responses. Our data indicate that introduction of DNA to target organs such as the liver in the case of malaria may improve the prospects for the development of an efficient vaccination strategy for the pre-erythrocytic antigens. To extend the present study as an application for clinical use, we used an anionic liposome containing hemagglutinating virus of Japan (HVJ-liposome) to deliver DNA vaccine into the liver intravenously. This gene transfer method has been known to be efficient for recovery from rat liver cirrhosis by inoculation of HVJ-liposome with the hepatocyte growth factor gene (37). This method, however, did not work well for a malaria DNA vaccine compared with direct immunization into the liver, suggesting the importance of efficient uptake of DNA vaccine by Kupffer cells in the liver (data not shown).

Further work is required to establish a novel DNA delivery system acceptable for clinical use. Moreover, the liver is an important and attractive target for the development of not only malaria vaccine, but gene therapy strategy. Widespread application of hepatic gene transfer in humans awaits the development of a safe and simple delivery system and inducible tissue-specific promoters that can direct efficient transgene expression *in vivo*.

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