Concrete Irrigation Canals - 2 -

The concrete irrigation canal project continued until 1985. By 1980, the total length of the concrete canals reached 2,053 km in Yamanashi.

Figure 75. The construction of a canal in 1965.

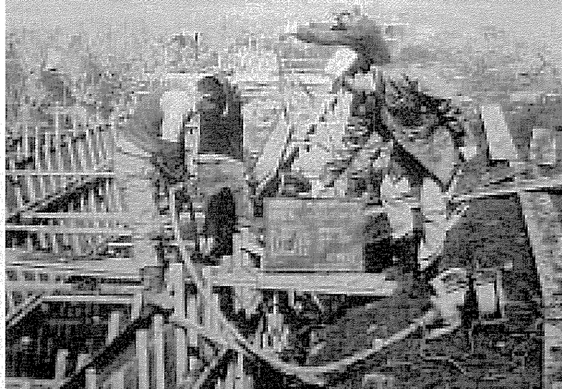


Figure 76. In Tamaho town in 1975.

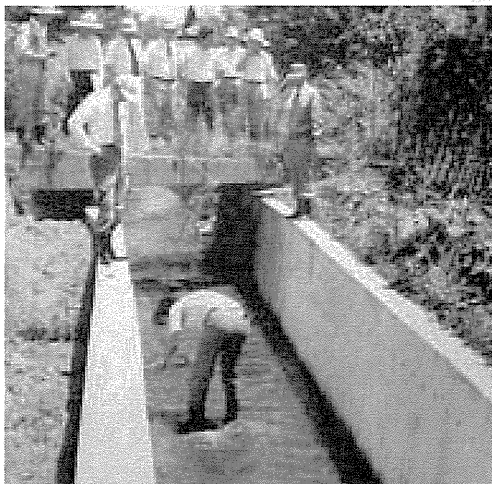


Figure 77. Snail check after the construction of the large canal in 1976.

Figure 78. Examination of a damaged canal in 1980 in Tamaho town.

11. Molluscicide by Fire - 1 -

Among the many trials using different methods, including hot-water, electricity, chemicals and fire, the major method adopted was chemicals. However, the mobile fire thrower fueled with acetylene gas was used as a complement of chemicals until 1955.

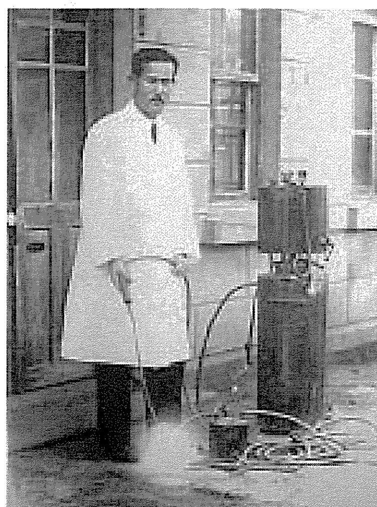


Figure 79. Dr. Hiroji Mitsui and his snail burner. The field trial in 1931 revealed the efficacy of this machine and was adopted the following year. Later on, a mobile canister was used until 1955 (Photo by Dr. Chuzo Mitsui).

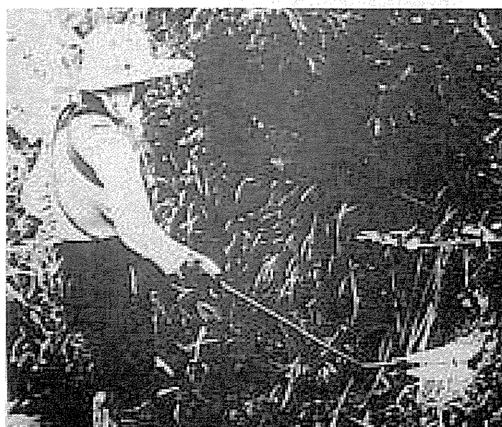


Figure 80. Field application of the burner in 1937.

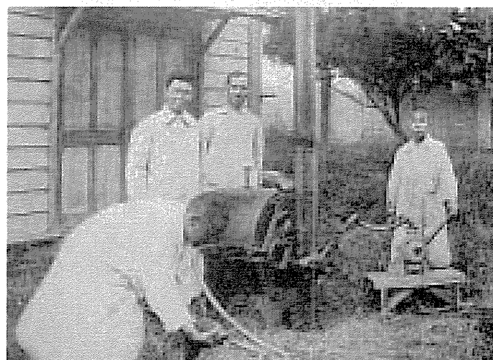


Figure 81. Prof. Fujinami and his hot water machine in 1919.

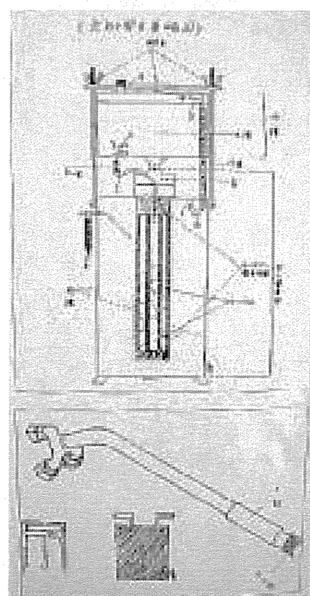
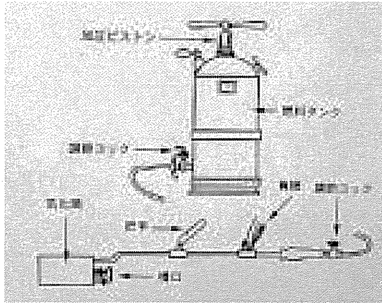


Figure 83. Dr.Mitsui's drawing of the structure of the burner.

Figure 82. Burning snails in 1951.

Molluscicide by Fire - 2 -

Acetylene gas was replaced by oil as the snail burner fuel in 1958, and the manual pump was replaced by a gasoline engine in 1974. The main body was also modified from back-pack style to cargo style with wheels.



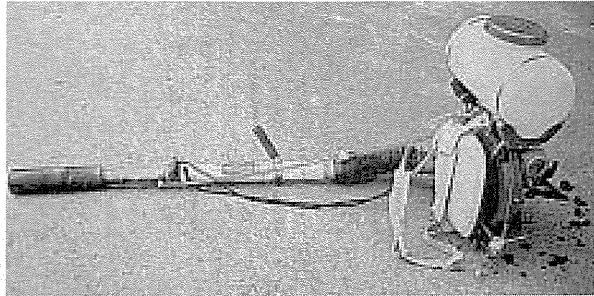


Figure 84. Back-pack style oil burner around 1974.

Figure 85. Molluscicide activity using a burner in a in 1960.

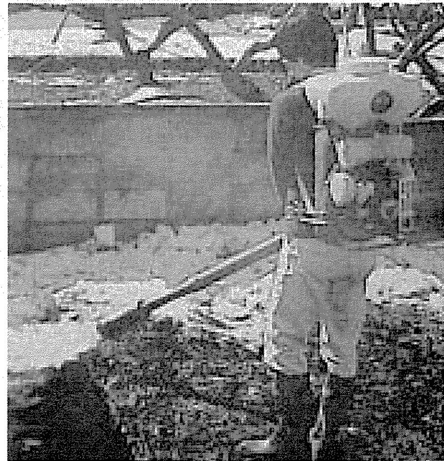


Figure 86. The modified oil burner in 1975.

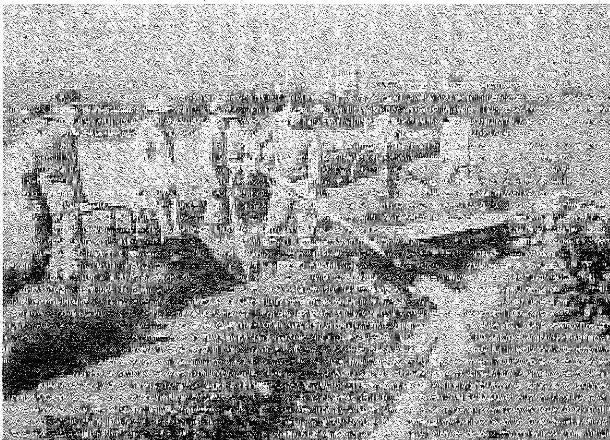


Figure 87. The fully modified burner was used for snail control in Tatsuoka town until as recently as 1982.

12. Molluscicide by Yurimin

The effectiveness of Yurimin was confirmed by Iijima in 1964. It was used with NaPCP until 1971 when NaPCP was prohibited due to its environmental toxicity. Yurimin was used until 1976.

Figure 88. A researcher examines the efficacy on snails in a laboratory at the Prefectural Institute of Hygiene in 1965.

Figure 89. A granular form of Yurimin was applied to the field (Ryuo town, 1968).

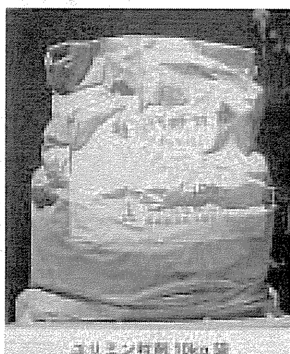


Figure 90. 10kg package of Yurimin

地方病撲滅に新作戦

ユリミン粒剤で殺貝

Figure 91. Newspapers reported the new molluscicide strategy using Yurimin.

Figure 92. In Hatta village in 1973.

13. Molluscicide by B-2

The Prefectural Institute of Hygiene recommended the anti-fungi compound B-2 as a molluscicide with high activity but low toxicity to humans, animals and fish. In 1976, it was used with Yurimin and was continuously used after the ban on Yurimin the following year.

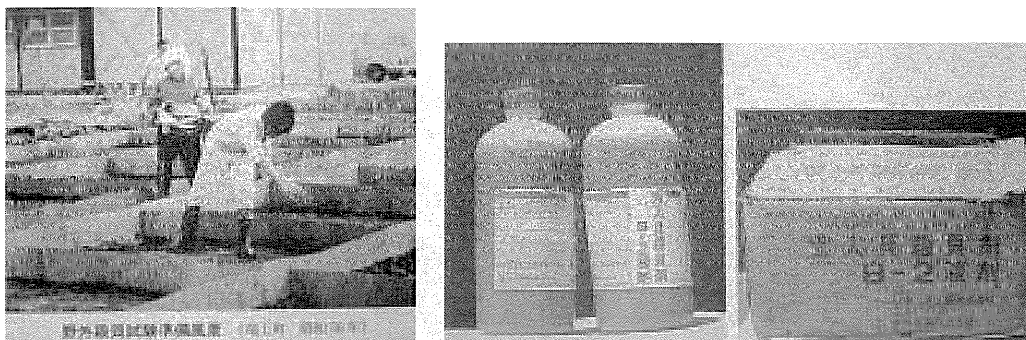


Figure 93. Preparations for field trials in Ryuo town in 1975.

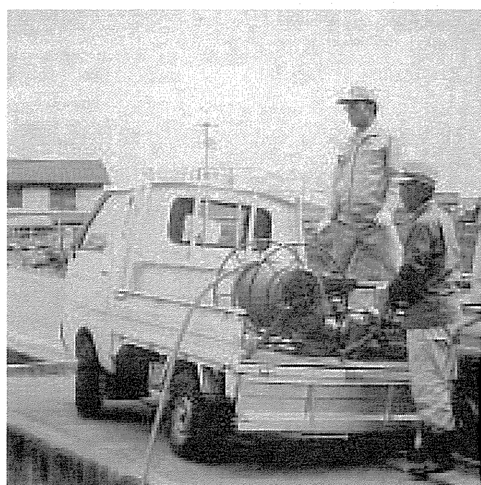


Figure 94. Mobile liquid tank and pump for spraying B-2 in Ryuo town in 1985.

殺貝剤を実験散布

Figure 95. Newspapers reported the replacement of banned Yurimin by B-2 in 1975 (Yamanashi Nichinichi Shimbun).

Figure 96. Liquid type B-2 was applied to be field by spray (Nakatomi town, 1990).

14. Health Education Activity - 1 -

After the discovery of the pathogen, mode of transmission and intermediate host, people realized that this disease was possible to prevent.

Figure 97. The department of schistosomiasis research in the medical association published a textbook for primary and junior high school students in 1917.

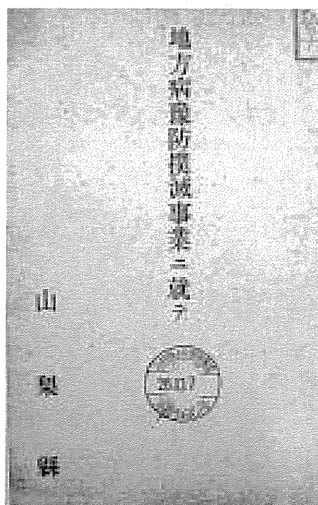


Figure 99. A booklet introducing the molluscicide project in Hiroshima Prefecture was distributed in Yamanashi in 1924 (Prefectural Library).

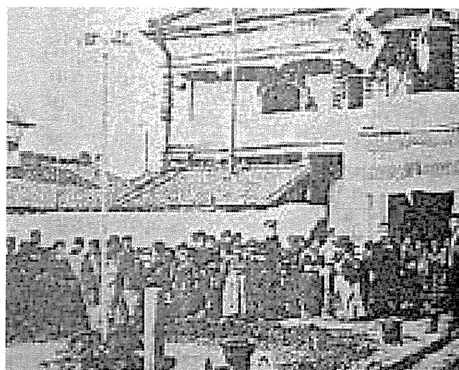


Figure 98. The venue of the "Health Exhibition" organized by the Yamanashi Association of Hygiene in 1913.

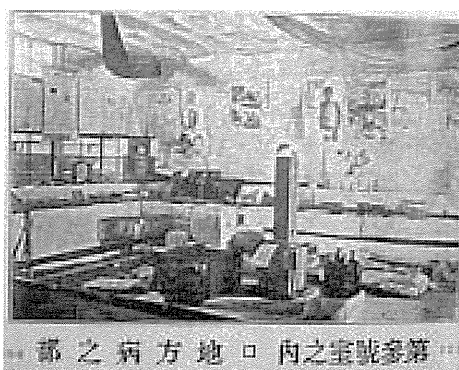


Figure 100. The exhibition of schistosomiasis (Prefectural Library).

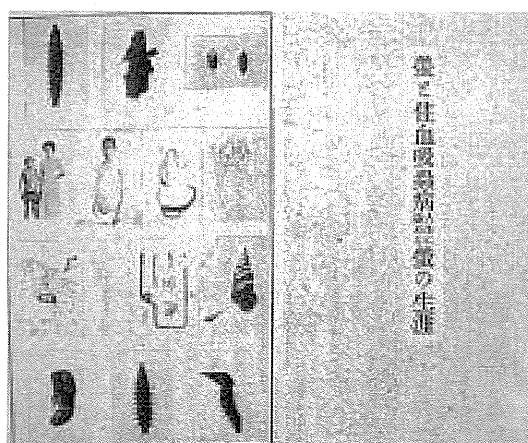


Figure 101. A book entitled "Fireflies and Schistosomiasis" was published by Mikinosuke Miyajima in 1918.

14. Health Education Activity - 2 -

From 1955, the prefecture strongly and successfully pushed forward the eradication program including health education.

Figure 102. Governor Hisashi Amano visits the endemic area to encourage villager activities (1957).

Figure 103. A comprehensive strategy including the revamping of agriculture and health care was initiated (1958).

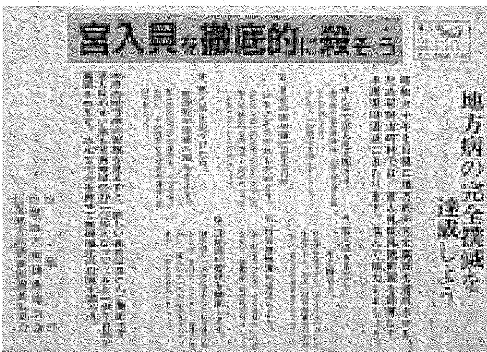


Figure 104. An advertisement calling for the complete elimination of snails (1979).

Figure 105. Advertisement cars were used in the health education campaign.
Above: 1955
below: 1970

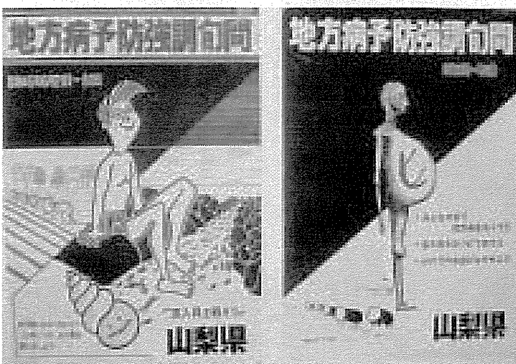


Figure 106. Posters advertising schistosomiasis prevention week in 1970.

15. Other controls : Predators

Carp, worms, nematodes, and trematodes were proposed and examined as predators, but none proved successful.

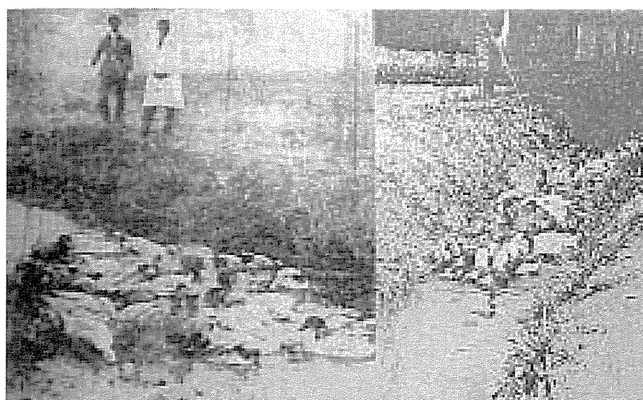
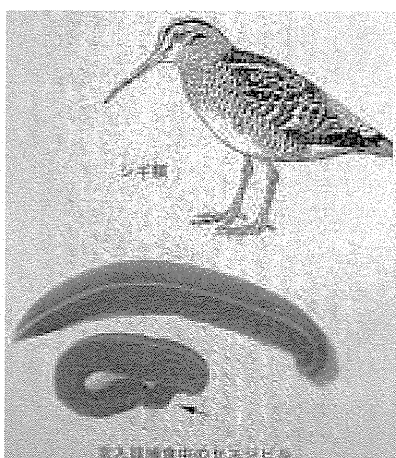


Figure 107. Kenzo Sugiura used ducks as a possible predator of snails.

Figure 108.
Kenzo Sugiura (1866-1933) devoted his life to all the aspects of anti-schistosomiasis research and medical practice.



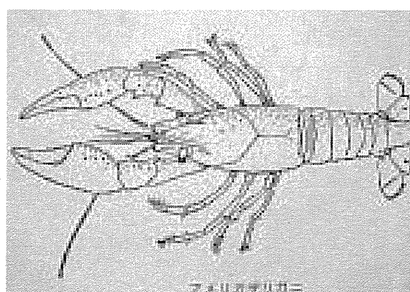


Figure 109. Firefly nymphs attack the oncomelania snail (Photo taken in 1938) (Courtesy of Dr. S. Hara).

16. Other controls: Ointments, fecal treatment

At early ages, ointment of vaseline and fecal treatment were recommended as controls. Later, rubber boots and gloves were recommended. After the war, Benlate was recommended for use as an ointment.

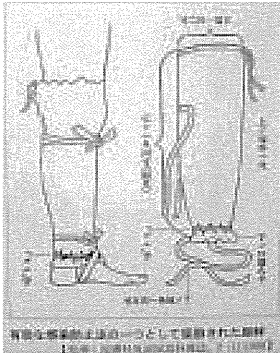


Figure 110. Matsura, Journal of Urology and Dermatology, 9 (11), 1909.

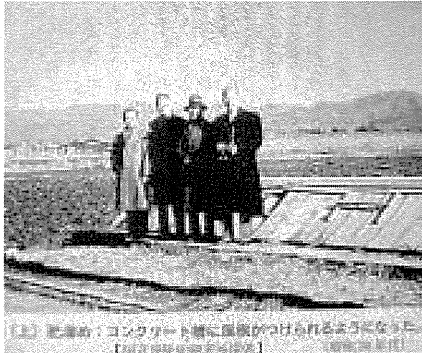


Figure 111. A cesspool was covered with a roof around 1955.

Figure 112. Benlate was popular around 1955.

Figure 113. Cesspools containing human feces in 1943 were not covered.

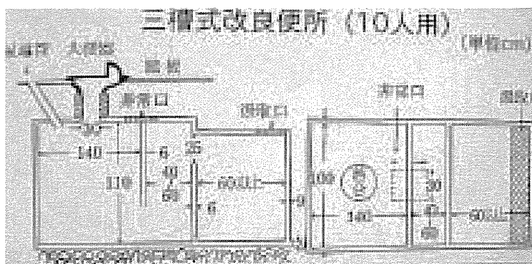


Figure 114. A modified toilet was designed by the local government after the Toilet Improvement Act (1929) and Parasitic Disease Prevention Law (1933) were approved, but it did not become popular.

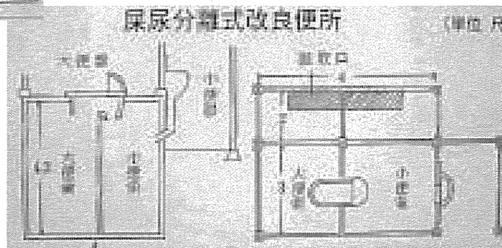


Figure 115. A modified toilet was designed to separate feces and urine and to prevent parasitic disease in 1954.

17. Stibnal, the anti-schistosomiasis drug

In 1922, sodium antimonyl tartrate, or Stibnal, was shown to be effective by Dr. Nishi.

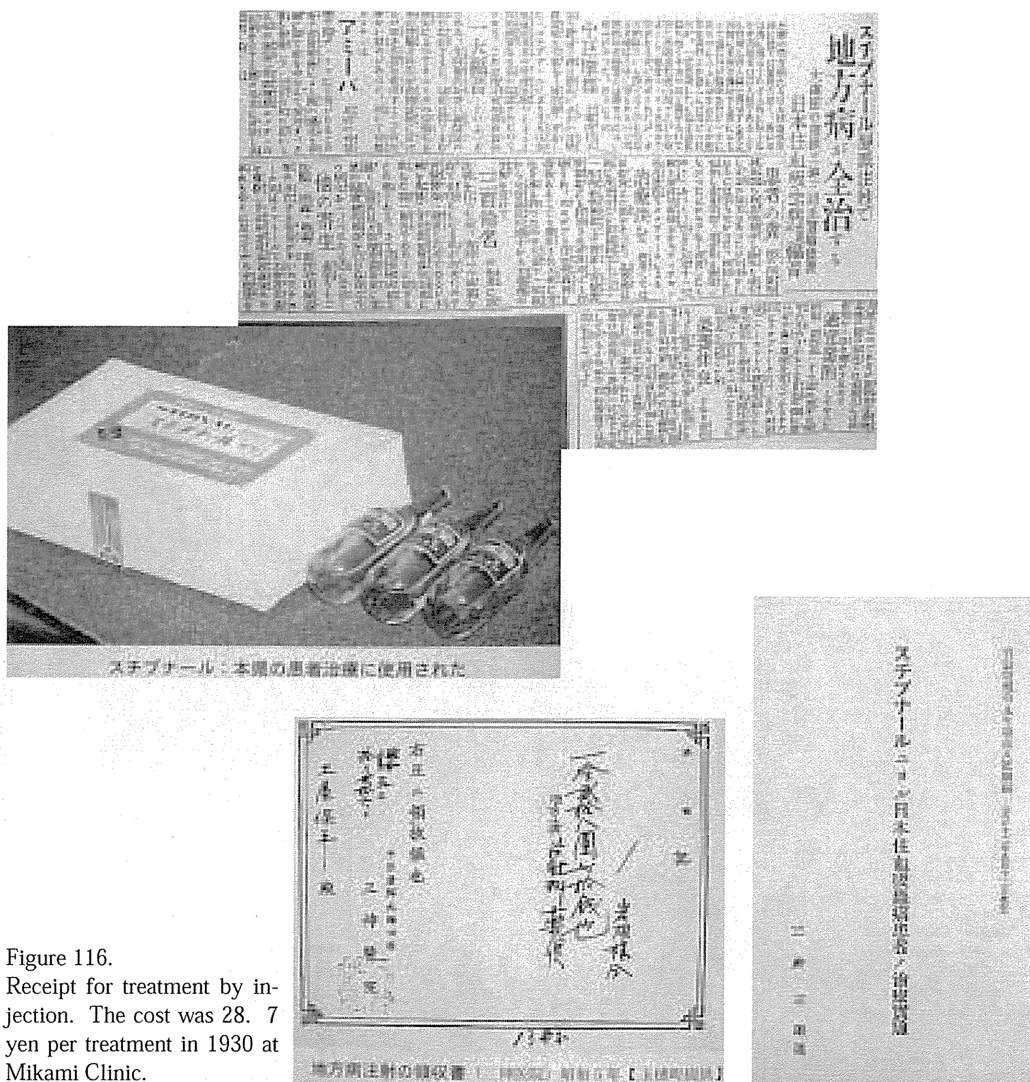


Figure 116.
Receipt for treatment by injection. The cost was 28. 7 yen per treatment in 1930 at Mikami Clinic.

Figure 117. Stimon was given by intramuscular injection and used for mass chemotherapy in 1947.

18. Skin Tests and Fecal Examinations

A skin test was developed by Dr. George Hunter of 406 MGL and Dr. Sabro. Sugiura in 1948.

In 1957, Shujo. Ohta applied the examination to primary school pupils using the 406 MGL antigen. This antigen was used until 1975.

Figure 118. Delayed-type hypersensitivity was provoked by the intradermal injection of the antigen (Hatta village in 1974).

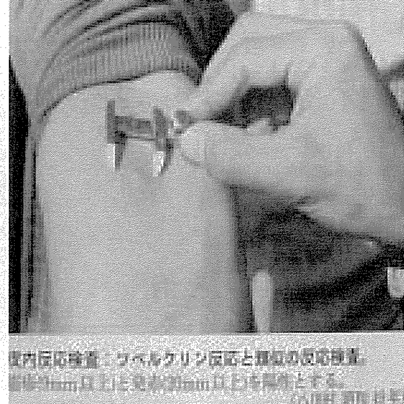


Figure 119.
Left: 406 MGL antigen was used until 1975.
Right: NIH of Japan antigen was used until 1985 when ELISA was applied for detection.

Figure 120. Fecal examination at the Prefectural Institute of Hygiene in 1971.

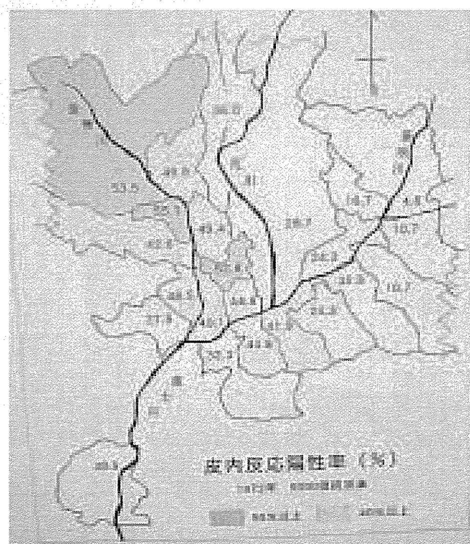


Figure 121. The prevalence of skin test positivity (%) in 1972.

19. Advanced Cases and Mortality

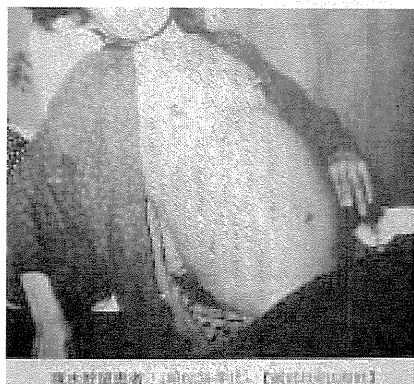


Figure 122. Ascites observed in a patient with chronic schistosomiasis in the 1950's.

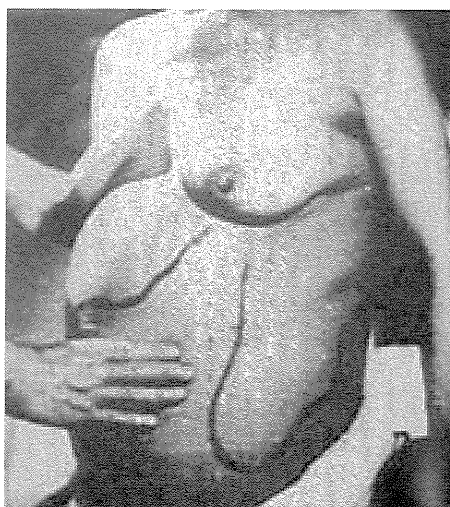


Figure 123. The enlarged liver and spleen were indicated by black lines (Photo taken around 1943).

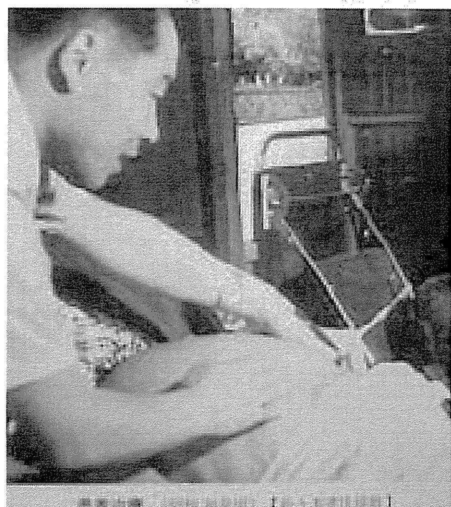


Figure 124. At the clinic (1961).

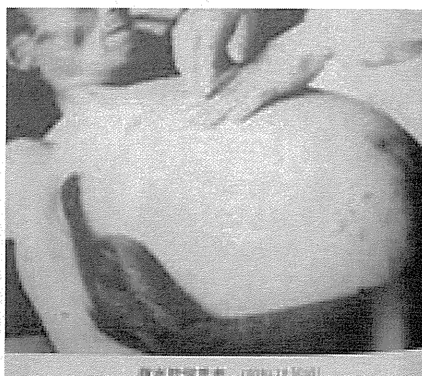


Figure 125. The ascites observed in chronic schistosomiasis, around 1943.

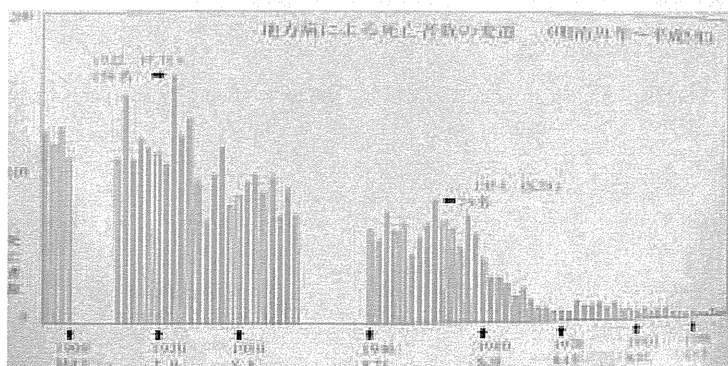


Figure 126.
The number of deaths per year from 1906 to 1993, in Yamanashi.

20. Growth Retardation

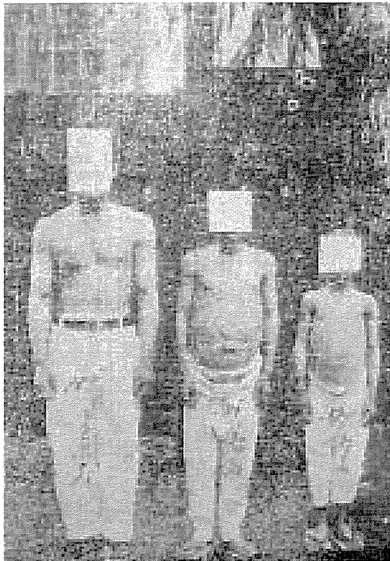


Figure 127. The comparison of stature between a healthy 18 year-old boy (left) and chronic schistosomiasis patients of 19 years (center) and 15 years of age (right) in 1928.

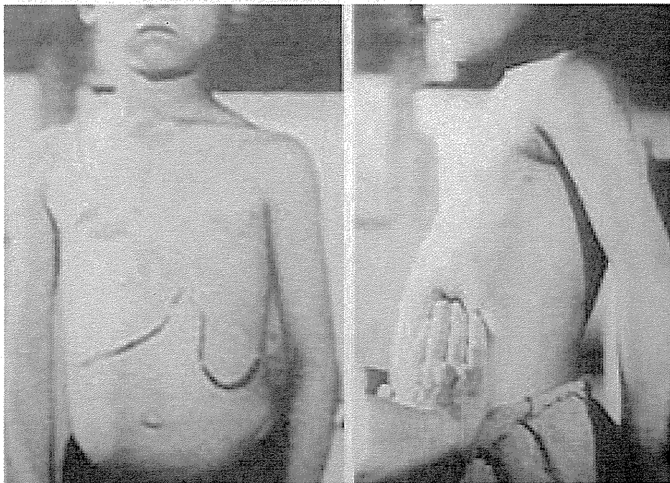


Figure 128. A primary school pupil showing hepato-splenomegaly (1943).

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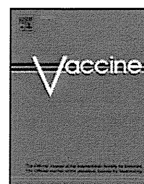

Immunogenicity of novel nanoparticle-coated MSP-1 C-terminus malaria DNA vaccine using different routes of administration

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ABSTRACT

An important aspect in optimizing DNA vaccination is antigen delivery to the site of action. In this way, any alternative delivery system having higher transfection efficiency and eventual superior antibody production needs to be further explored. The novel nanoparticle, pDNA/PEI/γ-PGA complex, is one of a promising delivery system, which is taken up by cells and is shown to have high transfection efficiency. The immunostimulatory effect of this novel nanoparticle (NP) coated plasmid encoding *Plasmodium yoelii* MSP1-C-terminus was examined. Groups of C57BL/6 mice were immunized either with NP-coated MSP-1 plasmid, naked plasmid or NP-coated blank plasmid, by three different routes of administration; intravenous (i.v.), intraperitoneal (i.p.) and subcutaneous (s.c). Mice were primed and boosted twice at 3-week intervals, then challenged 2 weeks after; and 100%, 100% and 50% mean of survival was observed in immunized mice with coated DNA vaccine by i.p., i.v. and s.c., respectively. Coated DNA vaccine showed significant immunogenicity and elicited protective levels of antigen specific IgG and its subclass antibody, an increased proportion of $CD4^+$ and $CD8^+$ T cells and INF-γ and IL-12 levels in the serum and cultured splenocyte supernatant, as well as INF-γ producing cells in the spleen. We demonstrate that, NP-coated MSP-1 DNA-based vaccine confers protection against lethal *P. yoelii* challenge in murine model across the various route of administration and may therefore, be considered a promising delivery system for vaccination.

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1. Introduction

Malaria is a major cause of disease and death, with half of the world's population at risk [1], and an estimated 243 million cases led to nearly 863,000 deaths in 2008 and most of them are children under 5 years of age [2,3]. Various strategies have been developed to prevent this burden, such as diagnosis and treatment, and vector control [4–6]. However, these strategies are still limited because of malaria parasite resistance to most antimalarial drugs and insecticide resistance in the anopheline mosquitoes that transmit the disease [5,7]. Together with other control methods, vaccination holds the promise of controlling and perhaps eventually eradicating malaria [8–11]. DNA vaccination is one of the novel approaches for developing new generation vaccines against malaria. Blood stage infection is established by the invasion of erythrocytes by merozoites [12,13], and a great deal of effort is focused on vaccines targeting this stage, because this approach can directly reduce morbidity and mortality associated with malarial disease in humans [14]. Hence, any intervention that could block merozoite invasion of erythrocyte can lead to control of malaria parasite replication [15,16]. Of the many proteins associated with merozoite, merozoite surface protein 1 (MSP-1), has become a major vaccine candidate [17,18]. This is because antibodies to MSP-1 C-terminal portion, was found at all stages of invasion from initial attachment through to complete invasion [13,19], and therefore considered a promising candidate for the development of a malaria vaccine [20].

Major problems and difficulties associated with malaria vaccine development have been highlighted [21–23], and one of them is the poor immunogenicity of genetically engineered

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antigens, and which required a delivery system and adjuvant to elicit their effect at targeted site of action. A delivery system which transports the antigen to site of action with an adjuvant that activates the cells via interaction with cell receptors and enhances the potency of the antigen, are important aspect in optimizing DNA vaccination [23–25]. Introduction of nanotechnology and the development of nano-carrier-based vaccines have started to receive a lot of attention in order to provide effective immunization through better targeting and triggering antibody response at the cellular level. Many studies demonstrated that, non-viral carrier systems are widely used as transfection agents to deliver nucleic acids for both in vitro and in vivo applications [26,27]. Recently, a few nanoparticle-based vaccines have shown to be effective in the induction of immune responses in animal models without the need for an adjuvant [28]. In this way, any alternative gene delivery systems having higher transfection efficiency and eventual superior antibody production needs to be further explored. The novel nanoparticle (pDNA/-PEI/γ-PGA complex) is one of the promising gene delivery system, which is taken by the cells via γ-PGA-specific receptor mediated pathway and showed high transfection efficiency and low toxicity [29]. Recently, application of this nanoparticle coating MSP-1 by intravenous delivery was shown to partially protect against lethal *Plasmodium yoelii* challenge in mouse model [30]. Also, in the past several years, attention was placed on routes of vaccine administration in order to induce strong immune response against pathogens. The choice of routes to deliver plasmid DNA for obtaining efficacious immunogenic response of the expressed antigen is restricted [31–34] and also influences the immune responses [32,33,35–37]. Therefore, determination of optimal routes to induce antibody and cellular responses are important steps in the development of vaccines against malaria infection.

It is because of the need for an improved delivery system, that in this study, we used nanoparticle formulation for the delivery of a characterized malaria blood stage vaccine candidate (MSP-1). The aim of this current study was to check the effect of nanoparticle coating on *P. yoelii* MSP-1 C-terminus plasmid on induction of immune response in mice using three different routes of administration.

2. Materials and methods

2.1. Construction and purification of MSP1-C terminus plasmid DNA

Sequence of *P. yoelii* MSP-1 (*PY05748*) from the *Plasmodium* genome database, PlasmoDB (www.plasmodb.com) was used for primer design. Primer set of MSP-1 fragment from region 4819–5286 bp, with BglII and BamHI restriction sites, were designed using Oligo Perfect™ Designer (www.invitrogen.com). Forward-5′-***AGATCT***ATGCTTGACGAATTTGTAGAACATGC-3′ and reverse 3′-***GGATCC***CTTATAATAAAATTGATAATCCCATAAAGCT-5′ (restriction sites underlined). *P. yoelii* blood-stage cDNA was used as template to PCR amplify the C-terminal MSP-1. The PCR-amplified product was directly cloned into a BglII and BamHI restriction sites of plasmid VR1020 (Vical, San Diego, CA) to obtain the construct pVR1020-MSP-1 C-terminus. Plasmid DNA, pVR1020-MSP-1 C-terminus was transformed into Top10 chemically competent *Escherichia coli* (Invitrogen, Carlsbad, CA). Ten (10) bacterial colonies were picked and plasmid purified using Qiagen miniprep kits (Qiagen, Maryland) to check the presence of expected fragment. The plasmid containing expected inserts were analyzed by restriction digestion and DNA sequence in the constructs was confirmed by automated DNA sequencing. For large scale DNA vaccine preparation, the plasmid DNA was purified using Qiagen Mega kits (Qiagen, Maryland, USA) according to the manufacturer's instructions. The purified plasmid DNA was resuspended in 5% glucose solution in appropriate concentration and kept until use at −80 °C.

2.2. Formulation and preparation of nanoparticle pDNA complex

Nanoparticle (PEI/γ-PGA) coated-plasmids were formulated as previously described [29]. Briefly, polyethylenimine (PEI) solution (pH 7.4) and pDNA, pVR1020-MSP-1 C-terminus or pVR1020 blank, were mixed by pipetting thoroughly and left for 15 min at room temperature and then gamma polyglutamic acid (γ-PGA) was mixed with the pDNA/PEI complex by pipetting and incubated at room temperature for 15 min. The ternary complex, pDNA/PEI/γ-PGA was constructed at a charge ratio, phosphate of pDNA:nitrogen of PEI:carboxylate of γ-PGA = 1:8:6, with 62.8 ± 0.3 nm particle size and − 14.8 ± 0.7 mV electric charge (ζ-potential). The size and ζ-potential of the complex was determined using Zetasizer Nano ZS (Malvern Instrument, Ltd., UK).

2.3. Immunization of mice

Three different routes of injection; intravenous (i.v.), intraperitoneal (i.p.) and subcutaneous (s.c.) were used to immunize groups of 6-week-old female C57BL/6 mice (SLC, Lab, Japan) with different formulations of the DNA vaccine designated as coated, naked and coated control. Mice in the coated group were immunized with 100 μg of coated-plasmid DNA (pVR1020-MSP-1/PEI/γ-PGA), naked group with 100 μg of pVR1020-MSP-1 C-terminus, and coated control group with 100 μg of pVR1020/PEI/γ-PGA. In the first experiment, ten (10) C57BL/6 mice were used in two groups, one for coated plasmid DNA and the other for naked, to observe the effect of coating. After the first experiment, we proceeded to the second experiment that was designed to observe the antigen driven immune response after the vaccination with the coated plasmid DNA. Mice were prime-immunized at day 0 and two subsequent boosters of 100 μg/mouse of plasmid DNA were given at day 21 and 42. Two weeks after the last immunization, pooled or individual sera were collected from all the mice, for ELISA and cytokines analysis, and 2 mice from each group were randomly picked and sacrificed and spleen cells prepared for flow cytometric analysis, ELISPOT and cytokines analysis.

2.4. Ethical statement

The study was conducted strictly according to the recommendation in the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology, Japan (Notice no. 71). All animal experiments were approved by the Nagasaki University, Board of Animal Research, according to Japanese Guideline for use of experimental animals (Permit Number 0811130716). All efforts were made to minimize suffering, and animals were humanely sacrificed under ketamine anesthesia.

2.5. Challenge infection and protection assay

Two weeks after the last immunization, mice were each challenged by intraperitoneal injection of 10^5 of lethal strain of *P. yoelii 17XL*-parasitized red blood cells (pRBCs) as described [30]. Blood smears from the tail snips were examined by microscopy for the presence of parasites after staining with Giemsa. Parasitaemia was monitored, from day 4 after challenge infection, daily until clearance of parasitaemia or otherwise death of mouse.

2.6. Parasite antigen preparation and recombinant P. yoelii MSP-1 C-terminus proteins

P. yoelii antigen was prepared as previously described [30]. Briefly, parasite protein extracts were prepared by incubation of *P. yoelii*-infected RBCs in 0.15% saponin in PBS for 30 min and washed 3 times in PBS by centrifuging at 2000 rpm. The parasite pellet was then solubilized in 1% Triton X-100 and the cells were broken by freeze and thaw in liquid nitrogen 3 times. The lysate was then centrifuged at 10,000 × *g* for 10 min and the supernatant was used as soluble lysate or as crude antigen for ELISA. $PyMSP1_{19}$ encompasses C-terminal region of PyMSP1 (Asp1658 to Gly1757) based on the sequence of *P. yoelii* MSP-1 (*PY05748*) was amplified by PCR from *P. yoelii* 17XNL gDNA and cloned between the XhoI and BamHI sites of plasmid pEU-E01-GST-TEV (a vector with an N-terminal glutathione S-transferase tag followed by a tobacco etch virus protease cleavage site; Cell Free Sciences, Matsuyama, Japan). The inserted nucleotide sequence was confirmed using the ABI PRISM 3130 Genetic Analyzer and the BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA). Recombinant protein $PyMSP1_{19}$ was produced with the wheat germ cell-free protein expression system by the bilayer translation reaction method described previously [38,39]. After synthesis, the $PyMSP1_{19}$ was affinity purified by passage through a glutathione–sepharose 4B column (GE Healthcare, Camarillo, CA) and eluted by on-column cleavage with AcTEV protease (Invitrogen, Carlsbad, CA) after extensive washing of the column with phosphate-buffered saline (PBS). Concentration of purified protein was determined using the Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA). Purified protein sample was then stored in aliquots at −80 °C until assayed using enzyme-linked immunosorbent assays (ELISA).

2.7. Measurement of antibodies titre by ELISA

Immunoglobulin G and its subtype antibody responses were assessed by ELISA from the pooled sera collected from immunized mice 14 days after last immunization as described previously [30]. Briefly, 96-well plates were coated with 100 μl of 0.5 μg/ml of rMSP1 C terminus or 18 μg/well of parasite antigen in coating buffer and kept overnight at 4 °C. Plates were washed three times with 400 μl/well of 0.05% Tween–PBS and then blocked for nonspecific binding using 340 μl/well of 0.1% blocking reagent (Roche Diagnostics, Mannheim, Germany) for 1 h at 37 °C. Plates were washed three times with 400 μl/well of 0.05% Tween–PBS and 100 μl of serially diluted pooled sera (1:20) was added and incubated at 37 °C for 3 h. Plates were then washed five (5) times with 400 μl/well of 0.05% Tween–PBS, and 100 μl of horse raddish peroxidase (HRP)-conjugated goat anti-mouse IgG and subclasses (Southern Biotechnology, Birmingham, AL) diluted with blocking buffer (1:4000 and 1:2500 for IgG and subtypes, respectively) was added and incubated for 1 h at room temperature. Plates were washed 5 times with 400 μl/well of 0.05% Tween–PBS and antigen–antibody reaction was visualized by the addition of 50 μl/well of 3, 3′, 5, 5′-tetramethylbenzidine (TMB) (Vector Laboratories, CA, USA). The color development reaction was stopped after 30 min by adding 50 μl of 1 N of H_2SO_4, and the absorbance was measured in an automated plate reader (Bio-Rad, Hercules, CA) at 450 nm.

2.8. Flow cytometric analysis of $CD4^+$ and $CD8^+$ T cells

Spleens were removed aseptically from each mouse and splenocytes prepared using two sterilized slide glass by gently pressing the spleens on the rough edge of the slide to get lymphocytes in 10 ml of RPMI 1640 medium containing 10% fetal calf serum (FCS). This was pipetted several times and filtered through gauze. The filtrate was then centrifuged at 1550 rpm for 5 min with 10% FCS–RPMI. Lysis buffer was added and incubated at room temperature for 15 min, to lyse any trace of red blood cells. After washing, 100 μl containing 1×10^6 splenocytes were prepared and transferred into flow cytometry tubes, stained with Fc-γ blocking antibody, and incubated for 15 min at 4 °C. Washing was done once with 800 μl of wash buffer and centrifuged at 3600 rpm for 90 s. Stained surface molecules (APC-CD3, FITC-CD4 and PE-CD8) for $CD4^+$ and $CD8^+$ T were detected by adding 2 μg/ml/test of APC-conjugated anti-CD3, FITC-conjugated anti-$CD4^+$ or PE-conjugated anti-$CD8^+$ and incubated at 4 °C for 30 min in the dark. Cells were washed twice and resuspended in 500 μl of FACs buffer and acquired on the FACS machine (Beckman Coulter Fullerton, CA, USA). Phenotype analyses were performed using the Kaluza software (BD Biosciences, USA).

2.9. Splenocyte culture supernatant and serum cytokine analyses

Cytokine (IL-4, IL-10, IL-12 and IFN-γ) levels in the supernatants of antigen stimulated splenocyte and sera from immunized mice were measured using Procarta® Cytokine Assay Mouse Plex Kits (Affymetrix, Inc. Santa Clara, CA, USA) in quadruplicate, according to the manufacturer's instructions. Briefly, after standard preparation, filter plate was wet with 150 μl/well of reading buffer and incubated at room temperature for 5 min. Fifty microliters (50 μl/well) of antibody beads were added to each well, and washed once with 150 μl of washing buffer. Twenty five microliter (25 μl/well) of serum standard buffer, and either 25 μl/well of serum samples or 50 μl/well of supernatant were added and incubated at room temperature for 1 h. Plates were washed three times, and 25 μl of premixed detection antibody was added, incubated for 30 min on a shaker at room temperature. After three washes, 50 μl of Streptavidin-PE (Affymetrix, Inc. Santa Clara, CA, USA) was added and incubated for 30 min. Three washes after, 120 μl/well of reading buffer was added to each sample well and read on a Luminex instrument, LABScan 100 (Luminex Corporation. Austin, USA).

2.10. Determination of IFN-γ and IL-4 producing T cells by ELISPOT assay

ELISPOT was carried out in duplicates for activated and non-activated cells using mouse IFN-γ ELISPOT and IL-4 ELISPOT kits (Mabtech AB, Sweden). IFN-γ ELISPOT and IL-4 ELISPOT assays were performed using spleen cells activated with *P. yoelii* antigen according to the manufacturer's instructions. Briefly, splenocytes collected from different group of immunized mice were incubated with 5 μg/ml of *P. yoelii* antigen or 1 μg/ml of concanavalin A (Sigma, USA), and kept at 37 °C in a 5% CO_2 atmosphere either for 24 h (IL4 assay) or 48 h (INF-γ assay). Ninety-six-well Multiscreen-IP membrane plate (MultiScreen, Millipore MAIP S45) was pre-wet with 15 μl of 35% of ethanol for maximum of 1 min. Plates were washed 5 times with 200 μl of sterile water per well and coated either with 100 μl at a concentration of 15 μg/ml of an anti-mouse recombinant IL-4 (11B11) (Mabtech AB, Sweden) or anti-mouse recombinant IFN-γ monoclonal antibody (AN18) (Mabtech AB, Sweden) and incubated at 4 °C overnight. The plate was washed 5 times with 200 μl per well of sterile PBS (pH 7.4). The plates were then blocked by adding 200 μl of 10% FCS–RPMI and incubated at room temperature for 30 min. Two million spleen cells (2×10^6) resuspended in 100 μl/well of serially diluted cell suspension (1:10) was added and incubated at 37 °C in a 5% CO_2 atmosphere overnight. Plates were washed and incubated with peroxidase-conjugated streptavidin diluted in PBS–0.5% FCS (1:800). Seventy-five microliters (75 μl/well) of 1-Step

NBT/BCIP Solution (Pierce, Rockford, IL, USA) was added for spot development, then plates were washed extensively in tap water and left to dry at room temperature before spots counting. All samples were tested in duplicates and each immunization experiment was independently repeated at least two times. Spot counting was done using Eliphoto Scan software (Minerva Tech K.K, Tokyo, Japan).

2.11. Statistical analysis

Data were analyzed with GraphPad prism (Software version 5.00, Inc; San Diego California USA). Survival curves were analyzed using Kaplan–Meier test. The Student *t*-test was used to analyze the difference in antibody response between mice in coated and naked groups. The Pearson rank correlation test was used to determine the relationship between survival rates and different parameters. Statistical significance was designated as $p < 0.05$.

3. Results

3.1. Comparison of the effects of nanoparticle (NP) coated and naked DNA vaccine

3.1.1. On the course of blood-stage infection

To determine whether immunization with nanoparticle-coated MSP-1 C-terminus plasmid have any effect on the course of blood-stage infection, two groups of mice were immunized three (3) times at 3-week intervals with either coated or naked MSP-1 C-terminus plasmid and finally challenged with 10^5 of lethal *P. yoelii 17XL*-parasitized red blood cells intraperitoneally. It was clearly observed that mice immunized with coated plasmid, across of all the 3 routes of immunization, have lower parasitaemia than the naked plasmid vaccinated group (Fig. 1A). Mice immunized with NP-coated MSP-1 C-terminus plasmid survived longer and were protected against lethal strain of *P. yoelii* challenge than those groups immunized with naked plasmid (Fig. 1A). However, mice immunized by s.c. route showed a partial protection with about 57% survival (Table 1).

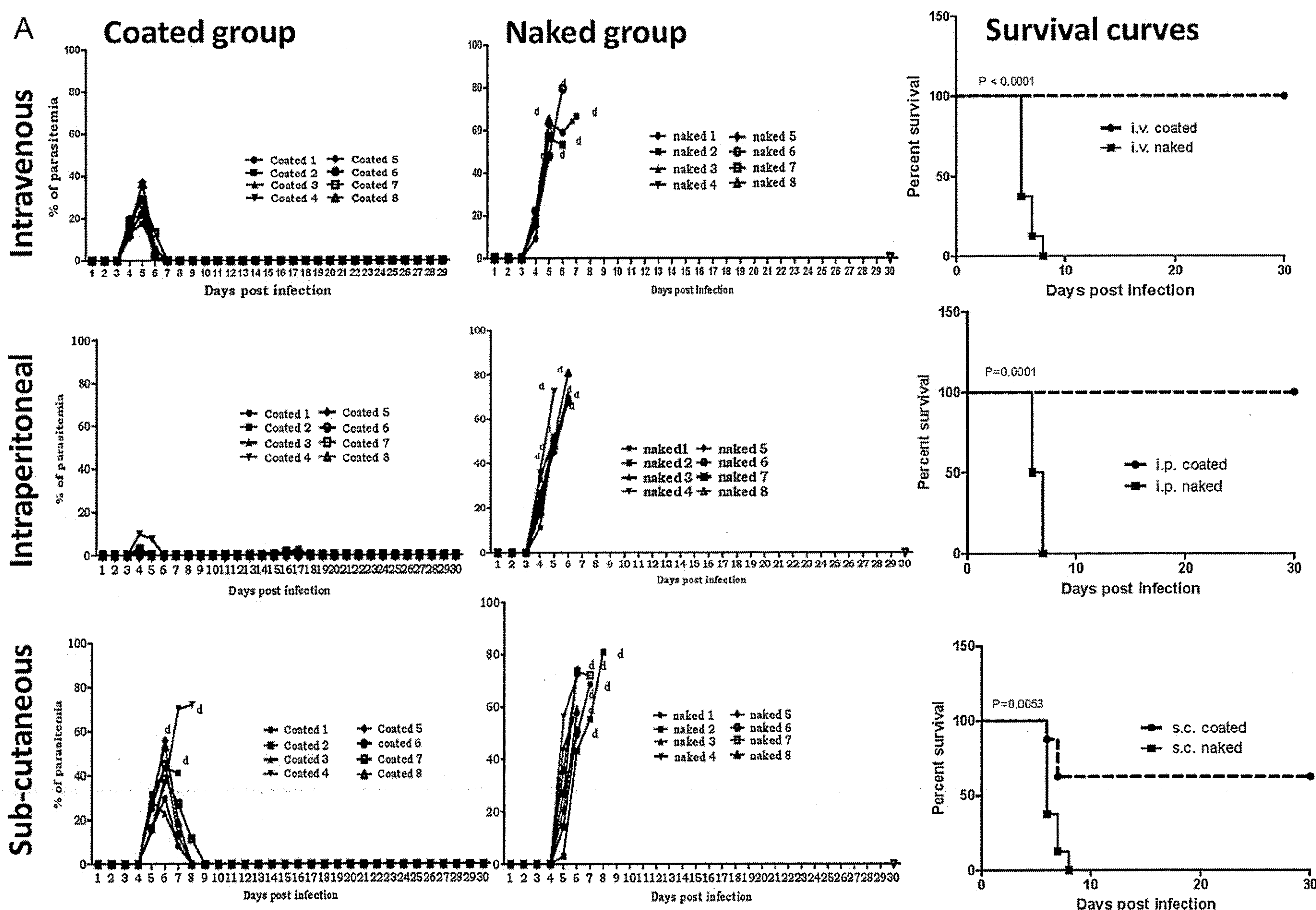

Fig. 1. Effect of coating on the course of parasitaemia and survival curves in group of immunized mice. (A) Parasitaemia profile and survival curve: 6 weeks old C57BL/6 mice were divided in two groups (coated and naked plasmid DNA). Mice were immunized 3 times at 3-week intervals by i.v., i.p. and s.c. routes of administration, then challenged by intraperitoneal injection of 10^5 pRBCs and paraitaemia monitored daily. Each line represents individual mouse with its parasitaemia profile. The notations coated 1–8, naked 1–8 represent individual mouse; d is indicated for death. (B) Stimulatory effect of coating on the course of parasitaemia and survival rates in group of immunized mice. (A) Parasitaemia profile. Six weeks old C57BL/6 mice were divided into two groups; NP-coated plasmid DNA and NP-coated blank plasmid, to observe the effect of coating on antigen driven immune responses. Mice were immunized 3 times at 3-week intervals by i.v., i.p. and s.c. routes of administration, then challenged and parasitaemia monitored daily. Each line represents individual parasitaemia profile. The notations coated 1–6, coated control 1–6 represent individual mouse; d is indicated for death. Survival curves analyzed by using Kaplan–Meier test. Statistical significance was designated as $p < 0.05$.

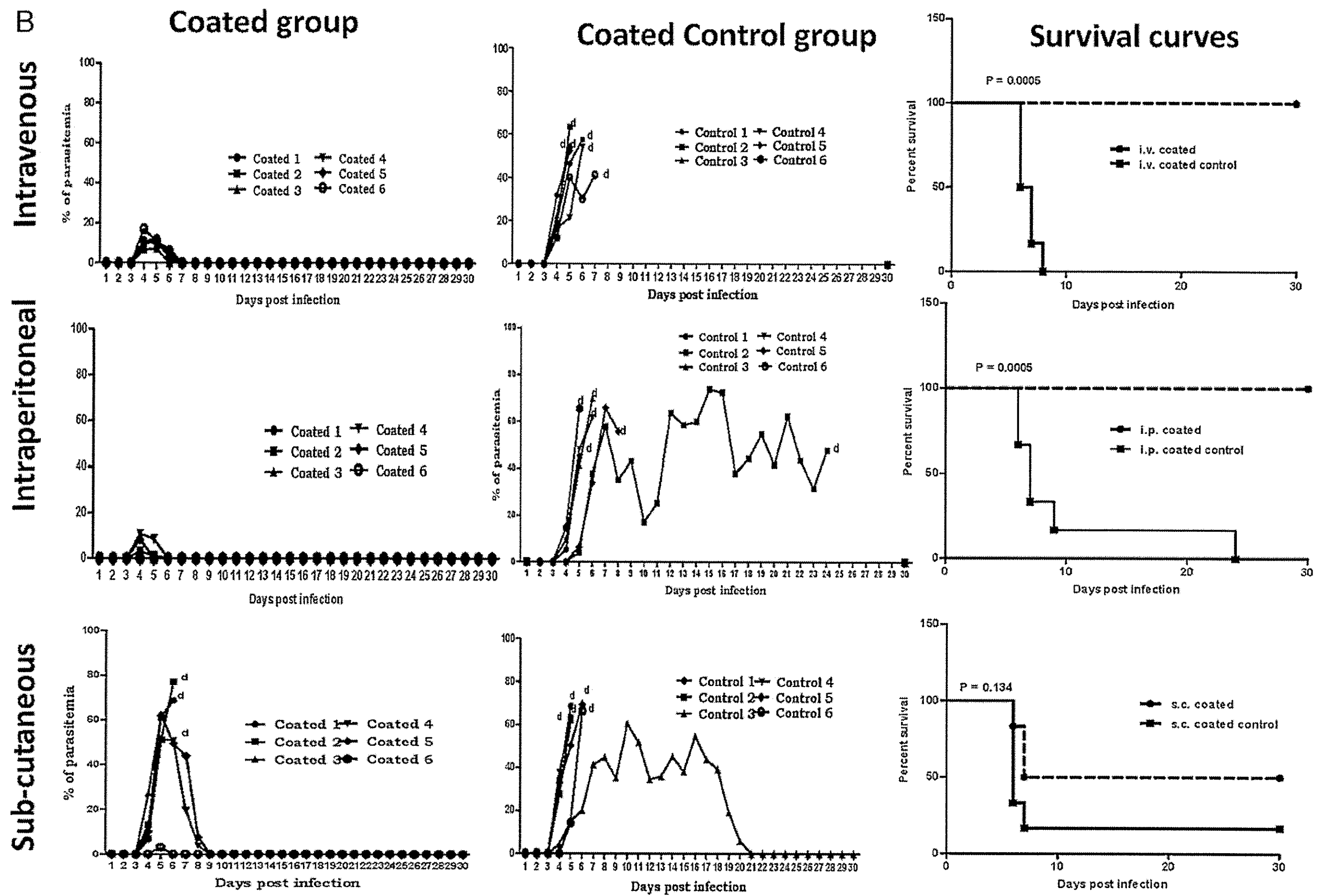

Fig. 1. *Continued.*

3.1.2. On antigen specific IgG and its subclass antibodies production

Sera were collected from immunized mice 2 weeks after the last immunization and recombinant MSP-1 antigen specific IgG and its subclass antibody titers were measured by ELISA. It was clearly observed that group of mice immunized with NP-coated MSP-1 C-terminus plasmid showed high levels of IgG and IgG1, IgG2a and IgG2b subtypes as compared to naked group, regardless of the three routes administered (Fig. 2A and B). To check the variations between individual mouse antibody productions within the group of immunized mice, individual mouse serum was analyzed by ELISA for IgG, IgG1, IgG2a and IgG2b (Fig. 2C). However, mean antibody titers obtained from individual mice and the pooled sera varied, this could be attributable to assay variability. We observed a significant difference of IgG2a and IgG2b subtypes antibody between coated and naked in vaccinated mice by i.p. and i.v. No significant difference was observed between coated and naked in group of mice vaccinated by s.c. route of administration. Similar results were obtained using crude antigen (Fig. S1).

Table 1
Summary of the total number of mice immunized in two different independent experiments depicting the percentage survival.

Routes	Group	Total number of mice	Number of survived	Percentage of survival
i.v.	Coated	14	14	100
	Naked	14	1	7.14
	Coated control	6	0	0
i.p.	Coated	14	14	100
	Naked	14	1	7.14
	Coated control	6	0	0
s.c.	Coated	14	8	57.14
	Naked	14	2	14.28
	Coated control	6	1	16.6

3.1.3. On splenic T cell populations

To check the antigen driven stimulatory effect by the NP-coated DNA vaccine, the number of $CD4^+$ and $CD8^+$ T cells in the spleen were estimated in two mice picked randomly from each group sacrificed 14 days after the last immunization. Flow cytometric analysis was performed on splenocytes by staining with surface molecules (CD3-APC, CD4-FITC and CD8-PE). The percentage of $CD4^+$ and $CD8^+$ T cells was higher in the spleen cells of mice from the coated group as compared naked group across of all the route administration (Fig. 3A).

3.1.4. On cytokines production

To observe the effect of NP coating on cytokine production, four (4) cytokines (IL-4, IL-10, IL-12p40 and IFN-γ) were estimated from cultured splenocyte supernatants and pooled sera from each group of immunized mice. Consistently, it appeared that IL-12p40 production in both serum and culture supernatant was significantly higher in mice immunized with coated DNA plasmid as compared to naked DNA plasmid across of all the three route of delivery. Also, IL-4, IL-10 and INF-γ appeared to be higher in mice immunized intraperitoneally with coated DNA plasmid (Fig. 4A and B).

3.2. Antigen driven immuno-stimulatory effect of NP-coated DNA vaccine compared with NP-coated blank plasmid DNA

On protection, antibody production and T cells proliferation

Two groups of mice were immunized three times at 3 weeks intervals with either coated plasmid or coated blank plasmid and finally challenged with 10^5 pRBCs. It was observed that group of mice immunized with coated MSP-1 plasmid showed low parasitaemia as compared to coated blank plasmid immunized mice (Fig. 1B). After challenge, all the mice in coated groups immunized by i.v. and i.p. were able to control their parasitaemia and recovered completely from lethal challenge, which was similar

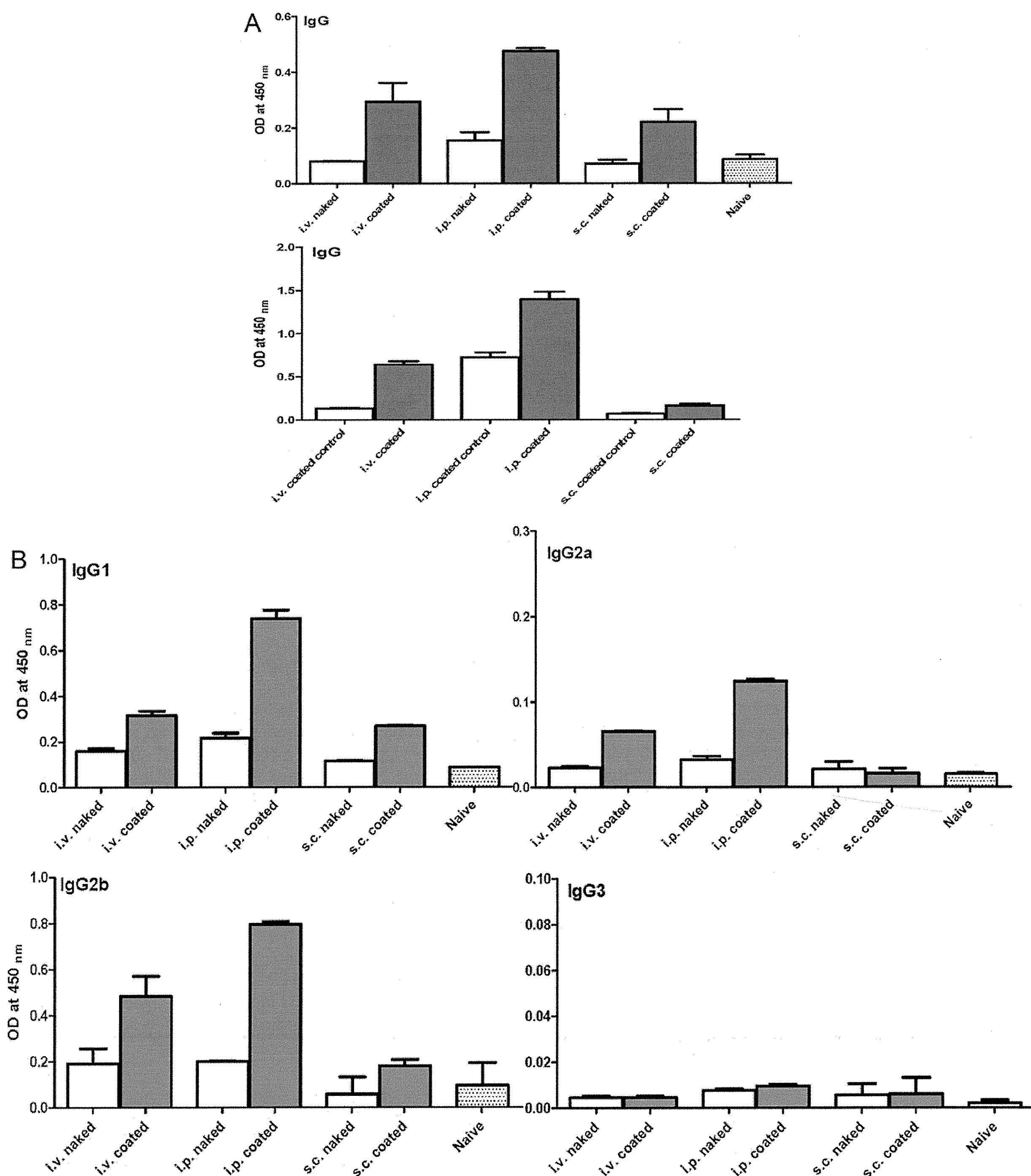

Fig. 2. Stimulatory effect of nanoparticle coating on IgG and its subclass antibody production. Two weeks after the last immunization, sera were collected from immunized mice in each group and IgG levels measured by ELISA as $OD_{450\,nm}$ at 1:40 dilution using rMSP-1. Values are mean ± SD of duplicate measurement. (A) Total IgG antibody titre in the pooled sera collected from immunized mice from the first and second experiment, upper and lower panel, respectively. (B) IgG subclasses antibody titre in the pooled sera collected from immunized mice from the first experiment. (C) Individual IgG and its subclass levels antibody titre in the group of immunized mice with coated plasmid (gray-shaded box plot) or naked (open box plot). Student test was used to find difference between coated and naked group. Statistical significance was designated as $p < 0.05$. (D) IgG subclasses antibody titre in the pooled sera collected from immunized mice from the second experiment.

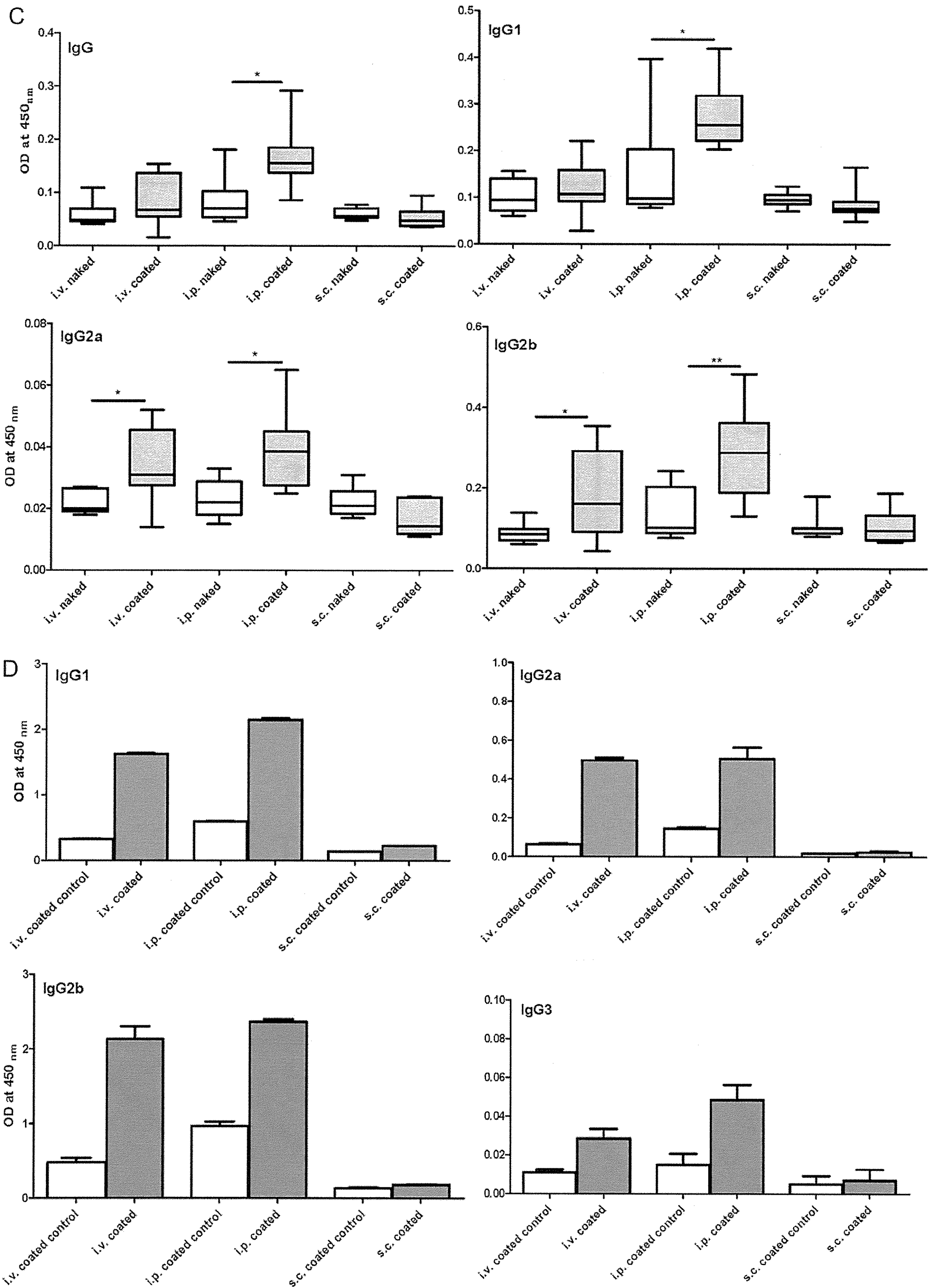

Fig. 2. *Continued.*