ISSN (Print) 0023-4001
ISSN (Online) 1738-0006

Korean J Parasitol Vol. 51, No. 6: 637-644, December 2013
http://dx.doi.org/10.3347/kjp.2013.51.6.637

▣ ORIGINAL ARTICLE

Modulation of Antibody Responses against *Gnathostoma spinigerum* in Mice Immunized with Crude Antigen Formulated in CpG Oligonucleotide and Montanide ISA720

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Abstract: This study aimed to investigate the antibody responses in mice immunized with *Gnathostoma spinigerum* crude antigen (GsAg) incorporated with the combined adjuvant, a synthetic oligonucleotide containing unmethylated CpG motif (CpG ODN 1826) and a stable water in oil emulsion (Montanide ISA720). Mice immunized with GsAg and combined adjuvant produced all antibody classes and subclasses to GsAg except IgA. IgG2a/2b/3 but not IgG1 subclasses were enhanced by immunization with CpG ODN 1826 when compared with the control groups immunized with non-CpG ODN and Montanide ISA or only with Montanide ISA, suggesting a biased induction of a Th1-type response by CpG ODN. After challenge infection with live *G. spinigerum* larvae, the levels of IgG2a/2b/3 antibody subclasses decreased immediately and continuously, while the IgG1 subclass remained at high levels. This also corresponded to a continuous decrease of the IgG2a/IgG1 ratio after infection. Only IgM and IgG1 antibodies, but not IgG2a/2b/3, were significantly produced in adjuvant control groups after infection. These findings suggest that *G. spinigerum* infection potently induces a Th2-type biased response.

Key words: *Gnathostoma spinigerum*, CpG oligonucleotide, Th1-type response, Th2-type response, nematode, immunity

INTRODUCTION

Gnathostoma spinigerum is a nematode parasite, of which larvae cause gnathostomiasis in humans and certain animals, and is prevalent mainly in Asia [1,2]. Recently, human gnathostomiasis has become an emerging disease among travellers from western countries who visit endemic areas [3,4]. Human beings are accidental hosts, infected by consuming raw or semi-cooked foods that are contaminated with the infective larvae. The parasite rarely develops into a mature worm in humans but can survive for a long time in the body. It usually migrates into the subcutaneous tissue and causes intermittent migratory swellings [5]. Sometimes *Gnathostoma* larvae reach the central nervous system, resulting in various signs and symptoms that may be life-threatening [6-9]. The anthelmintic drug, albendazole, has been used for the treatment of gnathostomiasis [10]. However, the efficacy of this drug treatment is not very satisfactory and frequent failure was reported after a long term follow-up study [11]. Therefore, vaccine development is an alternative approach for prevention and control of this disease.

Although strong antibody responses are induced by infection with *G. spinigerum* in humans and mice [12-17], protective immunity against challenge infection with the same parasite species remains unclear. This suggests that although the *Gnathostoma* antigens are immunogenic, the level of antibody responses may be insufficient for protection or may be biased by antibody isotype switching. Therefore, the use of an appropriate immuno-modulating strategy is necessary to obtain protective immunity by vaccination.

Unmethylated CpG oligonucleotides (CpG ODNs) are known to modulate both innate and adaptive immune responses through initiating Toll-like receptor 9 [18,19]. Activation of dendritic cells by CpG ODN induces cell maturation and production of proinflammatory cytokines such as interleukin (IL)-1, IL-6, TNF-α, and type 1 interferon, as well as T-helper 1 (Th1)-promoting cytokine IL-12 [20,21]. Delivery of

• Received 30 May 2013, revised 7 August 2013, acepted 11 October 2013.
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CpG ODN with various antigens can enhance antigen-specific cell-mediated and humoral immunity [22-24]. CpG ODNs have been used as an adjuvant for enhancing immunity against various parasitic infections, including malaria [25-29].

In this study, mice were immunized with crude antigens of *G. spinigerum* with the combined adjuvant of CpG ODN 1826 and Montanide ISA720. The antibody responses and protective effectss against *G. spinigerum* challenge infection was investigated.

MATERIALS AND METHODS

Mice and parasites

Male Swiss albino mice, weighing 25-30 g, were obtained from the National Laboratory Animal Centre, Salaya, Nakhon Pathom, Thailand, and kept in the Animal Unit of the Faculty of Medicine, Khon Kaen University. Five mice were randomly placed into each cage containing wood shavings. Rodent's chow and water were given ad libitum. All animal experiments were performed according the Guidelines for Animal Experimentation of the National Research Council of Thailand and the study was approved by the Animal Ethics Committee of Khon Kaen University (reference no. 0501.04/0013). *G. spinigerum* advanced third-stage larvae (AL3) were maintained in our laboratory according to procedures described previously [30] and used for antigen preparation and challenge infection.

Crude *G. spinigerum* antigen, oil-based adjuvant, and oligonucleotides (ODNs)

Crude somatic antigen of *G. spinigerum* AL3 was produced as described previously [17]. The ODNs used in this study were CpG 1826 (TCCATGACGTTCCTGACGTT; the underlined nucleotides represent the immunostimulatory residues) and the control non-CpG ODN 2138 (TCCATGAGCTTCCTGAGCTT) (Coley Pharmaceutical Group, Wellesley Hills, Massachusetts, USA). Montanide ISA720 (Seppic, Paris, France), an oil- based adjuvant, was also used.

Immunization and challenge infection

The experimental design is summarized in Table 1. Ten mice of each group were immunized with either crude *G. spinigerum* antigen (GsAg; 350 μg/mouse) or normal saline solution (NSS) (control). Crude antigen or NSS incorporated in Montanide ISA720 only, or in Montanide ISA720 with CpG ODN 1826 or non-CpG ODN 2138, was administered on days 0 and 21 post-immunization (PI) via subcutaneous injections and then on days 42 and 56 PI via intraperitoneal injections. Mice were challenged orally with 4 live *G. spinigerum* AL3 28 days after the last immunization (i.e. day 84 after the start of the experiment). All mice were sacrificed at day 196 after the start of the experiment. The worm burdens were determined by counting the *Gnathostoma* larvae in all organs under a light stereomicroscope (×15-45).

Antibody assay

Sera were collected 1 day before and then every 1-4 weeks after the first immunization, and *G. spinigerum*-specific antibody levels were assessed by ELISA. The dilutions of serum, horseradish peroxidase (HRP)-conjugated anti-mouse antibody, biotin-conjugated anti-mouse antibody, and avidin-HRP conjugate in each assay are shown in Table 2. A high binding capacity ELISA plate (Corning Incorporated, New York, USA)

Table 1. The experimental design for immunization

Group	Immunization types[a]
1	*Gnathostoma spinigerum* antigen in NSS+Montanide ISA720+CpG ODN 1826
2	*Gnathostoma spinigerum* antigen in NSS+Montanide ISA720+non-CpG ODN 2138
3	*Gnathostoma spinigerum* antigen in NSS+Montanide ISA720
4	NSS+Montanide ISA720+CpG ODN 1826
5	NSS+Montanide ISA720+non-CpG ODN 2138
6	NSS+Montanide ISA720
7	NSS

NSS, normal saline solution (0.85% NaCl in distilled water).
[a]Montanide ISA720 in groups 1-6 was emulsified with other solutions at a ratio of 7:3.

Table 2. Optimum dilutions of sera and conjugates used for determination of *Gnathostoma spinigerum*-specific antibody classes and subclasses by ELISA

Antibody detected	Dilution used			
	Serum	Biotinylated antibody	Avidin-peroxidase conjugate	HRP-conjugated antibody
IgG1	1:400	1:50,000	1:50,000	
IgG2a	1:400	1:50,000	1:50,000	
IgG2b	1:400			1:100,000
IgG3	1:200	1:5,000	1:5,000	
IgG	1:50			1:1,000
IgM	1:200	1:5,000	1:5,000	
IgE	1:200	1:2,000	1:1,000	
IgA	1:200	1:5,000	1:5,000	

was coated with 100 µl of 2.5 µg/ml of GsAg in a coating buffer overnight at 4℃. After 3 washes with 0.05% Tween 20 in PBS (PBST), the wells were blocked with 250 µl of 3% bovine serum albumin (BSA; fraction V; Sigma, St. Louis, Missouri, USA) at room temperature for 1 hr. After washing, 100 µl of optimum dilutions of serum were added. After incubation at 37℃ for 1 hr, the wells were washed. For IgG and IgG2b determination, HRP-conjugated anti-mouse IgG and IgG2b antibodies (Zymed Laboratories, South San Francisco, California, USA) were added, respectively. After incubation at 37℃ for 1 hr, the wells were washed and o-phenylenediamine (OPD; Sigma) substrate solution was added and incubated at room temperature for 30 min before adding 8 N H_2SO_4 to stop the reaction. The optical density (OD) was read at the wave-length of 490 nm by an ELISA reader. For IgM, IgE, IgA, IgG1, IgG2a, and IgG3 determination, biotin-conjugated anti-mouse IgM, IgE, IgA, IgG1, IgG2a, and IgG3 antibodies and avidin-HRP conjugate (BD Pharmingen, California, USA) were used, respectively.

Parasite burdens and histological study

At the end of the experiment, all animals were sacrificed by ether inhalation. Worm burden and recovery were determined by counting the worms in all organs by the compression method under a stereomicroscope.

Statistical analysis

The significance of the difference between pairs of groups was analyzed by the Student's *t*-test or Mann-Whitney Rank Sum Test as appropriate. The results in the different groups were compared using the non-parametric Kruskal-Wallis One Way Analysis of Variance on Rank.

RESULTS

Antibody response after immunization with crude *G. spinigerum* antigen

To examine the kinetics of the antibody responses after immunization, sera were collected from mice of all groups and assessed for IgG, IgM, IgE, IgA, and IgG isotypes (IgG1, IgG2a, IgG2b, and IgG3) specific for GsAg. Control mice in groups 4-7 (Table 1) did not produce antibodies to GsAg. Mice immunized with GsAg mixed with Montanide ISA720 and CpG ODN 1826 (group 1), or with Montanide ISA720 and non-CPG ODN 2138 (group 2), or with Montanide ISA720 alone (group 3) produced high levels of all antibody classes and IgG subclasses, except IgA antibody, specific to *G. spinigerum* antigen (Fig. 1). The IgG antibody responses in mice in groups 1-3 were not significantly different as determined immediately before the challenge infection (at week 4 after the last immunization) (mean OD ± SE; 1.95 ± 0.12, 2.04 ± 0.10, and 1.94 ± 0.10, respectively, $P > 0.05$; Fig. 1C), as were IgM and IgE antibody responses (mean OD ± SE: IgM; 0.98 ± 0.13, 1.29 ± 0.20, and 1.08 ± 0.12, respectively, $P > 0.05$; Fig. 1A; and mean OD ± SE: IgE; 0.30 ± 0.03, 0.34 ± 0.05, and 0.30 ± 0.05, respectively, $P > 0.05$; Fig. 1B).

The IgG1 antibody responses in mice immunized with GsAg mixed with Montanide ISA720 and CpG ODN (group 1) were statistically significantly lower than those in mice immunized with the GsAg in Montanide ISA720 and non-CpG ODN, or with GsAg in Montanide ISA720 at 4 weeks after the last immunization (mean OD ± SE; 1.66 ± 0.02 versus 1.82 ± 0.03, or 1.77 ± 0.04, respectively, $P < 0.05$; Fig. 1D). By contrast, IgG2a, IgG2b, and IgG3 antibody responses of mice immunized with GsAg mixed with Montanide ISA720 and CpG ODN were significantly higher than those of mice immunized with GsAg in Montanide ISA720 and non-CpG ODN or with GsAg in Montanide ISA720 at week 12 after the first immunization (mean OD ± SE of IgG2a; 1.12 ± 0.05, 0.56 ± 0.12, 0.48 ± 0.09, respectively, $P < 0.05$; Fig. 1E mean OD ± SE of IgG2b; 2.07 ± 0.17, 1.45 ± 0.15, 1.60 ± 0.14, respectively, $P < 0.05$; Fig. 1F mean O.D. ± SE of IgG3; 2.05 ± 0.19, 1.04 ± 0.27, 0.35 ± 0.12, respectively, $P < 0.05$ Fig. 1G). In addition, the ratio of IgG2a/IgG1 antibody levels of mice immunized with GsAg in Montanide ISA720 and CpG ODN was higher than those of the other groups (Fig. 2A).

Antibody responses of immunized mice after challenge infection with *G. spinigerum*

Following a *G. spinigerum* challenge, antibodies in all immunized mice were monitored (Fig. 3). Control mice immunized with adjuvant only produced some IgG and IgM, but little IgE, and no IgA antibodies. The IgG and IgM antibody levels in control mice reached a peak between week 3 and 6 after the challenge but the levels were far less than those of immunized mice. In contrast, the IgG antibody levels of immunized mice after challenge infection remained high for a few weeks then gradually decreased regardless of the types of adjuvant used. In contrast, the IgM levels of immunized mice increased during the first week after challenge infection then gradually decreased.

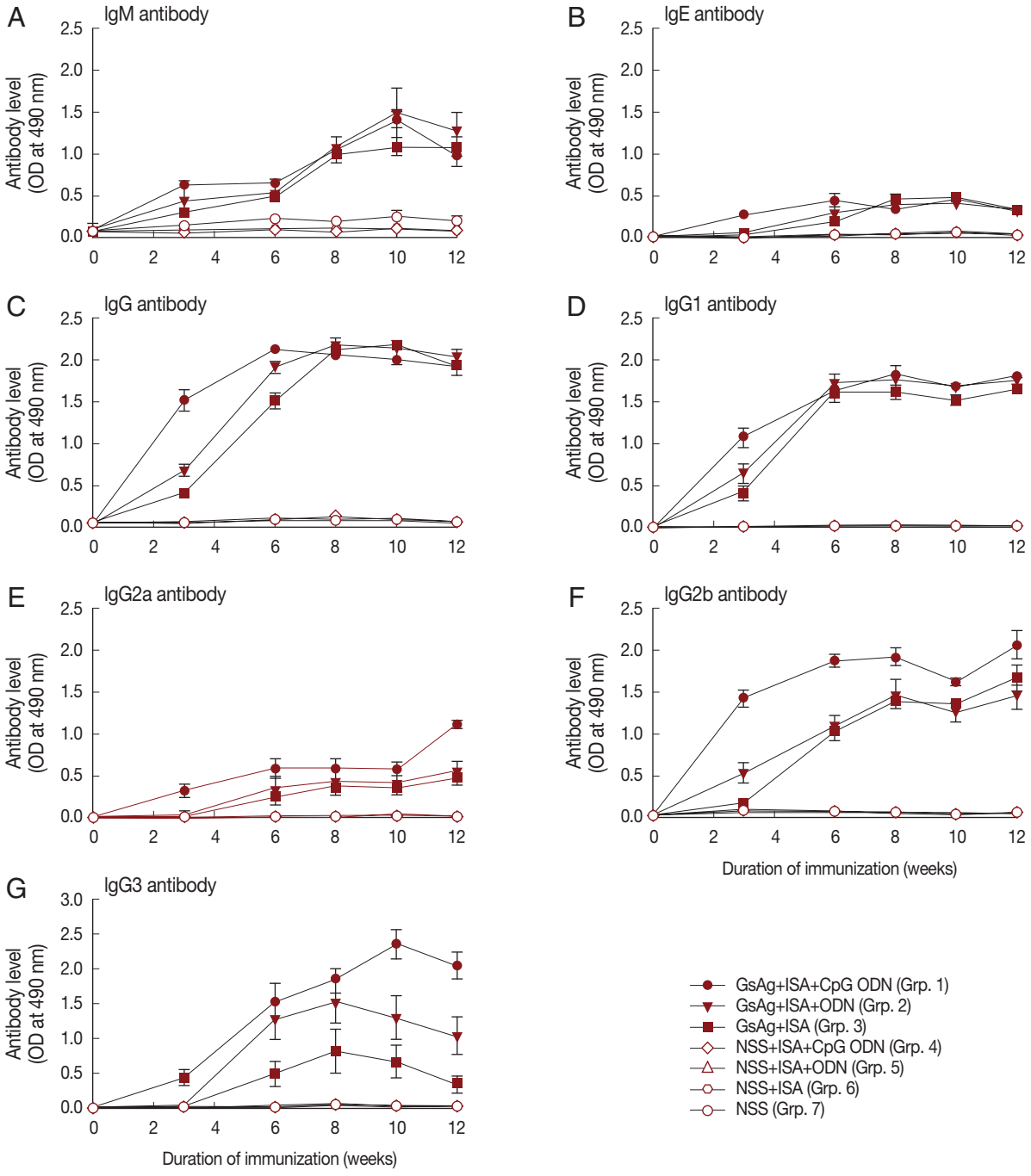

Fig. 1. Kinetics of *G. spinigerum*-specific antibody class and subclass levels during the course of immunization. Mice were immunized at weeks 0, 3, 6, and 8 with *G. spinigerum* crude antigen in NSS mixed with Montanide ISA720, with or without CpG ODN 1826 or non-CpG ODN 2138. Sera were collected 1 day before each immunization and every 2 weeks after the last immunization, diluted to the optimal level (see Table 2), and assessed for *G. spinigerum* -specific IgM, IgE, IgA, IgG, and their subclasses by ELISA. Data show mean ± SE of OD. Group (Grp.) 1 = GsAg+ISA+CpG ODN; Group 2 = GsAg+ISA+ODN; Group 3 = GsAg+ISA; Group 4 = NSS+ISA+CpG ODN; Group 5 = NSS+ISA+ODN; Group 6 = NSS+ISA; Group 7 = NSS.

In terms of IgG subclass responses, IgG1 antibody levels remained high, whereas those of IgG2a, IgG2b, and IgG3 decreased continuously. Actually the IgG3 antibody levels started to drop after the 4th week post challenge (Fig. 3G), except for the group immunized with GsAg in Montanide ISA720 and CpG ODN, which increased for 2 weeks before dropping. The

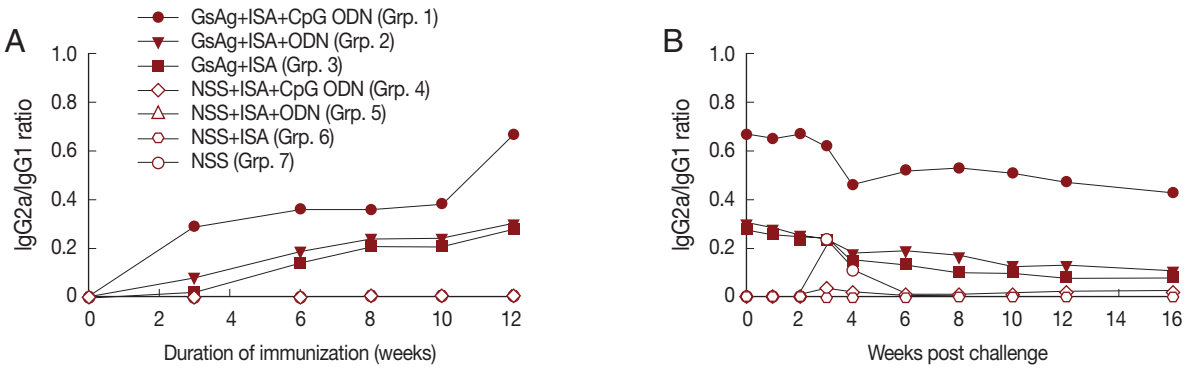

Fig. 2. The ratios of IgG2a/IgG1 antibody levels of mice during the course of immunization and post challenge infection. Group (Grp.) 1 = GsAg+ISA+CpG ODN; Group 2 = GsAg+ISA+ODN; Group 3 = GsAg+ISA; Group 4 = NSS+ISA+CpG ODN; Group 5 = NSS+ISA+ODN; Group 6 = NSS+ISA; Group 7 = NSS.

IgG2a/IgG1 ratios decreased over time after challenge infection (Fig. 2B).

Parasite burdens

We investigated the protective effect of the immunization by determining the worm recovery at week 16 post challenge. No significant differences of worm recovery were observed among groups of immunized and non-immunized mice (mean worm recovery ± SE of groups 1-7; 3. 6 ± 0.7, 3.9 ± 0.3, 3.8 ± 0.7, 3.9 ± 0.4, 3.1 ± 0.8, 3.1 ± 0.8, and 3.3 ± 0.7, respectively; $P > 0.05$).

DISCUSSION

In an attempt to overcome a round worm infection, we used an immunostimulatory oligodeoxynucleotide containing unmethylated CpG motif (CpG ODN 1826) to combine with oil-based Montanide ISA720, which has been proven to be safe in human immunization trials [31]. After challenging with *Gnathostoma* larvae, immunized mice were not protected as demonstrated by the worm recovery. Our study showed that mice immunized with *G. spinigerum* crude antigen produced all antibody classes and subclasses to *G. spinigerum* protein except IgA antibodies (Fig. 1). Neither the presence of CpG ODN 1826 nor non-CpG ODN in the immunization, increased the levels of the IgG1 antibody responses, relative to Montanide ISA alone (Fig. 1D), suggesting that CpG ODN did not enhance an IgG1 response. By contrast, IgG2a, IgG2b, and IgG3 antibody levels were consistently higher after immunization with CpG ODN, compared to the other adjuvant regimes (Fig. 1E-G), suggesting a role of CpG ODN in the induction of a Th1-type biased response.

After challenge infection with live *G. spinigerum* AL3, antibody responses were observed in all groups of mice. In the control groups treated with adjuvants only, IgM and IgG1 were detected 1 week after infection and sustained at high levels for up to 16 weeks of study (Fig. 3A, D). In contrast, the IgG2a, Ig2b, and IgG3 antibody responses to the infection were negligible (Fig. 3E-G). These findings may suggest that infection with live *G. spinigerum* larvae results in a strong Th2-type biased response [32]. This suggestion was also supported by the data of the groups of mice immunized with *G. spinigerum* crude antigen prior to infection. These groups showed sustained levels of IgG1 but continuously decreasing levels of IgG2a, IgG2b, and IgG3 (Fig. 3D-G), and opposite IgG2a/IgG1 ratios before and after infection (Fig. 2). In addition, it is possible that the failure of mice to develop protective immunity and the survival of parasite larvae after challenge infection observed in this experiment may be due to the escape mechanisms of this parasite by a biased regulation of the type of antibody responses, through which Th2-producing cells [33]. This needs to be further studied.

We conclude that the crude *Gnathostoma* larval antigen with CpG ODN can induce high immune responses with a shift to a Th1-type response after immunization but no protection is achieved together with the shift to Th2-response after infection. The development of vaccine for this disease may require more understanding of the mechanisms of immune regulation by the parasite.

ACKNOWLEDGMENTS

This research was funded by grants from the National Sci-

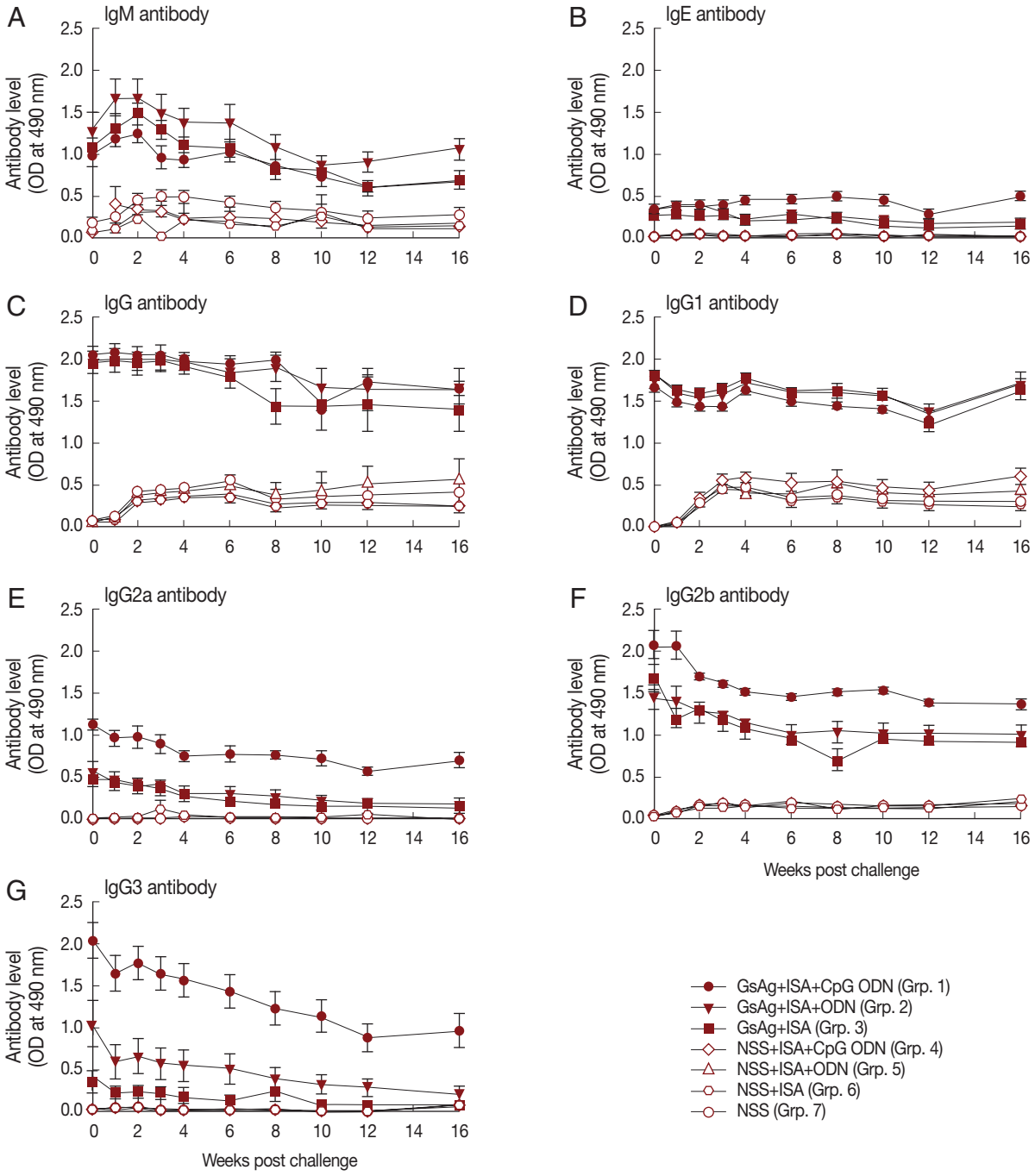

Fig. 3. Kinetics of *G. spinigerum*-specific antibody class and subclass levels post challenge infection with live *G. spinigerum* AL3. Four weeks after the last immunization, each mouse was challenged orally with 4 live *G. spinigerum* AL3. Sera were collected every 1, 2, or 4 weeks after challenge infection, diluted to optimum levels (see Table 2), and assessed for *G. spinigerum* -specific IgM, IgE, IgA, IgG, and their subclasses by ELISA. Data show mean ± SE of OD. Group (Grp.) 1 = GsAg+ISA+CpG ODN; Group 2 = GsAg+ISA+ODN; Group 3 = GsAg+ISA; Group 4 = NSS+ISA+CpG ODN; Group 5 = NSS+ISA+ODN; Group 6 = NSS+ISA; Group 7 = NSS.

ence and Technology Development Agency (Discovery Based Development Grant); the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission, Thailand through Health Cluster (SHeP GMS); the Faculty of Medicine, Khon Kaen University. Wanchai Maleewong and Pewpan M. Intapan

were supported by TRF Senior Research Scholar Grant, Thailand Research Fund grant no. RTA5580004. We wish to acknowledge the support of the Khon Kaen University Publication Clinic, Research and Technology Transfer Affairs, Khon Kaen University, for their assistance. We wish to thank Dr. Yukifumi Nawa for valuable comments and suggestions and Dr. Arthur M. Krieg for his providing the CpG ODN 1826 and the control non-CpG ODN 2138.

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