ROLES OF PARTIALLY PURIFIED ANTIGENS FROM *GNATHOSTOMA SPINIGERUM* LARVAE ON ANTIBODY PRODUCTION BY HUMAN B CELL CULTURE

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Abstract. A 24 kDa protein from advanced third stage *Gnathostoma spinigerum* larvae (GsAL3) is used for gnathostomiasis serodiagnosis. This study investigated whether partially purified protein antigen (Ag) from GsAL3 (Gnath Ag), prepared by simple gel filtration chromatography, could be used for serodiagnosis. Using DNA microarray analysis, significant gene expression related to immunoreactivity was examined in peripheral blood mononuclear cells (PBMC) cocultured with Gnath Ag. Antigenicity was then determined by its capacity to induce antibody production among purified naive B cells stimulated with Gnath Ag and anti-CD40. Seven and 14 days post-exposure, immunoglobulin levels (Igs) in culture supernatants were determined by enzyme-linked immunosorbent assay. The Gnath Ag stimulated PBMC had a significant increase in gene expression related to an innate immune response and decreased cell mediated immunity, but the expression of gene related antibody production was not markedly increased. The Gnath Ag stimulated naive B cells or lipopolysaccharide primed B cells to produce low levels of specific antibody. Our findings support the assertion that partially purified Gnath Ag possess low antigenicity for Ig induction. Further studies are needed to improve *G. spinigerum* larva Ag for serodiagnosis.

Keywords: *Gnathostoma spinigerum*, immunoglobulin, immunogenicity, B cell culture

INTRODUCTION

Human gnathostomiasis is mainly caused by advanced third stage larvae (GsAL3) or immature *Gnathostoma spinigerum* worms. After infection, GsAL3 commonly migrate through the pulmonary, ocular, genitourinary, gastrointestinal, auditory, and central nervous systems, causing heavy pain and localized edematous swelling, particularly periorbital swelling (Daengsvang, 1981; Herman and Chiodini, 2009). The 24 kDa molecular weight component of GsAL3 was first identified, and later used for serodiagnosis

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(Chaicumpa *et al*, 1999). The 24 kDa Ag is located in the body fluid, esophagus, and intestine of the larvae (Chaicumpa *et al*, 1999). A previous study demonstrated the 21 kDa protein band from GsAL3 was the most specific protein and was advantageous for serodiagnosis with 100% sensitivity using immunoblotting with IgG_4 from patient sera. While the 20 and 24 kDa protein bands may be additional markers to confirm infection the 21 kDa band is unclear (Anataphruti *et al*, 2005).

In the present study, partially purified GsAL3 Ag (Gnath Ag) was prepared by simple gel filtration chromatography (Dekumyoy *et al*, 2004). The profiles of the Gnath Ag included 20, 21 and 24 kDa proteins, previously reported as specific markers to diagnose gnathostomiasis (Chaicumpa *et al*, 1999; Anantaphruti *et al*, 2005). We attempted to determine whether Gnath Ag was appropriate for serodiagnosis. The immunogenicity of Gnath Ag was evaluated by: 1) screening for immune related gene expression among peripheral blood mononuclear cells stimulated with the Gnath Ag using DNA microarray, 2) determining Ig production by naive B cells cocultured with Gnath Ag by ELISA. Our findings demonstrate Gnath Ag has low antigenicity and induces low levels of specific IgM and IgG. Further studies are needed to improve the quality of Gnath Ag for serodiagnosis application.

MATERIALS AND METHODS

Approval for this study was obtained from The Ethics Committee, Faculty of Tropical Medicine, Mahidol University and The Thai Red Cross Society, Bangkok, Thailand. All participants involved in this study were informed the objectives of this study and gave written informed consent.

Preparation of partially purified *G. spinigerum* Ag (Gnath Ag)

Gnath Ag were prepared by separation of crude antigens using modified syringe column chromatography. Briefly, GsAL3 was separated from the livers of naturally infected eels using 1% acid-pepsin for digestion. The larvae were ground up under cold conditions, and then sonicated with an ultrasonicator (Heat Systems, New York City, NY,) for 10 minutes. The crude antigen in the supernatant was then collected by centrifugation at 10,000 rpm (Anantaphruti *et al*, 2005) and further purified through a modified syringe Sephacryl S-200 column (Dekumyoy *et al*, 2004). Fifteen eluted drops were collected at each fraction. The GsAL3 fractions containing 24 kDa Ag were screened and selected using direct enzyme-linked immunosorbent assay (ELISA) with specific antibody against the 24 kDa segment (Chaicumpa *et al*, 1991). Each fraction was pooled, determined for protein concentration and used as partially purified 24 kDa Gnath Ag protein for the present study. The protein profiles of the Gnath Ag were separated by one dimentional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% polyacrylamide slab gel and silver staining (The ProteoSilver™ Silver staining kit; Sigma-Alrich, St Louis, MO) as shown in Fig 1.

Preparation of peripheral blood mononuclear cells (PBMC)

The buffy coats of O positive blood obtained from healthy donors were purchased from the Thai Red Cross Society, Bangkok, Thailand. Peripheral blood mononuclear cells (PBMCs) were separated from the buffy coat by gradient centrifugation with Lymphoprep (Axis-Sheld Poc AS, Norway) according to the previous study (Boyum, 1968). The buffy coat

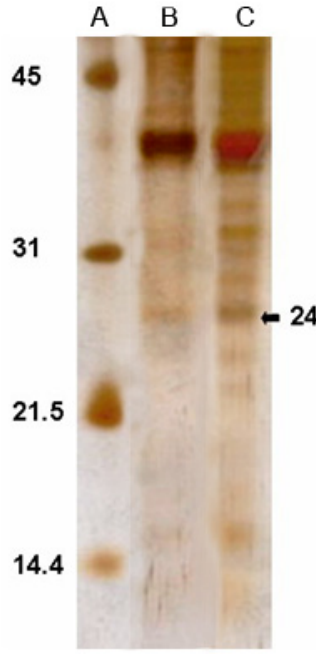

Fig 1–Silver stain analysis of Ag from the third stage larva of *Gnathostoma spinigerum*. Proteins were isolated by a modified syringe Sephacryl S-200 column. One microgram of the partially purified crude Ag (the Gnath Ag) (lane B) and the soluble crude Ag (lane C) were separated by one dimentional SDS-PAGE using 12% polyacrylamide slab gel and silver staining. Lane A is molecular weight standard.

used for each experiment had fewer than 1% memory cells (CD27+) determined by FACS Calibur flow cytometry using anti-human CD 27 antibody.

Preparation of naive B cells from PBMC

Purified naive B lymphocytes were magnetically obtained from PBMC using an LS column of MACS MicroBeads (Miltenyi Biotec, Gladbach, Germany) as described previously (Cerutti *et al*, 2002). Briefly, PBMCs were labeled with biotinylated anti-IgD antibody (Southern Biotech, Birmingham, AL). Positive cells were double stained with MACS anti-biotin Microbeads using a magnetic separation LS column to purify the positive cells. The purity of the naive B cells was approximately 90-95%, as assessed by expression of $CD20^{+}CD27^{-}$, determined by FACS Calibur flow cytometry using anti-human CD 20 and CD 27 antibodies (Dako, Glostrup, Denmark).

Determination of immune response related gene expression in PBMC cocultured with Gnath Ag by DNA microarray analysis

Five million normal PBMC ($CD27^{+}$ cells < 1%) were cultured in complete medium alone (RPMI1640 supplemented with 10% inactivated fetal bovine serum, 1% glutamine and 1 mg/ml of gentamicin) or with Gnath Ag (2.5 µg/ml) in an atmosphere of 90% N_2 and 5% CO_2 at 37°C. After 18 hours incubation, the RNA was extracted for microarray analysis (Affymetrix GeneChip Human Gene 1.0 ST Array) (Baechler *et al*, 2004). The data were analyzed by Agilent GeneSpring GX Software, version 10.0.

Naive B cell culture

The 90-95% purified naive B cells were cultured in complete RPMI 1640 medium. Anti-humanCD40 monoclonal antibody from Mabtech, Nacka, Sweden (instead of CD40 ligand from T cell coculture), recombinant interleukin-4 (rIL-4) (R&D systems, Minneapolis, MN) and Gnath Ag were used at concentrations of 10 µg/ml, 300 U/ml, and 1-5 µg/ml, respectively. After 7 and 14 days cultivation, the supernatant was collected and assayed for Ig levels by ELISA.

Lipopolysaccharide (LPS) primed naive B cell culture

The 90-95% naive B cells were incubated with 10 µg/ml LPS from *Escherichia coli* (serotype 055:B5) (Sigma) in complete RPMI 1640 medium for 6 hours as

described in a previous study with modification (Mättö *et al*, 2008). After washing twice with 0.01 M PBS, the LPS primed B cells were further cultured in medium alone, in medium with antiCD40 (10 µg/ml) only or with IL-4 (300 U/ml) or with Gnath Ag (5 µg/ml). After 7 and 14 days of cultivation, the supernatant was collected and assayed for Ig by ELISA.

Determination of total and specific IgM and IgG production in cultured supernatant by ELISA

Total IgM and IgG levels in cultured supernatant were determined by standard ELISA as reported previously (Cerutti *et al*, 2002). Assays of *G. spinigerum* specific Ig levels were modified from an earlier study (Watthanakulpanich *et al*, 2008) using plates coated with crude *G. spinigerum* Ag at a concentration of 1 µg/well. A positive Ag-Ab reaction was detected by peroxidase and its substrate 2, 2'-azino-bis (3 ethylbenzthiazoline-6-sulfonic acid). The optical density (OD) was measured at 405 nm against the blank well with a microplate ELISA reader (Tecan, Männedorf Switzerland).

Data analysis

Determination of gene expression was conducted by DNA microarray analysis and represents the normalized intensity values for gene expression with Gnath Ag induced PBMC compared to the control PBMC. The profiling of significantly up- and down-regulated gene expressions in the stimulated PBMC was determined with a fold change cut-off of ≥ 2 for the normalized intensity valves. The results of Ig production were obtained from 2-3 independent experiments. The total Ig levels in ng/ml are expressed as means and the amount of specific Ig is expressed as OD ratios (Michael and Bundy, 1992; Turner *et al*, 2004), calculated from means of the OD. The negative or background values from the culture in medium alone are represented as an OD ratio = 1.

RESULTS

Expression of up- and down-regulated genes related to immune response in PBMC induced by Gnath Ag

The immune response of the PBMC to the Gnath Ag was first screened by DNA microarray analysis. Within 18 hours of stimulation by Gnath Ag at a concentration of 2.5 µg/ml, the profiles of up- and down-regulated genes were recorded and are summarized in Fig 2. The data represents the valves of gene expression due to Gnath Ag induced in PBMC compared to the complete medium alone. To determine the appropriate gene profiles related to immune response due to Gnath Ag, we focused on the stimulated PBMC. Most of the marked up-regulated genes were involved in encoding for the innate immune response, inflammatory mediator production, cell signaling and the cell mediated immune response. The expression of genes related to the induction of mediators *eg*, chemokines, leukotrienes, heat shock protein and IL-1, and the cell mediated immune response, *eg*, NK cells or cell signalings, were major findings. While the genes encoding for functions related to the process of Ig production, such as T-cell receptors, MHC class II production and Ig light chain production, were only partly expressed.

The Ig production by Gnath Ag induced naive B cell culture

The 90-95% purified naive B cells were stimulated with Gnath Ag at concentrations of 1, 2 and 5 µg/ml in the presence of antiCD-40 instead of T cell coculture. The total and specific IgG level

Expression of genes related to the immune response in PBMC stimulated with partially purified crude *Gnathostoma* larva antigens (Gnath Ag).

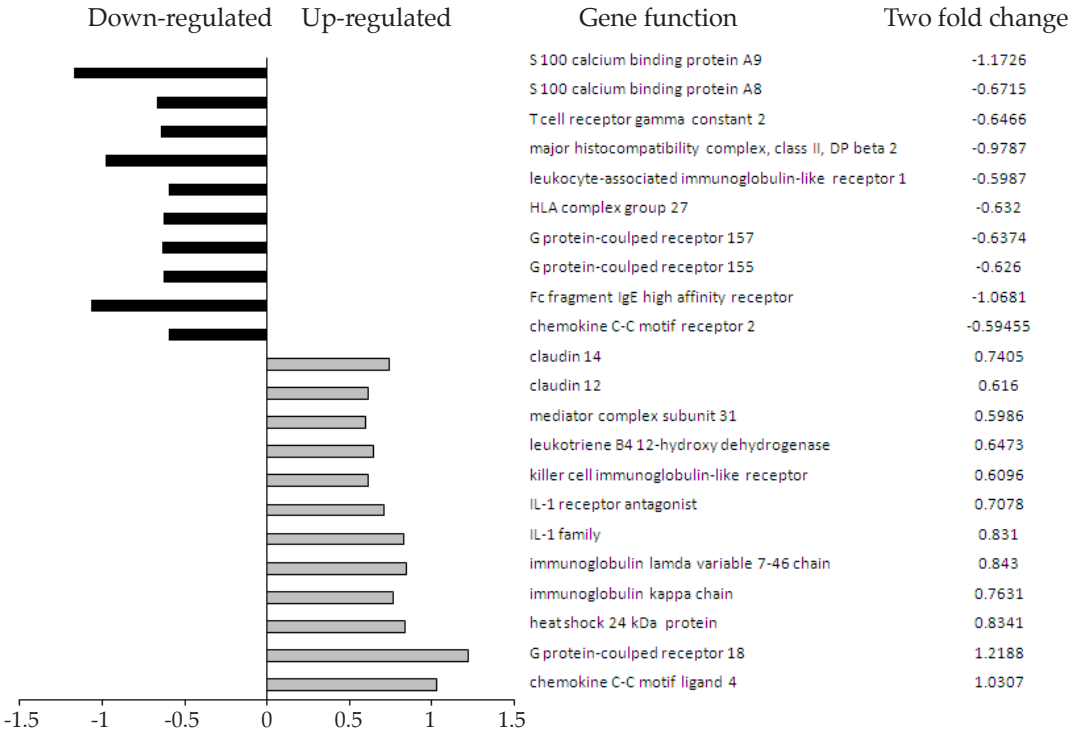

Fig 2–Down- and up-regulation of genes related to immune response in PBMC after coculture with Gnath Ag (2.5 µg/ml) for 18 hours. The RNA extracts from the stimulated PBMC and normal PBMC cultured in medium alone were performed DNA microarray analysis as indicated in Materials and Methods. Histogram represents two fold change of gene expression after stimulation in comparison to the normal PBMC. The data was from one duplicate experiment.

7 and 14 days after stimulation with Gnath Ag are shown in Fig 3A and B. Gnath Ag induced B cells to produce lower amounts of total IgG compared to those cultured with IL-4 and anti-CD40 (Fig 3A). All concentrations of Gnath Ag in the presence of anti-CD40 stimulated B cells to produce low levels of specific IgG in comparison to those cultured in medium or medium plus IL-4 and anti-CD40 during Days 7-14 of stimulation (Fig 3B). The total and specific IgM levels stimulated by Gnath Ag were not significantly different from the background negative control cultured in medium alone (data not shown).

The IgM and IgG production by LPS primed naive B cell culture induced by the Gnath Ag

After incubation with LPS (10 µg/ml) for 6 hours, the primed B cells were then cultured with Gnath Ag at a concentration

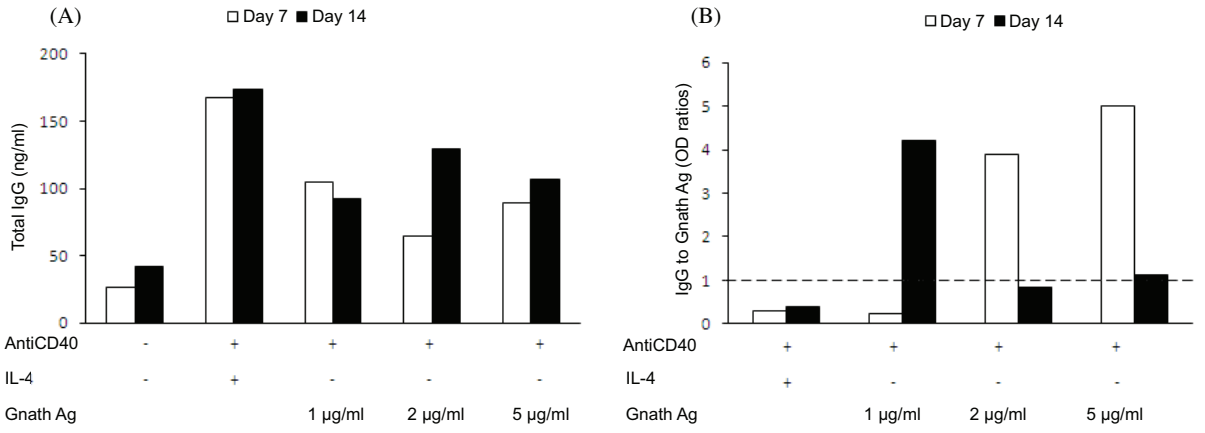

Fig 3–Levels of IgG in the supernatant of activated B cell cultures measured by ELISA. Naive B cells ($1x10^6$ cells/ml) were stimulated with the Gnath Ag (1, 2.5 or 5 µg/ml), IL-4 (300 U/ml) plus anti CD40 (10 µg/ml) or in the medium alone for 7 and 14 days. (A) the total IgG (ng/ml) levels and (B) the Gnath Ag specific IgG levels are expressed as OD ratios. The dotted line indicates negative or background values from those in medium alone (OD ratio = 1). The data was from three independent duplicate experiments.

of 5 µg/ml in the presence of antiCD40. The total and specific of Ig production in the culture supernatant were assayed on Days 7 and 14 of incubation as shown in Fig 4. The total IgM levels produced by LPS prime B cells stimulated with Gnath Ags was not higher than the background levels of those cultured in the medium alone (Fig 4A). Gnath Ag specific IgM was markedly increased on Day 14 comparing to the background but not different from those stimulated with IL-4 and anti-CD40 (Fig 4B). In contrast to the IgM production stimulated by Gnath Ag in LPS primed B cells, total and specific IgG markedly increased on Day 14 (Fig 4C and D).

DISCUSSION

In this study, Gnath Ag was obtained from the soluble fraction of crude GsAL3 Ag obtained by gel filtration chromatography. We used Sephacryl S-200 gel filtration, suitable for the size of the proteins (5-2,500 kDa). The Gnath Ag was more purified and could be separated by SDS-PAGE into fewer bands than the crude Ag (Fig 1). This finding is consistent with other studies (Saifullah *et al*, 2000; Ahmad *et al*, 2004; Dekumyoy *et al*, 2004; Revilla-Nuin *et al*, 2005). In our opinion, the immune response to crude Ag and Gnath Ag is different. Crude Ag is composed of various Ag from numerous macromolecules, *eg*, lipids, carbohydrates and proteins, which may be common to other helminthes (Nopparatana *et al*, 1991). A previous study demonstrated cross-reaction between patients infected with other helminthes using ELISA with crude Ag (Nopparatana *et al*, 1991).

The 24 kDa component from the GsAL3 was firstly identified, and later used to confirm gnathostomiasis (Chaicumpa *et al*, 1999; Luammaunwai *et al*, 2007). Based on previous studies, we

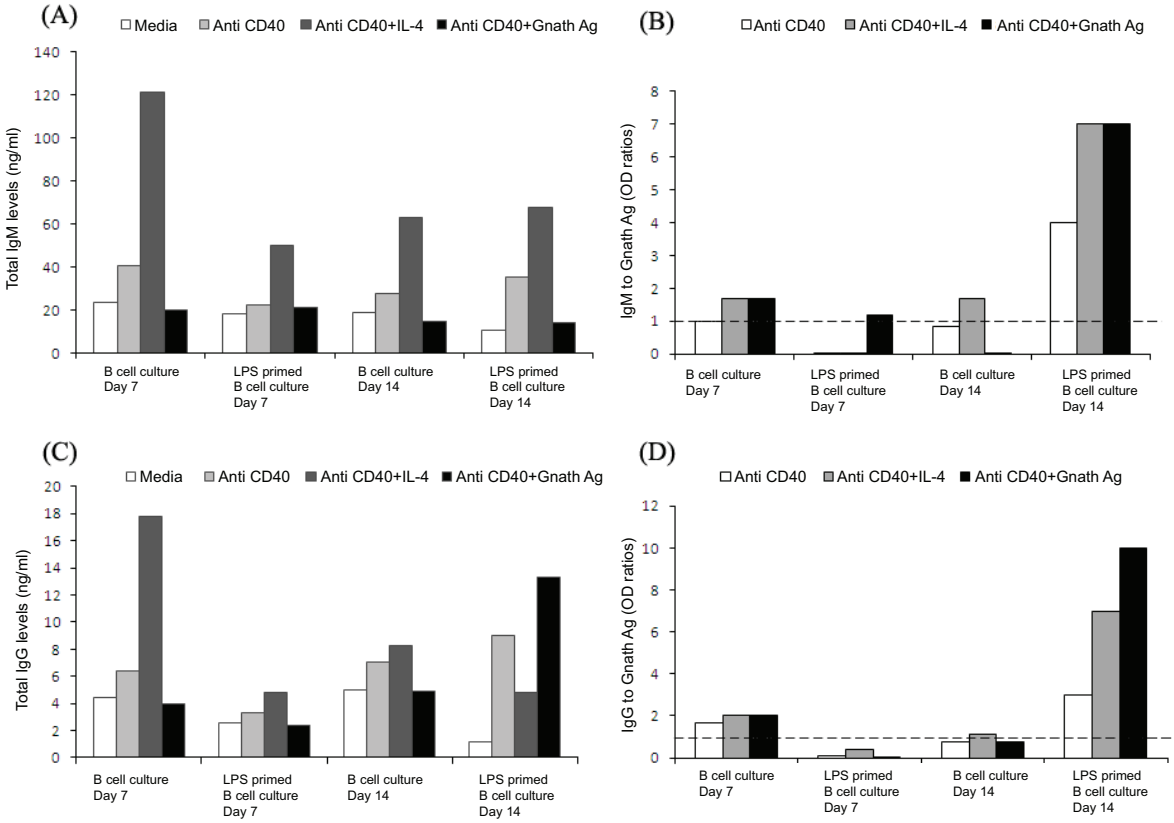

Fig 4–Levels of IgM and IgG produced by naive B cell and LPS primed naive B cell cultures. Naive B cells or 6 hour LPS primed naive B cell ($1x10^6$ cells/ml) were stimulated with the Gnath Ag (2.5 µg/ml), IL-4 (300 U/ml) plus anti CD40 (10 µg/ml), or in the medium alone with or without antiCD40 for 7 and 14 days. (A) and (C) the total IgM and IgG (ng/ml) levels, respectively. (B) and (D) the levels of specific IgM and IgG to Gnath Ag, respectively are expressed as OD ratios. The dotted line indicates negative or background values from those in medium alone (OD ratio = 1). Each data represents as mean from 2 independent duplicate experiments.

applied monoclonal Ab to the 24 kDa component to select fractions of crude somatic extract. The present study aimed to determine whether partially purified crude Ag (Gnath Ag) could be used as an alternative Ag for serodiagnosis of gnathostomiasis. The Gnath Ag was in the range of 45 kDa to 24 kDa (Fig 1). The immunogenicity of Gnath Ag is important to determine whether it is appropriate for serodiagnosis. Using DNA microarray analysis (Baechler *et al,* 2004), we investigated the properties of Gnath Ag focusing on the immune response of normal PBMC to Ag within 18 hours of cultivation. The profiling of significant up- and down-regulated gene expressions was not related to immune response. Only a few of the up- and down-regulated genes were involved in cell signaling and in the process of antibody production (Fig 2). In contrast to our unpublished data, normal PBMC cocultured with intact third stage larva or with its secretions (ES) for

18 hours showed gene expression significantly related to immune response. The PBMC stimulated with the larvae expressed 58 down-regulated genes and 29 up-regulated genes involving immune activity. In contrast to the up-regulated gene expressions, the down-regulated gene profiles possessed functions involving cytotoxic immunity, including gene groups of toll like receptors, Fc receptors for IgG and IgE, granzymes in cytoxic T cells and killer cell receptors (unpublished data).

In the present study, ineffective antigenicity of Gnath Ag may lead to inappropriate induction of specific antibody production. This finding may be explained by natural human gnathstomiasis. During infection, intact larvae migrate through the tissue of patients and excrete waste, destroying tissue during larval migration (Herman and Chiodini, 2009). The immune cells are exposed to the intact surface of larvae and waste materials but not the internal components of larvae obtained from soluble fractions of sonicated larval somatic extract.

The second part of our study, using purified naive B cell stimulated with Gnath Ag, was designed to support the finding of gene profiling. The purified naive B cell culture was used to limit other factors and focus only on activated B cells with ongoing Ig production (Cerutti *et al,* 2002; Deehan *et al*, 2002). Anti-CD40 was added to the culture instead of T cell cocultures for T-cell dependent immunity (Fear *et al,* 2004). The finding of Ig production by purified naive B cells stimulated with Gnath Ag supports the gene profiling data. The role of Gnath Ag was not predominantly related to the humoral immune response. Specific IgM from B cells against various concentrations of Gnath Ag (range 1 to 5 μg/ml) was not observed. The specific IgG had no significant change in levels in comparison to those cultured in medium alone or IL-4 and anti-CD40 (Fig 3). Although we pre-activated the naive B cells with LPS (Mättö *et al,* 2008) for 6 hours, the efficiency of the B cells in producing specific antibody was poor (Fig 4).

This poor response by B cells to produce antibodies indicates purified Gnath Ag may be needed to produce a test for serodiagnosis. Further studies are needed to improve the purification and efficiency of the Ag. Western blot analysis of Gnath Ag with the sera of gnathstomiasis patients (Anataphruti *et al,* 2005; Luammaunwai *et al*, 2007) might be useful to select target proteins for further recombinant tests or synthetic peptides, as has been demonstrated in other studies (He *et al*, 2009; Azzibrouck *et al*, 2010; Li *et al*, 2010; Luammaunwai *et al*, 2010; Du *et al*, 2011). In conclusion, the Gnath Ag is still not appropriate antigenic to induce specific Ab. Further studies, both in the animal models and *in vitro* study, are needed to improve the quality of the Ag for application of gnathostomiasis serodiagnosis.

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