Production of IgM by B Cells After Stimulation with GLIS from *Ganoderma lucidum* Mushroom

QINGJIU TANG[1,2] JINGSONG ZHANG[1,2] WERNER REUTTER[2] & HUA FAN [2]
[1]Edible Fungi Institute, Shanghai Academy of Agricultural Sciences, Shanghai, 201106, China; and [2]Institut fur Molekularbiologie und Biochemie, Freie Universitat, Berlin, Germany.
E-mail: tangqingjiu2003@yahoo.com.cn

Abstract: *Ganoderma lucidum*, a traditional Chinese medicine, harbors a well-known capacity to modulate immunoactivity and inhibit tumour cell growth. GLIS is a proteoglycan fraction purified from *G. lucidum* through different chromatographic steps. This fraction has been proven to induce the proliferation and differentiation of lymphocytes and activation of macrophages. In the presence of GLIS, B cells were activated into antibody producing plasma cells, and secreted significant amounts of IgM. With respect to the response to mouse spleen lymphocytes (MSLs), the relative amount of IgM obtained from B cells induced with GLIS was lower in the absence of accessory cells. When 0.5% macrophages (0.5%) were added to B cells, B cell survival and IgM secretion increased significantly after stimulation with GLIS. The interaction of B cells and macrophages was not due only to the substances secreted by macrophages, but also to a direct effect.

Key words : *Ganoderma lucidum*, GLIS, IgM, macrophages, B cells

1 Introduction

Currently, mushroom and plant polysaccharides have received attention as complementary and alternative medicines. These compounds have been called biological response modifiers, which modulate host biological responses against tumours. *G.lucidum* is called "Lingzhi" in China. It is a basidiomycete fungus, without lamellae, belonging to the family Polyporaceae. This medicinal mushroom has been widely used for the treatment of various diseases, including cancer, in China for more than a thousand years. Previous studies have reported a bioactive fraction (GLIS), isolated from the fruiting body of *G. lucidum* using successive chromatographic steps. GLIS is a proteoglycan and has a carbohydrate:protein ratio of 11.5:1. GLIS stimulated the proliferation of mouse spleen lymphocytes, resulting in a three- to four-fold increase in the percentage of B cells,[1] and also stimulated macrophages. In this work, the production of IgM by B cells after stimulation by GLIS was investigated.

2 Materials and Methods

2.1 Preparation of lymphocytes from mouse spleens

Balb^c mice, aged 8-10 weeks (ca. 28 ± 1 g), were used for lymphocyte preparation. Spleens were removed after killing the mice by cervical dislocation. Spleens were cut into several pieces, then pressed through a stainless steel mesh (100 mesh) into a culture plate using a sterile syringe plunger. The mesh was rinsed twice with PBS under sterile conditions. The spleen cell suspension was transferred into a new tube and sedimented for 10 min. The supernatant was pipetted into another tube and the cell clumps in the bottom of the tube were discarded. After centrifuged at 300 g for 6 min, cell pellets were washed twice with PBS. Cells (3×10^8) were resuspended for 10 min at room temperature in 1 ml Tris-HCl-buffer:NH_4Cl solution [mix 9 volumes of 0.83% (w/

v in water) NH_4Cl with 1 volume of Tris-HCl (2.06 w/v in water, pH 7.65), adjust to pH 7.2] in order to lyse red cells. Cells were counted in a Z series Counter (Counter Electronics, Miami, USA). The cell suspension was further diluted five-fold with medium. After mixing and centrifugation, the cell pellets were finally re-suspended in RPMI 1640 medium, containing 10% foetal calf serum, 100 IU/ml penicillin and 100 µg/ml streptomycin, for further experiments. Cell viability was assessed by trypan blue exclusion.

2.2 Separation of B cells from mouse spleen lymphocytes by magnetic cell sorting

Lymphocytes prepared from mouse spleens were washed, and the cell pellet re-suspended in 90 µl of buffer (PBS with 1% BSA) per 10^7 total cells. Magnetic cell sorting (MACS) CD19 MicroBeads (10 µl) were added to 10^7 total cells. The cell suspension was mixed well and incubated at 6°C for 20 min. The cells were then washed with 20 volumes of buffer and centrifuged at 300 g for 10 min. The supernatant was removed completely. Cell pellets were re-suspended in 500 µl of buffer per 10^8 cell, and the cell suspension was transferred to a LS^+ separation column which had been washed with 5 ml of PBS and placed in the MidiMACS magnet. The cell suspension was run through the column and the effluent was collected as non-B cells. Then the column was rinsed 3x with 3 ml buffer and then removed from the magnet separator. Finally, 5 ml of buffer was added to the reservoir of the LS column and the B cells were firmly flushed out using a plunger.

2.3 Preparation of bone marrow-derived macrophages (BMMs)

Bone marrow-derived macrophages were prepared according to the method described by Stanley.[2] $Balb^c$ mice of similar weight (28 ±1g) and age (8-10 weeks) were selected for obtaining bone marrow macrophages at random. Mice were killed by cervical dislocation, and the tibias and the femurs were removed by cutting the proximal end of the femur and the distal end of the tibia, leaving the other ends intact. A 23-gauge needle was inserted into the intact ends and bone marrow was flushed out through the cut ends with ice-cold DMEM. The marrow cells were dispensed by three passes through a 22-gauge needle and centrifuged (1200 g, 5 min, 4°C). The cell density was adjusted to 10^6 cells/ml in a complete DMEM medium containing 10% L929 (ATCC, Rockville, MD, CCL1) conditioned medium. After being incubated at 37°C for 3 days, the non-adherent cells were collected, and the adherent cells were discarded. The cells were then seeded into bacteria-culture dishes with 10% L929 conditioned medium, and incubated at 37°C for another three days. The non-adherent cells were washed away with sterile PBS. Adherent macrophages were collected for use by scraping with cold PBS, replacing the growth medium with complete medium.

2.4 Separation and purification of GLIS from *G. lucidum*

The crude extract of *G. lucidum* (GL) was prepared by extracting fruit bodies 3x with boiling water. The extract was dialysed and fractionated using a DEAE-Sephacel column (2.6 × 100 cm) and applying a linear gradient of NaCl in water (0 to 2 mol/l NaCl in 1500 ml). The fractions were monitored for peptide content by UV absorption at 280 nm, for carbohydrate content by the phenol-sulfuric acid reaction, and for immunostimulatory capacity using an appropriate bioassay. A fraction eluted with about 500 mmol/l NaCl was further fractionated using Sephacryl S-300 (Step 2) and S-400 (Step 4) gel filtration chromatography, and cation exchange chromatography on SP-Sepharose (Step 3), 9all from Amersham Pharmacia Biotech, Uppsala, Sweden). All the columns were eluted with water. A fraction obtained from the last step, that exhibited the strongest specific stimulation of lymphocyte proliferation, was designated GLIS.

2.5 B cell survival assay

B cells were isolated from MSLs or TMSLs according to the method described above. An aliquot (180 µl) of B cell suspension (2×10^6 cells/ml) was added to each well of a 96-well microplate and 20 µl of various test agents were added in triplicate. The cells were collected from triplicate wells daily on day 1 through day 8. B cells with or without treatment were centrifuged and washed twice with PBS. The cells were then labelled with 100 µl of 5 µg/ml propidium iodide for 30 min. After being washed with PBS 3 times, the cells were re-suspended in 0.3 ml PBS. Fluorescence analysis was performed using a FACScan analyzer. CELLQuest software was used to analyse the data for calculation of the percentage of dead cells.

2.6 Determination of immunoglobulin production

IgM or IgG in the culture supernatants of B cells was measured by enzyme-linked immunosorbent assay (ELISA). B cells were incubated with or without agents at the indicated concentration for 1 to 8 days. Aliquots of a suspension of each sample were used for the immunoglobulin ELISA-assay. Purified anti-mouse Ig (100 µl of 0.2µg/ml solution) (Boehringer Mannheim Biochemica Mannheim, Germany) was added to a 96-well microplate overnight at 4°C. Unbound antibodies were removed by washing 5x with PBS containing 0.05% Tween-20 (PBS-Tween). The plate was then incubated with 1% BSA in PBS at room temperature for 1 h to block the non-specific binding. After the plate was washed five times with PBS-Tween, 100 µl of cell-free supernatant, IgM or IgG standard samples were added and incubated for 2 h. The plate was washed 5x with PBS-Tween and 100 µl of biotin-labelled goat anti-mouse IgM or IgG (Amersham Pharmacia Biotech Uppsala, Sweden) in PBS-Tween containing 1% BSA were added to the well, and the plate was incubated for 2 h at room temperature. After washing the well 5x with PBS-Tween, each well was incubated with 200 µl of alkaline phosphatase-streptavidin (DIANOVA, Hamburg, Germany) for 1 h at room temperature. The plate was washed five times with PBS-Tween, each well was incubated with 200 µl of chromogenic substract solution (1 mg of *p*-nitrophenylphosphate disodium salt in 1 ml of diethanolamine buffer, pH 9.8) for 30 min, and stopped with 50 µl of 1 M Na_2CO_3. The absorbance was measured at 405 nm using a micro ELISA autoreader.

2.7 B cells co-cultured with macrophages or supernatants (SN) of macrophages

Macrophages were diluted to the different concentrations 10, 10^2, 10^3, 10^4 and 10^5 cells/ml in RPMI 1640 medium. B cells isolated from MSLs were diluted to 2×10^6 cells/ml using the medium which contained different macrophage concentrations. After being stimulated with different stimuli, the aliquots of a suspension of each sample were used for the immunoglobulin ELISA-assay.
Macrophages (15 ml; 1×10^5 cells/ml) were cultured in complete RPMI 1640 medium in a 9 cm plate and stimulated with 500 µg/ml GLIS or 50 µg/ml LPS. After 24 h, the culture supernatants (SN) were collected and filtered. B cells were diluted to 2×10^6 cells/ml using SN and added to 96-well plates. After being stimulated with different stimuli, aliquots of a suspension of each sample were used for the immunoglobulin ELISA-assay.

3 Results

3.1 Secretion of IgM by MSLs (mice spleen lymphocytes) after stimulation by GLIS

The effects of GLIS on B cells were examined by determining the increased concentration of IgM antibody in the medium. Figure 1 shows that MSLs secreted very high amounts of IgM after stimulation with GLIS. Furthermore, secretion of IgM increased daily until day 6. The effect of GLIS on the secretion of immunoglobulin was comparable to that of LPS.

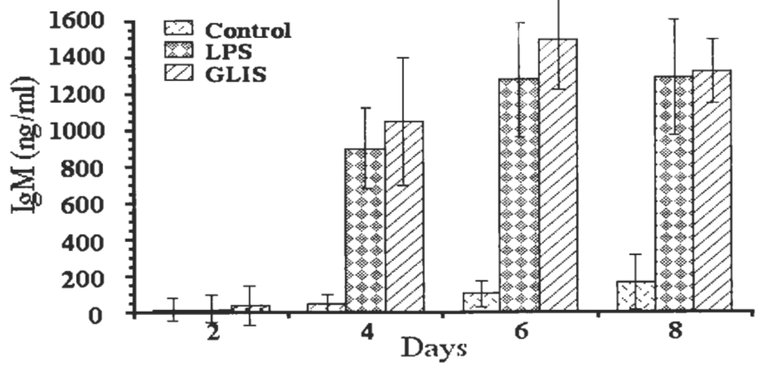

Fig 1. Secretion of IgM by MSLs after stimulation by GLIS

MSLs were incubated with 500 μg/ml of GLIS, 50 μg/ml of LPS or 0.9% NaCl as control. At the times indicated, aliquots of supernatants were taken for determination of the concentrations of IgM by ELISA. Results represent the means ± SD of 3 different experiments.

3.2 Secretion of IgM by B cells after stimulation by GLIS

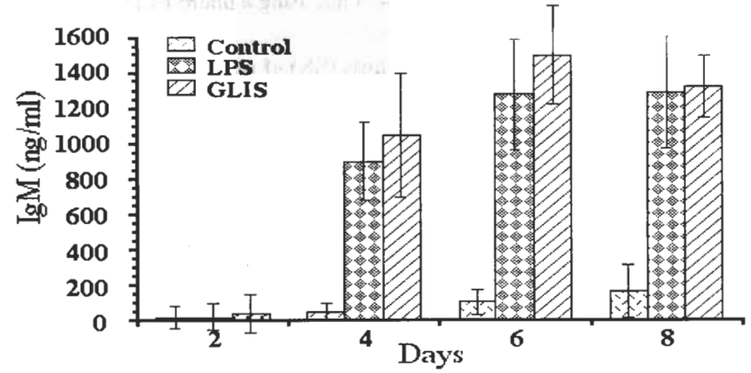

Fig 2. Secretion of IgM by B cells after stimulation by GLIS

Isolated B cells were incubated with 500 μg/ml of GLIS, 50 μg/ml of LPS, 0.9% NaCl as control. At the indicated times, aliquots of supernatants were taken for determination of the concentration of IgM by ELISA. Results represent the means ± SD of 3 different experiments.

3.3 Increase survival of B cells in the presence of macrophages after stimulation with GLIS

In the presence of macrophages, B cell survival was increased after stimulation with GLIS. The percentage of dead B cells was measured by FACS analysis with propidium iodide staining. Without GLIS stimulation, B

cells alone rarely survived for 4 days in the culture medium, but co-incubation with macrophages increased the survival rate of B cells to 9%. When B cells were stimulated by GLIS, almost 95% of the cells were dead by the fourth day, whereas 51% of the B cells were still viable in the presence of macrophages. This finding suggests that macrophages could increase the survival of B cells in the presence of GLIS (Figure 3).

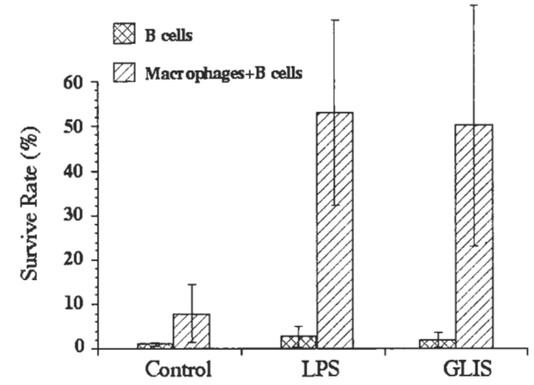

Fig 3. Viabilty of B cells by co-incubation with macrophages

B cells with or without 0.5% macrophages were stimulated with 500 μg/ml GLIS, 50 μg/ml LPS or 0.9% NaCl as control for 4 days in culture. Percentages of the dead B cells were measured by FACS analysis with propidium iodide staining. Results represent the means ± SD of 3 different experiments.

3.4 Secretion of IgM by B cells in presence of macrophages after stimulation with GLIS

Although the survival of B cells was increased by macrophages treated with GLIS, it remained to be determined if macrophages influenced B cells to increase IgM secretion. The capacity of IgM secretion by B cells was measured after stimulation with GLIS in the presence of 0.5% macrophages. The secretion of IgM was increased two-fold in the presence of macrophages stimulated by GLIS (Figure 4).

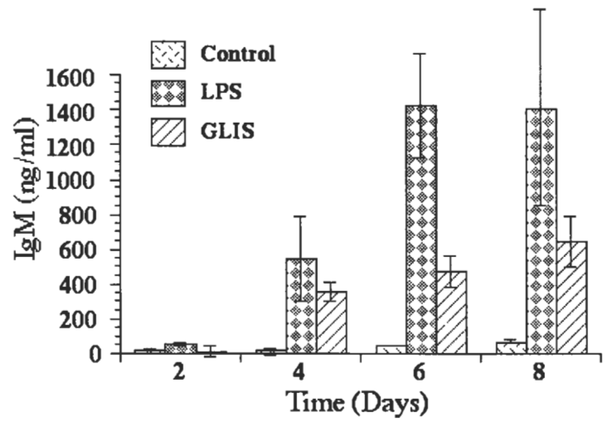

Fig 4. Secretion of IgM by B cells co-cultured with macrophages

B cells co-cultured with 10^4 macrophages were stimulated with 500 mg/ml GLIS. 50 μg/ml LPS or 0.9% NaCl as control for different time. At the indicated times, aliquots of supernatant were taken for determination of the concentration of IgM by ELISA. Results present means ± SD of 3 different experiments.

3.5 Effect of IL-6 or supernatants of macrophages on B cells

Macrophages could significantly increase IgM secretion by B cells after stimulation with GLIS but it was not clear if B cells interacted directly with macrophages or through various substances secreted by macrophages such as cytokines. It was found that IL-6 and the supernatant of macrophages could not prolong the survival of the B cells as do macrophages in the presence of GLIS. IL-6 could stimulate the B cells to secrete IgM, but could not increase the IgM secretion of B cells in the presence of GLIS or LPS. The supernatants of macrophages could not stimulate B cells to secrete IgM but, in the presence of the supernatant of macrophages which had been stimulated with GLIS, IgM secretion of B cells stimulated by GLIS was slightly enhanced (Figure 5B). The results suggest that the increase of B cell survival and IgM secretion is primarily influenced by direct interaction with macrophages.

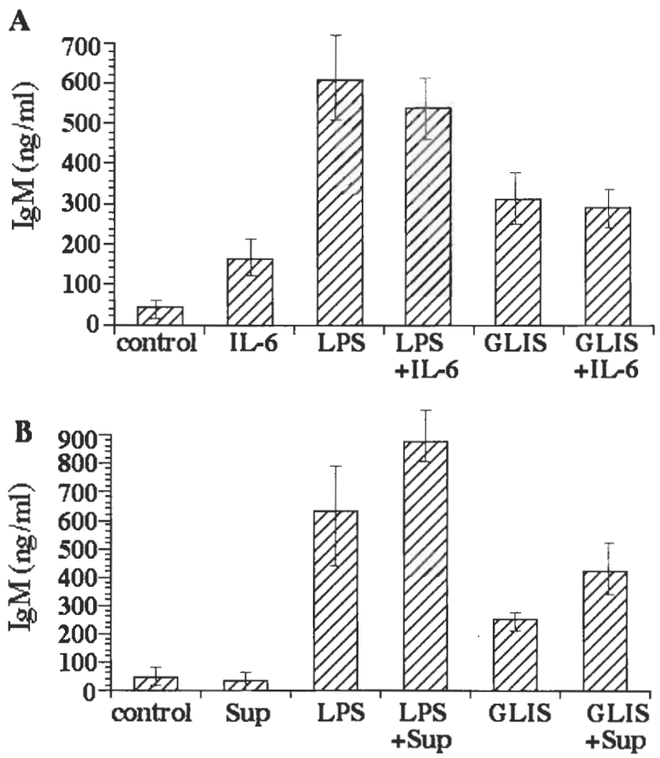

Fig 5. Secretion of IgM by purified B cells stimulated wtih GLIS in presence of IL-6 or the supernatant of macrophages

(A) B cells were stimulated by 500 μg/ml GLIS, 50 μg/ml LPS or 0.9 NaCl as control for 8 days in presence or absence of IL-6, the supernatant was used for detection of the IgM. (B) B cells were stimulated with 500 μg/ml GLIS, 50 μg/ml LPS or 0.9 NaCl as control for 8 days in presence or absence of the supernatant of macrophages for 8 days, supernatant were used for detection of IgM. Results represent the means ± SD of 3 different experiments.

4 Discussion

Our results showed that B cells could be stimulated by GLIS directly; the survival of B cell and production of IgM were significantly lower than those of mouse spleen cells. In the presence of macrophages, the survival of

B cells and IgM secretion was significantly increased after stimulation with GLIS. It seems that the effect of GLIS on spleen B cells includes interaction with macrophages.

Marginal zone macrophages are important for the induction of anti-virus TI-1 and TI-2 and anti-bacterial TI-2 antibody response.[3] Bondada et al.[4] considered that T independent antigens elicit antibody response in the absence of carrier specific T helper cells, but require signals from accessory cells (macrophages and dendritic cells) or specific cytokines. A relationship between adherent cells and B cells was also reported by Takemoto et al.[5] They found that the mitogenic activity of F-5-2, a pectic polysaccharide fraction isolated from medicinal plants, was abolished by removing the resident adherent cells from spleen cells. Re-addition of those cells to the culture system restored the activity of F-5-2. The adherent cells appear to be essential for the proliferation of spleen cells by F-5-2. For B cells stimulated by GLIS, macrophages are not essential but beneficial for IgM production. This indicates that the macrophages play an important role in maintaining survival or preventing apoptosis of B cells and force them to secrete IgM.

Although it has been studied extensively, questions still remain about the mechanism that regulates Ig secretion. Goodrich et al.[6] suggested the intestinal epithelial cells might secrete IL-6 and TGF-ß to regulate local B cell antibody secretion, and their effects may be highly dependent on the activation state of the epithelial cells. Guo et al.[7] found that when normal B cells from mice spleens were cultured with a pectic polysaccharide bupleuran 2IIc in the presence of anti-IL-6 neutralizing antibodies, the enhanced IgM secretion by bupleuran 2IIc was reduced. When B cells were stimulated with this sample, IL-6 secretion and transcription of IL-6 mRNA were enhanced. Among the cytokines capable of stimulating B lymphocytes, IL-6 was reported to induce B cell proliferation and the secretion of large amounts of IgM.[8] The present study showed that IL-6 can enhance the production of IgM, but has no synergetic effect with GLIS. The reason may be that IL-6 is produced by GLIS-stimulated B cells themselves.

References

[1] Zhang J, Tang Q, Zimmerman-Kordmann M, *et al*. Activation of B lymphocytes by GLIS, a bioactive proteoglycan from *Ganoderma lucidum*. Life Sci, 2002, 71:623-638.
[2] Stanley ER. Murine bone marrow-derived macrophages. Meths. Mol. Biol. 1997, 75:301-304.
[3] Spencer J, Perry ME, Dunn-Walters DK. Human marginal-zone B cells. Immunol Today, 1998, 19:421-426.
[4] Bondada S, Wu H, Robertson DA, *et al*. Accessory cell defect in unresponsiveness of neonates and aged to polysaccharide vaccines. Vaccine, 2000, 19:557-65.
[5] Takemoto N, Kiyohara H, Maruyama H, *et al*. A novel type of B-cell mitogen isolated from juzen-taiho-to (TJ-48), a Japanese traditional medicine. Int J Immunopharmacol, 1994, 16:919-29.
[6] Goodrich ME, McGee DW. Regulation of mucosal B cell immunoglobulin secretion by intestinal epithelial cell-derived cytokines. Cytokine, 1998, 10:948-955.
[7] Guo Y, Matsumoto T, Kikuchi Y, *et al*. Effects of a pectic polysaccharide from a medicinal herb, the roots of *Bupleurum falcatum* L. on interleukin 6 production of murine B cells and B cell lines. Immunopharmacol. 2000, 49:307-316.
[8] Markine-Goriaynoff D, Nguyen TD, Bigaignon G, *et al*. Distinct requirements for IL-6 in polyclonal and specific Ig production induced by microorganisms. Int Immunol. 2001,13:1185-1192.