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Characteristics of B-Cell-Specific Growth Substance Produced by *Bacillus licheniformis* E1

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A B cell-specific growth substance (BGS) was isolated from the slime layer of *Bacillus licheniformis* E1. Unlike LPS, the BGS was not affected by polymixin B, an inhibitor of LPS, or by TLR4, and resulted in the growth of B cells. When BALB/c mice were treated with the BGS, the B cell population was found to increase in both the bone marrow and the spleen, with a marked increase after 24 h in the bone marrow and after 48 h in the spleen. When using antibodies to B cell lineage-restricted surface molecules to analyze the B cell population changes resulting from treatment with the BGS, an increase in immature B cells (IgM^+ and $AA4.1^+$) and mature B cells (IgM^+ and IgD^+) was found in the bone marrow 24 h after treatment with the BGS, whereas a decrease in mature B cells and increase in IgG^+ B cells were found in the spleen. When the BGS and OVA antigen were injected into the peritoneal cavity of BALB/c mice, this resulted in a high OVA-specific antibody titer in the sera, similar to that induced by aluminum hydroxide. Therefore, it is anticipated that the mass production of the BGS by *B. licheniformis* E1 could be used for studies of B cells in immunology, and contribute to the development of a new adjuvant for vaccine manufacture.

Keywords: Adjuvant, *Bacillus licheniformis* E1, B cell-specific growth substance (BGS), immunomodulator

Immunomodulators are substances that can affect the host immune response by increasing or suppressing the growth, differentiation, and activities of immune cells [12, 18]. Proteins, glycoproteins, peptides, lipopolysaccharides (LPS), and lipid derivatives [33] are all well-known immunomodulators. Although some of these substances are derived from animals and plants, they are mainly produced by microorganisms.

Polysaccharide A (PS A) [34], β-(1-3)-glucans [25, 35], mannan [36], krestin (PSK), polysaccharopeptide (PSP) [23], and hyaluronic acid (HA) [30] are some of the currently known immunomodulators derived from microbes, and such polymers unusually increase the activities of T cells or antigen-presenting cells (APCs) [33].

Some substances function as T cell-independent antigens in the host immune system that differentiate B cells as short-lived effector cells with no immunologic memory by stimulating B cells without any help from T cells, and also function to induce the production of low-affinity IgM and IgG antibodies [1, 31]. Known examples include LPS from Gram-negative bacteria, the immunostimulating factor (ISTF) of *Actinobacillus actinomycetemcomitans*, macrophage-activating lipopeptide-2 (MALP-2) of *Mycoplasma fermentans*, and outer surface lipoprotein A (OspA), which is a lipoprotein from *Borrelia burgdorferi*. LPS is a typical bacterial B cell mitogen that induces the differentiation of B cells and also promotes the expression of IgM on the surface of B cells [21]. Meanwhile, ISTF not only proliferates B cells and APCs, but also induces the production of IL-6 and tumor necrosis factor-α (TNF-α) [17], MALP-2 increases the proliferation and differentiation of B cells through Toll-like receptor 2 (TLR2) in a T cell-independent manner [5], and OspA is known to proliferate B cells through CD40/CD40L interaction [31].

In a previous report, the current authors separated a B cell-specific growth substance from a slime layer of *Bacillus licheniformis* E1 [8]. This study investigated the impact of the growth substance on the different stages of B cell differentiation, while also evaluating the effectiveness of the growth substance as an adjuvant.

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Materials and Methods

Animals

BALB/c, C3H/HeJ, and C3H/HeN female mice between 6 and 7 weeks of age were purchased from Orientbio (Seoul, Korea) and Central Lab. Animal (Seoul, Korea). The animals were maintained and used in accordance with guidelines prepared by Yonsei University College of Medicine (Seoul, Korea), which has been fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC) for the care and use of laboratory animals.

Reagents

The aluminum hydroxide (Al), Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FICA), LPS (from *Escherichia coli*, serotype 0111:B4), ovalbumin (OVA), peroxidase substrate, and polymixin B (PMB) were all purchased from Sigma-Aldrich (Steinheim, Germany), and the EZ-link Sulfo-NHS-LC-biotinylation kit used to biotinylate the OVA was purchased from Pierce (Rockford, IL, U.S.A.).

Antibodies

The antibodies used for the cell staining and FACS analysis were as follows: phycoerythrin (PE) anti-mouse CD45R/B220, fluorescein isothiocyanate (FITC) anti-mouse CD3, FITC anti-mouse CD25 (IL-2R), FITC anti-mouse CD117 (c-*kit*), FITC anti-mouse IgM (BD Pharmingen, SanDiego, CA, U.S.A.), FITC anti-mouse IgG (Vector Laboratories, Burlingame, CA, U.S.A.), PE anti-mouse IgD, and PE anti-mouse C1qRp (AA4.1) (eBioscience, Burlingame, CA, U.S.A.).

The antibodies used for the ELISA were as follows: purified anti-mouse IgG, horseradish peroxidase (HRP)-conjugated anti-mouse IgG (BD Pharmingen), affiniPure rabbit anti-mouse IgM and IgG Abs (ImmunoResearch Laboratories, West Grove, PA, U.S.A.), purified mouse IgM and IgG, HRP-conjugated goat anti-mouse IgM and IgG (Southern Biotechnology Associates, Birmingham, AL, U.S.A.), and HRP-conjugated anti-biotin (Vector Laboratories).

Purification of BGS from *Bacillus licheniformis* E1

The purification of the concentrate from *B. licheniformis* E1 was performed as previously described [8]. Briefly, the *B. licheniformis* E1 was cultured in a brain heart infusion (BHI; Difco, Becton Dickinson, Sparks, MA, U.S.A.) broth on a shaker at 37°C for 48 h. The cultured supernatant was then spun down at 10,000 ×*g* at 4°C for 1 h, and then the harvested supernatant was boiled at 100°C for 30 min and spun down again in the same way as described above. The supernatant was concentrated using a DIAFLO ultrafiltration membrane (Filter code: YM 100; Millipore, Bedford, MA, U.S.A.). Five ml of the concentrate was then applied to a DEAE Sepharose Fast Flow column (25×330 mm) (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) previously equilibrated with a 0.05 M phosphate buffer (pH 7.4), and the column eluted with a linear gradient of 0.0–1.0 M NaCl in the phosphate buffer at a flow rate of 2 ml/min. Five ml of each fraction was collected and monitored at 280 nm, and their activities measured using a BALB/c mouse B cell proliferation assay. Those elutes showing mouse B cell proliferation activity were pooled and concentrated using the same ultrafiltration membrane. Thereafter, 1 ml of the concentrate was subjected to a Sephacryl S-500 column (16×900 mm) (GE Healthcare Bio-Sciences AB) previously equilibrated with the phosphate buffer and eluted with the same buffer at a flow rate of 1 ml/min. Three ml of each fraction was collected and monitored at 280 nm. Those elutes showing mouse B cell proliferation activity were pooled and concentrated using the same ultrafiltration membrane. The concentrate was again subjected to a Superose TM 6HR column (GE Healthcare Bio-Sciences AB), and the fractions monitored at 214 nm. The fractions at the peak were collected, pooled, concentrated, and freeze-dried. The resulting concentrate produced by *B. licheniformis* E1 is hereinafter referred to as the B cell-specific growth substance (BGS).

Isolation of Mouse T and B Cells

The lymphocytes were obtained from the mice spleens. A single cell suspension was prepared by gently teasing a spleen between two glass slides, and the red blood cells were lysed using an ACK lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA).

To isolate the T cells, the splenocytes were incubated in a MACS buffer (PBS containing 0.5% bovine serum albumin and 2 mM EDTA) with a biotin-antibody cocktail (biotin-conjugated monoclonal antibodies against CD45R, CD49b, CD11b, and Ter-119; Miltenyl Biotec, Auburn, CA, U.S.A.) for 10 min at 4°C, washed, and incubated with anti-biotin MicroBeads (Miltenyl Biotec) for 15 min at 4°C, according to the procedures provided by the manufacturer. The cells were then applied to a magnetic column (Miltenyl Biotec), and the effluent was collected and washed in an RPMI 1640 medium supplemented with 2 mM L-glutamine, 2.2 mM sodium bicarbonate, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL, Grand Island, NY, U.S.A.).

To isolate the B cells, biotin-conjugated monoclonal antibodies against CD43, CD4, and Ter-119 (Miltenyl Biotec) were used.

Splenocyte Proliferation Assay

The activity of the purified BGS was measured based on the proliferation of the splenocytes from the BALB/c mice. The fractionated T or B cells in a complete medium supplemented with RPMI 1640 and 10% heat-inactivated fetal calf serum (2.5×10^5 cells/well) were plated in 96-well round-bottomed microtiter plates (Corning, NY, U.S.A.). Fifty μl of the purified BGS was then added to each well and the plates were cultured for 48 h. The cell proliferation was measured using 6 h of ^{3}H-thymidine (^{3}H-TdR, New England Nuclear, Boston, MA, U.S.A.) incorporation.

Immunization

To induce a BGS-specific immune response, BALB/c mice were injected once intraperitoneally (i.p.) (day 0) with the purified BGS (25 μg/mouse). The mice were then sacrificed 1, 2, and 3 d after the i.p. immunization, and cell suspensions from the spleen and bone marrow from each group of mice used for FACS analysis.

For OVA immunization, BALB/c mice were injected with an initial intradermal (i.d.) immunization of OVA (100 μg/mouse) mixed with one of the following adjuvants: FCA adjuvant (100 μl/mouse), Al (20 μg/mouse), or the BGS (25 μg/mouse). The mice were then intradermally boosted with OVA (100 μg/mouse) mixed with FICA (100 μl/mouse), Al (20 μg/mouse), or the BGS (25 μg/mouse) 1, 2, and 3 weeks after the initial immunization. One week after the last immunization, blood was harvested through the vena cava of each mouse, and the sera stored at –20°C until the ELISA.

Isolation of Bone Marrow Cells

Bone marrow cells from BALB/c mice were flushed out of the femurs with an ice-cooled complete medium using a 10-ml syringe with a 21-gauge needle. The red blood cells were depleted using an ACK lysis buffer, and the bone marrow cells washed with an RPMI 1640 medium.

Thymidine Incorporation Assay

A total of 1×10^6 cells/ml were cultured in 200-μl volumes in 96-well round-bottom plates. The cells were cultured for 48 h and pulsed with 1 μCi per well of ^{3}H-TdR for the last 8 h before determining the ^{3}H-thymidine incorporation.

FACS Analysis

For the cytometric analysis of the bone marrow cells, splenocytes, and MACS-purified T or B cell suspensions, the cells were incubated with antibodies labeled with FITC and/or PE in a FACS buffer (PBS, 1% FCS, 0.05% sodium-azide) for 30 min on ice, and then washed twice with the same FACS buffer. The data were acquired using a FACSCalibur (Becton Dickinson, San Jose, U.S.A.) and analyzed using CellQuest software (Becton Dickinson).

ELISA Methods

The total IgM and IgG levels were measured using a capture-ELISA. Ninety-six-well ELISA plates (Nunc, Copenhagen, Denmark) were coated with 100 μl/well of affiniPure rabbit anti-mouse IgM (1 μg/ml) or IgG (2 μg/ml) Ab for 24 h and blocked with 150 μl of PBS containing 10% BSA for 2 h at room temperature. After washing the plates three times with PBS containing 0.05% Tween 20 (PBST), the BALB/c mouse sera diluted with PBS containing 10% BSA (100 μl/well) were added and incubated for 1 h. The plates were then washed five times, and 100 μl of 1/1,500 HRP-conjugated goat anti-mouse IgM or IgG Ab was added to each well. After incubating the plates at room temperature for 1 h, they were washed five times with PBST and reacted with a peroxidase substrate at 37°C for 30 min. The reaction was stopped by the addition of 2 N H_2SO_4 and the optical density (OD) at 450 nm read using an ELISA plate reader. The standard curves for IgM and IgG Ab were plotted using purified mouse IgM and IgG, respectively.

The OVA-specific IgG levels were measured using a capture-ELISA. The plates were coated with 100 μl/well of purified anti-mouse IgG mAb (2 μg/ml) for 24 h and blocked with 150 μl of PBS containing 10% BSA for 2 h at room temperature. After washing the plates three times with PBST, the BALB/c mouse sera diluted with PBS containing 10% BSA (100 μl/well) were added and incubated for 1 h. One hundred μl per well of biotinylated OVA (10 μg/ml) was then added and the incubation continued for 1 h at room temperature. The plates were then washed five times, and 100 μl of 1/1,500 HRP-conjugated goat anti-biotin mAb was added to each well. After incubating the plates at room temperature for 1 h, they were washed five times with PBST and reacted with a peroxidase substrate at 37°C for 30 min. The reaction was stopped by the addition of 2 N H_2SO_4 and the OD at 450 nm read using an ELISA plate reader.

Statistical Analysis

All the experiments were performed based on three separate trials and representative data are presented. All the data are expressed as the mean±SD (n=3). The differences were analyzed using the Student's *t*-test and the results considered significant with a *p*-value <0.05.

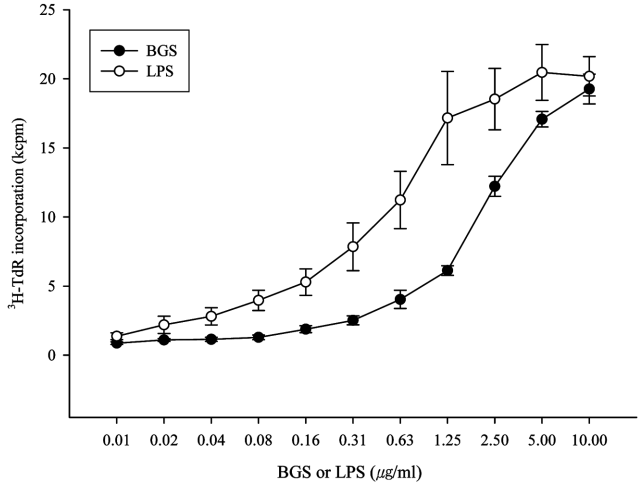

Fig. 1. Effect of BGS on proliferation of splenocytes.
Splenocytes (2×10^5 cells/200 μl) from BALB/c mice were incubated with various concentrations (horizontal axis) of the BGS or LPS for 48 h, followed by a 6 h ^{3}H-TdR incorporation assay. Each value represents the mean±SD of three trials.

RESULTS

Effect of BGS on Proliferation of Mouse Spleen Lymphocytes

Since LPS is a well-known B cell mitogen [15, 22], the effect of the BGS on the proliferation of BALB/c mice lymphocytes was compared with the effect of LPS. Lymphocytes isolated from the spleens of BALB/c mice were treated with various doses of the BGS or LPS, incubated for 48 h, and then measured using a ^{3}H-TdR incorporation assay. As shown in Fig. 1, the BGS and LPS both induced splenocyte proliferation, and their splenocyte proliferation

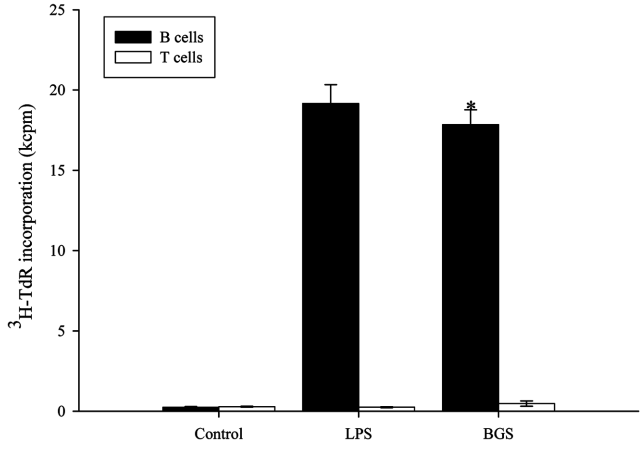

Fig. 2. BGS induced proliferation of B cells *in vitro*.
Splenic B or T cells were cultured for 48 h with the BGS (10 μg/ml) or LPS (5 μg/ml), and the cell proliferation was measured based on the 6 h ^{3}H-TdR incorporation. Each value represents the mean±SD of three trials. *, Statistical significance compared with "LPS" (p=0.2004).

activities were shown to be dose-dependent. The optimized concentration doses of the BGS and LPS for mouse splenocyte proliferation were 10 mg/ml and 5 mg/ml, respectively.

To determine which splenocytes were proliferated in response to the BGS and LPS, the T and B cell proliferation responses were measured using a ^{3}H-TdR incorporation assay. The T and B cells were treated with the optimized concentration of the BGS or LPS, and then incubated for 48 h. As expected, the BGS and LPS both induced B cell proliferation, whereas neither affected the T cell proliferation. The B cell proliferation measurements for the BGS and LPS were 18.66±1.80 and 20.15±2.19 kcpm, respectively; however, the statistical difference between the two was insignificant at p=0.2004 (Fig. 2).

Effect of PMB on B Cell Proliferation Activity of BGS

Although the BGS and LPS both had an affect on increasing the proliferation of B cells, they differ as regards their origin and structure. Thus, to determine whether their active ingredients affecting B cells also differed, the response of the BGS to PMB, an inhibitor of LPS [24], was tested. Splenic B cells isolated from BALB/c mice were treated with the BGS, LPS, BGS+PMB, or LPS+PMB, and incubated for 48 h, and the B cell proliferation was measured using a ^{3}H-TdR incorporation assay. The B cell proliferation measurement for the sample treated with BGS+PMB was 13.46±2.24 kcpm, which was slightly lower than the measurement for the sample treated with the BGS alone at 17.33±0.12 kcpm; however, the statistical difference between the two BGS samples was insignificant at p=0.1179 (Fig. 3).

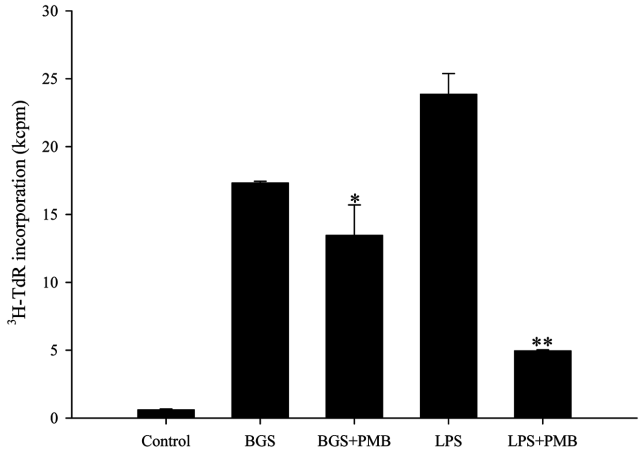

Fig. 3. PMB inhibited B cell proliferation activity of LPS, yet not that of BGS.
Splenic B cells isolated from BALB/c mice were treated with the BGS (10 μg/ml), LPS (5 μg/ml), BGS+PMB (5 U/ml), or LPS+PMB. The B cell proliferation was measured using a ^{3}H-TdR incorporation assay. Each value represents the mean±SD of three trials. *, Statistical significance compared with "BGS" (p=0.1179). **, Statistical significance compared with "LPS" (p=0.0172).

In contrast, the B cell proliferation measurement for the sample treated with LPS+PMB was 4.95±0.069 kcpm, which was considerably lower than the measurement for the sample treated with LPS alone, at 23.86±1.52 kcpm. Thus, the statistical difference was significant at p=0.0172 (Fig. 3).

BGS Induced Proliferation of B Cells Isolated from C3H/HeJ Mice Lacking Toll-Like Receptor 4

The B cell response to LPS can be induced by binding LPS with cell-surface Toll-like receptor 4 (TLR4) [7, 16, 27]. Thus, to determine whether the B cell proliferation activity of the BGS and LPS differed in terms of TLR4 binding, the effects of the BGS and LPS on splenic B cell proliferation were tested in C3H/HeJ mice lacking TLR4 [32] and C3H/HeN mice with intact TLR4. Splenic B cells from C3H/HeJ mice and C3H/HeN mice were treated with the BGS or LPS and incubated for 48 h. As shown in Fig. 4, the proliferation measurement for the splenic B cells from the C3H/HeJ mice treated with LPS showed a noticeable decrease at 11.99±1.39 kcpm when compared with the measurement for the splenic B cells from the C3H/HeN mice treated with LPS at 22.74±2.12 kcpm, representing a statistically significant difference at p=0.0021. However, in the case of treatment with the BGS, the proliferation measurements for the splenic B cells from the C3H/HeJ mice and C3H/HeN mice were 18.39±4.22 and 21.30±0.82 kcpm, respectively, representing a statistically insignificant difference at p=0.1609.

Change of B Cell Population in Bone Marrow and Spleen from BGS-Treated BALB/c Mice

To test the effect of the BGS on B cell proliferation *in vivo*, BALB/c mice were given a single dose (25 μg/mouse) of

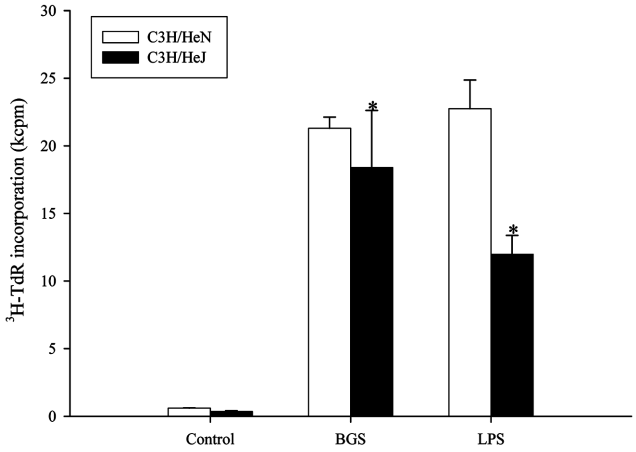

Fig. 4. B cell proliferation activity of BGS unaffected by TLR4.
Splenic B cells from C3H/HeN and C3H/HeJ mice were stimulated with the BGS (10 μg/ml) or LPS (5 μg/ml) for 48 h. The degree of lymphocyte proliferation was measured using a ^{3}H-TdR incorporation assay. Each value represents the mean±SD of three trials. *, Statistical significance compared with "C3H/HeN": p=0.1609 (BGS) and p=0.0021 (LPS).

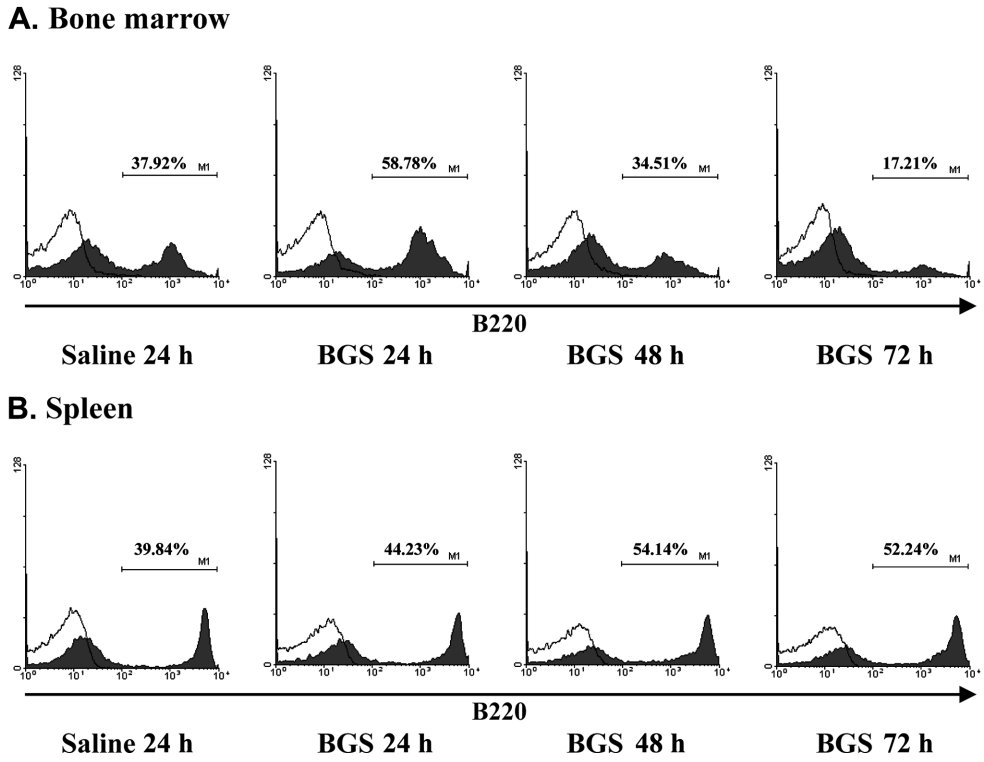

Fig. 5. BGS increased B cell population *in vivo*.
BALB/c mice were treated intraperitoneally with the BGS (25 μg/mouse) or saline. At different time intervals, mice from each group were sacrificed and B cells isolated from the bone marrow (**A**) and spleen (**B**). A FACS analysis was performed based on the staining of CD45R/B220-PE (filled area). Open area: isotype control. The results are representative of three independent experiments.

the BGS *via* the peritoneal cavity and then divided into three subgroups to be sacrificed after 24, 48, and 72 h. The control group treated with saline was sacrificed after 24 h. Cells harvested from the bone marrow and spleen were stained with CD45R/B22O-PE and then analyzed using FACSCalibur flow cytometry (Fig. 5).

The bone marrow B cell population in the BGS-treated group sacrificed after 24 h markedly increased to 58.78% when compared with that in the saline-treated control group at 37.92%. However, the bone marrow B cell population decreased to 34.51% and 17.21% in the BGS-treated groups sacrificed after 48 and 72 h, respectively (Fig. 5A).

The splenic B cell population in the BGS-treated group sacrificed after 24 h also increased to 44.23% when compared with that in the saline-treated control group sacrificed after 24 h at 39.84%. However, in contrast to the bone marrow B cell population, the splenic B cell population increased to 54.14% and 52.24% in the BGS-treated groups sacrificed after 48 and 72 h, respectively (Fig. 5B).

Target Stage of BGS in B Cell Lineage

To identify the target stage of the BGS during B cell development, BALB/c mice were treated with a single dose (25 μg/mouse) of the BGS *via* the peritoneal cavity, and then sacrificed after 24 or 48 h. For the control, another group of BALB/c mice were treated with saline, and sacrificed after 24 h. Bone marrow cells and splenocytes were isolated from each group, and MACS beads used to separate the B cells. The separated B cells were then stained with FITC-conjugated anti-CD117, -CD25, -IgM, -IgG, and PE-conjugated anti-AA4.1 and -IgD, respectively, on B cell lineage-restricted surface molecules, and finally analyzed using FACSCalibur flow cytometry, as shown in Figs. 6 and 7.

The transitions in the bone marrow-derived B cell lineage are shown in Fig. 6. The population of pro B cells ($CD117^+$), a type of B cell progenitor, was 6.89% and 8.01% in the BGS-treated groups sacrificed after 24 and 48 h, respectively, which was higher than the 3.81% population in the saline-treated control group. The population of pre B cells ($CD25^+$), another type of B cell progenitor, was 21.71% and 16.34% in the BGS-treated groups sacrificed after 24 and 48 h, respectively; however, this was not noticeably different from the 20.82% population in the saline-treated control group (Fig. 6A).

The population of immature B cells (IgM^+ and $AA4.1^+$) was 29.4% and 27.2% in the BGS-treated groups sacrificed after 24 and 48 h, respectively, which was slightly higher than the 21.2% population in the saline-treated control

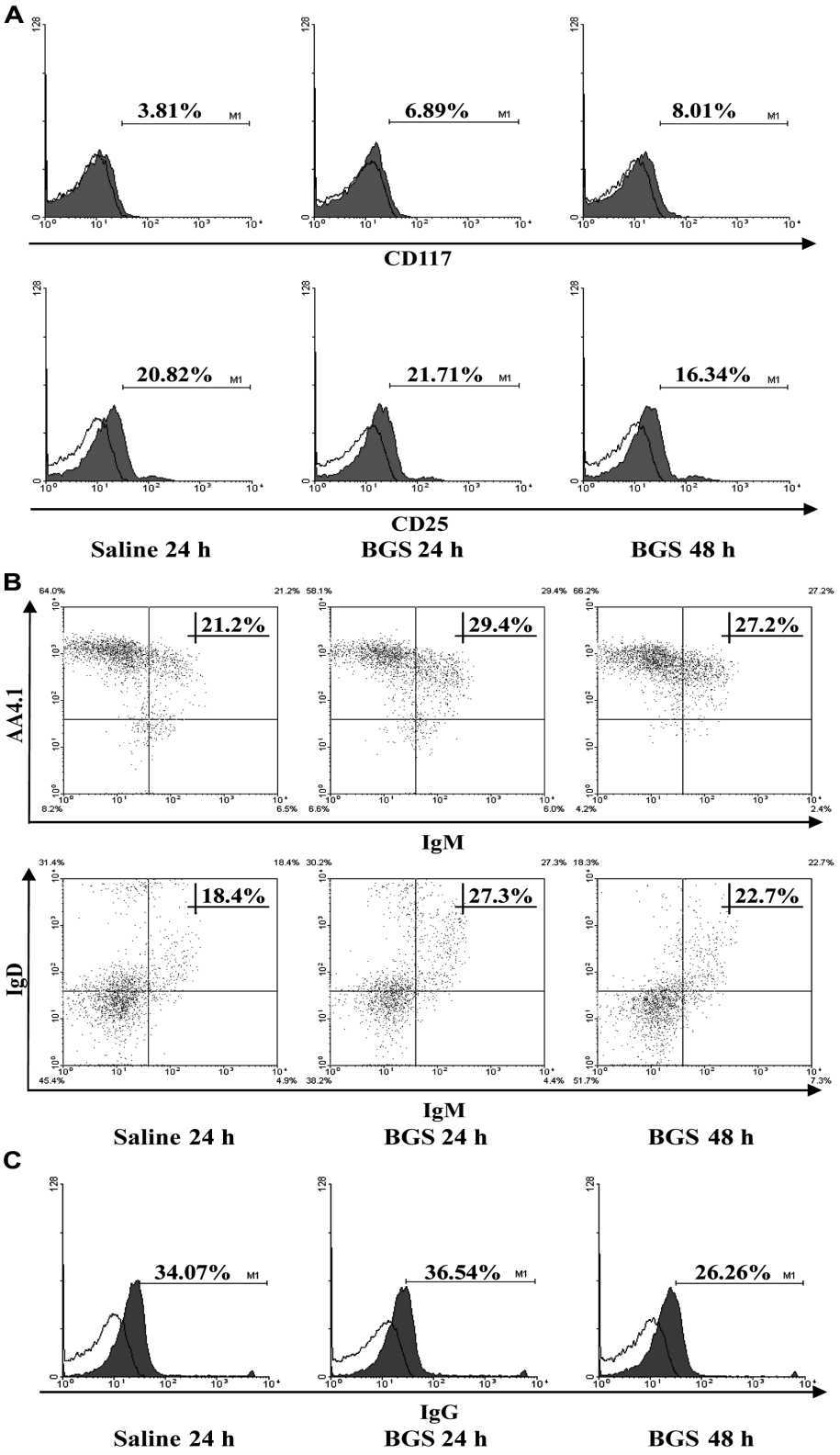

Fig. 6. Effect of BGS on early stage of B lineage cell *in vivo*.
BALB/c mice were treated intraperitoneally with the BGS (25 μg/mouse) or saline. At different time intervals, mice from each group were sacrificed and B cells isolated from the bone marrow. **A**. FACS analysis using antibodies (CD117-FITC, CD25-FITC; filled area). Open area: isotype control. **B**. FACS analysis using antibodies (IgM-FITC/AA4.1-PE, IgM-FITC/IgD-PE). **C**. FACS analysis based on staining of IgG-FITC (filled area). Open area: isotype control. The results are representative of three independent experiments.

group. A population increase was also measured for the mature B cells (IgM^+ and IgD^+) at 27.2% and 22.7% in the BGS-treated groups after 24 and 48 h, respectively, compared with 18.4% in the saline-treated control group (Fig. 6B).

In contrast, the population of IgG^+ B cells showed different percentage changes to the immature and mature B cells. After 24 h, the population of IgG^+ B cells in the BGS-treated group was 36.5%, which was very similar to the 34.1% population in the saline-treated control group. However, the IgG^+ B cell population in the BGS-treated group decreased to 26.2% after 48 h (Fig. 6C).

Since the spleen is a secondary lymphoid organ, the present investigation of the B cell lineage in mouse spleens treated with the BGS was limited to immature B cells and mature B cells, as shown in Fig. 7.

The population of splenic immature B cells was 15.2% in the BGS-treated group after 24 h, which was similar to the 13.4% population in the saline-treated control group. However, the splenic immature B cell population in the BGS-treated group increased to 19.9% after 48 h. Meanwhile, the splenic mature B cell populations were generally lower in the BGS-treated groups than in the saline-treated control group. For the saline-treated control group, the splenic mature B cell population was 46.2%, whereas it was only 33.6% and 36.9% in the BGS-treated groups after 24 and 48 h, respectively (Fig. 7A).

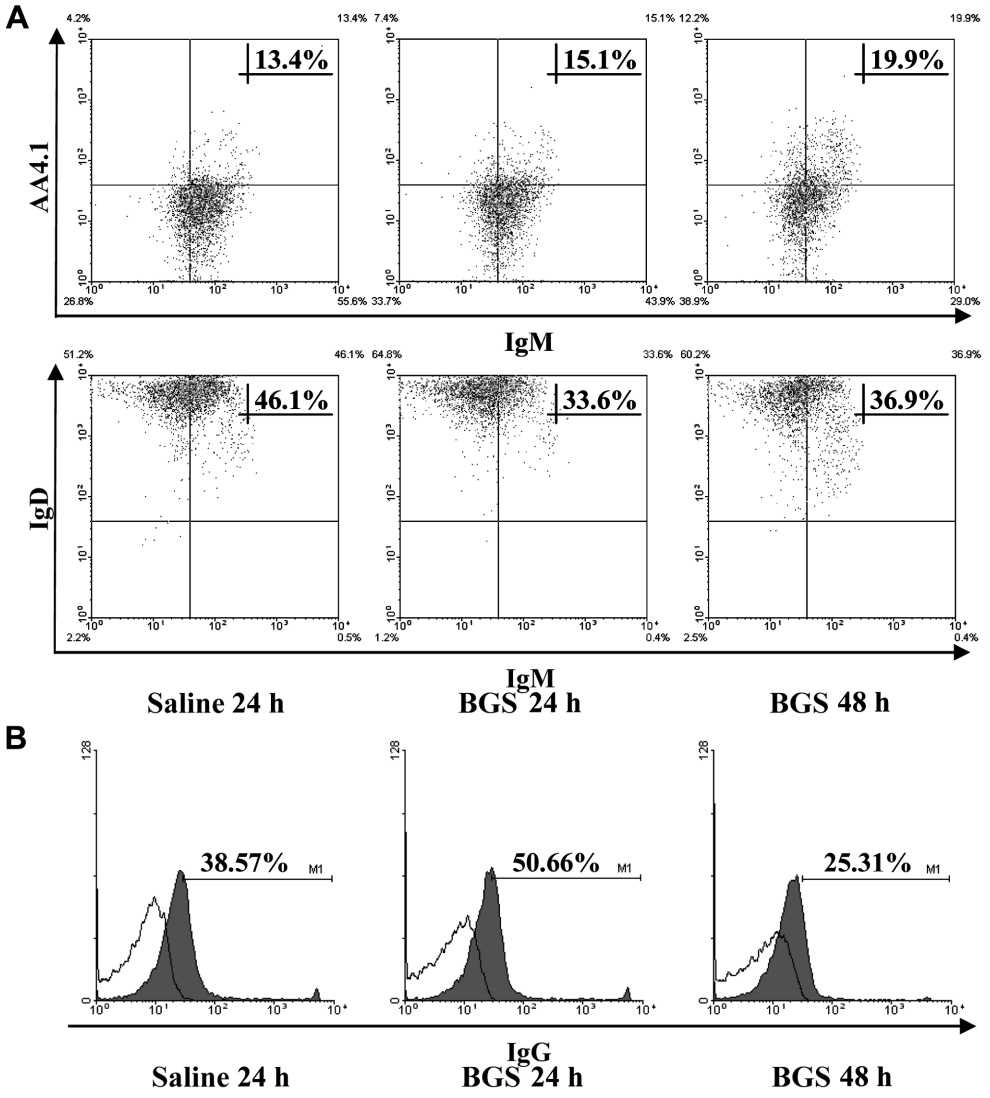

Fig. 7. Effect of BGS on splenic B cell differentiation *in vivo*.
BALB/c mice were treated intraperitoneally with the BGS (25 μg/mouse) or saline. At different time intervals, mice from each group were sacrificed and B cells isolated from the spleen. **A**. FACS analysis using antibodies (IgM-FITC/AA4.1-PE, or IgM-FITC/IgD-PE). **B**. FACS analysis based on staining of IgG-FITC (filled area). Open area: isotype control. The results are representative of three independent experiments.

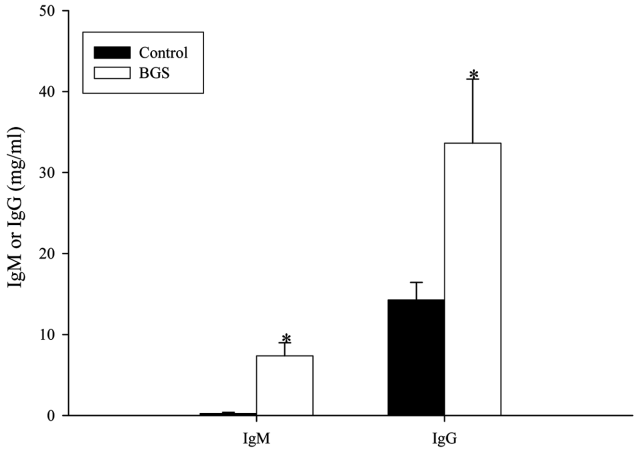

Fig. 8. BGS increased levels of serum IgM and IgG.
BALB/c mice were injected with the BGS (25 μg/mouse) *via* the peritoneal cavity. Three days later, 3 mice from each group were sacrificed and the blood was harvested. The immunoglobulin levels in the sera were measured by ELISA. Each value represents the mean±SD of three trials. *, Statistical significance compared with "control": p=0.0016 (IgM) and p=0.0083 (IgG).

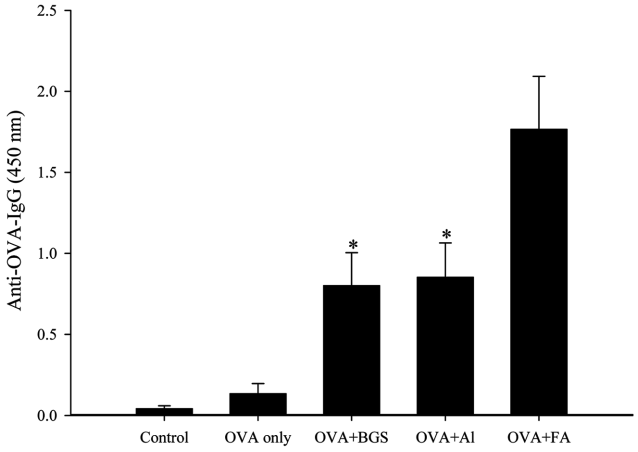

Fig. 9. BGS affected OVA-specific antibody production as adjuvant.
BALB/c mice were intradermally immunized with OVA (100 μg/mouse) or a mixture of OVA and an adjuvant (BGS, Al, or FA) once a week for 4 weeks. One week after the last immunization, mice from each group were sacrificed and the sera obtained. The levels of OVA-specific IgG in the sera were measured using ELISA. Each value represents the mean±SD of three trials. *, Statistical significance compared with "OVA only": p=0.0044 (OVA+BGS) and p=0.0035 (OVA+Al).

Finally, the population of splenic IgG^+ B cells in the saline-treated control group was 38.6%, whereas in the BGS-treated groups, the splenic IgG^+ B cell population was initially higher at 50.7% after 24 h, and then dropped to 25.3% after 48 h (Fig. 7B).

BGS Increased Concentration of IgM and IgG *In Vivo*

To investigate the effect of the BGS on the production of antibodies in B cells, BALB/c mice were treated with one dose of the BGS (25 μg/mouse) *via* the peritoneal cavity. The BGS-treated mice were then sacrificed after 72 h. The serum was separated from the blood, and the concentrations of IgM and IgG in the serum were measured. The concentrations of IgM and IgG for the saline-treated control group were 0.25±0.15 and 14.26± 2.18 mg/ml, respectively, whereas those for the BGS-treated group were noticeably higher at 7.38±1.60 and 33.63± 7.92 mg/ml, respectively (Fig. 8).

Adjuvant Effect of BGS

To examine whether the BGS might elicit adjuvant effects in terms of antigen-specific antibody production, the BGS was tested in comparison with other adjuvants. Groups of BALB/c mice were intradermally immunized with OVA, OVA+BGS, OVA+Al, or OVA+FA once a week for 4 weeks. One week after the last immunization, the OVA-specific IgG within the blood serum was measured, and the OVA-specific IgG antibody production found to be higher in the groups immunized with a mixture of OVA and one of the adjuvants than in the control group immunized with only OVA. As shown in Fig. 9, when used as an adjuvant, the BGS produced almost the same effect as Al, yet less than FA.

Discussion

It has already been reported that microorganisms produce a great variety of immunomodulators, most of which appear to increase the growth, differentiation, or activation of T cells, yet not B cells [33]. Thus, only a few immunomodulators, such as LPS and polysaccharide from streptococci, are known to be specific to B cells [12, 15, 22].

The current authors recently isolated a BGS from *B. licheniformis* that was identified as a glycoprotein with a molecular mass of 1,594 kDa [8]. Although the BGS and LPS do not share a common origin or structure, they both induce the growth of B cells. Thus, to determine whether the active ingredients causing the growth of B cells were similar, the effect of PMB, an LPS inhibitor, on the functional activity of the BGS was examined, along with the response of the BGS to TLR4, a receptor of LPS. When PMB+LPS was added, the growth of splenic B cells isolated from BALB/c mice was indeed inhibited, whereas PMB+BGS still induced B cell growth. Similarly, when splenic B cells from C3H/HeJ mice lacking TLR4 were used, LPS was unable to induce cell growth, whereas the BGS successfully induced cell growth. Consequently, in contrast to LPS, the activities of the BGS were not inhibited by PMB or TLR4 as a receptor, suggesting that its active ingredients are different.

When studying the effect of the BGS on the growth of B cells *in vivo*, the B cell population was shown to increase in both bone marrow cells and spleen cells from BALB/c mice treated with the BGS.

To examine the BGS target in B cell development, B cells were harvested and separated from the bone marrow and spleens of BGS-treated BALB/c mice. These B cells were then studied using anti-B cell lineage-restricted surface molecule antibodies. When looking at the B cell population in the bone marrow, the population of IgG^+ B cells showed no change, yet there was a decrease in the population of pro-B cells and an increase in the populations of immature B cells and mature B cells. In contrast, when examining the B cell population in the spleen, the population of IgG^+ B cells showed a significant increase when compared with the slight increase in the population of immature B cells and decrease in the population of mature B cells. Thus, when treated with the BGS, the B cell population increased, due to an increase of immature B cells and mature B cells. These outcomes also agree with results from previous studies, which found that B cell progenitors develop into immature B cells in the bone marrow and then migrate to the spleen, where they become mature B cells [3, 4], and that some of the short-lived immature B cells in the spleen are differentiated and become long-lived mature B cells [9, 20, 29].

Along with B cell development, V-D-J rearrangements lead to the formation of clonotypic BCR and surface expression of IgM molecules [6]. The surface IgM (sIgM) of B cells initiates class switch recombination (CSR) with the Cμ gene, a heavy-chain constant region, into Cγ, Cα, or Cε gene, and then by recombination, each gets to be a secondary heavy-chain isotype, such as IgG, IgA, or IgE [13, 26]. In particular, IgG begins to be manifested on the cell surface during the memory B cell stage, and it is known to be the most abundant form of Ig isotype amongst immunoglobulins [2, 10, 28]. In this study, the results showed no increase in IgG^+ B cells in the bone marrow of BGS-treated BALB/c mice, but a high increase of splenic IgG^+ B cells, indicating that the BGS may play a role in the differentiation of mature B cells into IgG^+ B cells. This possibility is also supported by the noticeable increase in the concentrations of IgM and IgG in the blood serum from the BGS-treated BALB/c mice.

Adjuvants are usually defined as compounds that can increase and/or modulate the intrinsic lower immunogenicity of an antigen. Adjuvants are therefore required to assist vaccines to induce potent and persistent immune responses, while also providing the additional benefits of reducing the number of injections and amount of antigen needed [11]. In the present study, the BGS was found to induce the growth of B cells *in vivo*, plus it increased the production of IgM and IgG, indicating that the BGS functions as an adjuvant. Thus, to investigate the adjuvant effects of the BGS, BALB/c mice were sensitized with various mixtures of OVA and the BGS. When the BGS was used as an adjuvant, high concentrations of anti-OVA antibodies were found in the mice. Although the adjuvant effect of the BGS was shown to be less than that of Freund's adjuvant, which is a highly effective adjuvant used predominantly in animal studies [19], the adjuvant effect of the BGS was almost similar to that of aluminum hydroxide, which is used in making vaccines for humans [14].

The overall safety concerns, including possible immediate and long-term side effects, of the BGS as an adjuvant have not yet been studied. Nonetheless, the antigen-specific antibody production demonstrated in this study indicates that the BGS may have potential as a new immunostimulator or adjuvant.

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