present data. The benefit of inhibition of IL-10 production for host defence has been previously demonstrated *in vivo*. IL-10-deficient mice displayed increased anti-mycobacterial immune responses and decreased bacterial burden (Murray & Young, 1999). In the absence of IL-10, antigen-specific memory T cells, which are efficiently produced by vaccination with BCG-SM for instance, may be fully activated for elimination of *M. leprae*. Although these are still preliminary findings, in one experiment BCG-SM more efficiently inhibited the multiplication of *M. leprae* in footpads of mice than in parent BCG. Therefore, BCG-SM may wipe out favourable conditions for the survival of *M. leprae*. The molecules that are present in the parental BCG and are associated with GM-CSF production remain undefined in the present study, but identification of these molecules may be useful to further enhance the T-cell-stimulating activity of BCG-SM. Also, the identification of such molecules may contribute greatly to the control of the pathogenic mycobacterial diseases using modified BCG.

In this study, we demonstrated that BCG-SM which can induce abundant GM-CSF production, may be more potent than parent BCG in immunostimulation and in the inhibition of IL-10 production, for preventing the survival of *M. leprae*.

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NOTE *Clinical Pathology*

Immunoadjuvant Activity of Crude Lectin Extracted from *Momordica Charantia* Seed

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ABSTRACT. The aim of this study is to investigate the immunoadjuvant activity of the crude *Momordica charantia* lectin (crMCL) extracted from seed using β-galactosidase (β-gal) as the model antigen. BALB/c mice were injected intramuscularly with β-gal alone or β-gal + crMCL for up to four immunizations at two-week intervals. After administration of 2 doses, the IgG-specific titer to β-gal was significantly higher in mice in the β-gal + crMCL group than in that from the animals from the β-gal alone group, while it was about the same in both groups after 1 dose. Our data suggest that crMCL may help raise antibodies under the prime and boost administration regimen and could be a potent vaccine adjuvant.

KEY WORDS: lectin, *Momordica charantia*, vaccine adjuvant.

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Vaccination is a cost-effective approach for controlling and preventing infectious diseases. Ideal vaccines should have impeccable safety records in all populations and elicit a high level of long-lived efficacy. In the practice of administering safer, inactivated vaccine, administration of antigen alone is not effective enough to achieve this goal. The use of effective vaccine adjuvants needed to enhance immune response to antigens has been investigated for the modern vaccine development [10].

Lectins are proteins or glycoproteins with carbohydrate binding specificity, which are found in both plants and animals and are involved in diverse biological functions, including immunomodulatory and immunoadjuvant properties, and some of these have been examined under clinical trial. Intranasal administration of mistletoe lectin I with ovalbumin (OVA) increased OVA-specific serum IgG and mucosal IgA [8]. Lectin isolated from Korean mistletoe (*Viscum album coloratum*) augmented keyhole limpet hemocyanine-specific IgG level [16]. However, there has been no study of the use of *Momordica charantia* seed lectin (MCL) as an immunoadjuvant.

Momordica charantia, a climber belonging to the family *Cucurbitaceae*, is commonly known as bitter gourd or bitter melon in English [4]. Fruit and seeds of bitter gourd are traditionally used as medicinal herbs and/or vegetables in Southeast Asian countries [14]. MCL, a galactose-specific glycoprotein present in the seeds of *Momordica charantia* with the $\alpha_2\beta_2$-type subunit architecture, exhibits strong type-I and weak type-2 ribosome inactivating protein activities as well as insulinomimetic activity [1, 2, 11, 12]. In this report, we aimed to elucidate the potency of the adjuvant activity of crude MCL (crMCL) on the enhancement of IgG immune response to co-administered model antigen β-galactosidase (β-gal) to provide a basis for the development of a more effective vaccine.

Extraction of crMCL was performed as described previously [15]. Briefly, *Momordica charantia* seeds were crushed into pieces, protein were subsequently extracted in phosphate-buffered saline (PBS, pH 7.4) and concentrated by ethanol precipitation, and the dried extract was re-suspended in PBS and used as crMCL. The protein concentration of crMCL was determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, U.S.A.) and quantified by the Bradford protein assay kit [3] (Bio-Rad, Hercules, CA, U.S.A.), using lyophilized bovine plasma gamma globulin as a standard according to the manufacturer's instructions. The extracted crMCL showed hemagglutinating (HA) activity against red blood cells from BALB/c mice, and the HA titre corresponded with the amount of protein of crMCL. The HA titer was defined as the last dilution that showed complete HA activity, and 13 g/l of crMCL correlated to 2^{13} HA units.

Female BALB/c (*H-2d*) mice, 6–8 weeks old, from Charles River, Japan, were housed in an air-conditioned animal facility at the University of Miyazaki with a light/dark cycle of 14/10 hr and maintained on food and water *ad libitum*. Mice were always adapted for more than one week after arrival to our laboratory before use in experiments. This experiment was approved by the Animal Care and Use Committee, University of Miyazaki.

For inoculation of the mice and blood sample collection, BALB/c mice were injected intramuscularly in the right hind legs with β-gal (50 μg/mouse) (Roche Diagnostics, Indianiapolis, IN, U.S.A.) with or without crMCL (130 μg/mouse = 10×2^{13} HA units) at weeks 0, 2, 4 and 6. Sera was

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collected by heart puncture under ether anesthesia two weeks after the last immunization and antibody titer was determined. Additionally, kidney, liver and spleen were collected from mice immunized with two doses for histopathological observation.

β-gal-specific antibody titers were determined by ELISA, as described previously [5]. Briefly, 96-well Nunc-immuno MaxiSorp assay plates were coated with 50 μl β-gal (5 μg/ml) in coating buffer (0.1 M Na_2HPO_4, pH 9.0) per well at 4°C overnight. After blocking the wells with 10% fetal calf serum in PBS, serial two-fold dilutions of sera were incubated for 2 hr at 37°C. Biotinylated γ-chain-specific goat anti-mouse IgG (Sigma-Aldrich, St.Louis, MO, U.S.A.), or, to determine antigen-specific IgG isotypes, mouse IgG1 antibody (polyclone, BETHYL, Montgomery, TX, U.S.A.), mouse IgG2a antibody (polyclone, BETHYL, Montgomery, TX, U.S.A.), mouse IgG2b antibody (polyclone, BETHYL, Montgomery, TX, U.S.A.), mouse IgG2c antibody (polyclone, BETHYL, Montgomery, TX, U.S.A.) or mouse IgG3 antibody (polyclone, BETHYL, Montgomery, TX, U.S.A.), was added as secondary antibodies. Color reactions were developed with streptavidin-biotinylated horseradish peroxidase complex (Amersham Biosciences, Buckinghamshire, U.K.) and ABTS Peroxidase Substrate Solution (Kirkegaard & Perry Laboratories, Gaithersburg, MD, U.S.A.). ABTS Peroxidase Stop Solution (Kirkegaard & Perry Laboratories, Gaithersburg, MD, U.S.A.) was added to stop the color development and the OD value of each well was determined using a microplate spectrophotometer (Benchmark Plus, Bio-Rad, Hercules, CA, U.S.A.) at 405 nm. Endpoint titers were expressed as the reciprocal log2 of the last dilution which produced an optical density at 405 nm of 0.1 unit above the values of the negative control, which was prepared from animals injected with PBS.

To determine the immunoadjuvant effect of crMCL, antigen-specific antibodies were examined in sera of the mice immunized with β-gal alone or β-gal + crMCL. After a single injection, β-gal-specific IgG antibody was detected in all immunized mice (Table 1) while no antibody titer for β-gal was detected in animals injected with crMCL only (data not shown). With two doses, anti-β-gal IgG antibody titer in the β-gal + crMCL group was significantly higher than that in the β-gal alone group ($P<0.05$). However, production of anti-β-gal IgG reached a plateau after the third inoculation, which was about the same after the fourth immunization. These results suggest that crMCL could significantly improve antigen-specific total IgG antibody response in the regimen of priming with the booster vaccination (2 doses).

To obtain preliminary insights into the nature of the immune responses after 2 doses, the IgG subclass was analyzed by ELISA. Significantly larger amounts of IgG1, IgG2b, IgG3 ($P<0.01$) and IgG2c ($P<0.05$) were seen in β-gal + crMCL-injected mice, compared with β-gal-injected mice, however, the IgG2a antibody level did not reach the detection limit (<3 log2) (Fig. 1). IgG1 was the predominant IgG subclass, followed by IgG2b in both β-gal alone and β-gal + crMCL-injected mice.

Table 1. β-gal-specific total IgG response (log_2) in sera from immunized mice

Immunization		β-gal alone	β-gal + crMCL
Priming	(1 dose)	12.5 ± 1.29	13.3 ± 0.96
Priming + boost	(2 doses)	14.5 ± 1.29	16.5 ± 0.58*
Priming + 2 boosts	(3 doses)	17.0 ± 0.00	17.0 ± 1.41
Priming + 3 boosts	(4 doses)	17.5 ± 0.58	17.3 ± 0.50

* Indicates a significant difference observed in the β-gal + crMCL group after two doses, compared with the β-gal group ($P<0.05$) by Student's *t*-test. Data are represented as mean ± S.D. (n=4).

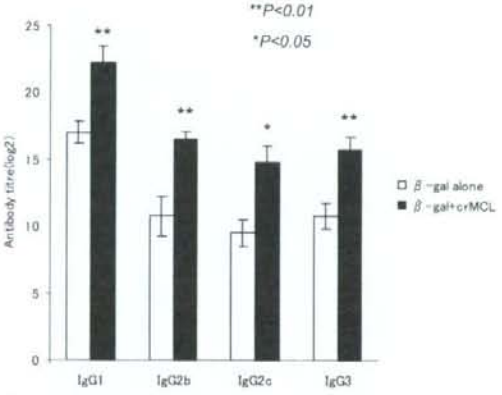

Fig. 1. Determination of IgG subclass response to β-gal in sera from mice immunized i.m. with two doses of β-gal (50 μg/mouse) alone or β-gal (50 μg/mouse) together with crMCL (130 μg/mouse). Sera were collected at two weeks after the second immunization for IgG subclasses by ELISA. After two doses, a significant difference was observed between the β-gal + crMCL group compared to β-gal alone by Student's *t*-test for each of the β-gal-specific IgG subclasses: IgG1, IgG2b, IgG3 ($P<0.01$) and IgG2c ($P<0.05$). IgG2a level did not reach the detection limit (<3 log_2) in any of the groups. The predominant IgG subclass was IgG1, followed by IgG2b. Data are represented as mean ± S.D. from four mice.

To examine the pathologic effects of crMCL, organs including the liver, spleen and kidney were examined histologically at 2 weeks after the second inoculation. No significant histopathological lesions were observed in any of the groups (data not shown). These findings indicate that crMCL has no obvious side effects on the organs of immunized mice for the doses of crMCL used in this study.

Vaccination is the first line of defense to reduce morbidity and mortality during a pandemic, such as influenza. However, commercial vaccines would be unsuitable in the current situation. To enhance the immunogenicity of a vaccine, one of strategies is to use an adjuvant with dose-sparing potential [13]. The data obtained in this study indicated that crMCL is a potent immunoadjuvant when admixed with model antigen β-gal via intramuscular administration. In this study, we used 50 μg of β-gal as a model antigen based on previous experiments [5]. Even with administration of

antigen alone, 3 doses were thought to be sufficient to achieve a maximum antigen-specific IgG level. Further investigation is required to determine whether the adjuvant activity of crMCL is more effective for lower amounts of antigen.

Serum IgG level to β-gal was significantly different in sera collected from mice following two immunizations; moreover, antigen-specific IgG subclass antibody response was effectively enhanced, as for IgG1 and IgG2b. This suggests that crMCL could actively induce a strong humoral immune response when followed by a booster immunization. This would be advantageous for the development of effective vaccination programs, as an unprimed population would likely require at least two doses to induce immunity [13]. Furthermore, Hehme *et al.* [6] reported that a two-dose regimen and an adjuvant system were required for a pandemic vaccine to elicit a satisfactory immune response. Therefore, this crMCL would be a promising adjuvant for vaccinations.

Here, we focused on antigen-specific IgG production enhanced by crMCL because we speculate that its effects are mainly on IgG. Induction of IgA may vary with lectin identity. Lavelle *et al.* [9] reported that several lectins poorly stimulated local specific IgA secretion as intranasal adjuvants because of insufficient binding to mucosa. Yoon *et al.* [16] indicated that Korean mistletoe lectin had no apparent effect on antigen-specific IgM production after subcutaneous injection. The effect of MCL on IgA and IgM induction remain to be determined.

The source of crMCL is seeds of bitter gourd, which are part of the normal diet. However, in studies on immune response by bitter gourd extract, Ike *et al.* [7] found that mice died after the intraperitoneal inoculation of whole or pulp juices of bitter gourd. Different procedures for extraction and route of administration may be responsible for this discrepancy, and these alternatives need to be considered from a safety prospective. In the present study, during the period of four immunizations, all animals at least survived without signs of severe illness prior to the sampling time point. Furthermore, histopathological analysis showed that no significant lesions after two doses in either group on various organs, including the liver, spleen and kidney from animals. These results suggest that crMCL used in this study does not cause severe side effects, although further analysis is required to ensure its safety.

In conclusion, our results show that antigen-specific IgG antibody is significantly enhanced by crMCL via intramuscular administration with the prime and boost regimen. This suggests that crMCL may be promising for use as a vaccine adjuvant. As lectin tested in this study was in the crude extract form, further study is needed to focus on purified MCL and to determine the specific effects, as well as its mechanisms, to stimulate immune responses.

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Characterization of lectin isolated from *Momordica charantia* seed as a B cell activator

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ABSTRACT

Lectin isolated from the seeds of *Momordica charantia* (MCL) is a galactose-specific glycoprotein. To investigate the effects of MCL on cell activation, we analyzed the responses of BALB/c splenocytes, thymocytes, T cells and B cells on MCL stimulation. Proliferation assays showed that MCL selectively stimulates the B cell subset of splenocytes ($p < 0.05$) in a dose and time dependent manner and that this activation proceeds without the involvement of T cells. Flow cytometric analysis revealed that the fluorescein isothiocyanate (FITC)-labeled MCL binds to B cells, which was inhibited by specific sugars, including galactose. Mouse immunoglobulin (Ig) was able to inhibit MCL-induced proliferation of mouse B cells, suggesting MCL stimulates B cell activation via membrane Ig in the B cell surface. Moreover, after 96-h co-culture, MCL triggered splenocytes to produce a large amount of non-specific IgM in culture supernatants ($p < 0.01$). Additionally, MCL was shown to up-regulate the cell activation marker CD86, in a B cell subpopulation distinct from that affected by LPS. These data suggest that MCL is a T cell-independent B cell activator and a polyclonal Ig inducer, and provide further information on the immunomodulatory effect of MCL.

1. Introduction

Lectins, which have been found in plants, animals and microorganisms, are a group of proteins or glycoproteins that specifically bind to cell surface carbohydrates. Since the interaction between lectins and carbohydrates has been shown to be involved in biological functions such as cell adhesion, migration, cell proliferation, differentiation and apoptosis, they have been widely utilized in biochemistry, cell biology and immunology studies. To date, lectins from a variety of species have been discovered, and their specific biological activities have been investigated, including lectins from concanavalin A (ConA) [1], European mistletoe (*Viscum album* L.) [2] and peanut agglutinin [3].

T and B lymphocytes are two principal players in immune response, having different responses to mitogen stimulation. Interestingly, the majority of lectins, from a variety of origins, stimulate T lymphocytes and are only rarely mitogenic towards B lymphocytes [4]. Based on advances in B cell biology, B cell activation is known to be regulated in either a T cell-dependent or T cell-independent manner. These aspects of B cell biology not only affect immune therapy strategies but also the conceptual framework of evolutionary immunology.

Momordica charantia lectin (MCL) is a galactose-specific protein found in the seeds of bitter gourd and has been the subject of active research for the past two decades. MCL is a tetrameric glycoprotein with $\alpha_2\beta_2$-type subunit architecture [5] that exhibits agglutinating activity towards human type-O red blood cells [6]. Additionally, our previous findings show that crude lectin extracted from *M. charantia* seeds (crMCL) has the ability to effectively enhance β-galactosidase-specific immunoglobulin G (IgG) [7], indicating its promise as an immune adjuvant for vaccine development. However, the stimulatory activities of MCL on immune cells remain unknown.

In this paper, we report the unexpected immunostimulatory effects of MCL on murine cells *in vitro*, leading to specific B cell proliferation and differentiation into antibody-secreting cells, without the involvement of T cells. We further determined that MCL selectively binds to B cells, and subsequently induces B cell proliferation

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via interaction with membrane Ig (mIg) with an up-regulation of cell activation markers in a specific B cell subpopulation. As this study suggests that the stimulatory function of MCL directly regulates B cell activation and highlights the different characteristics of MCL from LPS, these results may also have specific implications for the immunomodulatory effect of MCL.

2. Materials and methods

2.1. Animals

All experiments were performed with female BALB/c (*H-2d*) mice, 6–8 weeks old, from Charles River, Japan. Mice were housed in an air-conditioned animal facility at the University of Miyazaki with a light/dark cycle of 14/10 h and maintained on food and water *ad libitum*. Mice were adapted for more than 1 week after arrival to our laboratory and before use in experiments. Experiments were approved by the Animal Care and Use Committee, University of Miyazaki.

2.2. Preparation of MCL

M. charantia seeds (Ishihara foods Co. Ltd., Miyazaki, Japan) were crushed into pieces prior to protein extraction in phosphate-buffered saline (PBS, pH 7.4) and subsequent concentration by ethanol precipitation. The dried extracts were re-suspended in PBS as crMCL. Subsequently, crMCL was purified by galactose-linked Sepharose 6B affinity column (GE Healthcare Bio-Science, Piscataway, NJ), dialyzed against water and lyophilized, to produce MCL powder. MCL powder was then re-suspended in PBS and stored at −20 °C until use. MCL protein concentration was quantified by the Bradford protein assay kit (Bio-Rad, Hercules, CA) [8] using lyophilized bovine plasma gamma globulin as a standard, according to the manufacturer's instructions.

2.3. Cell preparation and culture

Spleen or thymus obtained from normal BALB/c mice was minced and passed through a fine steel mesh to obtain a homogeneous cell suspension. Erythrocytes were removed by hypotonic lysis using 0.1 × PBS and 2 × PBS. Cells were re-suspended in PRMI-1640 medium (Sigma–Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (JPH Biosciences, Lenexa, KS), 50 μM 2-mercaptoethanol (Sigma–Aldrich), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma–Aldrich) and cultured in a humidified incubator (37 °C, 5% CO_2). B cells were isolated from splenocytes of normal BALB/c mice by depletion of non-B cells (negative selection) using a B cell isolation kit (Miltenyi Biotec, Auburn, CA) with monoclonal antibodies against CD43 (Ly-48) (rat IgG2a), CD4 (L3T4) (rat IgG2b) and Ter-119 (rat IgG2b). T cells were obtained from splenocytes of normal BALB/c mice by depletion of non-T cell (negative selection) using a Pan T cell isolation kit (Miltenyi Biotec) with monoclonal antibodies against CD11b (Mac-1) (rat IgG2b), CD45R (B220) (rat IgG2a), DX5 (rat IgM) and Ter-119 (rat IgG2b). The purity of enriched B cells or T cells was determined by flow cytometric analysis using corresponding phycoerythrin (PE) rat anti-mouse CD19 (clone 1D3) antibody (BD Pharmingen, San Diego, CA) and allophycocyanin (APC) hamster anti-mouse CD3e (clone 145-2C11) antibody (BD Pharmingen). The separated T/B cell populations were confirmed to contain 92.9–99.5% and 98.5–99.7% enriched cells, respectively. The viability of all cells was >98%, as determined by Trypan blue exclusion.

2.4. Cell proliferation assay

Splenocytes, thymocytes, T cells or B cells (1×10^6 cells/ml) were cultured in triplicate in 96-microwell plates (100 μl/well) (Iwaki, Chiba, Japan) for 96 h with or without various concentrations of MCL in the presence or absence of mouse IgM (Southern-Biotech, Birmingham, AL) or albumin from chicken egg white (OVA; Sigma-Aldrich). ConA (5 μg/ml, Wako, Osaka, Japan) and lipopolysaccharide from *Salmonella enterica* serotype typhimurium (LPS; 10 μg/ml, Sigma–Aldrich) were used as positive controls for T and B cell mitogens, respectively. Cell proliferation was measured by ELISA with the incorporation of BrdU (Roche Applied Science, Basel, Switzerland).

2.5. Fluorescein isothiocyanate (FITC) labeling of MCL

MCL was labeled with FITC using ProtOn fluorescein labeling kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. Briefly, 100 μg of FITC was added to 100 μl of 0.1 M sodium phosphate containing 1 mg MCL powder and incubated for 30 min at room temperature. After reaction termination with 2 μl of 1 M ethanolamine, the reaction mixture was applied to a spin column to remove excess FITC and used as FITC-MCL. The protein concentration of FITC-MCL was calculated as 5 mg/ml according to the manufacturer's instructions and was confirmed to retain the original hemagglutinating activity towards human O type erythrocytes (data not shown).

2.6. MCL-cell binding assay

After pre-incubation with purified rat anti-mouse CD16/CD32 antibody (mouse BD Fc block, clone 2.4G2) (BD Pharmingen), a splenocyte suspension (1×10^6 cells) in 100 μl of 1% FBS–PBS was mixed with FITC-MCL together with APC hamster anti-mouse CD3e (clone 145-2C11) and PE rat anti-mouse CD19 (clone 1D3) antibodies and incubated at 4 °C for 30 min. After incubation, the suspension was washed once with 1% FBS–PBS and the cell pellet was then re-suspended in 500 μl 1% FBS-PBS for flow cytometric analysis. Data were acquired on a BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA).

2.7. Inhibition assay

To determine the inhibitory effects of various sugars on MCL-B cell binding, FITC-MCL was pre-incubated with the following sugars (1 M): D-(+)-mannose (Kanto Chemical, Tokyo, Japan), L-(−)-fucose (Nacalai Tesque, Kyoto, Japan), D(+)-galactose (Kanto Chemical), *N*-acetyl-D-galactosamine (Sigma–Aldrich) and methyl α-D-galactopyranoside (Sigma–Aldrich), for 2 h at 4 °C. Similarly, to assess the effect of Ig on MCL-B cell binding, FITC-MCL was pre-incubated with OVA or mouse IgM (1 mg/ml) for 2 h at 4 °C. The rest of the procedure was performed as for the MCL-cell binding assay.

2.8. Ig production

For Ig induction, 1 ml splenocyte suspensions (1×10^6 cells) were incubated in 24-microwell plates (Iwaki) with 100 μg/ml MCL for 96 h. The cell culture supernatants were harvested for total IgG or IgM assay by sandwich ELISA. Briefly, 96-well Nunc-immuno MaxiSorp assay plates (Thermo Fisher Scientific, Roskilde, Denmark) were coated with goat anti-mouse IgG (Sigma–Aldrich) or goat anti-mouse IgM (μ chain specific) (SouthernBiotech) in 0.1 M sodium phosphate buffer (pH 9.0) at 4 °C overnight. To assess MCL-specific Ig secretion, MCL was then coated on the 96-well plate. After blocking with 1% FBS-PBS at 37 °C for

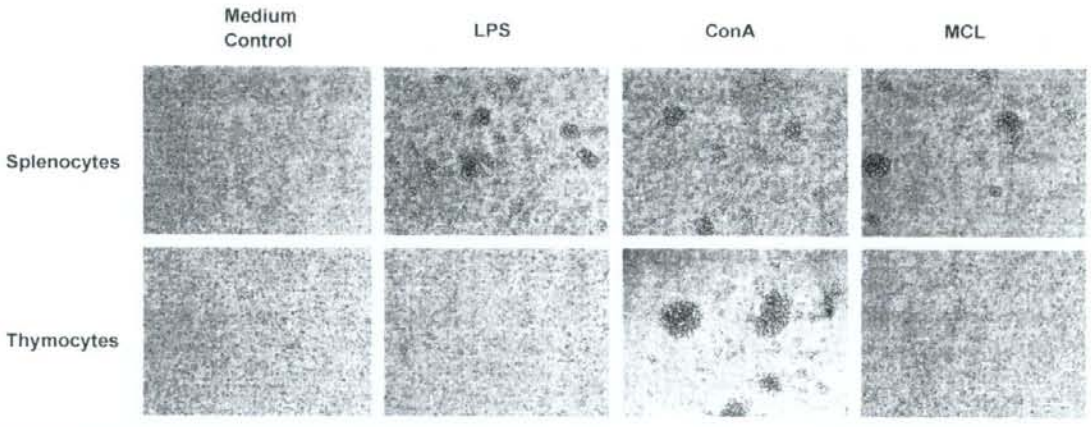

Fig. 1. MCL stimulated cell aggregation. Splenocytes and thymocytes from normal BALB/c mice were incubated for 96 h in medium alone or medium containing either ConA (5 μg/ml), LPS (10 μg/ml) or MCL (100 μg/ml). Scale bar: 50 μm.

1 h, serially diluted samples and standards were added to the appropriate wells and incubated at 37 °C for 2 h. Corresponding horseradish peroxidase-conjugated goat anti-mouse IgM (SouthernBiotech) and biotin-conjugated goat anti-mouse IgG (γ-chain specific) (Sigma–Aldrich) were used as the secondary antibodies. The color reactions were developed with streptavidin-biotinylated horseradish peroxidase complex (Amersham Biosciences, Buckinghamshire, U.K.) and ABTS peroxidase substrate solution (Kirkegaard & Perry Laboratories, Gaithersburg, MD). ABTS peroxidase stop solution (Kirkegaard & Perry Laboratories) was added to stop color development and the OD value of each well was determined using a microplate spectrophotometer (Bio-Rad) at 405 nm. The concentrations of IgG and IgM were measured with reference to standard curves using known amounts of mouse IgG isotype control (SouthernBiotech) or mouse IgM isotype control (SouthernBiotech).

To assess the effect of MCL stimulation on the differentiation of B cells into antibody-secreting cells, an ELISpot was performed as described previously [9] with some modifications. Briefly, ELISpot plates (Mabtech AB, Sweden) were coated with goat anti-mouse IgM (1 μg/ml) in PBS (pH 7.2) overnight at 4 °C, washed with PBS, and subsequently incubated with medium containing 10% FBS for 30 min at room temperature. Splenocytes or B cells in medium (10^5 cells) were added to each well and cultured in the presence or absence of ConA, LPS or MCL at 37 °C for 96 h. The cells were removed by washing 5 times with PBS. IgM-secreting cells were detected by incubating with horseradish peroxidase-conjugated goat anti-mouse IgM for 1 h at room temperature. The reaction was developed with TMB substrate until distinct spots were revealed in positive wells. The assays were performed in duplicate. The numbers of spots per well were calculated using ZoomBrowser EX ELISpot counting system (Minerva Tech, Tokyo, Japan).

2.9. Determination of cell surface marker expression

The level of cell surface activation markers was determined by flow cytometry. Purified B cells were incubated with either 100 μg/ml MCL, 5 μg/ml ConA, 10 μg/ml LPS or medium alone for 24 h at 37 °C. Cells were then stained with FITC rat anti-mouse CD86 (clone GL1) (BD Pharmingen) and PE rat anti-mouse CD19 (clone 1D3) or PE rat anti-mouse CD23 (clone B3B4) together with APC rat anti-mouse CD5 (clone 53-7.3) antibodies for analysis on a FACScanto II flow cytometer.

2.10. Statistical analysis

The statistical significance was analyzed using the Student's *t*-test with two-tailed distribution and two-sample equal variance (homoscedastic). Values were considered statistically significant at $p < 0.05$. All experiments were performed in triplicate.

3. Results

3.1. MCL specifically stimulated splenocyte proliferation

We assessed the cellular response to MCL stimulation at 96 h. Under microscopy, MCL was shown to strongly agglutinate splenocytes but not thymocytes, similar to results seen with LPS (Fig. 1). In contrast, ConA obviously stimulated aggregations of both splenocytes and thymocytes. Consistent with the morphological images, we observed that as with LPS, MCL significantly enhanced the proliferation of splenocytes ($p < 0.05$) but not thymocytes (Fig. 2A and B). Conversely, ConA induced both splenocyte and thymocyte proliferation. Next, we examined the time course of splenocyte proliferation (Fig. 2C) and performed cell number counts (Fig. 2D) in order to gain insight into the effect of MCL on cell activation. In comparison with the medium control, MCL significantly stimulated splenocyte numbers and proliferation throughout the entire culture period ($p < 0.01$). This phenomenon was both dose and time dependent. These data indicate that, similar to LPS, MCL possesses the ability to stimulate splenocyte, but not thymocyte, proliferation, and is contrary to the effects observed for ConA, which stimulated the proliferation of both.

3.2. MCL-B cell binding was inhibited by specific sugars

Initially, we attempted to evaluate MCL binding activity to splenocytes. As shown in Fig. 3A, it is apparent that FITC-MCL bound to splenocytes after co-incubation (right panel), with significant binding to $CD19^+$ B cells (38.9% versus 0.3%) and, to a much lesser extent, to $CD3e^+$ T cells (4.8% versus 0.1%), as compared to the control (left panel). This suggests that MCL selectively binds to B cells, and not to T cells.

We further investigated whether MCL-B cell binding was sugar-specific. As shown in Fig. 3B, D-(+)-galactose, *N*-acetyl-D-galactosamine and methyl α-D-galactopyranoside significantly

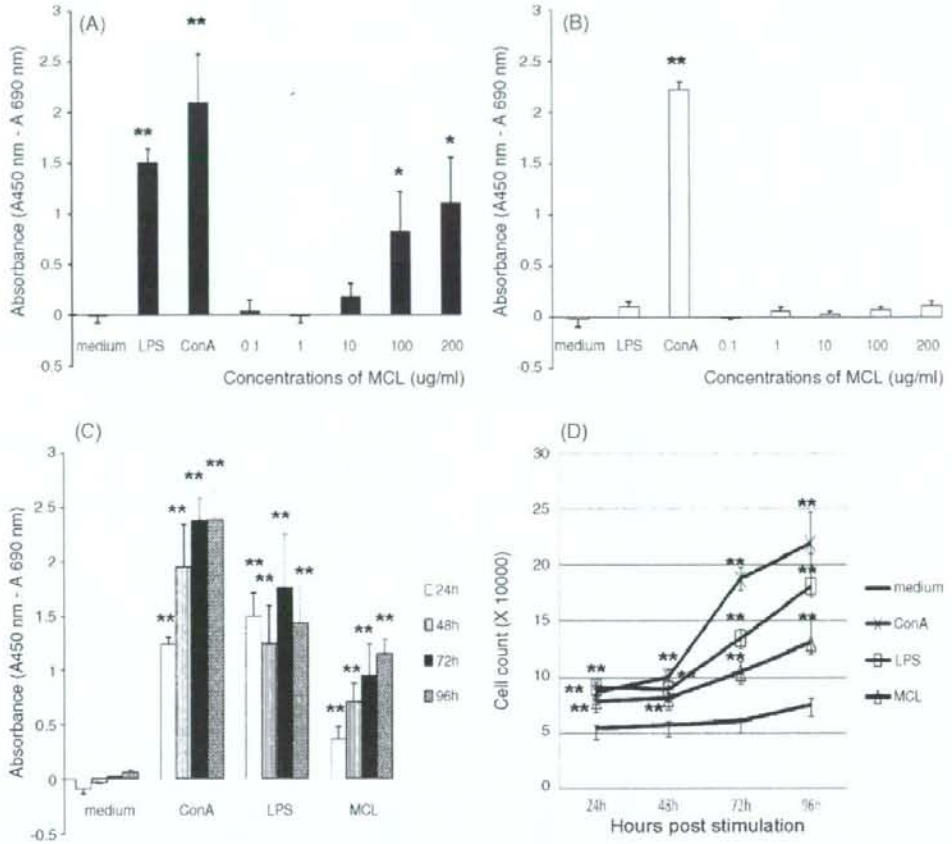

Fig. 2. MCL stimulated (A) splenocyte but not (B) thymocyte proliferation. Splenocytes or thymocytes from normal BALB/c mice were incubated with various concentrations of MCL for 96 h. The time course of splenocyte proliferation (C) and cell numbers (D) was analyzed with 200 μg/ml MCL for the entire culture period. Cell proliferation was determined by BrdU ELISA. Results are presented as average ± S.D. from three independent experiments. Asterisks represent statistical significance compared with the medium control (*$p < 0.05$, **$p < 0.01$).

decreased the percentage of MCL-B cell binding (6.20 ± 0.66%, 5.67 ± 1.27% and 5.63 ± 1.26%, respectively) as compared to cultures in the absence of sugar (13.63 ± 3.81%) ($p < 0.05$). These results indicate that MCL selectively binds to B cells and that this binding relies on the B cell surface carbohydrates that are specific for MCL.

The percentage of MCL-B cell binding increased with increasing concentrations of MCL, with almost complete binding at concentrations of 100 μg/ml (Fig. 3A). To observe the maximum inhibitory effect, we used a high concentration of sugar (1 M) and lowered the MCL concentration. Although different concentrations of FITC-MCL were used in the experiments, these results clearly reveal that MCL selectively binds to mouse B cells and that galactose as well as its derivatives are able to interfere with this binding.

3.3. MCL directly stimulated B cell proliferation

Cell proliferation is widely considered one of the most important features during B cell development. Fig. 4 shows that MCL readily increased B cell proliferation in a dose and time dependent manner ($p < 0.05$) but had no effect on T cell proliferation ($p > 0.05$). The stimulatory effect of MCL was significant at the 96-h time-point. A similar phenomenon was observed with the well-investigated B cell activator LPS, and while the known T cell mitogen, ConA, was indeed mitogenic for T cells, it had no effect on B cells. As in the case of LPS, MCL was shown to stimulate B cell proliferation in the absence of T cells. These results reveal that MCL is a T cell-independent B cell activator.

3.4. MCL increased IgM production in splenocyte culture supernatants

We further analyzed the effects of MCL on the differentiation of B cells into antibody-secreting cells. We observed that after 96-h treatment, the Ig detected in the splenocyte supernatant was predominantly IgM (Table 1, $p < 0.01$), while IgG remained below detectable limits (data not shown). This indicates that MCL has the

Table 1
Non-specific IgM production in splenocyte culture supernatants.

Treatment	IgM (ng/ml)
Medium control	27.83 ± 3.14
ConA	16.49 ± 23.67
LPS	5494.26 ± 906.04**
MCL	177.83 ± 16.65**

Results are presented as average ± S.D. from three independent experiments.
** Statistical significance compared with control ($p < 0.01$).

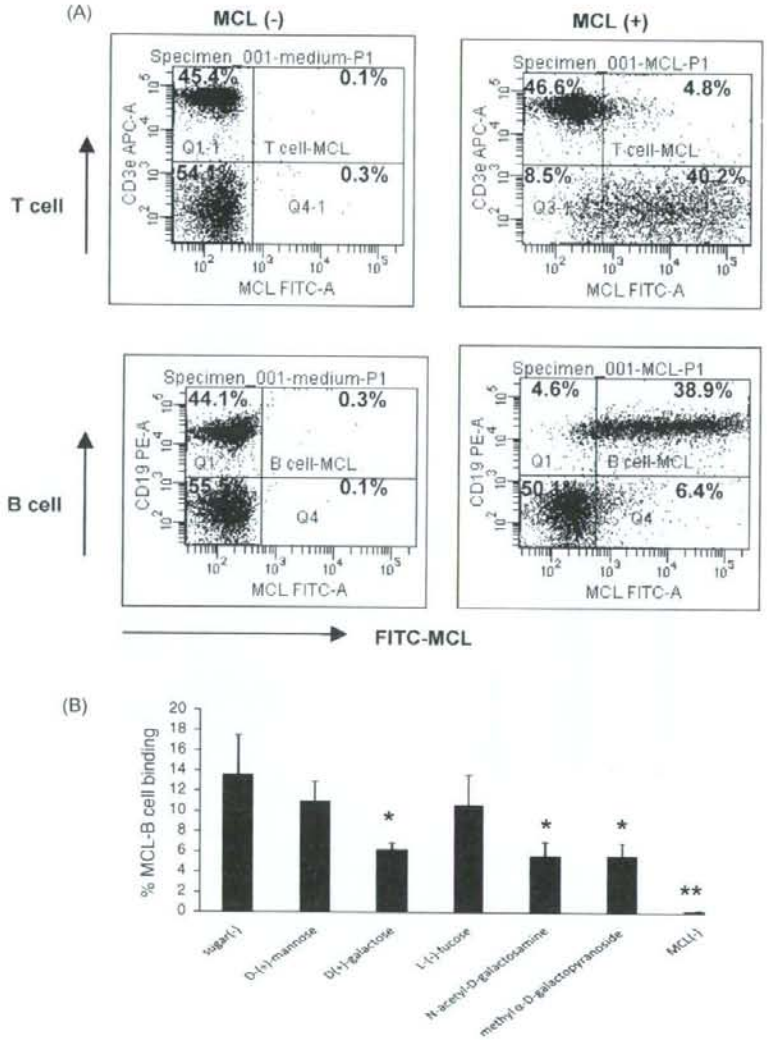

Fig. 3. MCL-cell binding assay was analyzed by flow cytometry. (A) MCL specifically bound to B cells. Splenocytes from normal BALB/c mice were incubated without (left panel) or with (right panel) 100 μg/ml FITC-MCL. Representative results of three replicates are shown. (B) MCL-B cell binding was inhibited by specific sugars. Splenocytes from normal BALB/c mice were incubated with a pre-incubated mixture of 25 μg/ml FITC-MCL and 1 M sugars. Results are presented as average ± S.D. from three independent experiments. Asterisks represent statistical significance compared to the culture devoid of sugar (*$p < 0.05$, **$p < 0.01$).

ability to enhance B cell differentiation and to lead to Ig production. To clarify whether the IgM antibodies were specific to MCL, MCL-specific IgM was determined for the same supernatants. Our results indicate that no MCL-specific IgM was detectable in the supernatants, as compared with the medium control (data not shown). As the positive control for the B cell activator used in this study, LPS induced detectable IgM and IgG antibodies in the supernatants. Thus, MCL could be a polyclonal Ig inducer with a dominant IgM isotype.

To further clarify the efficiency of B cell differentiation into plasma cells after MCL stimulation, we determined the presence of IgM-secreting cells in activated splenocytes at 96 h by ELISpot. Although ConA or LPS treatment resulted in IgM-secreting cell enhancement, no significant enhancement was detected with MCL stimulation, as compared with the control (data not shown). This result implies that MCL is a weaker B cell activator than LPS.

3.5. Membrane Ig of B cells was a candidate of functional receptors for MCL

Considering that many studies regards B cell mitogens report B cell activation is mediated through mIg, we determined if MCL also utilizes B cell mIg as a receptor. We tested Ig functions on MCL-B cell binding and MCL-stimulated cell proliferation. Although no significant difference was observed in blocking of MCL binding to B cells between OVA and mouse Ig treatment ($p > 0.05$, data not shown), the addition of mouse Ig to MCL treatment significantly suppressed MCL-induced proliferation of B cells ($p < 0.01$), while OVA did not

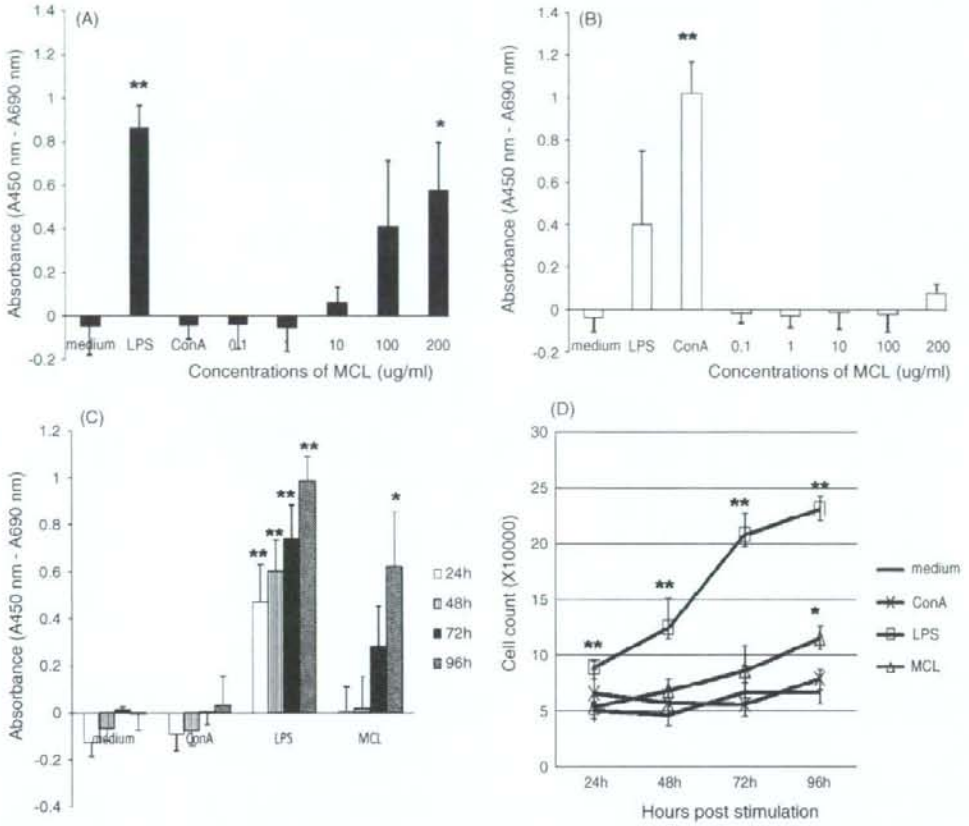

Fig. 4. MCL stimulated (A) B cell, but not (B) T cell proliferation. The sorted B or T cells from spleens of normal BALB/c mice were incubated with various concentrations of MCL for 96 h. The time course of B cell proliferation (C) and cell numbers (D) was analyzed with 200 μg/ml MCL for the entire culture period. Cell proliferation was determined by BrdU ELISA. Results are presented as average ± S.D. from three independent experiments. Asterisks represent statistical significance compared to control (*$p < 0.05$, **$p < 0.01$).

affect MCL-stimulated cell proliferation ($p > 0.05$), as compared to the culture with MCL alone (Fig. 5). These results indicate B cell activation signals are transduced via mIg in the B cell surface. Therefore, B cell mIg is shown to be a functional receptor for MCL as a B cell activator.

3.6. Expression of cell surface activation markers was determined

It is known that stimulated B cells exhibit a hyperexpression of various membrane activation markers normally associated with B cell activation. An up-regulation of the activation-associated

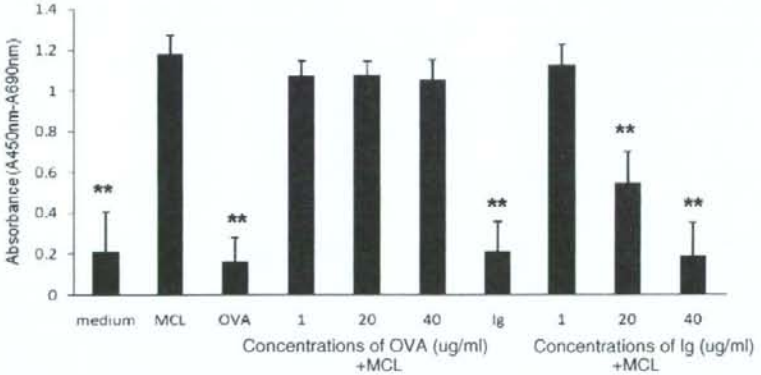

Fig. 5. MCL-induced B cell proliferation was suppressed by mouse Igs (IgM). BALB/c mouse splenocytes were incubated with either OVA or mouse IgM in the presence or absence of MCL (100 μg/ml) for 96 h. Cell proliferation was determined by BrdU ELISA. Results are presented as average ± S.D. from three independent experiments. Asterisks represent statistical significance compared to the culture with MCL treatment (*$p < 0.05$, **$p < 0.01$).

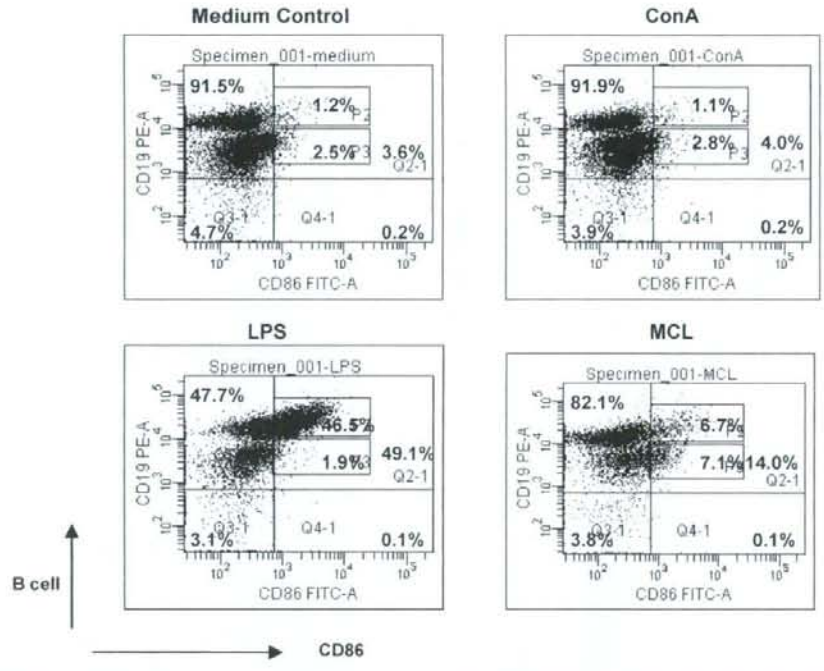

Fig. 6. MCL up-regulated expression of B cell activation markers. Purified B cells were treated with medium alone or medium containing either MCL (100 μg/ml), ConA (5 μg/ml) or LPS (10 μg/ml) for 24 h. At termination, cells were harvested and stained with FITC rat anti-mouse CD86 and PE rat anti-mouse CD19 or PE rat anti-mouse CD23 (clone B3B4) together with APC rat anti-mouse CD5 (clone 53-7.3) antibodies for use in flow cytometry. Representative results of three replicates are shown.

Table 2
Expression of CD86 in B cells after 24-h co-culture.

	Medium Control	ConA	LPS	MCL
$CD19^+CD86^+$ cell	3.08 ± 0.51%	3.30 ± 0.70%	46.40 ± 3.32%**	13.25 ± 1.26%**
$CD19^{+high}CD86^+$ cell	1.13 ± 0.25%	1.00 ± 0.27%	46.73 ± 4.28%**	9.28 ± 2.03%**
$CD19^{+low}CD86^+$ cell	2.05 ± 0.42%	2.13 ± 0.53%	1.75 ± 0.30%	5.25 ± 1.28%**
$CD5^+CD86^+$ cell	0.88 ± 0.62%	0.88 ± 0.13%	3.48 ± 0.88%**	2.48 ± 0.78%**
$CD23^+CD86^+$ cell	2.15 ± 1.70%	1.65 ± 0.42%	46.55 ± 3.67%**	8.83 ± 1.21%**,a

Results are presented as average ± S.D. from three independent experiments.
** Statistical significance compared with control ($p < 0.01$).
a Statistical significance compared with LPS treatment ($p < 0.01$).

molecule CD86, was detected after 24-h incubation with MCL. Flow cytometric analysis showed that, unlike cells cultured with the medium control, MCL induced an enhancement in the percentage of $CD86^+$ B cells (Fig. 6). After 24-h co-culture, we detected 14.0% $CD86^+$ B cells in the culture with MCL, 3.6% $CD86^+$ B cells in the control and 49.1% $CD86^+$ B cells in the culture stimulated with LPS. This result revealed that as with LPS, MCL is able to activate normal B cells and this activation involves CD86.

Although the effect of MCL on B cell activation seems to be weaker than that seen with LPS, it was noted that LPS and MCL stimulation had differing effects on CD86 expression in $CD19^{+high}$ and $CD19^{+low}$ cell populations (Fig. 6, Table 2). MCL stimulation increased $CD86^+$ cells to 9.28 ± 2.03% and 5.25 ± 1.28% of $CD19^{+high}$ ($p < 0.01$) and $CD19^{+low}$ ($p < 0.01$) cells, respectively; whereas LPS stimulation correspondingly resulted in increases of 46.73 ± 4.28% ($p < 0.01$) and 1.75 ± 0.30% ($p > 0.05$), as compared to 1.13 ± 0.25% and 2.05 ± 0.42% increases in the control, respectively (Table 2). MCL significantly activates both $CD19^{+high}$ and $CD19^{+low}$ cells ($p < 0.01$), whereas LPS stimulates only $CD19^{+high}$ cells ($p < 0.01$). To obtain preliminary insight into this phenomenon, we further assessed the expression of CD86 in $CD5^+$ B-1 cells and $CD23^+$ B-2 cells. Table 2 shows that MCL and LPS stimulation resulted in significant differences in $CD23^+CD86^+$ cells and $CD5^+CD86^+$ cells ($p < 0.01$), as compared to control. MCL and LPS induced identical levels of $CD5^+CD86^+$ cells ($p > 0.05$), but CD86 expression differed between them in $CD23^+CD86^+$ cells ($p < 0.01$). Overall, these data suggest that the mechanism of MCL B cell activation is distinct from that of LPS.

4. Discussion

Lymphocytes are the main reactive immune cells after pathogen invasion. Activation and differentiation of lymphocytes are associated with the interaction of host and pathogen. Advances in the study of lectins mitogenic to B cells suggest that they have effects on B cells that are characterized by special activation modes. It is well known that the mitogenic substance from *Ulex europeus* seeds (*Ulex* mitogen) primarily affects B cells, stimulating B cell proliferation and inducing IgM-secreting cells [10]. *Staphylococcus aureus* Cowan I (SAC) was confirmed to be a T cell-independent pure B

cell mitogen that acts directly on B cells by cross-linking of the Ig receptors on B cell surface without requirement for the process of self-recognition through the interaction of T and B cells [11]. Here, MCL is shown to follow a similar activation mode as of *Ulex* mitogen or SAC to stimulate B cell activation without T cell or any accessory cell help. Moreover, the inhibition assays of MCL-cell binding and the non-proliferative response of the sorted T cells to MCL implies that this stimulation could be the direct result of MCL binding to B cells. Additionally, it is interesting that lectins have varying sugar specificities, even though they possess similar capacities to activate B cells. As with MCL, the Pa1 isolectin of pokeweed mitogen, from *Phytolacca americana*, was shown to be mitogenic to B cells, but was *N*-acetyllactosamine specific [12].

Lectins are cell agglutinins which bind to sugar residues of specific configurations and sequences [13]. The carbohydrate–lectin interaction mediates the effects of lectin on cells. Moreover, T and B cells play distinctive but interdependent roles in immune protection, and can be distinguished based on their characteristic surface membrane composition. Boldt et al. demonstrated that ConA showed different binding abilities to human peripheral blood containing two subpopulations of lymphocytes, and consequently induced different responses [1]. In contrast, Blackedge et al. found that ConA did not discriminate between T and B cells in unseparated human peripheral blood lymphocytes [14]. Additionally, using flow cytometric analysis, Malin-Berdel et al. reported on the relative surface binding of 11 lectins (including ConA) to human peripheral blood T and B cells and human T- and B-cell lines. Only lectin from *Lens culinaris* (LCA), which preferentially bound to the two B-cell lines, exhibited selective binding to the undifferentiated cells [15]. Malin-Berdel et al. [15] postulated that the conflicting results possibly reflect the influence of cell volume on the evaluation of cell-bound fluorescence [16] or may be due to the use of much higher lectin concentrations [17] and different reaction conditions that make additional binding sites accessible [18]. In our study, MCL significantly bound to B cells and this reaction was effectively impaired by the addition of inhibitory sugars. These data suggest that the binding of MCL to the cell surface is associated with specific cell surface carbohydrates. As it is well known that in murine models, B cells contain more galactose residues than T cells [19], we could postulate that MCL selectively binds to B cells through an interaction with galactose residues on the B cell surface.

It is known that SAC relies on interaction of mIg molecules for B cell activation, which may transduce synergized signals for more efficient activation [11,20]. As MCL and SAC share a similar mode of action on B cell activation, we considered mIg as a possible receptor candidate. As expected, our results showed that Ig effectively blocks the cell proliferation by MCL, in contrast to OVA control. Although the inhibition of MCL-B cell binding by OVA was observed at the same extent as that by Ig, it is likely that interaction of MCL with mIg triggers B cell activation. Thus, the B cell response to MCL stimulation is thought to be mediated, at least in part, through mIg in the B cell surface. Other potential B cell receptors for MCL should be investigated in future experiments.

On the other hand, lectins are considered mitogenic or non-mitogenic according to their activity towards mouse splenocytes. Advances in lectinology report that binding alone is not sufficient for cell activation, since certain lectins are non-mitogenic even though they bind well to cells [21], indicating that lectins may utilize different receptors or binding sites for activation. It is revealed that specific receptor requirements would be the key to discriminating between galactose-specific lectins having B cell mitogenic activity, as with MCL, and others deficient in mitogenic activity.

The process of resting B cells maturing into Ig-secreting cells involves three distinct stages: activation, proliferation and differentiation. Here, MCL activated splenocyte proliferation and induced a marked increase in a dominant IgM isotype post 96-h treatment. Notably, this IgM was not specific to MCL, which is similar to other B cell activators [9,22]. Advances in B cell biology suggest that B-1 cells might be responsible for the majority of IgM secretion, which plays an important role in innate immunity by secreting large amounts of IgM class natural antibodies [23]. Although $CD5^+$ B-1 cells and $CD23^+$ B-2 cells were shown to express the activation marker CD86 after MCL stimulation, further studies will be undertaken to determine which subpopulation of B cells are targeted by MCL.

B cell activation involves the expression of co-stimulatory molecules such as: MHC class II, CD40, CD25 and B7 (CD80/CD86) [24,25]. A number of factors may up-regulate the expression of MHC class II antigens on B cells, including the cross-linking of surface Ig by antigens and cytokine stimulation. Here, we observed that MCL treatment promoted the expression of CD86 (B7-2), a ligand for CD28 and CD152, in enriched B cells. CD86 is an accessory molecule that plays an important role in T cell-B cell co-stimulatory interactions and its expression is increased by T cell- or B cell-specific stimuli. This observation corroborates other evidence for MCL as a B cell activator. Furthermore, we observed that LPS stimulated mainly $CD19^{+high}$ cell subsets, while MCL elevated the expression of CD86 in both $CD19^{+low}$ and $CD19^{+high}$ cell subsets. This discrepancy implies that MCL may trigger different B cell subpopulations or induce activation effects on B cells that differ from that of LPS. Culton et al. reported that $CD19^{+high}$ B cells are memory B cells, while $CD19^{+low}$ B cells are naive B cells [26]. The formation of memory B cells leads to Ig isotype switching from IgM to IgG, and a rapid production of high antibody titers during the secondary antibody response [27]. Low CD19 levels can decrease sensitivity to activation through mIg and may escape tolerance induction, since it is dependent upon mIg signal strength. These $CD19^{+high}$ B cells may be responsible for the high level of IgM production and the detectable IgG levels as a result of LPS treatment, and $CD19^{+low}$ B cells may be associated with low IgM levels as a result of MCL treatment. A comparison of CD86 expression in B cell subpopulations ($CD5^+$ B-1 cells and $CD23^+$ B-2 cells) also strengthens the evidence illustrating differences in effects of B cell activation between MCL and LPS. These findings highlight that MCL exhibits B cell activator characteristics that are distinct from that of LPS, which should be further confirmed in future experiments.

Compared to LPS, MCL seems to have a weaker effect on B cells, including its mitogenic activity, expression of activation markers and Ig production. In contrast to LPS, which summits B cell activation within a short time, it is necessary for MCL to stimulate B cells for 96 h or in a high concentration in order to reach significant cell proliferation.

In conclusion, our present results show that MCL has distinct immunostimulatory effects on murine cells, and selectively activates B cell subsets of splenocytes. Moreover, this stimulation occurred without T cell involvement, which is inhibited by mouse Ig, implying mIg in the B cell surface plays a critical role in the process. MCL also induced B cells to secrete non-specific IgM in the supernatants, demonstrating that MCL acts as a T cell-independent B cell activator. Finally, MCL is able to up-regulate the expression of CD86 in a special B cell subpopulation, exhibiting effects on B cells distinct from that of LPS.

Acknowledgments

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CONCISE ARTICLE

Relationship between pathogenicity for humans and *stx* genotype in Shiga toxin-producing *Escherichia coli* serotype O157

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Abstract To examine the reason why people infected with Shiga toxin (Stx) producing *Escherichia coli* (STEC) O157 strains develop varying clinical manifestations, 65 STEC O157 isolates originating from 64 different occurrences of infection in Miyazaki Prefecture in 2001–2003 and their 79 infected individuals were analyzed by *stx* genotyping, quantitative analysis of reversed passive latex agglutination (RPLA), genomic DNA analysis using pulsed-field gel electrophoresis (PFGE), and clinical manifestations. The isolates were found to carry the following *stx* genes: *stx2vha* alone (60.0%), *stx1/stx2* (27.7%), *stx1/stx2vha* (6.1%), *stx2* alone (3.1%), and *stx2/stx2vha* (3.1%). No strain carried the *stx1* gene alone. STEC strains carrying *stx2* were more frequently associated with clinical manifestations of hemolytic-uremic syndrome (HUS) or bloody diarrhea than those carrying *stx2vha*. Clusters of PFGE banding patterns were correlated well with the *stx* genotypes. We conclude that *stx* genotype is one of the important factors of clinical outcome of STEC O157 infection and that pathogenicity for humans was higher in the *stx2* genotype strains than in the *stx2vha* genotype strains, as reported previously by other researchers. Further, we newly found that four clusters identified by PFGE using restriction enzyme *Xba*I, *stx* genotypes and clinical manifestations were well correlated with each other.

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Introduction

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) is an emerging food-borne pathogen of clinical and public health concern and a cause of various symptoms such as diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome (HUS) in humans. Shiga toxins (Stx1 and Stx2) have a prominent role in the pathogenesis of STEC and are classified with their restrictive variants according to the nomenclature proposed by Calderwood et al. [1]. Stx1-related toxins include Stx1, Stx1c, and Stx1d, whereas Stx2-related toxins include Stx2, Stx2c, Stx2d, Stx2e, Stx2f, and Stx2g [2, 3]. Stx2c (a variant investigated in the present study) has several subtypes: Stx2vha, Stx2vhb, and others [4]. These toxins are encoded by genes designated *stx1*, *stx1c*, *stx1d*, *stx2*, *stx2c* (including *stx2vha*, *stx2vha*, etc.), *stx2d*, *stx2e*, *stx2f*, and *stx2g*, respectively. STEC strains can be classified into several *stx* genotypes, and some reports indicate that *stx* genotype is associated with the virulence level of STEC [5, 6].

There are many serotypes of STEC strains, but the one most associated with epidemic cases is O157. The STEC O157 strains have been reported to produce Stx1, Stx2, and Stx2c (Stx2vha and Stx2vhb) [5]. The pathogenesis of STEC infection in humans depends on many bacterial virulence factors including Stxs, enterohemolysin, intimin (encoded by *eae* gene), and host factors such as age [7].

In Miyazaki Prefecture in Japan, many human STEC infections have occurred recently, and this may be

connected to livestock breeding, a major activity in this area. Misawa et al. detected *stx* genes by PCR in 76.3% of calves in Miyazaki [8]. The dominant serotype of human-origin STEC in Miyazaki is O157, and clinical manifestations vary from no symptoms to serious systemic complications such as HUS.

The aim of this study is to examine why infected people with STEC O157 develop various clinical manifestations, and for this purpose, STEC O157 strains isolated in Miyazaki Prefecture were examined for *stx* genotypes and the amount of Stxs, and the results were analyzed in correlation with clinical manifestations of STEC O157 infections. To further obtain genetic information, 65 STEC O157 isolates were analyzed by pulsed-field gel electrophoresis (PFGE).

Materials and methods

Fecal samples collected from 79 infected subjects in Miyazaki Prefecture in 2001–2003 and 65 STEC O157 isolates in 64 different occurrences [one group outbreak (n=7), six family outbreaks (n=15), 24 sporadic cases, and 33 healthy carriers] were analyzed for the following: *stx* genotype analysis, quantitative analysis using reversed passive latex agglutination (RPLA), genomic DNA analysis using pulsed-field gel electrophoresis (PFGE), and clinical symptoms. Samples were collected from symptomatic and asymptomic subjects in family and group outbreaks. The subjects were all living in Miyazaki Prefecture, 1–84 years old (20 subjects: 1–8 years old; 59 people: 11≤ years old) and were classified into four categories on the basis of clinical manifestations: HUS (HUS), bloody diarrhea without HUS (BD), non-bloody (but watery) diarrhea without HUS (WD), and asymptomatic (none) (Fig. 1).

Escherichia coli (*E. coli*) strains were isolated from fecal samples with selective media of CT-SMAC (Oxoid Ltd., Hampshire, England), CHROMagar O157 TAM (CHROMagar, Paris, France), and DHL (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). The *stx* genes were examined with PCR as described by Karch and Meyer [9]. This PCR can detect *stx1*, *stx2*, *stx2c* (*stx2vha* and *stx2vhb*), *stx2d*, *stx2e*, and *stx2g*, but not *stx2f* genes [3]. STEC isolates were also serotyped with commercial *E. coli* antisera (Denka-seiken Co., Ltd., Tokyo, Japan). Enterohemolytic activity and possession of *eae* gene were confirmed in all O157 strains [7].

stx genes were typed by PCR or PCR and subsequent restriction fragment length polymorphism (RFLP) analysis, according to previously described methods [4, 9, 10]. The *stx* genes were classified into *stx1*, *stx2*, and four *stx2* variant groups including *stx2c*, *stx2d*, *stx2e*, and *stx2f* using PCR [10], as follows: 1 μl of boiled bacterial supernatant (DNA solution) was added to 24 μl of PCR reagent including 10×PCR buffer, dNTPs, appropriate primer, *TaKaRa Ex Taq* (TaKaRa Bio Inc., Shiga, Japan), and ultrapure water in 0.2-ml microtubes, followed by amplification for 25 cycles using a thermal cycler (PE Applied Biosystems 9700, Foster City, CA). The amplification products were electrophoresed on a 2% agarose gel (NuSieve 3:1 Agarose; Cambrex Bio Science Rockland, Inc., Rockland, ME) and visualized using a UV transilluminator after ethidium bromide staining.

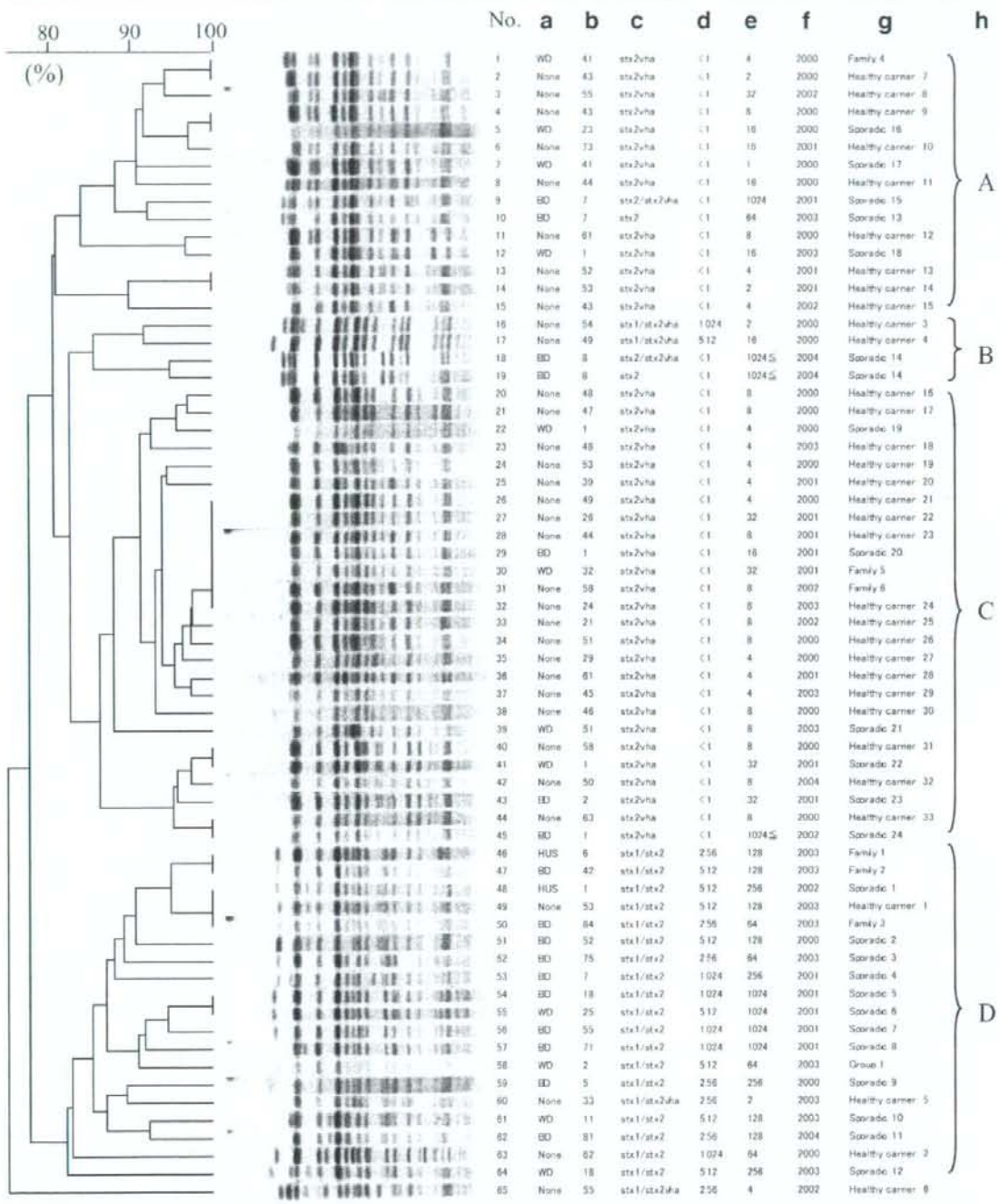
Furthermore, PCR and subsequent RFLP analysis according to the method described by Tyler et al. [4] were used to distinguish between *stx2* and *stx2vha* and *stx2vhb*. The digestion of PCR amplicons by three restriction enzymes *Hae*III, *Nci*I, and *Rsa*I (TaKaRa Bio Inc.) was performed as directed by the manufacturer.

PFGE was performed according to the protocol used by the PFGE-based national network set up to monitor the spread of O157 in Japan [11]. Briefly, bacterial cultures grown in Trypto-Soya broth (Nissui Pharmaceutical Co., Ltd.) at 37°C for 16–18 h were embedded in agarose plugs and were treated with Proteinase K (1mg/plug, Wako Pure Chemical Industries, Ltd., Osaka, Japan). After removing Proteinase K, genomic DNA embedded in plugs were digested with 30 U of restriction enzyme *Xba*I (Boehringer Mannheim, Mannheim, Germany) overnight at 37°C. Agarose gels (1% SeaKem Gold Agarose; Cambrex Bio Science Rockland, Inc.) were electrophoresed using a CHEF DR III apparatus (Bio-Rad Laboratories, Richmond, CA) under the following conditions: 0.5× Tris-Borate-EDTA buffer at 14°C, 6 V/cm for 22 h, with a linearly ramped switching time from 2.2 to 54.2 s. Images of gels stained with ethidium bromide were analyzed using Fingerprinting II software (Bio-Rad Laboratories).

Bacteria were grown to 2.4×10^8~6.6×10^8 cfu/ml in CAYE broth (Denka Seiken Co. Ltd., Tokyo, Japan) at 37°C for 24 h with shaking. They were further incubated for 30 min in the presence or absence of polymyxin B (final concentration of 5,000 U/ml). Culture supernatants were then obtained by centrifugation (13,000 g, 5 min) and filtered through 0.22-μm pore size membrane filter units (Millex-GV; Millipore, Bedford, MA). The filtrate was used as the Stx extract for RPLA.

Stx1 and Stx2 were quantitated using a Commercial RPLA test kit (VTEC-RPLA, Denka Seiken Co. Ltd.,

Fig. 1 Relation between clusters grouped on the basis of PFGE DNA fingerprints and the characteristics of 65 strains of STEC O157. **a**: Clinical manifestation (HUS, HUS; bloody diarrhea without HUS, BD; non-bloody watery diarrhea without HUS,WD; asymptomatic, none); **b**: age; **c**: *stx* genotype; **d**: Stx1 RPLA titer (polymyxin B-treated); **e**: Stx2 RPLA titer (polymyxin B-treated); **f**: year of isolation; **g**: origin; **h**: PFGE cluster



Tokyo, Japan), according to the manufacturer's instructions [12]. Briefly, serial two-fold dilutions of Stx extract from 1 to 1,024 times (25 μl) were mixed with equal volumes of latex particles sensitized with rabbit polyclonal anti-Stx1 IgG or anti-Stx2 IgG in 96-well V-bottom microtiter plates. The plates were incubated at room temperature and examined for latex agglutination for 20–24 h. The titers of Stx1 and Stx2 were expressed as the reciprocal of the

highest dilution that caused agglutination by the respective latex reagent.

Results and discussion

Stx2vha has been designated as a Stx2c subtype to date; however, it is shown as a Stx2d subtype in the second edition of Bergey's Manual [7]. In this study, we adopt the former definition to avoid confusion.

The *stx* genes of the 65 causative strains in 64 occurrences were investigated, and only *stx1*, *stx2*, and *stx2vha*, but not *stx2vhb*, *stx2d*, *stx2e*, and *stx2f* were detected. In practice, the combination of these three genes generated five genotypes: *stx2* alone, *stx2vha* alone, both *stx1* and *stx2* (hereafter, *stx1/stx2*), both *stx1* and *stx2vha* (hereafter, *stx1/stx2vha*), and both *stx2* and *stx2vha* (hereafter, *stx2/stx2vha*). The two most frequent genotypes were *stx2vha* (detected in 39 strains; 60.0%) and *stx1/stx2* (detected in 18 strains; 27.7%). The *stx1/stx2vha*, *stx2* alone, and *stx2/stx2vha* were detected in four (6.1%), two (3.1%), and two isolates (3.1%), respectively (Table 1). Consequently, 21 isolates (32.3 %) carried *stx2* genes, alone or in combination with other *stx* genes, and 45 strains (69.2 %) possessed *stx2vha* genes. No strain possessing only *stx1* gene was observed (*stx1* gene was detected only in combination with *stx2* gene or *stx2vha* gene, and no *stx1* variant gene was detected). While *stx2/stx2vha*, the most frequently occurring genotype in Belgium and Finland (29% and 64%, respectively) [2, 5], was detected with low frequency in Miyazaki, *stx2vha* and *stx1/stx2* were common in Osaka (15% and 57%, respectively) [13]. However, *stx2vha* appeared to be more common in Miyazaki than in Osaka. The high frequency of the *stx2vha* genotype in Miyazaki might be the result of environmental factors (such as breeding of livestock) or the source of specimens (many more samples from asymptomatic adults were used than samples from children). Our results suggest that the pattern of *stx* genotype distribution in Belgium [5] or in Finland [2] might be epidemiologically different from that in Miyazaki, though the reason is not clear.

The amount of Stx was determined by RPLA, and the relationship between *stx* genotype and their toxic activities was investigated. The RPLA Stx titers for the 65 representative isolates are shown in Fig. 1. Using Stx1 reagent, polymyxin B (an antibiotic that releases the *stxs* from the

Table 1 Numbers and sources of STEC O157 isolates in the present study and clinical manifestations of infected people

Occurrences (source of O157 isolates)	Causative strains				No. of people presenting with clinical manifestations*			
	stx genotype	Frequency % (no. of strains /no. of total strains)	No. of causative strains in the occurrences	No. of infected people in the occurrences	HUS	Bloody diarrhea without HUS	Non-bloody diarrhea without HUS	Asymptomatic
Group 1	*stx1/stx2*	27.7% (18/65)	1	7	3 (3)	13 (5)	6(3)	5
Family 1–3			3	6				
Sporadic 1–12			12	12				
Healthy carrier 1–2			2	2				
Family 4–6	*stx2vha*	60.0% (39/65)	3	9	0	3 (3)	10 (3)	32
Sporadic 16–24			9	9				
Healthy carrier 7–33			27	27				
Sporadic 13	*stx2*	3.1% (2/65)	1	1	-**	1 (1)	-	-
Sporadic 14***	*stx2*		1	1	-	1 (1)	-	-
Sporadic 14***	*stx2/stx2vha*	3.1% (2/65)	1	1	-	1 (1)	-	-
Sporadic 15	*stx2/stx2vha*		1	1	-	1 (1)	-	-
Healthy carrier 3–6	*stx1/stx2vha*	6.15% (4/65)	4	4	-	-	-	4
Total 64 occurrences		100%	65	79	3 (3)	19 (11)	16 (6)	41

*Number in a parenthesis represents the number of patients 0–8 years of age

**None

***Carried stx2 genotype strain and stx2/stx2vha genotype strain

periplasmic space of bacteria and increases specific activity of toxin extracts) -untreated cultures of isolates carrying the *stx1* gene had low Stx1-RPLA titers (8–64, data not shown), whereas all extracts of polymyxin B-treated cultures of them had high titers (256–1,024). Using Stx2 reagent, RPLA titers in strains carrying only *stx2* were significantly different from those carrying only *stx2vha*, as follows: isolates with *stx2*-only genotypes (such as *stx2* and *stx1/stx2*) showed high Stx2 titers of 64–1,024≤. By contrast, isolates with *stx2vha*-only genotypes (such as *stx2vha* and *stx1/stx2vha*) showed low Stx2 RPLA titers of 1–32, except one isolate with a remarkably high Stx2 RPLA titer of 1,024≤. The mean titer of Stx2vha was about 1/32 of the mean titer of Stx2. Little difference was observed between polymyxin B-treated and -non-treated cultures [12]. RPLA is based on antigen-antibody reaction between Stx (Stx1 or Stx2) and anti-Stx (anti-Stx1 or anti-Stx2) latex reagent. Compared with Stx1 or Stx2, Stx variants react with anti-Stx latex reagent more weakly because of their structural difference. Karmalli et al. reported that anti-Stx2 latex reagent also reacted with Stx2c, though it was 30-fold less sensitive to Stx2c than to Stx2 [12]. In this way, a remarkable difference of RPLA titer among bacterial strains did not depend on the amount of the toxin produced by bacteria, but depended on the variety of Stx2 subtypes. However, the Vero cell cytotoxicity (CD_{50}) also varied depending on the kind of Stx, and extracts of the *stx2vha* genotype isolates had an average of eight-fold lower titer than did extracts of the *stx2* genotype isolates (data not shown). By comparing the RPLA titer with the Vero cell cytotoxicity titer, the Stx2 RPLA titer was shown to correlated well with the Vero cell cytotoxicity, and above all, in Stx extract of isolates not treated with polymyxin B (Stx1 level was low; data not shown). Although RPLA and Vero cell assay are theoretically based on different reactions, between-method correlation was demonstrated for results involving isolates carrying *stx2* and/or *stx2vha* genes. Accordingly, the Stx2 RPLA titers might be related to Stx2-mediated virulence as well as Vero cell cytotoxicity.

The genotypes of causative strain in group and family outbreaks and in sporadic cases were *stx1/stx2*, *stx2vha*, *stx2* alone, and *stx2/stx2vha*, whereas genotypes mainly found in healthy carries were *stx2vha* and *stx1/stx2vha*. People infected with *stx2*-carrying strains (*stx2* alone and *stx1/stx2* genotypes) developed HUS or bloody diarrhea with higher frequency than people with *stx2vha*-carrying strains (*stx2vha* alone and *stx1/stx2vha* genotype) (X^2= 27.6). However, in 0- to 8-year-old patients, a significant difference in development to HUS or bloody diarrhea was not recognized between *stx2*-carrying strains and *stx2vha*-carrying strains (X^2=1.1), though it was not clear because of the limited number of specimens. On the other hand, all four people infected with the isolates carrying the *stx1/stx2vha* genotype were asymptomatic, and therefore, *stx1* seems to have lower virulence for humans. We confirmed that *stx* genotype was one of the important risk factors of disease severity, and further, that pathogenicity to humans was higher for the *stx2* genotype isolates than the *stx2vha* genotype isolates, as described previously by Nishikawa et al. [13] and Friedlich et al. [6].

However, Friedlich et al. [6] have shown that *stx2c* is the only *stx2* variant associated with HUS; however, the risk of developing HUS was significantly lower after infection with *stx2c*-bearing STEC strains than after infection with *stx2*-bearing STEC strains. Eklund et al. and Friedrich et al. found that *stx2c*-bearing STEC infections can progress to HUS [5, 6]. Taken together, these results indicate that patients infected with *stx2vha* genotype strains also are at some risk of developing severe symptoms.

The pathogenicity of STEC for humans is complex and depends on the virulence characteristics of the infecting STEC strain and host factors (such as age), although Stxs are the major virulence factors. Recently, Paton et al. suggested that a novel subtilase cytotoxin, SubAB, may contribute to the pathogenesis of human disease [14]. Accordingly, the risk for developing serious symptoms should be evaluated in consideration of various factors, such as *stx* genotype, *eae* gene, enterohemolysin, subtilase cytotoxin, and age of host [5].

DNA fingerprint pattern by PFGE analysis for the 65 causative strains in 64 occurrences are shown in Fig. 1. We first used PFGE to analyze epidemiological relevancy, but then we found that PFGE identifies clusters that were highly correlated with pathogenic genotype. The STEC O157 strains in Miyazaki Prefecture were divided into four large clusters designated A, B, C, and D, with one exception. The clusters bore some relationship to *stx* genotypes. Clusters A and C mainly comprised strains with *stx2vha* genotype and cluster D mainly comprised strains with *stx1/stx2* genotype. Cluster B comprised strains with various genotypes including *stx2*, *stx2/stx2vha*, and *stx1/stx2vha*. In this way, the PFGE pattern clusters were closely associated with *stx* genotype.

stx genotype, PFGE, and clinical manifestations were well correlated with each other and were also suggested to be correlated with pathogenecity, and therefore, determining the *stx* genotype of STEC O157 might be helpful in assessing the risk of development of severe symptoms.

In the present study, one strain of the *stx2vha* genotype, which belongs to cluster C (no. 45 in Fig. 1), was associated with very high RPLA titer (and also with high Vero cell cytotoxicity, data not shown). This strain seemed to be highly pathogenic (the patient presented with bloody diarrhea), although the DNA fingerprint of this strain was similar to those of other *stx2vha* genotype strains isolated

from asymptomatic patients. Why this *stx2vha* genotype strain was associated with high RPLA titer (and Vero cell cytotoxicity) is not clear.

Only STEC O157 strains were studied for the diversity of clinical manifestation of infected people in this study. However, non-O157 strains of STEC are also related to enteritis and serious complications. In addition, Kulkarni et al. [15] indicated that co-infection with different STEC serotypes may affect clinical outcome. In the future, we plan to investigate *stx* gene subtypes, virulence-related factors, and the contribution of non-O157 serotype strains to O157-related disease.

In conclusion, based on analysis of *stx* genotype, RPLA results, and clinical manifestations, we confirmed that *stx* genotype was one of the important risk factors of disease severity and that *stx2vha* genotype was less virulent than other *stx2* genotypes, as reported previously by other researchers [5, 6]. We newly found that three factors (clusters identified by PFGE using restriction enzyme *XbaI*, *stx* genotypes, and clinical manifestations) were correlated well each other and were also suggested to be correlated with pathogenesis. Accordingly, in STEC O157 infection, determining the *stx* genotype of the bacteria might be helpful in assessing the risk of developing severe symptoms.

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