Histological examination

The localization of infused IEL cells to intestinal mucosa was assessed immunohistochemically by using the labelled streptavidin–biotin (LSAB) method. Forty minutes after infusion of CFSE-labelled IELs, the small intestine was removed and fixed in a periodate, lysine–paraformaldehyde (PLP) solution. The samples were embedded in OCT compound (Miles, Elkhart, IN, USA) before being frozen in dry ice and acetone. Cryostat sections were reacted with MoAb of factor VIII (rabbit polyclonal; Dako, Carpinteria, CA, USA) or CD34 (RAM34; PharMingen, San Diego, CA, USA) overnight at 4°C after they were incubated in 5% normal goat serum in PBS. Sections were incubated with a second antibody, rhodamine-conjugated antirat IgG antibody (Chemicon International, Temecula, CA, USA) for 1 h at room temperature. The fluorescent preparations were examined using a laser-scan microscope (Carl Zeiss, Jena, Germany) at 488 nm for CFSE and 543 nm for rhodamine.

Administration of antibodies and OVA feeding

In some experiments lymphocytes were preincubated with MoAbs, which functionally block adhesion molecules. Antibodies against αE-integrin (M290, rat IgG2a), β7-integrin (FIB27, rat IgG2a) and CD11a (M17/4, rat IgG2a) were purchased from PharMingen Co. (San Diego, CA, USA) and 1×10^7 cells were incubated in 100 µg/ml of MoAbs for 30 min before the infusion of T lymphocytes. In other experiments, anti-MAdCAM-1 MoAb (MECA367, PharMingen, 2 mg/kg) dissolved in 0·2 ml of saline was infused from a jugular vein at 30 min before the injection of T lymphocytes. As controls, isotype-matched irrelevant antibodies were also used under the same conditions.

In another set of experiments, OVA 23–3 Tg mice were fed with OVA at a dose of 200 mg/day for 3 days and the alteration of migration patterns of IEL cell lines (stimulated and unstimulated) in the villus microvessels was compared with that in unfed mice.

Statistics

All results are expressed as means ± s.d. The differences among groups were evaluated by one-way analysis of variance (ANOVA) and Fisher's post-hoc test. The cut-off for statistical significance was set at $P < 0·05$.

Results

Expression of surface antigens and characteristics of the IEL cell line

IELs from OVA 23–3 mice were stimulated with antigen-presenting cells and OVA323-339 peptide every week. One IEL line from the OVA23-3 mice was obtained several weeks after the initiation of the culture, and the growing cells were expanded and then used for analysis. Figure 1 shows the expression of surface markers of the IEL cell line obtained by flow cytometry. The cell line expressed TCRαβ and CD4 but not CD8α. We also detected a Thy1 molecule, which is a pan-T cell marker. Expression of various adhesion molecules (L-selectin, α4-integrin, αE-integrin, β7-integrin and CD11a) on the surface of the IEL-line cells was determined. As shown in Fig. 1b, the IEL line cells always showed a strong expression of αE- and β7-integrin molecules on their surface. There was also expression of CD11a. However, there was no expression of L-selectin and α4-integrin molecules in the IEL cell line. However, the extent of the expression of αE- and β7-integrin molecules was decreased significantly when these cells were stimulated with a specific antigen, OVA.

The cell line was cultured with intact OVA and ConA in the presence of mitomycin C-treated BALB/c splenocytes. Figure 1c shows the proliferation of this cell line in response to OVA and ConA as determined by [^{3}H]-thymidine uptake. OVA stimulation showed a significant proliferation of the IEL cell line at concentrations greater than 3 µM. In contrast, ConA did not induce such proliferation even at a concentration of 10 µg/ml, by which the proliferation of splenocytes is usually strongly stimulated. The unresponsiveness of the IEL line to ConA was also confirmed by microscopic observation (data not shown). Next we analysed the cytokine production by the cell line. Secretion of three different cytokines (IFN-γ, IL-4 and IL-5) was determined after stimulation with OVA. ELISA of the culture supernatants showed that the cell line produced only IL-5 ($3·0 \pm 0·60$ ng/ml; $n = 5$) without OVA stimulation. On the other hand, antigen stimulation induced all of these cytokines, and the IEL cell line secreted $88 \pm 9·2$ U/ml IFN-γ ($n = 4$), 170 ± 18 pg/ml IL-4 ($n = 4$) and $11 \pm 2·1$ ng/ml IL-5 ($P < 0·05$ *versus* without OVA; $n = 4$) after stimulation with OVA at a concentration of 10 µM.

Migration of IELs in villus mucosa

We investigated the migration of IEL line cells to the villus mucosa by observation from the mucosal side. Figure 2 shows a microscopic picture of T lymphocytes adhered to the archade microvessels of a villus tip in the ileal mucosa. As shown in Fig. 2a and c, a significant number of IEL line cells accumulated in the microvessels of the lamina propria of the villi 20 min after injection. IELs adhered to the microvessels of the villus mucosa without rolling. Some IEL cells were also observed at the base of crypt, but few IELs were present inside the submucosal venules (data not shown). On the other hand, in Peyer's patches only a few IEL cells showed 'rolling' behaviour (less than 5%), and almost no IEL cell adherence was observed in postcapillary venules of Peyer's patches during the observation, as shown in Fig. 2d. Figure 3 illustrates the time–course change in the number of sticking IEL line cells with and without activation in the villus mucosa of the OVA23-3 mice. The number of

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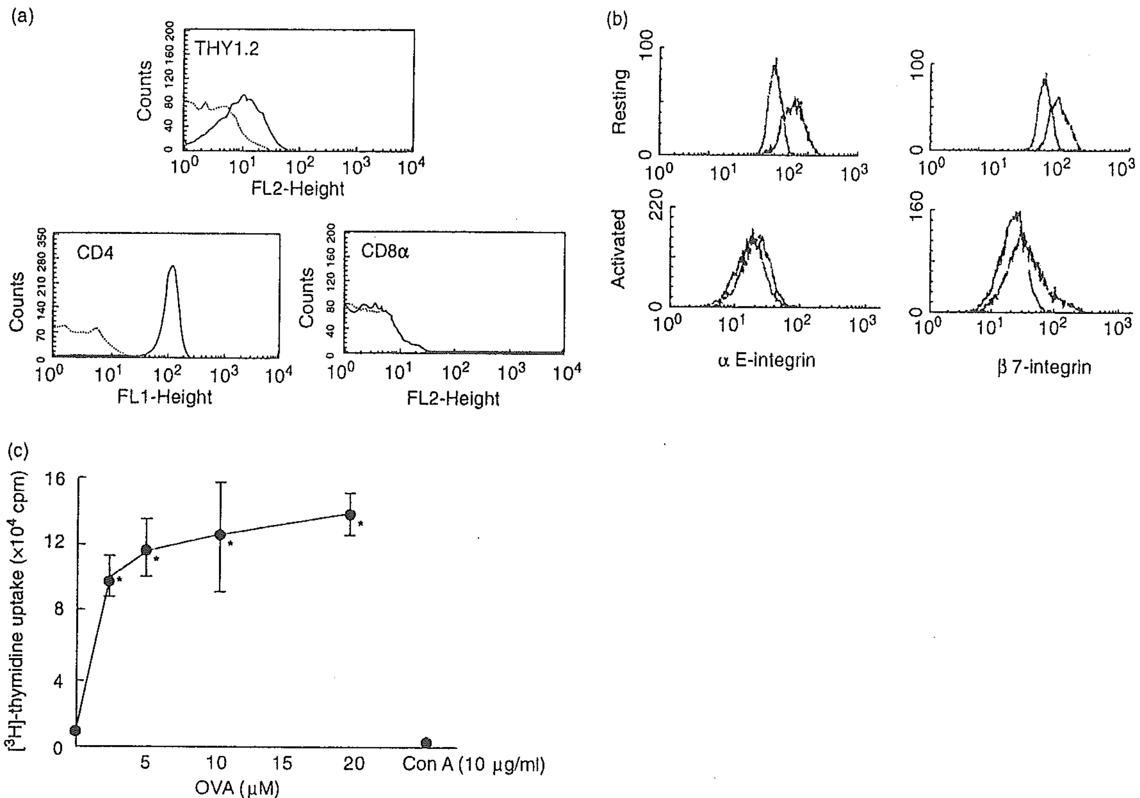

Fig. 1. Expression of surface antigens on intraepithelial lymphocyte (IEL) cell line established from ovalbumin (OVA)23-3 transgenic mice; 2×10^5 lymphocytes were first incubated with antimouse monoclonal antibodies against Thy1·2 (30-H12), pan T cell receptor (TCR) β chain (H57-597), CD4 (YTS191·1.2), CD8a (53.6.7), αE-intergin (M290), β7-integrin (Fib27), L-selectin (MEL-14) and CD11a (M17/4). They were then incubated with 1 ml of fluorescein isothiocyanate (FITC)-labelled antirat IgG and antihamster IgG. Flow cytometric analysis was performed using FACSort (Becton Dickinson). Data on viable cells, as determined by forward light-scatter intensity, were obtained using CONSORT software. Representative data from at least four individual measurements are shown. (a) Thy 1·2, CD4 and CD8α expression on resting IEL cell line (b); αE and β7 expression on resting and activated IEL cell line. For IEL cell line activation, cells were stimulated by a specific antigen, OVA (20 μM), for 20 h. (c) Antigen-specific and mitogenic proliferation of IEL cell line. Uptake of [^{3}H]-thymidine was examined as described in Materials and methods. Proliferation of IEL cell line in response to different concentrations of ovalbumin (OVA, 1–20 μM) and concanavalin A (ConA, 10 μg/ml) was determined. Values are means ± s.d. from six experiments. *$P < 0·05$ *versus* OVA 0 μM.

IEL cells accumulated in the microvessels of the villus tips increased rapidly, especially within the first 10 min, reached a maximum at 20 min, and then showed no significant change during the observation. When the IEL cells were activated with OVA, the number of adherent cells was significantly smaller compared to that of resting cells at any given time. However, the total number of IEL cells that had entered villus microvessels did not differ significantly between with and without activation in the OVA23-3 mice (control, 22·4 ± 3·1/min; with activation, 19·1 ± 3·8/min). To confirm whether injected IEL cells were within the epithelium of the villi, we examined the tissue section of intestinal mucosa 40 min after the administration of CFSE-labelled cells. Figure 4 shows the adhesion site of infused IELs and the location of Factor-VIII$^+$ or CD34$^+$ microvessels. It has been demonstrated that these cells coincided well with the lamina propria microvessels of ileal villi.

Figure 5 shows the inhibitory effect of the function-blocking of adhesion molecules on the sticking of unstimulated IEL line cells to archade microvessels of the villus mucosa at 20 min. The number of sticking lymphocytes in the control group was 24·0 ± 2·1 cells/mm^2, but this number decreased significantly as a result of pretreatment with MoAb, which blocks β7-integrins. Preinfusion of an anti-MAdCAM-1 antibody into the mice also significantly inhibited this cell interaction. However, the inhibitory effect of these antibodies was found to be partial, and more than 50% of lymphocytes remained adherent after administration of antiβ7 and anti-MAdCAM-1, respectively. Moreover, the combined blocking of β7-integrin with MAdCAM-1 further attenuated

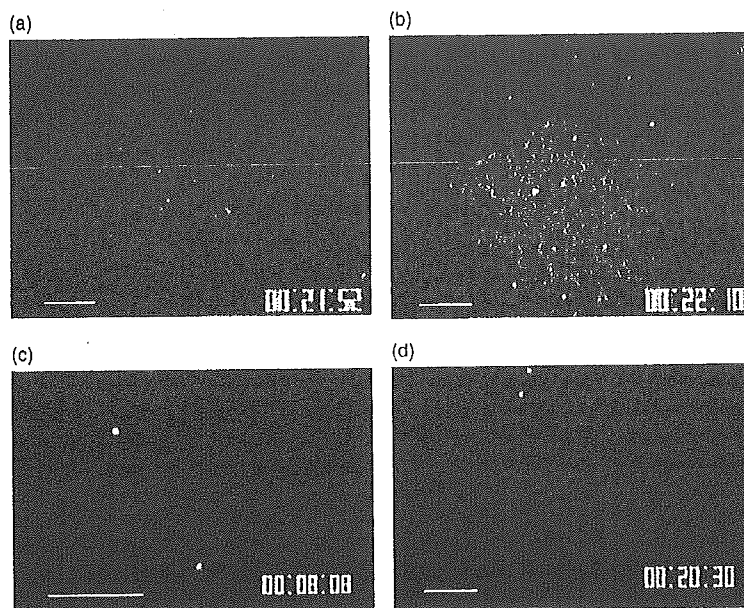

Fig. 2. Representative images of the distribution of a carboxyfluorescein diacetate succinimidyl ester (CFSE)-labelled resting intraepithelial lymphocyte (IEL) cell line in control mice (a) and in ovalbumin (OVA)-fed mice (b) Following adherance to the microvessels of a villus tip of the ileal mucosa at 20 min after infusion (× 10). Bar represents 100 µm. (c) Higher magnification image of labelled IEL cell line in control mice adhered to arcade microvessels of villus tips (× 20). Bar represents 100 µm. (d) Observation of CFSE-labelled resting IEL cell line postcapillary venules of Peyer's patches 20 min after infusion. There were few sticking IELs in this area (× 10).

the sticking of IELs in this area, although it only partially blocked the IEL adhesion. On the other hand, antibodies against either αE-integrin or CD11a did not significantly inhibit the IEL accumulation.

Figure 6 compares the number of IEL cells that adhered to the microvessels of the villus tips in the OVA23-3 control mice to that in the OVA-fed mice 20 min after the injection. Figure 2b shows a microscopic picture of resting IELs adhered to the microvessels of the villi in the ileal mucosa of the OVA-fed mice. Although the number of resting IEL cells increased gradually in the microvessels of the OVA-fed mice, showing a time-course change similar to that in the control mice (data not shown), the number of sticking cells was significantly lower than that in the control transgenic mice. Similarly, in the case of stimulated IELs, only a small number of activated IELs were observed in the microvessels of the OVA-fed mice and this number did not increase significantly during the observation (Fig. 6).

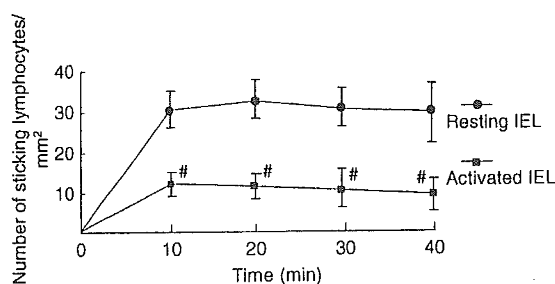

Fig. 3. Time-course change in the number of sticking intraepithelial lymphocyte (IEL) line cells to villus mucosa in control ovalbumin (OVA)23-3 mice. The number of cells accumulated in the microvessels of villus tips is compared between unstimulated (resting) and activated (stimulated with OVA, 20 µM) IEL cell lines. The lymphocytes located in the 1-mm² observation field were counted. #$P < 0 \cdot 05$, compared with resting IELs. Values are means ± s.d. for six animals.

Discussion

In the present study, we established an Ag-specific IEL line from OVA23-3 TCR-Tg mice. To our knowledge, this is the first report on the establishment of an antigen-specific IEL line. This is because there has been no suitable method to induce expansion of IEL clones, which would respond to a specific antigen, as no appropriate antigens could be found to stimulate IELs. In this study we used Tg mice whose TCR restriction and specific ligands are well known and we also used, instead of an intact antigen, an OVA323-339 peptide which can cause significant stimulation of the culture at an early stage. The cell line established from OVA23-3 expressed Thy1·2, TCR αβ and CD4, but not CD8α. In our preliminary study, we also observed that $CD4^+$ IELs freshly isolated from the Tg mice proliferated more strongly than $CD4^-$ IELs. These results suggest that in OVA23-3 mice, $CD4^+$ CD8 IELs have the capacity to proliferate selectively for a long time. This preferential selection of $CD4^+$ IELs may be due to the TCR in these Tg mice being restricted to $I\text{-}A^d$, major histocompatibility complex (MHC) class II molecules [15].

The cell line expressed TCR αβ and CD4 in a way similar to that of peripheral T cells, and did not express TCRγδ and

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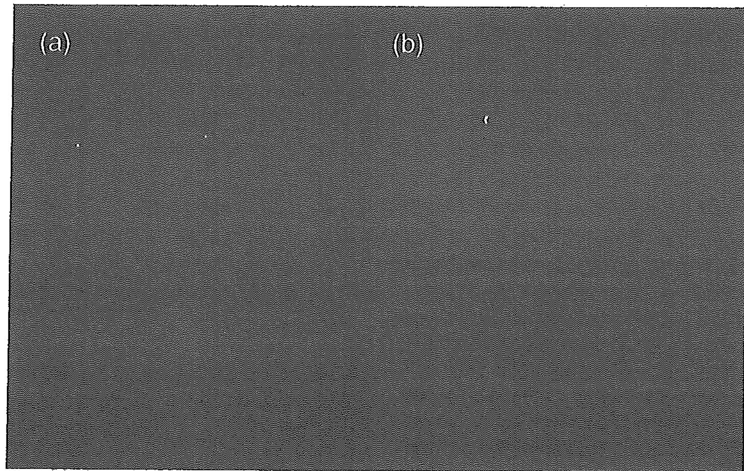

Fig. 4. Representative pictures of simultaneous observation of carboxyfluorescein diacetate succinimidyl ester (CFSE)-labelled intraepithelial lymphocyte (IEL) (green) and factor VIII-positive (a) or CD34-positive (b) microvessels (red fluorescence) in small intestinal villi as determined by immunohistochemistry. The lysine–paraformaldehyde (PLP)-fixed sections 40 min after IEL infusion were observed (× 100).

CD8αα, which are the more common phenotypes of IELs. However, the cell line also expressed αEβ7 integrin, which is a characteristic adhesion molecule expressed in 80–90% of IELs. We also showed that the cell line could not respond to ConA, in spite of its proliferative capacity to specific Ag. These observations suggest that the IEL cell line we established does, in fact, possess the characteristics specific to IELs and that these characteristics differ from those of peripheral T cells. Note also that we used a cell line with some characteristics of mature IELs for demonstrating possible mechanisms of localization to the intestinal villi, whereas we do not know what the characteristics of the circulating IEL precursor is. Therefore, there is a possibility that the circulatory precursor could express undiscovered adhesion molecules which are down-regulated on mucosal entry.

The ELISA of culture supernatants showed that this cell line can produce IFN-γ, IL-4 and IL-5. The secretion of these cytokines was dose-dependently stimulated by a specific antigen. $CD4^+$ T cells have largely been grouped into two distinct subsets, Th1 cells producing IL-2 and IFN-γ, and Th2 cells producing IL-4 and IL-5 [23]. It appears that IEL cell lines produce both types of cytokines, suggesting that these

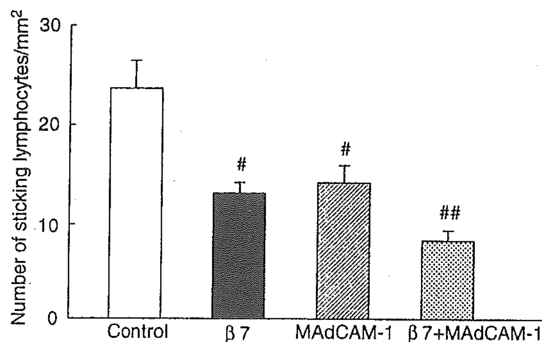

Fig. 5. The inhibitory effect of function-blocking of adhesion molecules on the sticking of resting intraepithelial lymphocyte (IEL) line cells to microvessels of villus mucosa at 20 min. The effect of monoclonal antibody against β7-integrin (Fib27) and MAdCAM-1 (MECA367)-treatment on sticking of IELs was investigated. IELs were treated with monoclonal antibody (100 μg/ml) against β7-integrin before infusion. In some experiments, the animals were pretreated (30 min before lymphocyte infusion) with a monoclonal antibody against MAdCAM-1 (2 mg/kg). The combined effect of functional blocking of β7-integrin and MAdCAM-1 molecules was also examined. $\#P < 0{\cdot}05$, compared with controls. $\#\#P < 0{\cdot}05$, compared with β7-integrin- and MAdCAM-1-blocking alone. Values are means ± s.d. for six animals.

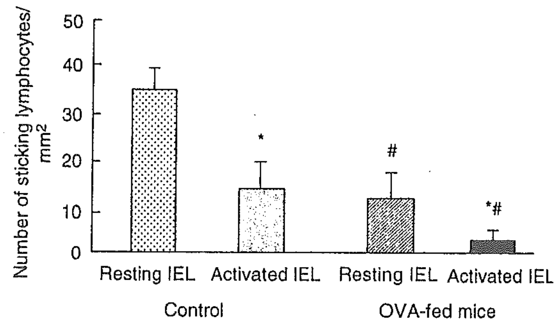

Fig. 6. Comparison of the number of intraepithelial lymphocyte (IEL) line cells adhered to the microvessels of villus tips in ovalbumin (OVA)23-3 control mice with that in OVA-fed mice, 20 min after injection. OVA 23-3 transgenic mice were fed with OVA at a dose of 200 mg/day for 3 days (OVA-fed mice), and the number of adhered IEL cell lines (resting and activated) was compared with that in unfed mice (control). $\#P < 0{\cdot}05$, compared with unfed controls. Values are means ± s.d. for six animals. $*P < 0{\cdot}05$, compared with resting IELs.

cell lines consist of a mixed population of Th1 and Th2 helper T cells. This is in accordance with the previous finding by Fujihashi *et al.*, who reported that $CD4^+$ IELs produced IL-4, IL-5 and IFN-γ [24].

In the present study, we have demonstrated that there is a significant accumulation of IEL line cells in the villus mucosa of the small intestine, not in the HEVs of Peyer's patches. We have reported previously that freshly isolated IELs from the intestinal mucosa of BALB/c mice showed very little interaction with Peyer's patch–HEVs, due possibly to a lack of L-selectin and weak expression of α4-integrin molecules [18], which is similar to the present results. In this study, accumulation of IEL line cells was observed along the archade microvessels near the epithelial cells of villus tips. This adhesion was significant, and was abrogated partially by antiβ7-integrin and by anti-MAdCAM-1 antibodies. Because the involvement of α4-integrin in IEL migration was almost negligible in this study, we do not know the exact reason for the paradoxical finding that migration is not inhibited by an anti-αE integrin, but is inhibited by anti-MAdCAM-1. Several studies have shown that αEβ7 is not a ligand for MAdCAM-1, and αEβ7 is not believed to participate in lymphocyte–endothelial cell interaction in the vascular endothelium [25,26]. Instead it has been shown that αEβ7 mediates adhesion of lymphocytes to epithelial cells [4,5], and it is also speculated that this integrin may be involved in the migration of lymphocytes to epithelial sites [27]. Therefore, there is a possibility that β7 and MAdCAM-1 could be functioning separately in this situation, although the counter ligands for β7-integrin or MAdCAM-1 in IEL interaction with the villus mucosa remain to be identified. An additive inhibitory effect of antiβ7-integrin and anti-MAdCAM-1 antibody on IEL adhesion could support this possibility, although there is another possibility that each antibody was not completely neutralizing. Moreover, it should be also noted that even the combination of both antiβ7 integrin and anti-MAdCAM-1 antibodies reduced the IEL adherence to microvessels of villi to only about 65% of what it was in the controls, suggesting that other mechanisms account for lymphocyte migration in this site. These other mechanisms may include the G-protein-independent mechanisms, such as capillary plugging.

In this study, we demonstrated that the pattern of IEL migration to the villus mucosa changed significantly after stimulation by a specific antigen. When the IEL line cells were activated with OVA, the adhesion of the antigen-stimulated IELs to the villus mucosa was significantly attenuated compared to that of the resting cells, with decreased surface expression of both αE- and β7-integrins. Because we found that an antiβ7-integrin antibody decreased the adherence of IEL line cells to villus microvessels, while an anti-αE antibody did not, the decreased cell surface expression of β7-integrin might be responsible for the decreased migration of antigen-stimulated IELs to the intestinal mucosa. One can speculate that changes in the expression of adhesion molecules could also interfere with the IEL interaction and migration to intestinal epithelial cells. We also demonstrated that in Tg, OVA-fed mice, the number of IEL line cells that accumulated in the villus mucosa was significantly lower than that in the non-fed control mice both in terms of resting and activated cells. In particular, in the case of antigen-activated IELs, the cell migration into the villus mucosa in the OVA-fed mice was almost completely eliminated. The exact mechanism of this inhibition is not known, but there is a possibility that factors other than adhesion molecules, including neurohumoral factors and chemokines, can also be involved in this inhibition. The immune hyporesponsiveness following an oral administration of an antigen is dose-dependent, and high-dose (200 mg) feeding of OVA in Tg-mice could lead to clonal anergy or clonal deletion [28,29], although in a Tg mouse model T cells specific to certain determinants on a self-antigen have been found to be less susceptible to tolerance [30]. The reduction of antigen-reactive T cells in lymphoid tissues was accompanied by a marked increase in the percentage of apoptotic cells following an antigen feeding [28]. These shut-down effects of antigen-activated IEL migration to the antigen-sensitized villus mucosa might be due to the early elimination of these infused cells from the circulating population. However, we found that the total influx of IEL line cells to the villus mucosa did not decrease compared to that in the controls.

In this study we have shown decreased migration of IELs to the intestinal mucosa after antigen activation, which can alter the population of these antigen-reactive T cells in the villus mucosa. The significance of the decreased migration of antigen-activated IELs and their exact role in allergic conditions and oral tolerance are subjects for future investigation.

Acknowledgements

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FULL PAPER *Immunology*

Effects of Sugar Cane Extract on the Modulation of Immunity in Pigs

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ABSTRACT. The experiment was aimed to test the efficacy of sugar cane extract (SCE) on the modulation of pig immunity under field conditions. The SCE preparation consisted of sugar cane extract (20%) and oilcake of rice bran (80%). SCE (500 mg/kg of body weight per day) was fed to weanling pigs on 3 consecutive days per week for 4 weeks. The results showed a significant enhancement of cytotoxicity of natural killer (NK) cells and phagocytosis by neutrophils and monocytes, compared to untreated pigs. The enhancement of NK cell function may have protected against porcine reproductive respiratory syndrome (PRRS), as there was a reduction in seroconversion rates in treated pigs. Moreover, SCE-treated pigs showed a 7.87% growth enhancement compared with untreated controls. Thus SCE produces an immunostimulative effect on porcine innate immunity that may provide protection against pathogens.

KEY WORDS: growth promotion, immunomodulation, leukocyte function, pig, sugar cane extract.

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Sugar cane extract (SCE) is a natural product which has displayed a wide range of biological effects including immunostimulation [8], anti-thrombosis activity [17], anti-inflammatory activity [14], vaccine adjuvant [7], anti-oxidant activity [18, 21], modulation of acetylcholine release [2], and anti-stress effects [3]. Study on mice inoculated with a minimum lethal dose of pseudorabies virus following with 3 consecutive days of oral administration with SCE showed a significant decrease in mortality (Koge, unpublished). Other studies have indicated SCE can enhance resistance to secondary bacterial infection [1, 19]. Studies on chickens indicate that SCE has an adjuvant effect on the activation of antibody and cell-mediated immune responses and provides a protective effect against *Eimeria tenella* infection [6]. These results suggest that SCE may positively regulate host natural immunity against viral, bacterial, and protozoal infections, via effects on the levels of macrophages, neutrophils and natural killer (NK) cells.

Porcine respiratory disease complex (PRDC) has become the major problem in the most intensive pig farms, causing massive economic loss. The causing factors of PRDC involve a complicated synergic interaction between host immunocompetence, polymicrobial infections and various stressors [23]. Pseudorabies, influenza virus, porcine reproductive respiratory syndrome (PRRS) virus, and *Mycoplasma hyopneumoniae* have been thought as primary agents of PRDC [10, 23]. However, antibiotics are commonly used to treat or prevent secondary bacterial infections of PRDC. As the abuse of antibiotics in veterinary medicine has been of particular concern from the viewpoint of public health, major strategies against infection are to improve the managerial systems and environmental conditions and to modulate host immunocompetence. Although studies of SCE on mice and chickens have shown enhancement of host defenses, the activity of SCE on the pig immune system and the efficacy of SCE applied in field conditions is still not documented. Therefore, the aim of this experiment was to investigate the effect of SCE on pig natural immunity, particularly on the function of neutrophils, monocytes, and NK cells. The efficacy of SCE application in farmed pigs was also evaluated.

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MATERIALS AND METHODS

Sugar cane extract (SCE): Shin Mitsui Sugar Co., Ltd., Japan has prepared four kinds of sugar cane extracts. Extract 1 from sugar cane juice consists of components adsorbed to a synthetic adsorbent resin. Extract 2 consists of volatile components from sugar cane juice adsorbed to a synthetic adsorbent resin. Extract 3 from sugar cane bagasse is obtained by hot water extraction. Extract 4 consists of crude protein (16.9%), fat (0.5%), ash (36.1%) and nitrogen-free extracts (46.5%) [8], prepared from sugar cane juice by chromatographic separation on an ion exchange column. Extract 4 was concentrated to ca. 40% solids containing ca. 4% sugars (glucose, fructose and sucrose). In this study, Extract 4 was used as SCE. SCE for oral administration was prepared by adsorption of Extract 4 to oilcake of rice bran, which was then dried. The ratio of SCE without glucose, fructose and sucrose: oilcake of rice bran was 1:4. Table 1 shows the composition of this material.

Experimental pigs, SCE administration, and sampling:

Table 1. Composition of sugar cane extract (Extract 4) for feed material

Data-item	% of sample	% of solid	Remarks
Moisture	6.7	–	135°C, 2 hr
Crude protein	18.5	19.8	Kjeldahl method
Crude fat	4.5	4.8	Diethyl ether extraction
Crude fiber (Insoluble fibers)	7.0	7.5	Filtration method
Ash	17.5	18.8	Direct incineration
Nitrogen-free extracts	45.8	49.1	*
Total	100.0	100	

*=100–(moisture + crude protein + crude fat + crude fiber +ash).

The experimental pigs for the kinetic study were purchased from a small farrow-to-finish pig farm that had been monitored for several years and had a history of good performance (>20 pigs/sow/year in the previous three years). Weanling pigs were raised under controlled conditions prior to and during the kinetic study. To test the effect of duration of SCE administration, pigs (N=5) were weighed and fed with SCE (500 mg/kg of body weight/day) for 1, 3, 5, or 7 consecutive days. To test the dose effect, 500, 1,000, 1,500 or 2,000 mg/kg of body weight/day was administered in feed for three consecutive days. Pigs fed with no SCE additive served as controls. Functional assays of leukocytes were conducted on day 4.

The effect of SCE on weanling pigs in the field was conducted on a farrow-to-finish pig farm of about 300 sows and 2,700 fattening pigs. The pig farm had a mild to moderate severity of PRDC. In this experiment, 180 weanling (five-week-old) pigs were randomly allocated into two groups, control (untreated, N=90) and SCE-treated (N=90), and moved into the isolated nursery unit. Ten pigs in each group were ear tagged for blood sampling. Pigs in the SCE-treated group were treated with SCE (500 mg/kg of body weight per day) in feed for three consecutive days per week for four weeks. The administration of SCE in feed was adjusted every two weeks, depending on the increasing body weight. Assays for leukocyte functions were performed at 1, 2 and 4 weeks post SCE administration. The growth performance, mortality, and the frequency of therapy in those pigs were recorded.

PRRS antibody detection: The presence of PRRS antibody in the control and SCE-treated pigs in the field test was assayed in 9-week-old pigs using a PRRS ELISA antibody kit (IDEXX Laboratories, Inc. U.S.A.), according to the manufacturer's instructions.

Leukocyte preparation: Blood samples were drawn from the jugular vein into heparinized tubes. Total white blood cell (WBC) was counted with an electronic haematology counter (Sysmex F-800). Heparinized blood was sedimented with 2% dextran in phosphate-buffered saline (PBS). After 15 min sedimentation, the upper layer of supernatant was separated by Ficoll-Paque (Pharmacia Biotech) gradient to obtain peripheral blood mononuclear cells (PBMCs) as previously described [11]. PBMCs were counted and the viability was determined by trypan blue exclusion method. PBMCs were suspended and adjusted to 1×10^7 cells/m*l* in RPMI 1640 medium (Gibco BRL, life Technologies, Inc.) containing 100 U/m*l* penicillin, 100 μg/m*l* streptomycin (Sigma, St. Louis, MO), and supplemented with 10% heat inactivated fetal bovine serum (Gibco BRL, Life Technologies, Inc.) and 2 mM L-glutamine (complete media; CM).

NK cells cytotoxicity: The assay was performed using a time-resolved fluorometer (TRF) system as previously described [11]. Briefly, the target cells, human erythromyelocytic leukemia cell line (K562) growing in logarithmic phase, were harvested and labeled with 2 μl of enhancing ligand bisacetoxymethyl 2,2': 6', 2"-terpyridine- 6,6"-dicarboxylate (BATDA) (Wallac Labelling Service) according to the manufacturer's instructions. Effector cells were obtained from PBMC after depletion of adherent cells in culture flasks for 30 min. The effector to target ratio was set at 100:1 and the mixture was incubated at 37°C in a 5% CO_2 incubator for 4 hr. Cytotoxic activity was calculated by the release of the fluorescent dye that had been chelated with europium (EuTDA) from dead target cells. The percent specific release of fluorescence of EuTDA was measured in a time-resolved fluorometer (1234 DELFIA, Wallac, Turku, Finland) and calculated as: [(Experimental release - spontaneous release)/(maximum release - spontaneous release)] × 100%. Throughout the experiment, counts of spontaneous release were ranged between 18% and 25%.

Phagocytosis by monocytes and neutrophils: Phagocytosis by monocytes and neutrophils was performed in whole blood and assayed by flow cytometry. A stock of FITC (fluorescein isothiocyanate) -labelled *Salmonella choleraesuis* (SC; ATCC 10743) stored in 20% glycerol (4×10^9 CFU/m*l*) was resusciated and opsonized with antiserum containing polyclonal antibody to *Salmonella*. One hundred microliters of blood was incubated with 25 μl of SC in a microtube (Bibby Sterilin Ltd, England; 50 × 6 mm round base) (leukocyte:bacterium ratio=1:25) at 37°C for 30 min. After incubation, RBCs were lysed in a buffer containing 0.899% ammonium chloride, 0.1% potassium hydrogen carbon and 0.0037% disodium EDTA in distilled water (DW) then washed in chilled FACS washing buffer (PBS containing 0.1% (w/v) bovine serum albumin and 0.01% (w/v) sodium azide). Cells were fixed in 1% paraformaldehyde in PBS and stained with monoclonal antibody (74–22–15;

ATCC) to SWC3, a surface molecule of phagocytes, followed by goat anti-mouse IgG-phycoerythrin (PE) conjugate, $F(ab')_2$ (1:100; Sigma Chemical Co.). Ten thousand PE-positive cells were collected by flow cytometry (Becton Dickinson Immunocytometry system; BDIS) using a CellQuest software (BDIS). Cell populations enriched for neutrophils and monocytes were gated on a side scatter (SSC) and SWC3 (FL-2) scatter plot. The background control was set on phagocytes which had not been fed bacterium. The percentage of FITC-positive cells (FL1) and mean fluorescence were measured against a 1% positive cell gated on background control. The phagocytic activities of cells were expressed as a phagocytic index (PI) calculated as: [(% positive × mean channel fluorescence) / 100].

Statistical analysis: Statistical analyses of the data were calculated using analysis of variance (ANOVA) and Duncan's multiple-range tests with *P* value of <0.05 being used to determine significance.

RESULTS

Kinetic effects of SCE administration on leukocyte function: As data on the effect of SCE in pigs is lacking, the kinetic effect of SCE dose (500, 1,000, 1,500, and 2,000 mg/kg/day) and duration (1, 3, 5, and 7 consecutive days) of SCE administration were evaluated in pigs in a controlled environment. The results showed that both the cytotoxic activity of NK cells and phagocytosis by monocytes were significantly increased in pigs treated with SCE for 1, 3, 5, and 7 days, compared with untreated control pigs (p<0.05). The duration of SCE administration did not produce any significant differences (Fig. 1A, 1B). Enhancement of phagocytic activity was not noted in neutrophils (p>0.05) (Fig. 1C).

To further understand the effect of dose of SCE, a preliminary study was conducted on pigs in a controlled environment. Five-week-old pigs were fed with different dosages of SCE (500 mg, 1,000 mg, 1,500 mg, or 2,000 mg/kg of body weight per day) for 3 consecutive days. Leukocyte function was assayed on day 4. The results showed a significant enhancement of NK cell cytotoxicity in those pigs fed with 500 or 1,000 mg SCE/kg/day compared with controls (p<0.05). However, the enhancement of NK cell function was not seen in pigs fed with high dosages of SCE (1,500 mg/kg and 2,000 mg/kg) (Fig. 2A). Phagocytic function of monocytes was significantly enhanced in pigs fed with SCE at a dose of 1,000 mg/kg (p<0.05), but not in pigs fed with SCE at doses of 500, 1,500 or 2,000 mg/kg/day (p>0.05) (Fig. 2B). Meanwhile, the administration of different doses of SCE (500 mg/kg, 1,000 mg/kg, or 1,500 mg/kg) did not cause any changes in phagocytosis by neutrophils. Controversially, there was a significant decrease (p<0.05) in phagocytic function of neutrophils in pigs fed with high dose of SCE (2,000 mg/kg), relative to control pigs (Fig. 2C).

Efficacy of SCE administration on immunomodulation under field conditions: Based on the results of kinetic study at a commercial pig farm, weanling pigs were fed with 500 mg/kg/day of SCE, and effects on innate immunity were evaluated. The cytotoxicity of NK cells in pigs fed with SCE for 1, 2, and 4 weeks was significantly enhanced compared to control groups (p<0.05) (Fig. 3A).

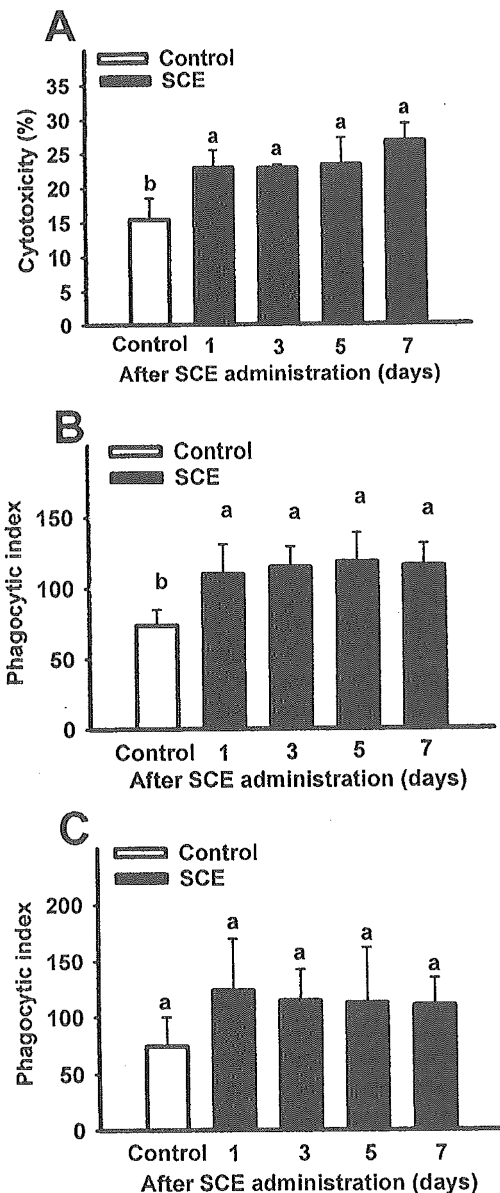

Fig. 1. Effect of duration of SCE administration on leukocyte activity. Five pigs in each group were fed with SCE (500 mg/kg/body weight/day) for 1, 3, 5, and 7 days. Leukocyte activity assays, including cytotoxicity of NK cells (A), phagocytosis by monocytes (B) and neutrophils (C), were performed at the same days. Different superscripted letters indicate a significant differences (p<0.05) between the control group (open bar) and the SCE-treated group (solid bar).

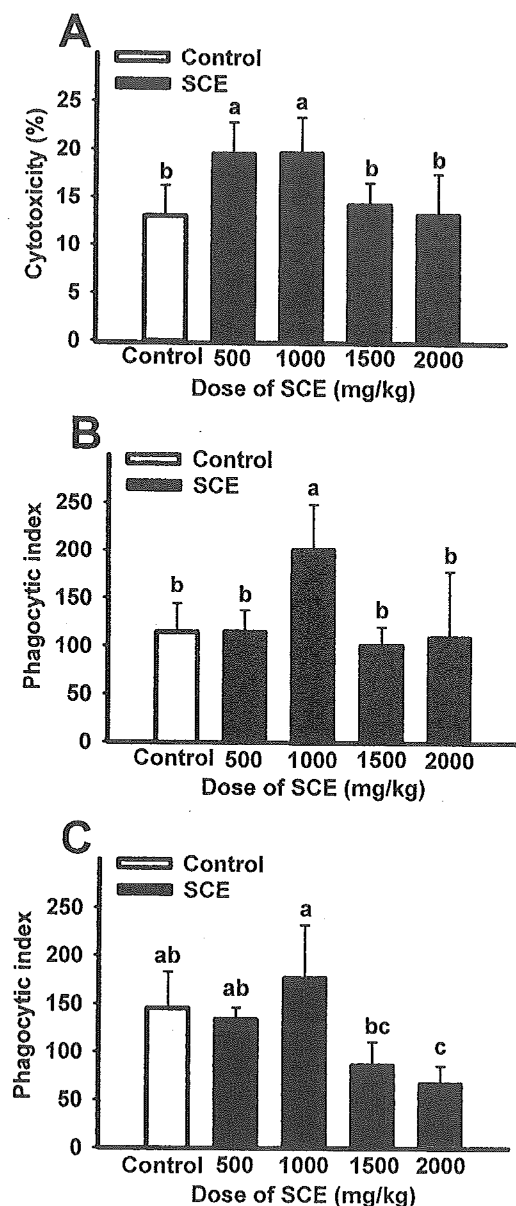

Fig. 2. Effect of SCE dose on leukocyte activity. Five pigs in each group were fed with different dosages of SCE (0–2,000 mg/kg/body weight/day) for 3 consecutive days. Leukocyte activity assays, including cytotoxicity of NK cells (A), phagocytosis of monocytes (B) and neutrophils (C), were performed at day 4. Different superscripted letters indicate a significant differences ($p<0.05$) between the control group (open bar) and the SCE-treated group (solid bar).

The phagocytic activity of monocytes from pigs fed with SCE for 2 and 4 weeks showed a 58.24% and 49.55% increase, respectively, which was significantly different from controls ($p<0.05$) (Fig. 3B).

Analyses of phagocytic activity of neutrophils, showed an increase in pigs fed with SCE for 2 weeks (76.46% increase) and 4 weeks (49.32% increase), similar to the trend seen in monocytes (Fig. 3C).

Anti-viral effect of SCE under field conditions: To address whether SCE can lead to a restriction of viral replication by enhancing NK cell activity, the effect of SCE on infection by PRRS virus was evaluated. As the trial farm had been contaminated with PRRS virus, piglets would be exposed to the virus and develop antibody later. In this experiment pigs had not received PRRS vaccine. Weanling pigs were fed with SCE (500 mg/kg/day) for 3 consecutive days per week for 4 weeks, and blood samples were collected for PRRS antibody detection. Pigs fed with SCE showed 8.33% seroconversion, which is much lower than the 25% seroconversion rate observed in control pigs (Fig. 4). However, the prevalence of PRRS antibodies did not differ between the treated and control groups.

Effect of SCE on growth and health: Growth performance including morbidity, mortality and body weight was evaluated between 5 and 14 week-old pigs. Two pigs in each group were culled due to diarrhoea, dermatitis or respiratory distress. There was no obvious difference in morbidity and mortality between the SCE-treated and the control groups. However, the average body weight of SCE-treated pigs increased by 7.87% compared with the control group, but this difference was not statistically significant ($p>0.05$) (Fig. 5).

DISCUSSION

Some plant extracts and probiotics have been found to have a wide range of physiological functions. Those products have been widely used as supplements in animal foods to boost innate immunity against infections [9, 15, 24]. By-products of sugar production from sugar cane have been reported to have a wide range of biological activities [12, 14, 17, 22], especially antioxidative activities, phylactic activities, and other physiological functions [1, 18, 19, 21]. Protective phylactic effects against viral and bacterial infections could be exploited to reduce the use of antibiotics in the pig industry. The results reported here, showing that SCE induces an up-regulation of leukocyte functions, highlights the potential benefits of SCE to the pig industry.

In the chicken model, a dose of SCE between 500 and 1,500 mg/kg of body weight produces adjuvant and phylactic effects against viral, bacterial, and protozoal infections [6–8]. Decreasing the dose of SCE may reduce the protective effect against infections. The administration of SCE in pigs has not been documented previously. Our results demonstrated a significant effect on NK cell cytotoxicity and the phagocytic activity of monocytes at 500 and 1,000 mg/kg/body weight of SCE. However, in contrast to studies on chickens, no significant enhancement of phagocytic activity of neutrophils was observed in pigs ($p>0.05$) [8]. The lack of effect of SCE on neutrophil activity in this study may be due to sample sizes, species differences between pigs and chickens, and high variation in phagocytic activity of neu-

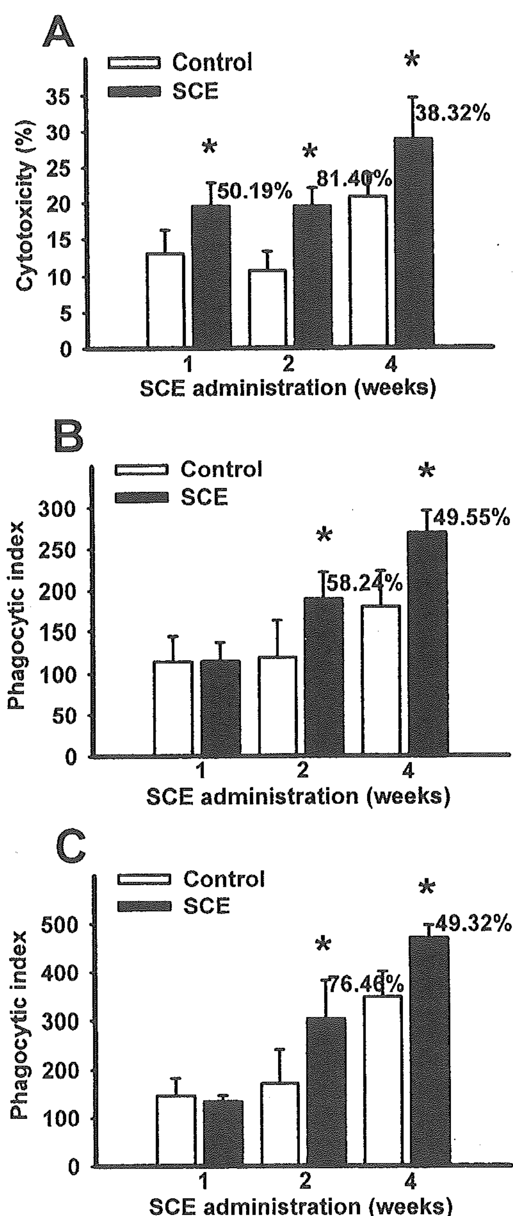

Fig. 3. Effect of prolonged treatment with SCE on leukocyte function. Weanling pigs were fed with SCE (500 mg/kg/ body weight/day) for 3 consecutive days per week for 4 weeks. Pigs fed without SCE served as controls. Leukocyte activity assays, including cytotoxicity of NK cells (A), phagocytosis of monocytes (B) and neutrophils (C), were performed at week 1, 2 and 4. Asterisk (*) indicates a significant difference ($p<0.05$) between the untreated control group (open bar) and the SCE-treated group (solid bar).

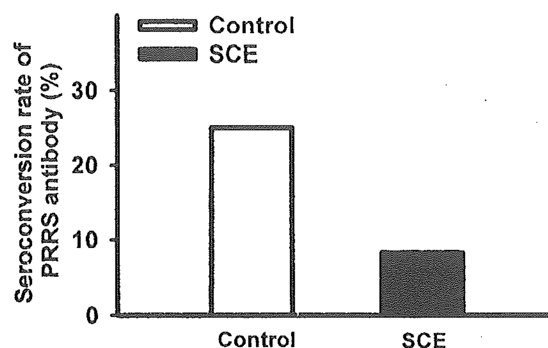

Fig. 4. Effect of SCE on PRRS virus infection. Weanling pigs were fed with SCE (500 mg/kg/day) for 3 consecutive days per week between the ages of 5 and 9 weeks. Blood samples were collected at the end of week 9. The presence of PRRS antibody was assayed using an ELISA kit.

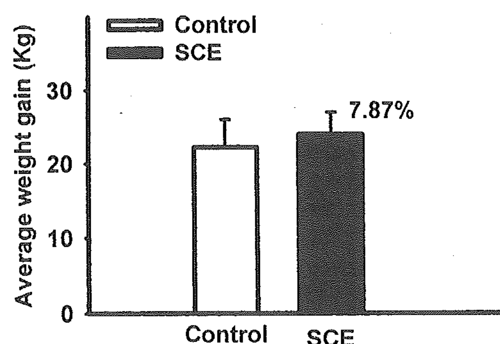

Fig. 5. Evaluation of SCE-treatment on growth performance of pigs. SCE was administered for 3 consecutive days per week between the ages of 5 and 9 weeks, and the body weight was measured in 14-week-old pigs. There was a 7.87% increase in growth in the SCE-treated group compared to control group, but this was not statistically significant ($p>0.05$).

trophils in pigs [5]. Moreover, between pigs and chickens, phagocytosis by porcine neutrophils may be positively regulated with a longer treatment, as noted under field conditions. The enhancement of leukocyte function in pigs fed with SCE at dosages of 500 and 1,000 mg/kg body weight, but not with 1,500 and 2,000 mg/kg body weight, suggests that some functions may decrease with high dosages. The reason behind this is not clear, however similar phenomena have been observed in other studies. High dosages of β-glucan caused overstimulation of prostaglandin E production and down-regulation of β-glucan receptor expression, resulting in the inhibition of phagocytosis [13]. High doses of another immunostimulant from *Ganodema lucidum* caused a decrease in leukocyte function [15]. Moreover, if SCE contains high levels of mannan, high dosages of the preparation may competitively bind the mannan receptor leading to down regulation of phagocytosis by macrophages and the production of IL-12, TNF-α and IFN-γ by lymphocytes [20]. Therefore, an appropriate dose and duration of SCE administration may be important for the efficient enhancement of porcine leukocyte function.

As weanling pigs face the gradual decay of maternal antibodies, there is increased risk of viral infection, particularly PRRS virus or porcine *circovirus* infection, which may persist in nursery units. The pilot studies showed that SCE has an immunostimulative effect at particular dosages, and under field conditions the mortality and morbidity rates in both control and SCE-treated groups were low, possibly due in part to depopulation and sanitation of the nursery before this experiment. It seems logical that good managerial and sanitary procedures are among basic requirements for disease control. Although leukocyte functional assays also confirmed that SCE could significantly enhance innate immunity including NK cells, monocyte and neutrophil functions at 2 weeks post SCE administration, phagocytosis by neutrophils and monocytes had not increased by 1-week post SCE administration. Variation among individuals, the presence of stressors, and managerial factors in field conditions may have influenced this result. Moreover, the cytotoxicity of NK cells of pigs at 4 weeks post experiment in both SCE treated and untreated control pigs was greatly enhanced compared with cytotoxicity at 1 and 2 weeks post-treatment. The increase in cytotoxicity may relate to increasing age as has been previously reported [11]. The significant enhancement of innate immunity after SCE stimulation may relate to cytokine release from activated leukocytes, in turn amplifying leukocyte functions [13, 16, 20].

PRRS has been thought of as an important primary pathogen in PRDC [23]. The serological and pathological data show a high prevalence of PRRS antibodies in most pig farms in Taiwan [4]. This may reflect exposure to PRRS virus under nursery conditions. As levels of cytotoxicity of NK cells are consistently increased after administration of SCE, the anti-viral activity of SCE towards PRRS is of particular interest. Both groups of experimental pigs were raised in the same pig house and exposed to the same air conditions. The seroconversion rate was greatly decreased in SCE-treated pigs. A repeated experiment at another pig farm also showed similar results (data not shown), confirming the antiviral effect of SCE in pigs. This suggests that the administration of SCE can enhance NK cell activity against early PRRS virus infection, and may contribute to decreasing the incidence of PRDC in pig farms.

Pigs may be gradually infected with PRRS virus and other pathogens at the nursery stage and develop antibodies later, as discussed above. Therefore, to evaluate the influence of natural virus infection, the effect of SCE on growth performance was evaluated at the end of the experiment, being week 14. In the farmed pigs, SCE-treated animals displayed 7.87% enhancement of growth compared to control pigs, but this was not a statistically significant difference. Molasses has been widely used in feed to improve pig appetite and SCE also shows a similar taste improvement that may contribute to increasing pig growth rate. Additionally, the reduction of infections and severity of pulmonary lesions after SCE administration may have further contributed to improving growth. A similar improvement of growth has been reported in chickens fed SCE through the positive regulation of host natural immunity against bacterial and protozoal infections [6, 8].

In conclusion, SCE has a broad biological effect in raising innate immunity to infections. Besides the improvement of managerial and environmental systems, and vaccination against infections, the administration of immunostimulants in feed may be an alternative method of preventing and reducing infections in pigs.

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RESEARCH NOTE

Food Science and Biotechnology

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Effects of Intraperitoneal Administration of *Lactococcus lactis* ssp. *lactis* Cellular Fraction on Immune Response

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Abstract Cellular components of *Lactococcus lactis* ssp. *lactis* (heat-killed whole cells, cytoplasm, and cell walls) were tested for their *in vivo* immunopotentiating activities. Peritoneal macrophages from mice injected intraperitoneally with cell-wall fractions exhibited significantly greater phagocytic activity than groups injected with whole cells or cytoplasm fraction. Cytotoxicity of natural-killer cells was highest in cytoplasm fractions. Production of cytokines (IFN-γ, IL-2, IL-6, and IL-12) in spleen cells was significantly higher when cellular components were injected intraperitoneally, and tended to be higher in whole-cell and cytoplasm groups than in cell-wall group. These results demonstrate lactic acid bacteria whole cells and their cytoplasm and cell-wall fractions have immunopotentiating activities.

Key words: Cytokine, *Lactococcus lactis* ssp. *lactis*, NK cell activity, Phagocytic activity, Immunopotentiating activity

Introduction

An immunopotentiator is a substance that directly or indirectly enhances a particular immunological function by modifying one or more components of the immunoregulatory networks. Immunopotentiators of bacterial origin have been used in vaccination and immunotherapy (1-2). Many lactic acid bacteria (LAB), which occur widely throughout nature, are beneficial for preventing or treating intestinal disorders and for enhancing host immune responses (3-6). Considerable attention has also been focused on the cancer-preventing activity of LAB, which inhibits tumor formation in the gastrointestinal tract of humans and animals. Because this activity is thought to be mediated by the activation of the host's immune system, many studies have investigated the effects of LAB and fermented milk on the immune system (7). The oral administration of LAB and fermented milk increases mitogenic responses (8), peritoneal macrophage activity (9), antibody responses to sheep red blood cells (10), and protection against intestinal infection (4). Animal studies have confirmed that yogurt and fermented milk containing probiotic bacteria inhibit tumor formation and proliferation (11-12). However, most reports on the antitumor and immunopotentiating activities of LAB have focused on LAB whole cells and their peptidoglycans, with little attention being paid to the soluble fraction of *Bifidobacterium* species (2, 13), despite the potential in food applications differing between soluble and insoluble materials.

Results of a previously described study on the *in vitro* immunopotentiating activity of the cellular component of *Lactococcus lactis*, reported for the first time by our group, suggest that *L. lactis* cytoplasm and cell-wall fractions as well as whole cells are capable of stimulating lymphocytes and macrophages to produce several cytokines (14). Based on these observations, in the present study, we have demonstrated systemic and mucosal immune responses of mice injected intraperitoneally with whole cells, cell-wall, and cytoplasm fractions of *L. lactis* ssp. *lactis*. These data could elucidate the interaction of the cellular components of LAB with the immune system.

Materials and Methods

Culture of *L. lactis* ssp. *lactis* *L. lactis* was cultured in M17 media (Difco, Detroit, MI, USA) for 18 hr at 30°C. After cultivation, the cells were harvested in a refrigerated centrifuge (Vision, Seoul, Korea), washed three times with distilled water, and lyophilized for storage. The lyophilized cells were resuspended in distilled water at 10 mg/ml and sonicated with a cell disruptor (Sonics and Materials, Danbury, CT, USA) for 30 min on ice. After the suspension was centrifuged at 800 × g for 30 min at 5°C, the pellet was removed. A cell-wall fraction of the pellet and a cytoplasm fraction of the supernatant were obtained from the supernatant using an ultracentrifuge (Hitachi, Tokyo, Japan) at 70,000 × g for 30 min.

Experimental animals Six-week-old male BALB/c mice (Clea Japan, Tokyo, Japan), were housed in plastic cages in an air-conditioned room and given food and water *ad libitum*.

Administration of *L. lactis* cellular components The mice were given the desired dose of cellular components dissolved in PBS. *L. lactis* cellular components were delivered by intraperitoneal injection on days 0, 2, and 4 at 500 μg/mouse/day, and PBS was used for controls. Each mouse was killed 1 day after completing the administra-

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tion, after which peritoneal exudate cells (PEC) and spleen cells were isolated.

Evaluation of the phagocytic activity of PEC The phagocytic activity of PEC was evaluated in an *in vitro* culture with fluorescent microparticles (15). PEC was isolated from the peritoneal cavity of the mice by lavage with 5 ml HBSS after the intraperitoneal injection of the cellular component. After centrifugation, the cell pellet was washed twice with HBSS and resuspended in 1 ml HBSS-HEPES. Twenty microliters of Fluoresbrite carboxylate microspheres (2.0 μm; Polyscience, Warrington, PA, USA) diluted 100-fold with HBSS-HEPES was added to the PEC suspension, which was then incubated for 1 hr at 37 °C. After stopping the reaction by adding 2 ml cold EDTA-PBS and collecting a cell pellet by centrifugation, the pellet was resuspended in 300 μl EDTA-PBS, and the phagocytic activity was measured using flow cytometry.

Analysis of natural-killer-cell activity The CytoTox 96 nonradioactive cytotoxicity assay kit (Promega, Madison, WI, USA) was used to assess the natural-killer (NK) activity of spleen cells. The CytoTox 96 assay quantitatively measures lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis. Concentration of released LDH in culture supernatants was measured with an enzymatic assay. Briefly, the isolated spleen cells were used as effector cells, and cells from the mouse Moloney leukemia cell line, Yac-1, were used as the target cells. Yac-1 cells (2×10^4 cells/ml) in RPMI-1640 without phenol red were seeded in round-bottomed tissue culture plates. Subsequently, 0.1 ml spleen cells treated with cellular components was added at appropriate concentrations. The assay plates were incubated for 4 hr in a humidified chamber at 37°C and 5% CO_2, and, after 4 hr, centrifuged at $250 \times g$ for 4 min. The supernatants were transferred, and the substrate mix was added to each well. After incubation for 30 min, the absorbance was measured at 490 nm. The NK-cell activity was calculated as a percentage of effector-cell-specific lysis. The percentage of specific LDH release was calculated according to the following formula:
Specific lysis (%) = (experimental-effector spontaneous - target spontaneous)/(target maximum - target spontaneous) × 100

Quantification of cytokine production Spleen, Peyer's patch, and mesenteric lymph-node cells were added to each well of a 24-well plate and cultured in the presence of Con A (5 μg/ml) or LPS (20 ng/ml) for 48 hr at 37°C: Con A was used for measuring IFN-γ, IL-2, and IL-4 productions; and LPS was used for IL-6, IL-12, and TNF-α. Cell-free supernatant fractions were harvested and stored at -20°C until assayed.

Concentration of cytokines in culture supernatant was determined using a sandwich ELISA. Briefly, microtiter plates were coated overnight at 4°C with purified rat anti-mouse cytokine-capture antibody at 50 μl/well (Pharmingen, San Diego, CA, USA) in 0.1 M sodium bicarbonate buffer (pH 8.2). The plates were then washed three times with PBS containing 0.2% Tween-20 (PBS-T), blocked with 100 μl of 1% (w/v) bovine serum albumin (BSA) in PBS for 30 min at 37°C, and washed three times with PBS-T. Standard murine cytokines or samples were diluted in PBS-T solution containing 1% BSA, and 50-μl aliquots of the mixture were added to the appropriate wells. The plates were incubated overnight at 4°C and washed four times with PBS-T. Subsequently, 50 μl biotinylated rat antimouse cytokine-capture monoclonal antibody diluted in BSA-PBS was added to each well. The plates were incubated at room temperature for 60 min and washed six times with PBS-T. Fifty microliters of streptavidin–alkaline-phosphatase conjugate diluted in BSA-PBS was added to each well. The plates were incubated for 30 min at room temperature, washed with PBS-T, and, to each well, added with 50 μl substrate (*p*-nitrophenylphosphate). The absorbance was read at 405 nm on a microplate reader (Bio-Rad, Hercules, CA, USA), and cytokine concentrations were quantified using a standard curve.

Statistics Significant differences between the experimental and control groups were determined using Fisher's protected least-significant difference (PLSD) test. All results are presented as means ± SD values. Tests were considered significant at $P<0.05$.

Results and Discussion

Phagocytic and NK cells are the major effectors of natural immunity, and numerous reports have been published on the relation between LAB and natural immunity (16). The phagocytic activity of peritoneal macrophages is shown in Fig. 1 as the phagocytic uptake of fluorescent microparticles by PEC. The microparticles incorporated into cells were counted with a flow cytometer (Fig. 1A). The results are expressed as the mean percentages of cells in which one, two or more than two particles were incorporated. Peritoneal macrophages from mice injected with cell-wall fractions exhibited significantly greater phagocytic activity than the other groups. Although the production of TNF-α and IL-6 in peritoneal macrophages was not affected by LAB cellular components (Fig. 1C), the percentage of cells incorporating three or more particles in the group injected with cell walls was three times higher than that of PEC from the control mice (PBS group). NK activity was assessed using spleen cells; the cytotoxicity of NK cells was about two times higher in cytoplasm groups than in the other groups (Fig. 2). Enhanced NK cytotoxicity has been reported to prevent cancer, and these cells may play an important role in the regulation of tumor development and metastasis (17). The primary target of LAB for their immunostimulatory effect was shown to be NK cells (18), and NK-cell activation by *Lactobacillus casei* has also been reported (19-20).

The production of cytokines by splenocytes in response to Con A or LPS was enhanced by intraperitoneal injection of cellular fractions of *L. lactis*. The production of IFN-γ, IL-2, IL-6, and IL-12 was significantly higher in spleen cells from the cytoplasm-fraction-injected group than in PBS-injected controls (Fig. 3). The injection of the whole-cell preparation enhanced the secretion of IL-2, IL-6, and IL-12, while the cell-wall fraction only enhanced the secretion of IL-6 and IL-12. The production of IL-4 and TNF-α did not differ significantly with the type of

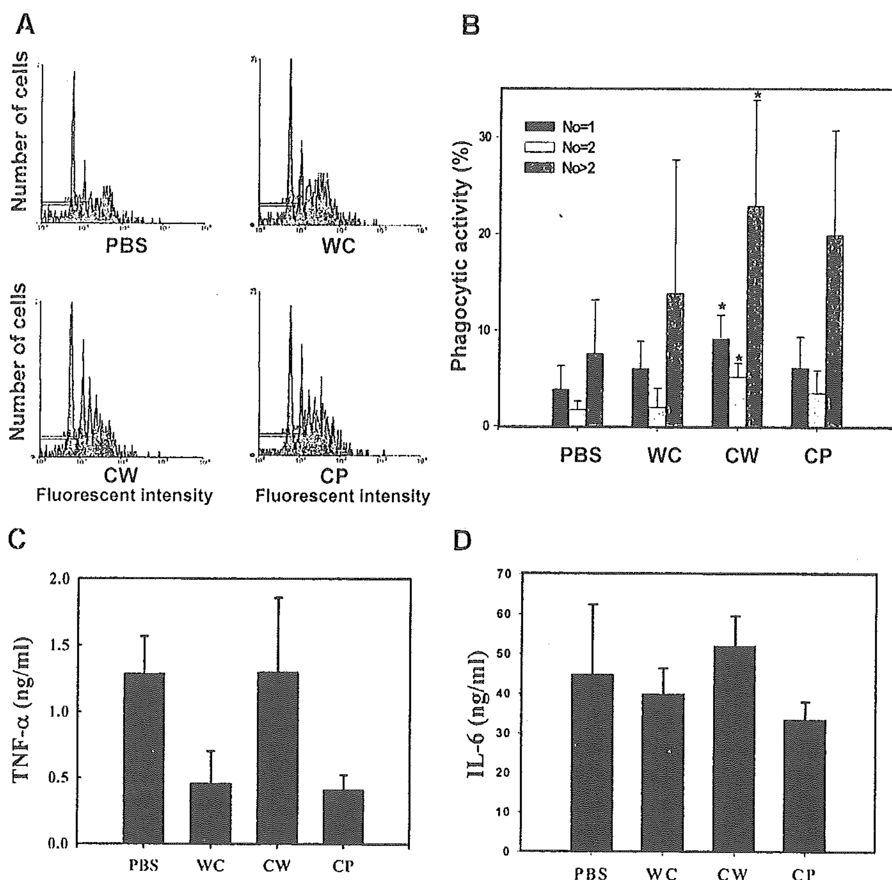

Fig. 1. Phagocytic activity of PEC obtained from mice injected intraperitoneally with heat-killed whole cells (WC), cell walls (CW), and cytoplasm (CP) of *L. lactis* at 500 μg/mouse on days 0, 2, and 4. On day 5, the phagocytic activity was measured (A, B) and cytokine production in PEC was analyzed (C). (A) Typical result of flow cytometry. (B) Course analysis of PEC phagocytic activity ("No." is the number of particles per cell). (C) TNF-α production in PEC. (D) IL-6 production in PEC. Data are shown as mean ± SD values (n=6, *$P < 0.05$).

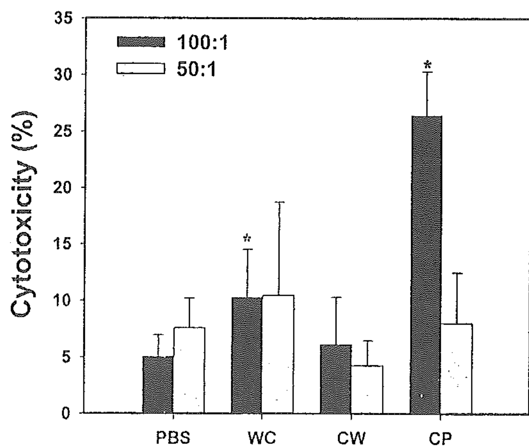

Fig. 2. Percentage cytotoxicity of the natural-killer activity of spleen cells obtained from mice injected intraperitoneally with WC, CW, and CP of *L. lactis* against Yac-1 cells. Effector: target cell ratios were 100:1 and 50:1. The cytotoxicity was measured using LDH assays. Data are shown as mean ± SD values (n=6, *$P < 0.05$)

fraction injected (Fig. 3). Macrophages are the main IL-12-, IL-6-, and TNF-α-producing cells, and these are important target cells for the antitumor or immunomodulating effects of some microorganisms. In particular, IL-12 potently stimulates cytotoxic T cells and NK cells, and enhances the production of several cytokines, including IFN-γ, IL-2, and TNF-α. In the present study, the injection of cellular components of *L. lactis* mainly augmented the production of IFN-γ, IL-2, IL-6, and IL-12, which is consistent with the results of previous studies (21-25). Most studies to date on the immunopotentiating activity of LAB have focused on whole LAB cells and their peptidoglycans (26), with little attention being paid to the soluble fraction, despite the potential in food applications differing between soluble and insoluble materials. Several recent studies have examined the effects of LAB cytoplasm on immunopotentiating activity (14). For example, the polysaccharide fraction of *B. adolescentis* cytoplasm stimulates Peyer's patches and lymph-nodes lymphocytes *in vitro* (2, 27). Another species of *Bifidobacterium*, *B. breve* (either whole cells or a cell-wall preparation) accelerates the *in vitro* proliferation of Peyer's-patch cells, particularly B cells (13). Takahashi *et al.* (28) investigated the interaction of cell fractions of LAB and the immune system using *B. longum* and *Lb. acidophilus*. Tejada-Simon and Pestka (29) reported that LAB and their cell-free extracts exhibit mitogenic and polyclonal-activating properties when cultured with cells of the immune system. Lee *et al.* (30) also reported that *Bifidobacterium* whole cells and cell-free extracts differentially induce cytokine

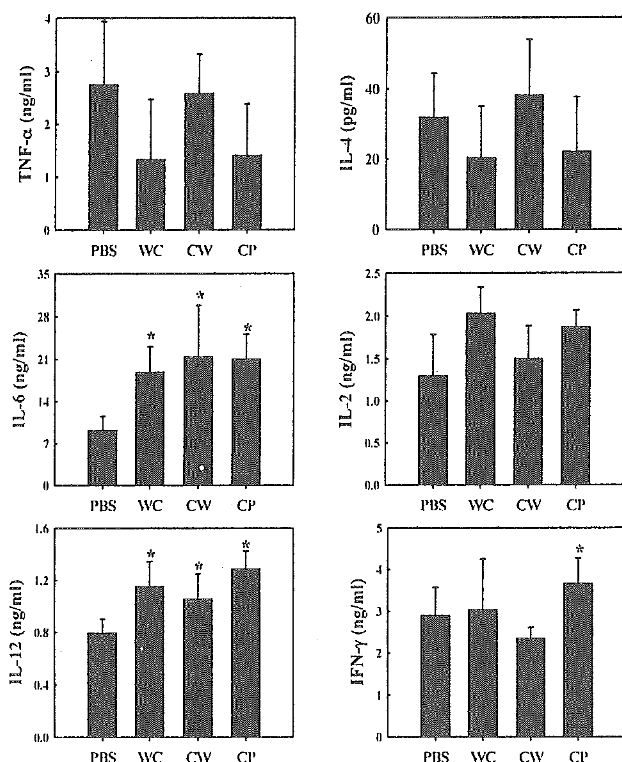

Fig. 3. Production of cytokines by spleen cells from mice injected intraperitoneally with WC, CW, and CP of *L. lactis*. Spleen cells were cultured with mitogen (Con A or LPS) for 48 h. The concentrations of cytokines (IL-2, IL-4, and IFN-γ for Con-A-stimulated cultures, and IL-6, IL-12, and TNF-α for LPS-stimulated cultures) in culture supernatant were measured by ELISA, and the data are shown as mean ± SD values (n=6, $*P < 0.05$)

production in murine macrophages. The present study has demonstrated that LAB whole cells as well as their cytoplasm and cell-wall fractions have immunostimulating activities, a finding which is important for understanding the mechanisms underlying the immunoregulatory function of LAB and their potential applications.

Acknowledgments

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Complete refolding of bovine β-lactoglobulin requires disulfide bond formation under strict conditions

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Abstract

β-Lactoglobulin (β-LG) denatured with 6 M guanidine hydrochloride (GdnHCl) containing a reducing agent and subsequently dialysed against phosphate-buffered saline (PBS) resulted in incomplete refolding of this protein despite the fact that the biological activity for retinol-binding was recovered to almost the same degree as that of the native molecule [Hattori, M., Ametani, A., Katakura, Y., Shimizu, M., Kaminogawa, S. J., Biol. Chem. 268 (1993) 22414–22419]. The enzyme probe method, evaluation of hydrophilicity values, in-gel mobility on SDS-PAGE, and evaluation of disulfide bonds with the Ellman method showed exposure of the hydrophobic region(s) and incorrect disulfide bond formation in such dialyzed β-LG molecules. We reveal in this present work that complete refolding could be attained by diluting denatured β-LG with PBS containing a reducing agent, before slow reoxidation of the sulfhydryl groups upon dialysis for gradient removal of the reducing agent in 6 steps. Complete renaturation was confirmed by analyzing the retinol-binding activity, CD spectra, intrinsic fluorescence, binding ability of monoclonal antibodies (mAbs), and SDS-PAGE. Step-by-step disulfide bond formation was considered to be critical for the complete refolding of denatured β-LG. Our method can contribute to establish a procedure for complete refolding of useful recombinant proteins in vitro without such biological aids as chaperones.
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Keywords: β-Lactoglobulin; Disulfide bond formation; Refolding

1. Introduction

Unfolding and refolding of proteins have been widely investigated [1] to elucidate the mechanisms for the three-dimensional structure formation of polymer molecules and the maturation of a protein newly synthesized in cells, as well as to produce biologically active recombinant proteins by recombinant DNA technology. Although polypeptide synthesis, structural formation of microdomains, modification of amino acid residues, and assembly of polymers occur in a complicated manner with the biological formation of a protein molecule, the production of biologically active proteins from an aggregated polypeptide in the inclusion bodies of recombinant bacteria requires the refolding of already synthesized polypeptide chains. Refolding processes that can only endow polypeptides with biological activities may not be enough to produce protein pharmaceuticals. Considering the potential for inducing anaphylactic reactions, recombinant proteins as administered pharmaceuticals should have the structurally native form. However, the methods to achieve complete refolding of proteins have not yet been well established. We found in the previous study

Abbreviations: β-LG, β-lactoglobulin; RCM-β-LG, reduced and carboxymethylated β-lactoglobulin; PBS, phosphate-buffered saline; GSH, reduced form of glutathione; GSSG, oxidized form of glutathione; K_{AS}, equilibrium constant; K'_d, dissociation constant; mAb, monoclonal antibody; MES, 2-(*N*-morpholino) ethanesulfonic acid; GdnHCl, guanidine hydrochloride

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[2] that when bovine β-lactoglobulin (β-LG) was treated with 6 M guanidine hydrochloride containing a reducing agent and subsequently dialysed against PBS, the molecule regained a level of retinol-binding activity similar to that of the native material. However, our panel of monoclonal antibodies could detect structural differences between such renatured and native molecules. Subramaniam et al. [3] have also found that the conformational stability of the renatured β-LG obtained by a method similar to ours was lower than that of native β-LG by evaluating the room-temperature tryptophan phosphorescence.

Bovine β-LG is a globular protein of MW 18,000 as a monomer containing two disulfide bridges as well as a free cysteine residue and can exist as a dimer or tetramer [4]. The structure of β-LG has been widely studied by X-ray crystallography [5–8], NMR [9–11], and other physicochemical methods. This protein consists of nine antiparallel β-sheets and one α-helix to form a calyx-shaped β-barrel structure and is classified as a member of the lipocalin superfamily [12,13]. The physiological function of β-LG is tentatively considered to be the binding and transportation of small hydrophobic ligands such as retinol and fatty acids [14]. Utilizing its structural data and advantage of smallness as a protein molecule, β-LG has also been studied as a model of unfolding [15–18] and refolding [2,10,19–21]. The refolding process of β-LG is unique in that non-native α-helix intermediates appear during an early phase. Elucidation of the β-LG folding mechanism can be used as a model to study the α-helix/β-sheet transition of other proteins such as prion proteins [10,21].

We study in this present work the structural features of β-LG treated for renaturation and develop a new method to achieve complete refolding of β-LG. Many useful proteins that are applicable as pharmaceuticals, including the lipocalin protein family, have a β-barrel structure [22], so the renaturation method we develop in this study can be applied to establish a production procedure for protein pharmaceuticals using recombinant DNA technology.

2. Materials and methods

2.1. Preparation of β-LG and RCM-β-LG

Fresh skim milk from a Holstein cow of the genotype AA for β-LG was supplied by our dairy farm. Crude β-LG was prepared by the method of Armstrong et al. [23] and purified by DEAE-Sephacel (Amersham Pharmacia Biotech, Buckinghamshire, UK) ion-exchange chromatography using linear gradient elution from 0 to 0.5 M of NaCl in a 0.05 M imidazole–HCl buffer (pH 6.7). A single band was obtained for the purified β-LG by PAGE both with and without SDS. RCM-β-LG was prepared by reducing the disulfide bonds in β-LG with 2-mercaptoethanol and then carboxymethylating the free sulfhydryl groups with sodium iodoacetate as described previously [2].

2.2. Preparation and purification of monoclonal antibodies (mAbs)

The ascites fluid containing each mAb was obtained as previously described [2,15,24]. Each mAb was purified with a Bakerbond ABx column (8 ID×250 mm; J. T. Baker, Phillipsburg, NJ, USA) to which each ascites diluted with 4 parts of 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) at pH 5.6 was applied. Elution was achieved with a linear gradient from 10 mM MES (pH 5.6) to 250 mM KH_2PO_4 (pH 6.8).

2.3. Denaturation and renaturation of β-LG

β-LG at 2.0 or 0.2 mg/ml was incubated for 3 h at 25 °C in phosphate-buffered saline (PBS; 0.11 M phosphate buffer at pH 7.1 containing 0.04 M NaCl and 0.02% NaN_3) containing 6 M guanidine hydrochloride (GdnHCl) in the presence of 0.14 M 2-mercaptoethanol. The subsequent renaturation treatment was initiated by the procedures summarized in Table 1. A medium bottle (500 ml, Pyrex) with a cap was used in the dialysis step of methods 6–11, and the head space was filled with N_2 gas.

2.4. Measurement of the retinol-binding activity

The retinol-binding activity of β-LG was measured by fluorescence titration [25]. In brief, retinol in ethanol was added to a 2.0 ml solution in a cuvette containing 0.2 or 0.02 mg of protein in PBS. Small increments (5 μl at a time) of the retinol in ethanol at about 100 or 10 μM were added to the cuvette with a micropipette. The mixture was thoroughly mixed and then allowed to equilibrate for 1 min before recording the fluorescence intensity. The fluorescence was measured by a Shimadzu RF-5300PC fluorescence spectrophotometer (Kyoto, Japan) with excitation at 330 nm and emission at 470 nm. The apparent dissociation constant (K'_d) was calculated according to the method of Cogan et al. [26] from the results of fluorescence titration.

2.5. Spectroscopic analyses

CD spectra of 0.1% β-LG in PBS were recorded at 25 °C with a J-720WI automatic recording spectro-polarimeter (JASCO, Tokyo, Japan) in a cell of 1.0 mm path length. The fluorescence intensity of β-LG dissolved in PBS at 0.001% was measured under an excitation wavelength of 283 nm by means of an RF-5300PC fluorescence spectrophotometer (Shimadzu, Kyoto, Japan).

2.6. SDS-PAGE

SDS-PAGE was carried out with 7.5% polyacrylamide according to the method of Weber and Osborn [27]. Protein bands were stained with a Plusone silver staining kit, protein