trafficking within microvessels supplying blood to the gut mucosa remains unclear.

Much remains to be clarified with regard to the functions and homing patterns of IELs and their molecular basis. One reason for the difficulty in studying IELs is the lack of appropriate cell lines established from these populations. The difficulty of establishing IEL cell lines is due to the poor proliferative capacity and a lack of knowledge about their adequate ligands. In the present study, we have established an antigen-specific IEL cell line from ovalbumin (OVA)23-3 mice expressing a transgenic (Tg) TCRαβ specific to OVA323-339 [15]. The majority of T cells in these mice expressed a TCR specific to this epitope, which gave us an advantage in establishing the antigen-specific cell line. Using this cell line, we analysed the functional characteristics of cells, such as their cytokine production and antigen-specific proliferation.

Recent *in situ* microscopy experiments with intestinal mucosa have demonstrated that lymphocyte homing involves organ-specific multi-step cascades of adhesion and signalling events in specialized blood vessels, termed high endothelial venules (HEV), as well as in the villus microvessels [16–18]. In this study, using the established IEL cell line we carried out an intravital microscopic procedure to monitor the dynamic process of lymphocyte migration in order to (1) investigate whether adhesion of IEL cells occurs in the villus mucosa of the small intestine and, if it does, examine a possible contribution of various adhesion molecules to this IEL–endothelial cell adhesive interaction, and (2) compare how recruitment of naive and antigen-specifically stimulated IEL cells differs in the villus mucosa of the small intestine.

Methods

OVA23-3 Tg mice and isolation of IEL

The process of establishing the OVA23-3 Tg mouse line we used has been described previously [15]. The mice carried a gene encoding TCR αβ (Vα3.1/Vβ15) derived from an OVA-specific $CD4^+$ T cell clone, 7-3-7. The animals were housed and bred within animal facilities at the University of Tokyo. We obtained transgenic mice from the F_1 generation of a cross between BALB/c (Clea Japan, Inc., Tokyo, Japan) and heterozygous transgenic mice. The care and use of laboratory animals were in accordance with the guidelines of the National Institute of Health.

IELs were isolated from Tg mice of both sexes, 8–24 weeks of age, by using modified procedures as described previously [19]. Briefly, an inverted intestine was cut into four segments and the segments were transferred into a 50-ml conical tube containing 45 ml of 5% fetal calf serum (FCS) in Ca^2, Mg^2-free Hanks's balanced salt solution (HBSS; Gibco Laboratories, Grand Island, NY, USA). The tube was shaken in an orbital shaker at 150 r.p.m. in the horizontal position for 45 min at 37°C. Cell suspensions were collected and passed through a glass-wool column to remove cell debris and adherent cells. Subsequently, the cells were suspended in 30% (wt/vol) Percoll (Pharmacia Biotech, Uppsala, Sweden) and centrifuged for 20 min at 600 *g*. After the centrifugation, the cells at the bottom of the solution were subjected to Percoll discontinuous-gradient centrifugation and IELs were recovered at the interface of 44% and 70% Percoll (>95% were $CD3^+$). The obtained cell suspensions were washed and stored on ice in RPMI (pH 7·4) with 5% FCS until used.

Establishment of IEL cell line and antigen stimulation

IELs from OVA23-3 mice (10^6 cells/ml) were stimulated every week with mitomycin C-treated $CD4^-$ BALB/c splenocytes (2×10^6 cells/ml) in a culture medium with 5 mM OVA323-339 peptide and a 10% culture supernatant of ConA-stimulated rat splenocytes [20]. The method of mitomycin C treatment was as follows: 10^7 splenocytes/ml were incubated with 50 mg/ml of mitomycin C (Sigma) for 45 min at 37°C and washed with an RPMI-1640 medium (Nissui Pharmaceutical, Tokyo, Japan) three times. The medium for the cell culture was RPMI-1640 containing 100 U/ml penicillin, 100 mg/ml streptomycin, 5×10^{-5} M 2-mercaptoethanol, and 10% FCS (Cansera International, Rexdale, Canada). Several weeks after the initiation of the culture, the growing cells were expanded and then an antigen-specific IEL line from the OVA23-3 mice was established. The IEL cell line was induced to rest by changing only the culture medium without antigen-presenting cells once a week for 4 weeks after the final antigen stimulation.

An antigen-stimulated IEL cell line was obtained by removing $CD4^-$ splenocytes by magnetic cell sorting (MACS) soon after the final antigen stimulation. In brief, the IELs (1×10^7) with $CD4^-$ splenocytes were suspended in 90 µl of phosphate buffered saline (PBS) containing 0·5% bovine serum albumin and 5 mM ethylenediaminetetra acetic acid (EDTA) and incubated in 10 µl of antimouse CD4 (L3T4)-labelled MACS microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) for 15 min at 6°C. After that, the treated IELs were passed through a separation column (type MS, Miltenyi Biotech) placed in the magnetic field of a MACS separator. The magnetically labelled $CD4^+$ IEL cells were retained in the column, while the unlabelled $CD4^-$ splenocytes ran through. After removing the column from the magnetic field, the retained $CD4^+$ IEL cells could be eluted as an antigen-stimulated IEL cell line. The $CD4^+$ IEL cells were washed and, until used, resuspended in RPMI-1640 with 5% FCS stored on ice.

Cell proliferation and cytokine production assay

The cell line was plated in 96-well plates at 1 or 2×10^5 cells/well with OVA or ConA (10 mg/ml) and at 4×10^5 cells/well with antigen-presenting cells (APC) (mitomycin C-treated

 © 2005 British Society for Immunology,

BALB/c splenocytes) in a total volume of 200 ml. After 24 and 48 h, 0·5 mCi of [^{3}H]thymidine was added to each well. The cells were harvested 20 h later and the [^{3}H]thymidine incorporation was measured by scintillation counting. Cytokines in the culture supernatants were detected using a two-site sandwich enzyme-linked immunosorbent assay (ELISA) as described previously [21]. Briefly, for assays of interleukin (IL)-4, IL-5 and interferon (IFN)-γ, Maxisorp immunoplates (Nunc Roskilde, Denmark) were coated with a rat antimouse IL-4 (BVD4–1D11, PharMingen, San Diego, CA, USA), a rat antimouse IL-5 and a rat antimouse IFN-γ antibody (XMG1.2). This was followed by incubation with alkaline phosphatase-streptavidin. A substrate (p-nitrophenyl phosphate) was added and the colour development was stopped by the addition of 5 N NaOH. The absorbance was determined at 405 nm.

Flow cytometry

Cells (2×10^5/sample) were washed with HBSS containing 5% FCS and 0·2% NaN_3 (the flow cytometry buffer) and centrifuged at 4°C, 400 *g* for 5 min. Each antibody was diluted appropriately with the flow cytometry buffer and then 25 μl was added to each cell preparation. The cells were stained on ice for 20 min, with mixing every 5 min. If the used antibodies were conjugated to biotin, the cells, after having been washed twice, were further stained with streptavidinylated fluorochrome. The samples were washed again, and 500–700 μl of flow cytometry buffer were added for analysis. Flow cytometry was performed using a FACSort machine (Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA). Aggregated cells were excluded from the analysis by gating a forward and a side scatter.

The MoAbs used in this study were as follows: an anti-pan TCR β chain (H57-597) conjugated to biotin and anti-Thy 1·2 (30-H12) conjugated directly to R-phycoerythrin (R-PE) were purchased from PharMingen, FITC-anti-CD4 (YTS191.1.2) and R-PE-anti-CD8α (53.6.7) were purchased from Gibco BRL (Gaithersburg, MD, USA); streptavidin-R-PE was purchased from Gibco BRL, anti-αEβ7 (2E7) was presented by Lefrancois *et al.* (22), and FITC-antihamster IgG (H + L) was purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL, USA). Antibodies against mouse L-selectin (MEL-14, rat IgG2a), α4-integrin (R1-2, rat IgG2b), αE-integrin (M290), β7-integrin (FIB27), CD11a (M17/4) and CD3 (145–2C11, hamster IgG) were purchased from PharMingen.

Lymphocyte labelling with carboxyfluorescein diacetate succinimidyl ester (CFSE)

CFDSE (Molecular Probes, Eugene, OR, USA) was dissolved in dimethylsulphoxide to 15·6 mm and a small aliquot (300 μl) was stored in a cuvette sealed with argon gas at −80°C until the experiments were conducted. Lymphocytes (1×10^7) were incubated in CFSE solution (20 μl of stock solution was diluted with 20 ml of RPMI-1640) for 30 min at 37°C. The labelled lymphocytes were centrifuged immediately through a cushion of heat-inactivated fetal bovine serum and washed twice with a cold suspension medium. The cells were resuspended in 0·2 ml of the medium and used within 30 min.

Intravital observation of lymphocyte migration in intestinal mucosa

The intestinal villi were observed from the mucosal surface and lymphocyte migration was also observed. After an intraperitoneal injection of pentobarbital sodium (50 mg/kg), the abdomen was opened via a midline incision. A 7-cm ileal segment ending at the caecal valve was gently extended onto a plate and a longitudinal incision of about 2 cm was made in the middle of the segment by microcautery along its antimesenteric border. The intestine was kept warm and moist by continuous superfusion with physiological saline warmed to 37°C. The adjacent intestinal segment and mesentery were covered with absorbent cotton soaked with Krebs–Ringer solution.

Suitable areas of villus tips were observed from the mucosal surface by an inverted fluorescence microscope (Diaphot TMD-2S, Nikon, Tokyo, Japan) and the observation was recorded by using a videotape recording system. The same area of ileal mucosa was always examined throughout the observation period. The behaviour of fluorescently labelled lymphocytes was visualized on a television monitor by using a fluorescence microscope equipped with a silicon intensified target image tube (SIT) camera with a contrast-enhancing unit (C-2400–08, Hamamatsu Photonics Co., Shizuoka, Japan) according to a method described previously [16,18]. In this setting, the tip of each villus was observed as an oblique circle, and archade microvessels in the villi were also observed. In another set of experiments, a 5-cm ileal segment ending at the caecal valve was chosen for observation of Peyer's patches. Two small incisions in the bowel wall were made and the luminal pressure of the gut loop was maintained at 10 cmH_2O with physiological saline. The microcirculation in Peyer's patches was observed through the serosa by microscope. Epi-illumination was achieved by using filters for excitation at 470–490 nm and for emission at 520 nm. Lymphocytes (1×10^7 dissolved in 1 ml) were injected into a jugular vein of the recipient mice for 3 min. The cell kinetics of the infused lymphocytes, their interaction with microvascular beds and their accumulation in the villus mucosa or Peyer's patches were monitored and recorded continuously on S-VHS videotapes for the first 20 min and then, at 10-min intervals, for 40 min. Lymphocytes adhering to the microvessels of the villus mucosa or Peyer's patches and remaining in the same position without movement for more than 30 s were defined as 'sticking' lymphocytes. The number of sticking lymphocytes was determined in a 1-mm^2 area observed in a video image.

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 $P < 0{\cdot}05$.

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 ± 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 ± 9·2 U/ml IFN-γ ($n = 4$), 170 ± 18 pg/ml IL-4 ($n = 4$) and 11 ± 2·1 ng/ml IL-5 ($P < 0{\cdot}05$ *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

© 2005 British Society for Immunology,

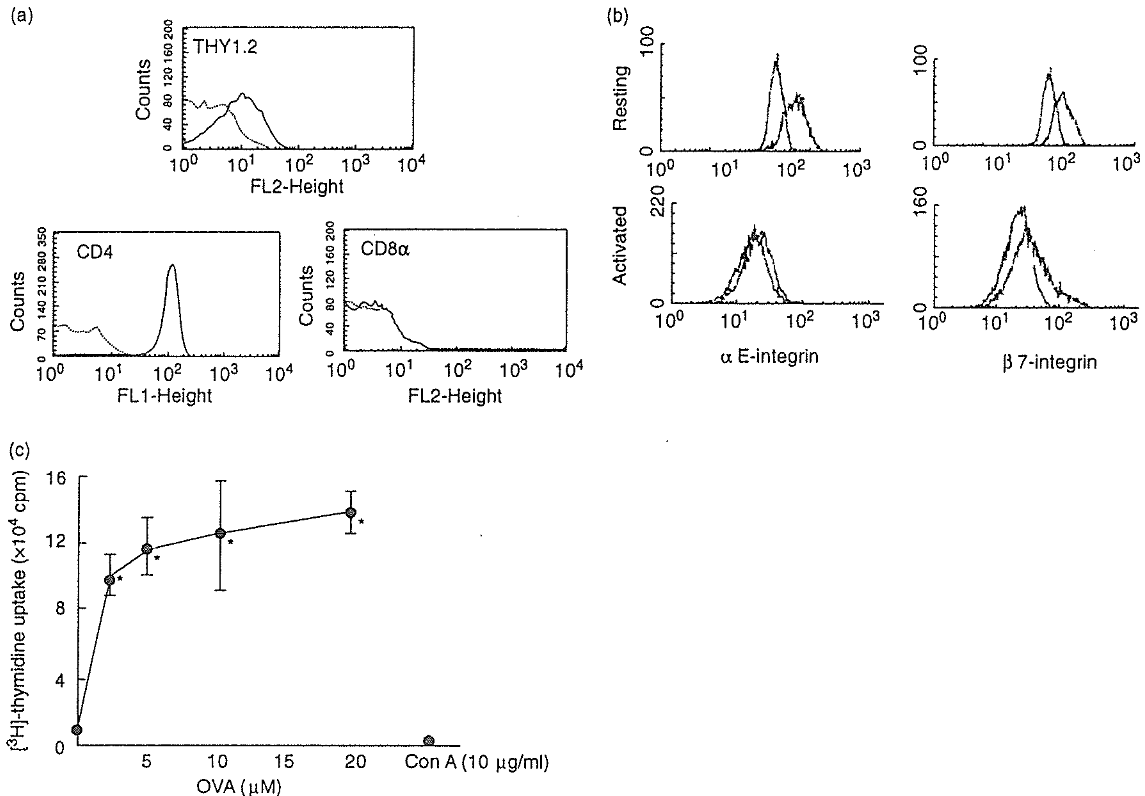

Fig. 1. Expression of surface antigens on intraepithelial lymphocyte (IEL) cell line established from ovalbumin (OVA)23-3 transgenic mice; 2×10^5 lymphocytes were first incubated with antimouse monoclonal antibodies against Thy1·2 (30-H12), pan T cell receptor (TCR) β chain (H57-597), CD4 (YTS191·1.2), 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. $^*P < 0{\cdot}05$ *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

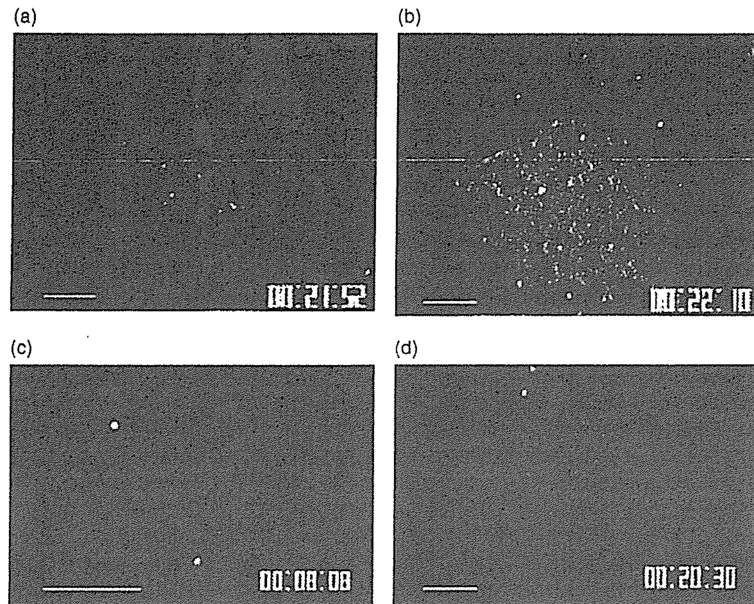

Fig. 2. Representative images of the distribution of a carboxyfluorescein diacetate succinimidyl ester (CFSE)-labelled resting intraepithelial lymphocyte (IEL) cell line in control mice (a) and in ovalbumin (OVA)-fed mice (b) Following adherance to the microvessels of a villus tip of the ileal mucosa 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).

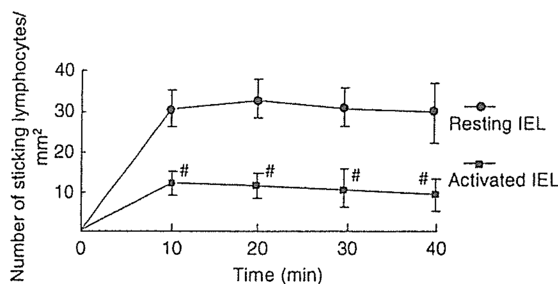

Fig. 3. Time–course change in the number of sticking intraepithelial lymphocyte (IEL) line cells to villus mucosa in control ovalbumin (OVA)23-3 mice. The number of cells accumulated in the microvessels of villus tips is compared between unstimulated (resting) and activated (stimulated with OVA, 20 μM) 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

© 2005 British Society for Immunology,

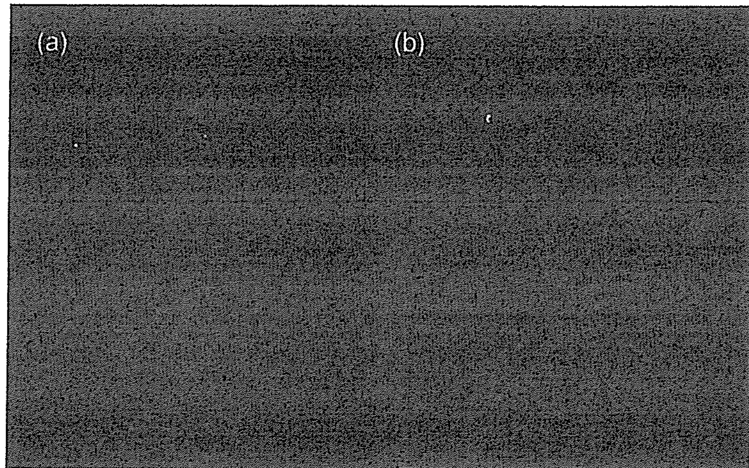

Fig. 4. Representative pictures of simultaneous observation of carboxyfluorescein diacetate succinimidyl ester (CFSE)-labelled intraepithelial lymphocyte (IEL) (green) and factor VIII-positive (a) or CD34-positive (b) microvessels (red fluorescence) in small intestinal villi as determined by immunohistochemistry. 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

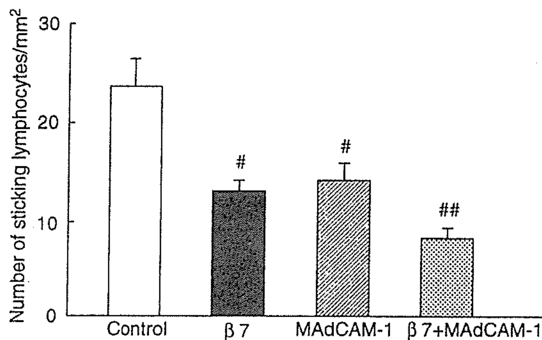

Fig. 5. The inhibitory effect of function-blocking of adhesion molecules on the sticking of resting intraepithelial lymphocyte (IEL) line cells to microvessels of villus mucosa at 20 min. The effect of monoclonal antibody against β7-integrin (Fib27) and MAdCAM-1 (MECA367)-treatment on sticking of IELs 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. #$P < 0.05$, compared with controls. ##$P < 0.05$, compared with β7-integrin- and MAdCAM-1-blocking alone. Values are means ± s.d. for six animals.

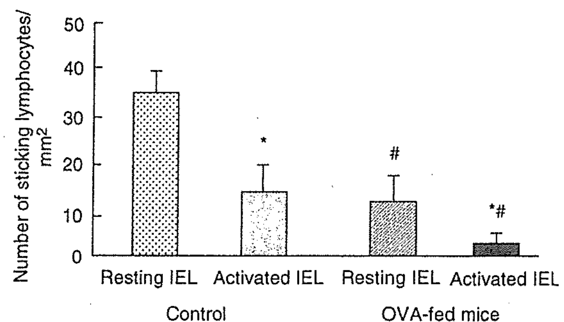

Fig. 6. Comparison of the number of intraepithelial lymphocyte (IEL) line cells adhered to the microvessels of villus tips in ovalbumin (OVA)23-3 control mice with that in OVA-fed mice, 20 min after injection. OVA 23–3 transgenic mice were fed with OVA at a dose of 200 mg/day for 3 days (OVA-fed mice), and the number of adhered IEL cell lines (resting and activated) was compared with that in unfed mice (control). #$P < 0.05$, compared with unfed controls. Values are means ± s.d. for six animals. *$P < 0.05$, 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

This study was supported in part by Grants-in-Aid for Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science and Technology and by grants from the Keio University School of Medicine, the National Defense Medical College and the University of Tokyo. This study was also supported by Funds for Food Allergy from the Japanese Ministry of Health, Labour and Welfare.

References

1 Guy-Grand D, Vassalli P. Gut intraepithelial T lymphocytes. Curr Opin Immunol 1993; 5:247–52.

2 Dillon SB, MacDonald TT. Functional characterization of ConA-responsive Lyt2-positive mouse small intestinal intraepithelial lymphocytes. Immunology 1986; 59:389–96.

3 Mosley RL, Whetsell M, Klein JR. Proliferative properties of murine intestinal intraepithelial lymphocytes (IEL): IEL expressing TCR αβ or TCR γδ are largely unresponsive signals mediated via conventional stimulation of the CD3–TCR complex. Int Immunol 1991; 3:563–9.

4 Cepak KL, Parker CM, Madara JL, Brenner MB. Integrin αEβ7 mediates adhesion of T lymphocytes to epithelial cells. J Immunol 1993; 150:3459–70.

5 Roberts K, Kilshaw SJ. The mucosal T cell integrin αM290β7 recognizes a ligand on mucosal epithelial cell lines. Eur J Immunol 1993; 23:1630–5.

6 Reinmann J, Rudolphi A. Co-expression of CD8α in CD4+ T cell receptor αβ+ T cells migrating into the murine small intestinal epithelial layer. Eur J Immunol 1995; 25:1580–8.

7 Rakasz E, Rigby S, de Andres B *et al.* Homing of transgenic γδ T cells into murine vaginal epithelium. Int Immunol 1998; 10:1509–17.

8 Morrissey PJ, Charrier K, Horovitz DA, Fletcher FA, Watson JD. Analysis of the intra-epithelial lymphocyte compartment in SCID mice that received co-isogenic CD4+ T cells. Evidence that mature post-thymic CD4+ T cells can be induced to express CD8α *in vivo.* J Immunol 1995; 154:2678–86.

9 Bradley LM, Watson SR. Lymphocyte migration into tissue: the paradigm derived from CD4 subsets. Curr Opin Immunol 1996; 8:312–20.

10 Hamann A, Rebstock S. Migration of activated lymphocytes. Curr Top Microbiol Immunol 1993; 184:109–24.

11 Mackey CR. Homing of naive, memory and effector lymphocytes. Curr Opin Immunol 1993; 5:423–7.

12 Butcher EC, Picker LJ. Lymphocyte homing and homeostasis. Science 1996; 272:60–6.

13 Salmi M, Andrew DP, Butcher EC, Jalkanen S. Dual binding capacity of mucosal immunoblasts to mucosal and synovial endothelium in humans: dissection of the molecular mechanisms. J Exp Med 1995; 181:137–49.

14 Picker LJ, Martin RJ, Trumble A *et al.* Differential expression of lymphocyte homing receptors by human memory/effector T cells in pulmonary versus cutaneous immune effector sites. Eur J Immunol 1994; 24:1269–77.

15 Sato T, Sasahara T, Nakamura Y *et al.* Naive T cells can mediate delayed-type hypersensitivity response in T cell receptor transgenic mice. Eur J Immunol 1994; 24:1512–16.

16 Miura S, Tsuzuki Y, Kurose I *et al.* Endotoxin stimulates lymphocyte–endothelial interactions in rat intestinal Peyer's patches and villus mucosa. Am J Physiol 1996; 271:G282–92.

17 Hokari R, Miura S, Fujimori H *et al.* Altered migration of gut-derived T lymphocytes after activation with concanavalin A. Am J Physiol 1999; 277:G763–72.

18 Koseki S, Miura S, Fujimori H *et al.* *In situ* demonstration of intraepithelial lymphocyte adhesion to villus microvessels of the small intestine. Int Immunol 2001; 13:1165–74.

19 Ishikawa H, Li Y, Abeliovich A, Yamamoto S, Kaufmann SHE, Tonegawa S. Cytotoxic and interferon γ-producing activities of γδ T cells in the mouse intestinal epithelium are strain dependent. Proc Natl Acad Sci USA 1993; 90:8204–8.

20 Hisatsune T, Enomoto A, Nishijima K *et al.* CD8+ suppressor T cell clone capable of inhibiting the antigen- and anti-T cell receptor-induced proliferation of Th clones without cytolytic activity. J Immunol 1990; 145:2421–6.

21 Yoshida T, Hachimura S, Kaminogawa S. The oral administration of low-dose antigen induces activation followed by tolerization, while high-dose antigen induces tolerance without activation. Clin Immunol Immunopathol 1997; 82:207–15.

22 Lefrancois L, Barrett TA, Havran WL, Puddington L. Developmental expression of the alpha IEL beta 7 integrin on T cell receptor gamma delta and T cell receptor alpha beta T cells. Eur J Immunol 1994; 24:635–40.

23 Mosmann TR, Coffman RL. Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol 1989; 7:145–73.

24 Fujihashi K, Yamamoto M, McGhee JR, Kiyono H. Alpha beta T cell receptor-positive intraepithelial lymphocytes with CD4+, CD8 and CD4+, CD8+ phenotypes from orally immunized mice provide Th2-like function for B cell responses. J Immunol 1993; 151:6681–91.

25 Rott LS, Briskin MJ, Andrew DP, Berg EL, Butcher EC. A fundamental subdivision of circulating lymphocytes defined by adhesion to mucosal addressin cell adhesion molecule-1. Comparison with vascular cell adhesion molecule-1 and correlation with β7 integrins and memory differentiation. J Immunol 1996; 156:3727–36.

26 Strauch UG, Lifka A, Goßlar U, Kilshaw PJ, Clements J, Holzmann B. Distinct binding specificities of integrins α4β7 (LPAM-1), α4β1 (VLA-4), and αIELβ7. Int Immunol 1994; 6:263–75.

27 Parker CM, Cepek KL, Russell GJ *et al.* A family of β7 integrins on human mucosal lymphocytes. Proc Natl Acad Sci USA 1992; 89:1924–8.

28 Chen Y, Inobe J, Marks R, Gonnella P, Kuchroo VK, Weiner HL. Peripheral deletion of antigen-reactive T cells in oral tolerance. Nature 1995; 376:177–80.

29 Gonnella PA, Chen Y, Inobe J, Komagata Y, Quartulli M, Weiner HL. *In situ* immune response in gut-associated lymphoid tissue (GALT) following oral antigen in TCR-transgenic mice. J Immunol 1998; 160:4708–18.

30 Cibotti R, Kanellopoulos JM, Cabaniols JM *et al.* Tolerance to a self-protein involves its immunodominant but does not involve its subdominant determinants. Proc Natl Acad Sci USA 1992; 89:416–20.

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

Dan-Yuan LO[1,2,4], Ter-Hsin CHEN[3], Maw-Sheng CHIEN[1], Kenji KOGE[5], Akira HOSONO[6], Shuichi KAMINOGAWA[6] and Wei-Cheng LEE[1]*

[1]Graduate Institute of Veterinary Pathology, [2]Department of Veterinary Medicine, [3]Graduate Institute of Veterinary Public Health, College of Veterinary Medicine, National Chung Hsing University, 250 Kuo Kuang Rd, 402 Taichung, [4]Department of Veterinary Medicine, National Chia Yi University, 300 University Rd, 600 Chiayi, Taiwan, R.O.C., [5]Chigasaki Laboratory, Shin Mitsui Sugar Co., Ltd., 1–2–14 Honson, Chigasaki, Kanagawa 253–0042 and [6]Department of Food Science and Technology College of Bioresource Sciences, Nihon University, Kanagawa, 252–8510, Japan

(Received 8 December 2004/Accepted 21 February 2005)

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.

J. Vet. Med. Sci. 67(6): 591–597, 2005

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.

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:

* CORRESPONDENCE TO: LEE, W. C., Graduate Institute of Veterinary Pathology, College of Veterinary Medicine, National Chung Hsing University, 250 Kuo Kuang Road, 402 Taichung, Taiwan, R.O.C.

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).

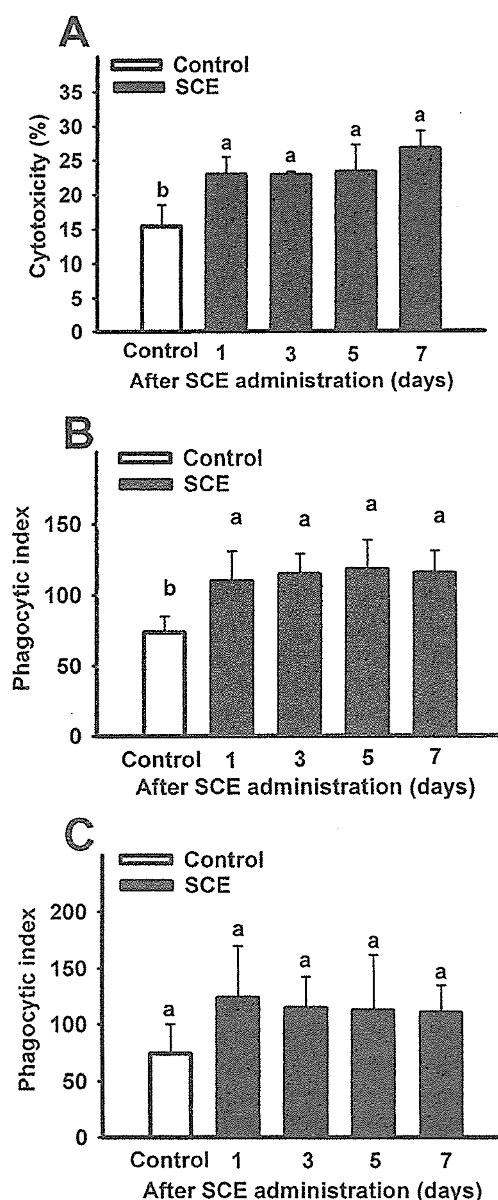

Fig. 1. Effect of duration of SCE administration on leukocyte activity. Five pigs in each group were fed with SCE (500 mg/kg/body weight/day) for 1, 3, 5, and 7 days. Leukocyte activity assays, including cytotoxicity of NK cells (A), phagocytosis by monocytes (B) and neutrophils (C), were performed at 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).

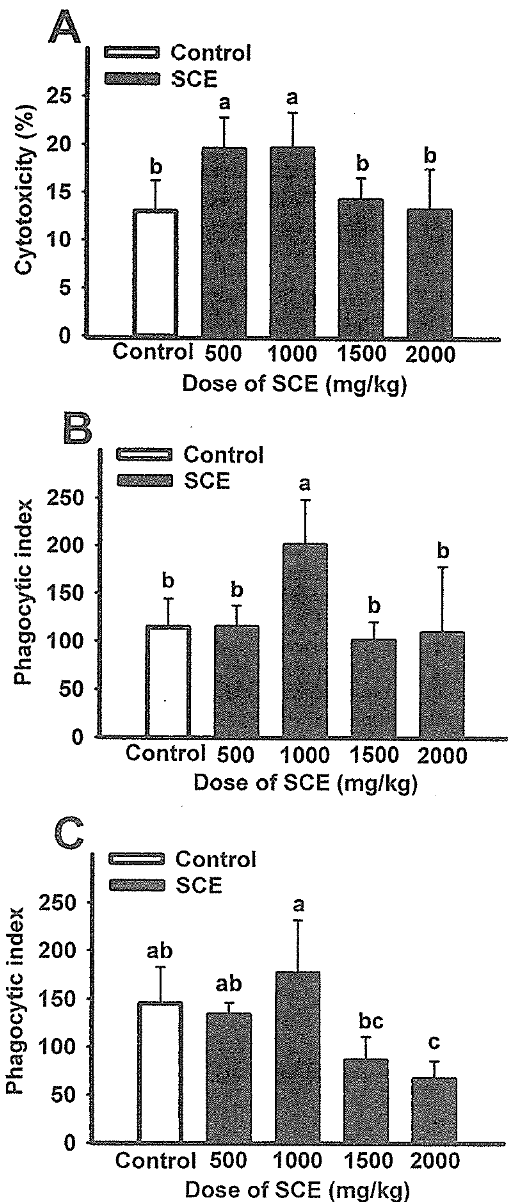

Fig. 2. Effect of SCE dose on leukocyte activity. Five pigs in each group were fed with different dosages of SCE (0–2,000 mg/kg/body weight/day) for 3 consecutive days. Leukocyte activity assays, including cytotoxicity of NK cells (A), phagocytosis of monocytes (B) and neutrophils (C), were performed 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-

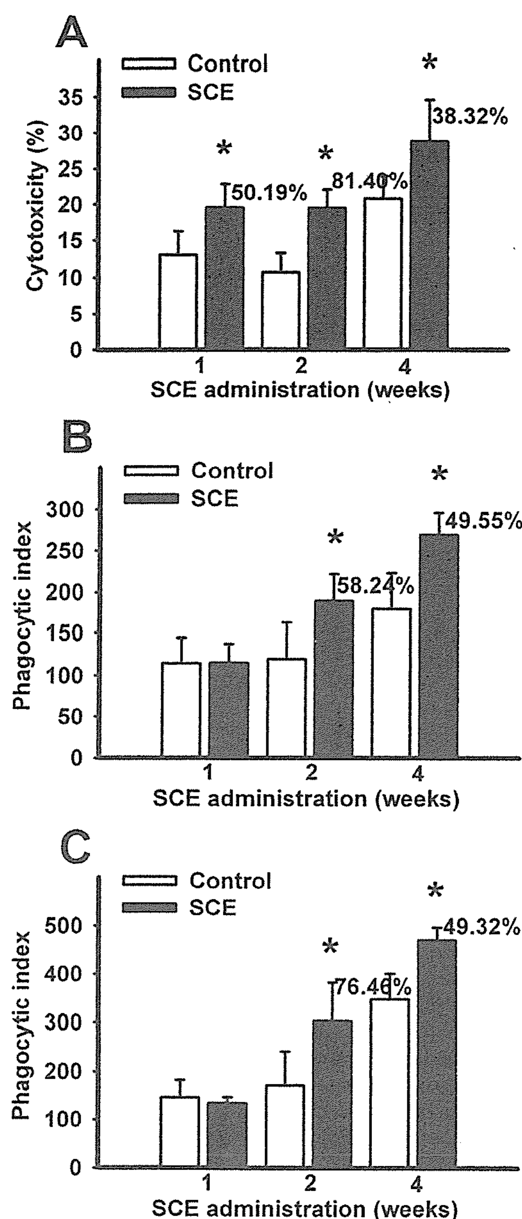

Fig. 3. Effect of prolonged treatment with SCE on leukocyte function. Weanling pigs were fed with SCE (500 mg/kg/ body weight/day) for 3 consecutive days per week for 4 weeks. Pigs fed without SCE served as controls. Leukocyte activity assays, including cytotoxicity of NK cells (A), phagocytosis of monocytes (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).

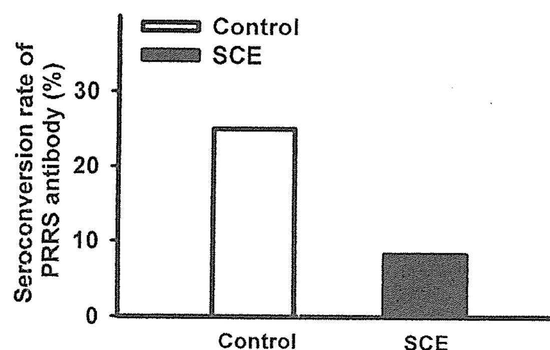

Fig. 4. Effect of SCE on PRRS virus infection. Weanling pigs were fed with SCE (500 mg/kg/day) for 3 consecutive days per week between the ages of 5 and 9 weeks. Blood samples were collected at the end of week 9. The presence of PRRS antibody was assayed using an ELISA kit.

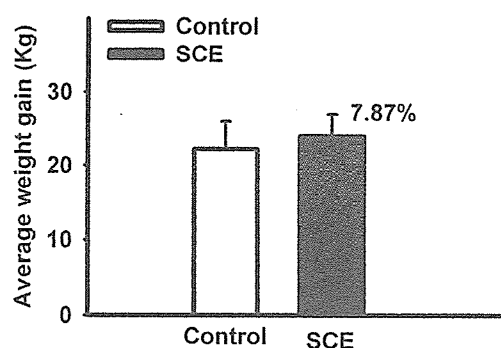

Fig. 5. Evaluation of SCE-treatment on growth performance of pigs. SCE was administered for 3 consecutive days per week between the ages of 5 and 9 weeks, and the body weight was measured in 14-week-old pigs. There was a 7.87% increase in growth in the SCE-treated group compared to control group, but 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.

ACKNOWLEDGEMENTS. This research was financially supported by the grant (91AS-7.2.1-BQ-B2) from the Council of Agriculture of Taiwan, Republic of China. Partial funding provided by Suchiang Chemical & Pharmaceutical Co., Ltd. was very much appreciated. The authors also thank Chia-Cheng Yo for his assistance in the field testing.

REFERENCES

1. Abdel-Nasser, M., Safwat, M. S. and Ali, M. Z. 1983. Detection of antibacterial substances in some plant residues and their effect on certain micro-organisms. *Zentralbl. Microbiol.* **138**: 63–69.
2. Barocci, S., Re, L., Capitani, C., Vivani, C., Ricci, M., Rinaldi, L., Paolucci, G., Scarpantonio, A., Leon-Fernandez, O. S. and Morales, M. A. 1999. Effects of some extracts on the acetylcholine release at the mouse neuromuscular junction. *Pharmacol. Res.* **39**: 239–245.
3. Brekhman, I. I., Nesterenko, I. F., Khasina, E. I. and Zorikov, P. S. 1978. Effect of yellow cane sugar on the performance and the degree of stress manifestations in animals. *Vopr. Pitan.* **6**: 69–70.
4. Chung, W. B., Wu, T. C., Lin, M. W., Huang, C. I., Chang, C. C., Chang, W. F. and Yang, P. C. 1997. Porcine reproductive and respiratory syndrome (PRRS) in Taiwan III. Epidemiologic survey. *J. Chin. Soc. Vet. Sci.* **23**: 43–50.
5. du Manoir, J. M., Albright, B. N., Stevenson, G., Thompson, S. H., Mitchell, G. B., Clark, M. E. and Caswell, J. L. 2002. Variability of neutrophil and pulmonary alveolar macrophage function in swine. *Vet. Immunol. Immunopathol.* **89**: 175–186.
6. El-Abasy, M., Motobu, M., Na, K. J., Shimura, K., Nakamura, K., Koge, K., Onodera, T. and Hirota, Y. 2003. Protective effects of sugar cane extracts (SCE) on *Eimeria tenella* infection in chickens. *J. Vet. Med. Sci.* **65**: 865–871.
7. El-Abasy, M., Motobu, M., Sameshima, T., Koge, K., Onodera, T. and Hirota, Y. 2003. Adjuvant effects of sugar cane extracts (SCE) in chickens. *J. Vet. Med. Sci.* **65**: 117–119.
8. El-Abasy, M., Motobu, M., Shimura, K., Na, K. J., Kang, C. B., Koge, K., Onodera, T. and Hirota, Y. 2002. Immunostimulating and growth-promoting effects of sugar cane extracts (SCE) in chickens. *J. Vet. Med. Sci.* **64**: 1061–1063.
9. Ghoneum, M. and Jewett, A. 2000. Production of tumor necrosis factor-alpha and interferon-gamma from human peripheral blood lymphocytes by MGN-3, a modified arabinoxylan from rice bran, and its synergy with interleukin-2 *in vitro*. *Cancer Detect. Prev.* **24**: 314–324.
10. Halbur, P. G. 1998. Porcine respiratory disease. *Proc. Int. Pig Vet. Soc. Congr.* **15**: 1–10.
11. Huang, Y. M., Chien, M. S., Liu, C. I., Lin, C. C., Shien, H. K. and Lee, W. C. 2000. Evaluation of the cytotoxicity activity of swine natural killer cells using non-radioactive assays based on flow cytometer and time-resolved fluorometer. *J. Chin. Soc.*

Vet. Sci. **26**: 297–308.

12. Koge, K., Nagai, Y., Mizutani, T., Suzuki, M. and Araki, S. 2001. Inhibitory effects of sugar cane extracts on liver injuries in mice. *Nippon Shokuhin Kagaku Kogaku Kaishi* **48**: 231–237.
13. Konopski, Z., Seljelid, R. and Eskeland, T. 1993. Cytokines and PGE2 modulate the phagocytic function of the beta-glucan receptor in macrophages. *Scand. J. Immunol.* **37**: 578–592.
14. Ledon, N., Casaco, A., Rodriguez, V., Cruz, J., Gonzalez, R., Tolon, Z., Cano, M. and Rojas, E. 2003. Anti-inflammatory and analgesic effects of a mixture of fatty acids isolated and purified from sugar cane wax oil. *Planta Med.* **69**: 367–369.
15. Lee, S. S., Wei, Y. H., Chen, C. F., Wang, S. Y. and Chen, K. Y. 1995. Antitumor effects of *Ganodema lucidum*. *J. Chin. Med.* **6**: 1–12.
16. Miura, T., Ohno, N., Miura, N. N., Adachi, Y., Shimada, S. and Yadomae, T. 1999. Antigen-specific response of murine immune system toward a yeast β-glucan preparation, zymosan. *FEMS Immunol. Med. Microbiol.* **24**: 131–139.
17. Molina, V., Arruzazabala, M. L., Carbajal, D., Mas, R. and Valdes, S. 2000. Antiplatelet and antithrombotic effect of D-003. *Pharmacol. Res.* **42**: 137–143.
18. Nakasone, Y., Takara, K., Wada, K., Tanaka, J. and Yogi, S. 1996. Antioxidative compounds isolated from *Kokuto*, non-centrifuged cane sugar. *Biosci. Biotechnol. Biochem.* **60**: 1714–1716.
19. Pryce, M. J., Aston, W. P. and Chadwick, J. S. 1990. Cane sugar factor as an inducing agent of immunity in Galleria mellonella. *Dev. Comp. Immunol.* **14**: 369–378.
20. Shibata, Y., Metzger, W. J. and Myrvik, Q. N. 1997. Chitin particle-induced cell-mediated immunity is inhibited by soluble mannan. *J. Immunol.* **159**: 2462–2467.
21. Takara, K., Matsui, D., Wada, K., Ichiba, T. and Nakasone, Y. 2002. New antioxidative phenolic glycosides isolated from *Kokuto* non-centrifuged cane sugar. *Biosci. Biotechnol. Biochem.* **66**: 29–35.
22. Tamaki, H., Man, S. L., Ohta, Y., Katsuyama, N. and Chinen, I. 2003. Inhibition of osteoporosis in rats fed with sugar cane wax. *Biosci. Biotechnol. Biochem.* **67**: 423–425.
23. Thacker, E. L. 2001. Immunology of the porcine respiratory disease complex. *Vet. Clin. North Am. Food Anim. Pract.* **17**: 551–565.
24. Yamamoto, Y., Shirono, H., Kono, K. and Ohashi, Y. 1997. Immunopotentiating activity of the water-soluble lignin rich fraction prepared from LEM-the extract of the solid culture medium of Lentinus edodes mycelia. *Biosci. Biotechnol. Biochem.* **61**: 1909–1912.

Food Sci. Biotechnol. Vol. 14, No. 3, pp. 405 ~ 409 (2005)

RESEARCH NOTE

Food Science and Biotechnology

© The Korean Society of Food Science and Technology

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

Ji Yeon Kim, Seongkyu Lee[1], Do-Won Jeong[2], Satoshi Hachimura[1], Shuichi Kaminogawa[3] and Hyong Joo Lee[2]*

Department of Food Standard Evaluation, Korea Food & Drug Administration, Seoul 122-704, Korea

[1]Department of Applied Biological Chemistry, The University of Tokyo, Tokyo, Japan

[2]School of Agricultural Biotechnology, and Center of Agricultural Biomaterials, Seoul National University, Seoul 151-742, Korea

[3]Department of Food Science and Technology, College of Bioresource Science, Nihon University, Tokyo, Japan

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.

*Corresponding author: Tel: 82-2-880-4853; Fax: 82-2-873-5095
E-mail: leehyjo@snu.ac.kr

Received February 25, 2005; accepted April 19, 2005

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-

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

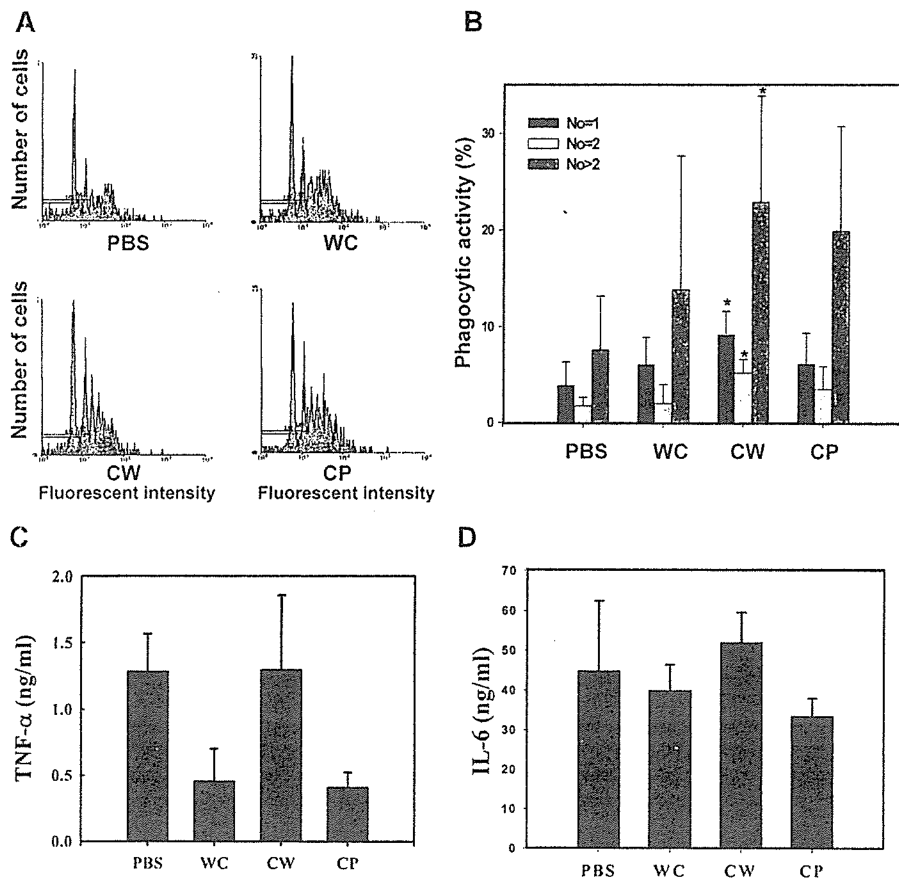

Fig. 1. Phagocytic activity of PEC obtained from mice injected intraperitoneally with heat-killed whole cells (WC), cell walls (CW), and cytoplasm (CP) of *L. lactis* at 500 μg/mouse on days 0, 2, and 4. On day 5, the phagocytic activity was measured (A, B) and cytokine production in PEC was analyzed (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, *$P < 0.05$).

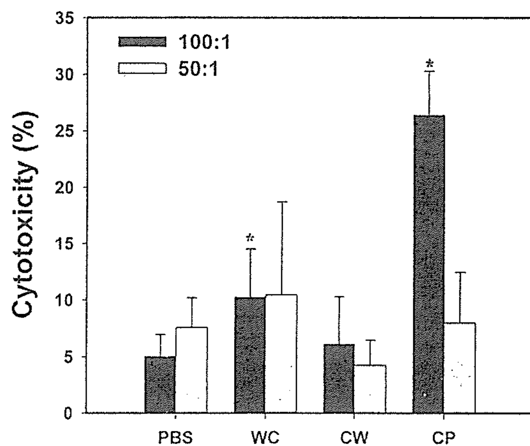

Fig. 2. Percentage cytotoxicity of the natural-killer activity of spleen cells obtained from mice injected intraperitoneally with WC, CW, and CP of *L. lactis* against Yac-1 cells. Effector: target cell ratios were 100:1 and 50:1. The cytotoxicity was measured using LDH assays. Data are shown as mean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

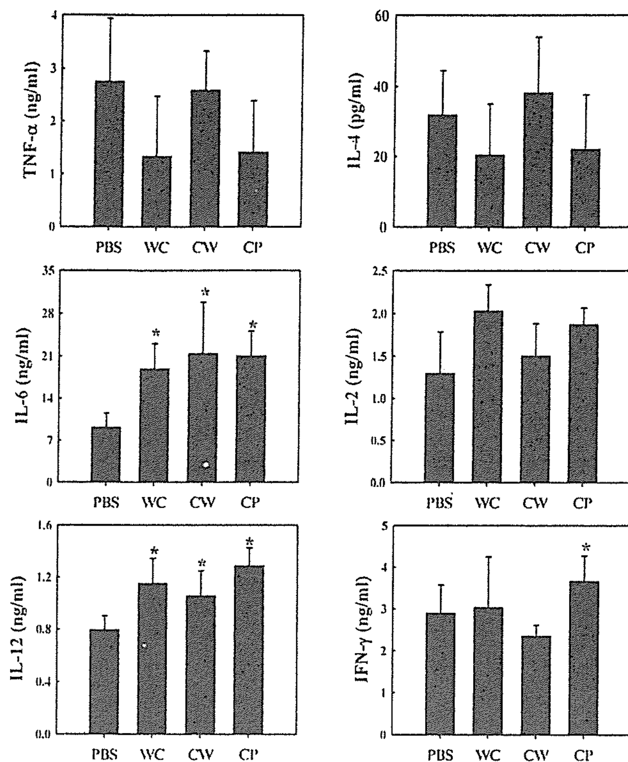

Fig. 3. Production of cytokines by spleen cells from mice injected intraperitoneally with WC, CW, and CP of *L. lactis*. Spleen cells were cultured with mitogen (Con A or LPS) for 48 h. The concentrations of cytokines (IL-2, IL-4, and IFN-γ for Con-A-stimulated cultures, and IL-6, IL-12, and TNF-α for 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

This work was supported by the BK21 program of the Korean Ministry of Education.

References

1. Fauci AS, Rosenberg SA, Sherwin SA, Dinarello CA, Longo DL, Lane HC. Immunomodulators in clinical medicine. Ann. Internal. Med. 106: 421-433 (1987)
2. Lee J, Ametani A, Enomoto A, Sato Y, Motoshima H, Ike F, Kaminogawa S. Screening for the immunopotentiating activity of food microorganisms and enhancement of the immune response by *Bifidobacterium adolescentis* M101-4. Biosci. Biotechnol. Biochem. 57: 2127-2132 (1993)
3. Perdigon G, Alvarez S, Holgado AAPR. Immunoadjuvant activity of oral *Lactobacillus casei*: influence of dose on the secretory immune response and protective capacity in intestinal infections. J. Dairy Res. 58: 485-496 (1991)
4. Perdigon. G, Macias MEN, Alvarez S, Oliver G, Holgado AAPR. Prevention of gastrointestinal infection using immunobiological methods with milk fermented with *Lactobacillus casei* and *Lactobacillus acidophilus*. J. Dairy Res. 57: 255-264 (1990)
5. Oh S, Lee J-H, Kim G-T, Shin J-G, Baek Y-J. Anti-cariogenic activity of a bacteriocin by *Lactococcus* sp. HY449. Food Sci. Biotechnol. 12: 9-12 (2003)
6. Lee N-K, Kim T-H, Choi S-Y, Lee SK, Paik H-D. Identifiaction and probiotic properties of *Lactococcus lactis* NK24 isolated from Jeot-gal, a Korean fermented food. Food Sci. Biotechnol. 13: 411-416 (2004)
7. Rafter JJ. The role of lactic acid bacteria in colon cancer prevention. Scand. J. Gastroenterol. 30: 497-502 (1995)
8. De Simone C, Vesely R, Negri R, Bianchi SB, Zanzoglu S, Cilli A, Lucci L. Enhancement of immune response of murine Peyer's patches by a diet supplemented with yogurt. Immunopharmacol. Immunotoxicol. 9: 87-100 (1987)
9. Perdigon G, De Macias, MEN, Alvarez S, Oliver G, De Ruiz Holgado AP. Effect of perorally administered lactobacilli on macrophage activation in mice. Infect. Immun. 53: 404-416 (1986)
10. Perdigon G, De Macias, MEN, Alvarez S, Oliver G, De Ruiz Holgado AP. Systemic augmentation of the immune response in mice by feeding fermented milks with *Lactobacillus casei* and *Lactobacillus acidophilus*. Immunol. 63: 17-23 (1988)
11. Balansky R, Gyosheva B, Ganchev G, Mircheva Z, Minkova S, Georgiev G. Inhibitory effects of freeze-dried milk fermented by selected *Lactobacillus bulgaricus* strains on carcinogenesis induced by 1,2-dimethylhydrazine in rats and by diethylnitrosamine in hamsters. Cancer Lett. 147: 125-137 (1999)
12. Reddy BS, Rivenson A. Inhibitory effect of *Bifidobacterium longum* on colon, mammary and liver carcinogenesis induced by 2-amino-3-methylimidazole[4,5-f]quinoline, a food mutagen. Cancer Res. 53: 3914-3918 (1993)
13. Yasui H, Ohwaki M. Enhancement of immune response in Peyer's patch cells cultured with *Bifidobacterium breve*. J. Dairy Sci. 74: 1187-1195 (1991)
14. Kim JY, Lee S, Hachimura S, Kaminogawa S, Lee HJ. *In vitro* immunopotentiating activity of cellular component of *Lactococcus lactis* ssp. *lactis*. J. Microbiol. Biotechnol. 13: 202-206 (2003)
15. Yoshizawa Y, Tsunehiro J, Nomura K, Itoh M, Fukui F, Ametani A, Kaminogawa S. *In vivo*macrophage-stimulation activity of the enzyme-degraded water-soluble polysaccharide fraction from a marine alga (*Gracilaria verrucosa*). Biosci. Biotechnol. Biochem. 60: 1667-1671 (1996)
16. Gill HS, Rutherfurd KJ, Prasad J, Gopal PK. Enhancement of natural and acquired immunity by *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) and *Bifidobacterium lactis* (HN019). Br. J. Nutr. 83: 167-176 (2000)
17. Kelly JM, Darcy PK, Markby JL, Godfrey DI, Takeda K, Yagita H, Smyth MJ. Induction of tumor-specific Tcell memory by NK cell-mediated tumor rejection. Nature Immunol. 3: 83-90 (2002)
18. Haller D, Blum S, Bode C, Hammes W, Schiffrin EJ. Activation of human peripheral blood mononuclear cells by nonpathogenic bacteria *in vitro*: evidence of NK cells as primary targets. Infect. Immun. 68: 752-759 (2000)
19. Kato I, Yokokura T, Mutai M. Augmentation of mouse natural killer cell activity by *Lactobacillus casei* and its surface antigens. Microbiol. Immunol. 28: 209-217 (1984)
20. Takagi A, Matsuzaki T, Sato M, Nomoto K, Morotomi M, Yokokura T. Enhancement of natural killer cytotoxicity delayed murine carcinogenesis by a probiotic microorganism. Carcinogenesis. 22: 599-605 (2001)
21. Cross ML, Mortensen RR, Kudsk J, Gill HS. Dietary intake of *Lactobacillus rhamnosus* HN001 enhances production of both Th1 and Th2 cytokines in antigen-primed mice. Med. Microbiol. Immunol. 191: 49-53 (2002)
22. Hessle C, Hanson LA, Wold AE. Lactobacilli from human gastrointestinal mucosa are strong stimulators of IL-12 production. Clin. Exp. Immunol. 116: 276-282 (1999)
23. Kato I, Tanaka K, Yokokura T. Lactic acid bacterium potently induces the production of interleukin-12 and interferon- by mouse splenocytes. Int. J.Immunopharmacol. 21: 121-131 (1999)
24. Murosaki S, Muroyama K, Yamamoto Y, Yoshikai Y. Antitumor effect of heat-killed *Lactobacillus plantarum* L-137 through restoration of impaired interleukin-12 production in tumor-beating mice. Cancer Immunol. Immunother. 49: 157-164 (2000)

25. Yasutake N, Matsuzaki T, Kimura K, Hashimoto S, Yokokura T, Yoshikai Y. The role of tumor necrosis factor (TNF)- in the antitumor effect of intrapleural injection of *Lactobacillis casei* strain Shirota in mice. Med. Microbiol. Immunol. 188: 9-14 (1999)
26. Hamann L, EL-Samalouti V, Ulmer AJ, Flad HD, Rietschel ET. Components of gut bacteria as immunomodulators. Int. J. Food Microbiol. 41: 141-154 (1998)
27. Hosono A, Lee J, Ametani A, Natsume M, Hirayama M, Adachi T, Kaminogawa S. Characterization of a water-soluble polysaccharide fraction with immunopotentiating activity from *Bifidobacterium adolescentis* M101-4. Biosci. Biotechnol. Biochem. 61: 312-316 (1997)
28. Takahashi T, Oka T, Iwana H, Kuwata T, Yamamoto Y. Immune response of mice to orally administered lactic acid bacteria. Biosci. Biotechnol. Biochem. 57: 1557-1560 (1993)
29. Tejada-Simon MV, Pestka JJ. Proinflammatory cytokine and nitric oxide induction in murine macrophages by cell wall and cytoplasmic extracts of lactic acid bacteria. J. Food Prot. 62: 1435-1444 (1999)
30. Lee MJ, Zang Z, Choi EY, Shin HK, Ji GE. Cytoskeleton recognization and cytokine production of macrophages bifidobacterial cells and cell-free extracts. J. Microbiol. Biotechnol. 12: 398-405 (2002)