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Establishment and Evaluation of an *in vitro* M Cell Model using C2BBe1 Cells and Raji Cells

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In vitro **M cell models, consisting of co-cultures of Caco-2 cells and lymphoid cells, were developed and examined to observe bacterial transport. However, under our experimental conditions, the differentiation of Caco-2 cells into M cell-like cells could not be induced efficiently. To obtain a functionally stable M cell model based on human cells, C2BBe1 cells were screened and co-cultured with human Raji cells. In our co-cultures, increased sialyl Lewis A antigen expression and decreased *Ulex europeaus* agglutinin 1 binding were observed. Regarding the functional properties of the model, microsphere and lactic acid bacteria transport across the C2BBe1 co-cultures were increased compared with the levels seen in monocultures. The C2BBe1 monolayers that were co-cultured with Raji cells exhibited some M cell features; therefore, we consider our M cell model to be useful for investigating the interactions of bacteria with M cells.**

Key words: M cell; C2BBe1; Raji; co-culture

INTRODUCTION

Immunization via the oral route offers several important advantages. In particular, unlike parenteral routes, specific immune responses to vaccine antigen are induced in the mucosa *(1)*. Therefore, a variety of oral vaccines that were generated from genetically modified bacteria have been reported *(2)*. We generated recombinant lactic acid bacteria (LAB) for use in an oral vaccine. These recombinants induced protective immunity and exhibited adjuvant properties *(3, 4)*. However, no practical oral vaccines that have used LAB as an antigen delivery vehicle have been established. The first step in the induction of protective intestinal immune responses is the uptake and transport of antigens to gut-associated lymphoid tissue (GALT). Hence, it is thought that efficient recombinant LAB transport to immuno-competent cells is necessary for effective vaccination.

It is generally thought that M cells, which are located in the follicle-associated epithelium (FAE) of Peyer's patch, play a major role in the uptake of luminal antigens *(5)*. M cells have a characteristic morphology and different functions compared with other intestinal enterocytes. M cells lack a well-organized brush border, have a thick glycocalyx, and display low levels of digestive enzymes, such as alkaline phosphatase and sucrase-isomaltase *(6–9)*. In addition, M cells have intraepithelial pockets containing lymphocytes, macrophages, and dendritic cells. The antigens internalized by M cells are transferred to these underlying immune cells, and antigen-specific immune responses are initiated *(10)*. Therefore, it is considered that these processes are key triggers of the induction of intestinal mucosal immunity. In addition, M cells are targeted by invasive pathogens, which exploit their uptake mechanisms to gain access to the body *(11)*. However, the uptake mechanisms of M cells are little known except for those of a few pathogens such as *Yersinia* and type-I-piliated bacteria *(12, 13)*. Due to the low number of M cells in the human intestine and the difficulty in culturing M cells, the characterization of M cells including their antigen uptake mechanisms has not advanced very far in *in vivo* or *in vitro* studies *(14, 15)*.

The human colon carcinoma cell line Caco-2 is widely used as a model of intestinal epithelial cells in studies of bacterial adhesion, invasion, and drug absorption *(16–18)*. In 1997, Kernéis et al. co-cultured Caco-2 cells with isolated murine Peyer's patch lymphocytes and proposed an *in vitro* human FAE model *(19)*. In this model, Caco-2 cells showed similar features to M cells, such as apical microvilli disorganization, the disappearance digestive enzymes, and the ability to transport microspheres and *Vibrio*. Based on this model, a human intestinal M cell model was established using co-cultures of Caco-2 cells and human Raji B cells instead of murine cells *(20)*. Subsequently, further M cell models with improved culture conditions have been developed, for example

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using Caco-2 subclones, and used to evaluate the transport of several pathogens and proteins *(21–23)*.

C2BBe1 cells were cloned from Caco-2 cells, and this cell line shows a more homogeneous brush border expression than the parental Caco-2 cells *(24)*. C2BBe1 cells have also been co-cultured with murine Peyer's patch lymphocytes in order to establish an M cell model *(25)*. In this study, to obtain a functionally stable M cell model based on human cells, we attempted to establish an M cell model by co-culturing C2BBe1 cells with Raji B cells.

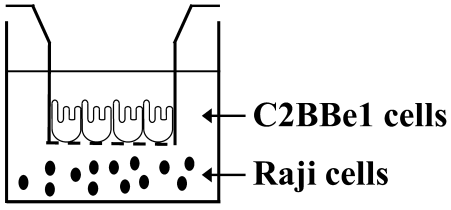

Fig. 1. Schematic of C2BBe1 cell and Raji cell co-culture model. Raji cells were added to basolateral side of C2BBe1 cell monolayers and co-cultured for 3–5 days.

MATERIALS AND METHODS

Bacterial strain and culture

Recombinant *Lactobacillus casei* IGM393 harboring pLPEmpty was grown in MRS broth (Difco) containing 5 μg/ml of erythromycin at 37 °C *(3)*.

Cell culture conditions

C2BBe1 cells were obtained from the American Type Culture Collection. The cells were cultured in Dulbecco's modified Eagle medium (DMEM; Sigma) supplemented with 10 % fetal bovine serum (FBS; JRD), 1 × Glutamax I (Gibco BRL), 1 × nonessential amino acids (Gibco BRL), penicillin (100 U /ml), and streptomycin (100 μg/ml) (Gibco BRL). The human Burkitt's lymphoma cell line Raji (RCB1647) was provided by RIKEN BRC through the National Bio-Resource Project of MEXT, Japan. The Raji cells were cultivated in RPMI1640 (Sigma) supplemented with 10 % FBS, 1 × nonessential amino acids, 1 × Glutamax I, penicillin (100 U /ml), and streptomycin (100 μg/ml) (Gibco BRL). All cells were grown in a humidified 5 % CO_2 atmosphere at 37 °C.

Induction of M cell features in C2BBe1 cells co-cultured with Raji cells

The induction of M cells from C2BBe1 cells was performed according to the methods of Corr et al. *(25)*. C2BBe1 cells were seeded (1×10^5 cells) onto transwell membranes (12-mm membrane diameter, 3.0-μm pore size, Corning) and cultured until they had fully differentiated. The medium was changed every 2 days. The transepithelial electrical resistance (TEER) of the C2BBe1 cells was measured with a Millicell-ERS (MILLIPORE) to confirm their differentiation and the integrity of the monolayer. After the TEER value of the C2BBe1 monolayer had reached 250 $\Omega \times cm^2$, Raji cells were added to the basolateral compartment (Fig. 1). The co-cultures were maintained for 3–6 days. The upper medium was changed every day.

Immunofluorescence

For immunofluorescence microscopy, samples were washed three times with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS. After fixation, the samples were quenched with 50 mM NH_4Cl for 10 min and washed with PBS. The samples were then blocked with 2% bovine serum albumin in PBS for 60 min, before being incubated with a sialyl Lewis A (SLAA) antibody, β1 integrin antibody, or FITC-conjugated *Ulex europeaus* (UEA-1) for 60 min at room temperature. Then, the samples were washed and incubated for 60 min with an Alexa Fluor 488 goat anti-mouse IgG1 antibody (dilution: 1:2000). Transwell membranes were removed with a scalpel and mounted on glass slides. The slides were observed by fluorescence microscopy (Biozero; KEYENCE), and the stained area was measured by imageJ software *(26)*.

Fluorescent microsphere transport

Microsphere transport was observed in Hank's Balanced Salt Solution (HBSS) buffered to pH 7.4. After equilibration, the HBSS on the donor side was replaced with 500 μl of prewarmed microsphere suspension. The number of particles transported across the cell monolayer was then quantified in a Fluorescent Activated Cell Scan (FACScan, Becton-Dickinson).

Measurement of Lactobacillus casei IGM393 transport

L. casei IGM393 that had been cultured overnight were collected by centrifugation, washed three times with PBS, and resuspended in DMEM, before the bacterial cell concentrations were adjusted to 2×10^8 CFU/ml. A 500-μl volume of the bacterial suspension was added to the apical side of the C2BBe1 monolayers and incubated for 3 h. The basolateral media were then sampled and spread onto MRS-agar plates to estimate the number of colony-forming units.

Statistical analysis

Data were evaluated with Student's t-test and p values of less than 0.01 were considered statistically significant.

RESULTS

Monitoring the transepithelial electrical resistance of C2BBe1 monolayers during growth on a transwell membrane

The differentiation of C2BBe1 cells and the integrity of the monolayers were confirmed by measuring their transepithelial electrical resistance. The TEER values of the C2BBe1 cells had reached 300 $\Omega \times cm^2$ at 21 days (Fig. 2). After the C2BBe1 cells had been co-cultured with Raji cells, the TEER values of the co-cultures were similar to those of the monocultures (Fig. 3).

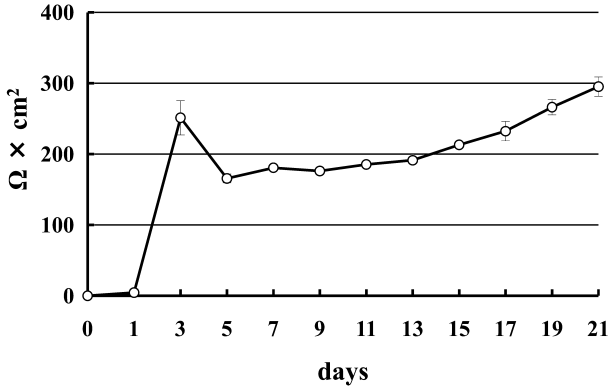

Fig. 2. TEER values of C2BBe1 monolayers grown on transwell membranes.

Expression of M cell markers

To investigate the effects on the C2BBe1 monolayer of co-culture with Raji cells, the expression levels of characteristic phenotypic markers of human M cells were examined. The expression of SLAA was increased by approximately 3-fold in the co-cultures compared with the monocultures (Fig. 4), and the binding of UEA-1 was decreased in apical membrane of the co-cultures (Fig. 5). There was no clear difference in the apical localization of β1 integrin in the C2BBe1 monolayers between the monoculture and co-culture conditions.

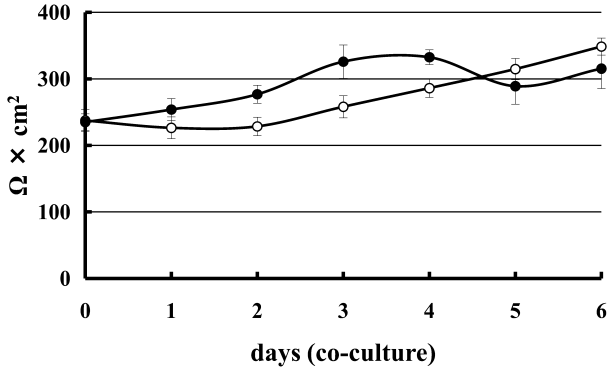

Fig. 3. Comparison of the TEER values of C2BBe1 monocultures and co-cultures. After the TEER values of the monolayer had reached 250 $\Omega \times cm^2$, Raji cells were added to the basolateral compartment. The TEER values of co-cultures were measured everyday (closed circles). Monocultures of C2BBe1 monolayers were used as controls (open circles).

Transport of fluorescent microspheres

In order to confirm that the C2BBe1 cells had acquired M cell functional features, the number of transported fluorescent microspheres was measured. The transport of particles was increased 100-fold in the co-cultures compared to the C2BBe1 monocultures (Fig. 6).

Quantification of L. casei IGM393 transport across C2BBe1 monolayers

The ability of the *in vitro* M cell model to translocate *L. casei* IGM393 was examined. *L. casei* IGM393 were added to the apical side of the C2BBe1 monolayers. The C2BBe1 monolayers cultured with Raji cells had transported 10^3 CFU *L. casei* IGM393 after 3 h incubation at 37°C (Fig. 7). On the other hand, little bacterial transport was observed in the C2BBe1 monolayers cultured alone.

DISCUSSION

Observations of the internalization of the bacteria into non-phagocytic cells have mainly been performed using epithelial cell monolayers. However, in the intestine, a number of bacteria invade the host through M cells, and the morphology and function of M cells are markedly different from those of epithelial cells. Hence, a simple epithelial cell monolayer is insufficient as an M cell model, and a model system resembling M cells is necessary to observe bacterial internalization *in vitro*.

In vitro M cell models have been generated by co-culturing a variety of Caco-2 subclones with mouse Peyer's patch or human B cells. We attempted to establish an M cell model using Caco-2 cells in a preliminary study. However, as the Caco-2 monolayer was unstable during co-culture, we found it difficult to establish an M cell model using this technique. Therefore, Caco-2 clones were screened to see if they could be used to produce a stable model.

C2BBe1 cells form a polarized monolayer with an

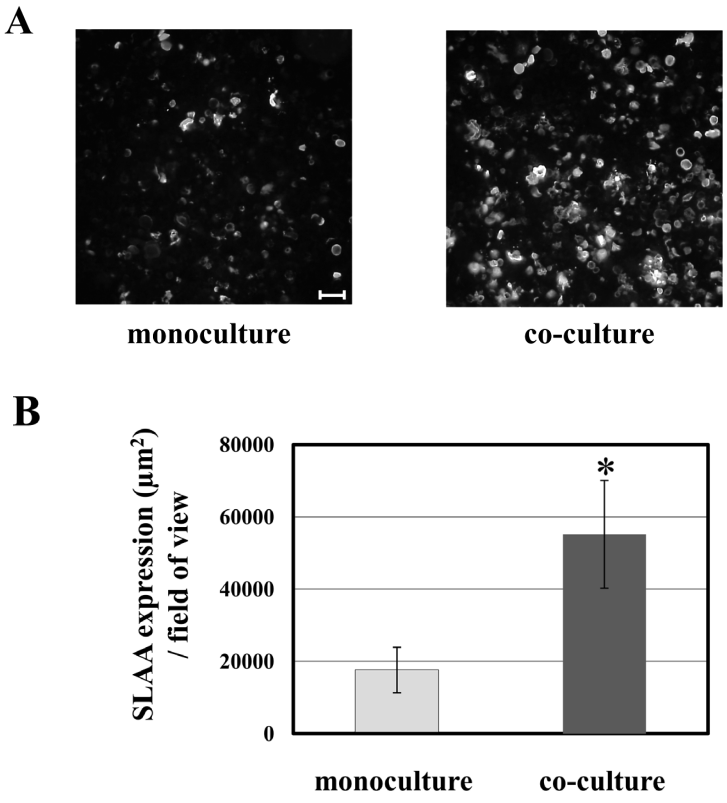

Fig. 4. Observation and quantification of sialyl Lewis A antigen expression. A) Immunohistochemistry of SLAA expression in C2BBe1 monocultures and co-cultures was observed by fluorescence microscopy. Scale bar: 50 μm. B) SLAA expression in the apical membranes of co-cultures compared with that observed in monocultures.

apical brush border that is morphologically comparable with that of the human colon and have been used to investigate bacterial adhesion and invasion *(27, 28)*. In the present study, we investigated whether human Raji B cells can induce C2BBe1 cells to differentiate into M cell-like cells.

First, the TEER values of C2BBe1 cells cultured on transwell membranes were measured as an indicator of cell monolayer integrity because we consider careful monitoring to be important for the establishment of a stable and reproducible model *(29)*. The TEER value increased rapidly within 3 days of the cells being seeded on the transwell membranes and gradually increased thereafter (Fig. 2). The C2BBe1 cells grew slowly and more stably over the long-term than other Caco-2 clones (data not shown). After the TEER value had reached 250 $\Omega \times cm^2$, Raji cells were added to the basolateral compartments of the C2BBe1 monolayers. Monolayers of other Caco-2 clones could not be used because the TEER values of their co-cultures were extremely low, and the integrity of the differentiated monolayers was lost (data not shown). On the other hand, the TEER values of C2BBe1 co-cultures were between 250 and 300 $\Omega \times cm^2$, which was similar to that of the C2BBe1 monocultures (Fig. 3). The reduction in the Caco-2 cell co-culture TEER has been suggested to be due to the conversion of Caco-2 cells into M cells, whereas the C2BBe1 co-cultures seemed to maintain their integrity *(21)*.

In order to investigate the effects of Raji cells on C2BBe1 monolayers, the expression of M cell markers was examined. Several M cell markers have been reported, and in our experiment we observed that the apical expression of SLAA was significantly increased in co-cultures compared to monocultures (Fig. 4). The binding of UEA-1, which is a mouse and rabbit M cell marker, was decreased in the apical membranes of the co-cultures (Fig. 5). These results were also observed in a number of human M cell models *(20, 30)*. On the other hand, we were not able to find clear differences in the localization of β1 integrin between the co-cultures and

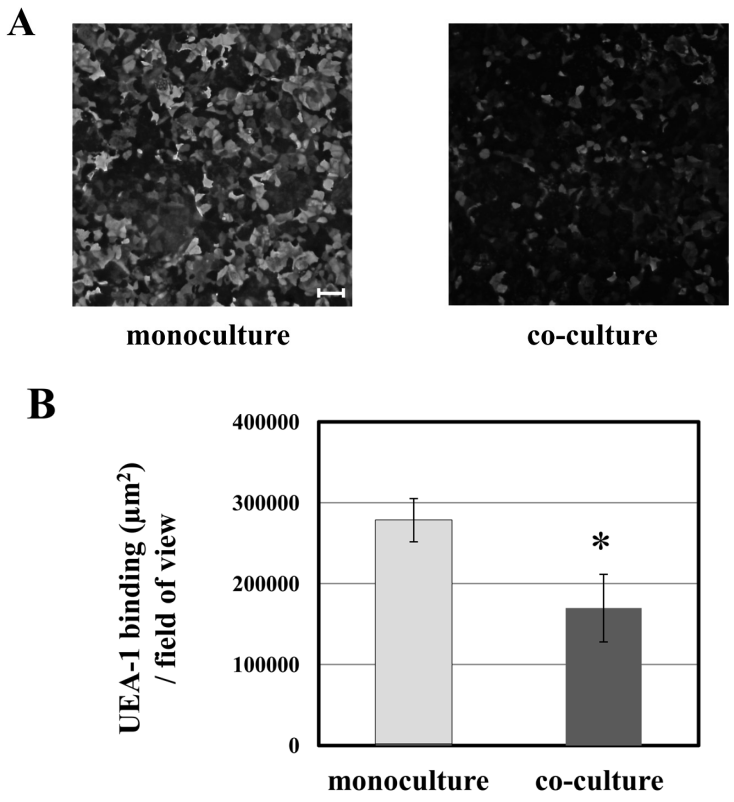

Fig. 5. Binding of UEA-1 lectin to C2BBe1 cells in monocultures and co-cultures. A) The binding of UEA-1 conjugated with FITC to C2BBe1 cell monolayers was observed by fluorescence microscopy. Scale bar: 50 μm. B) UEA-1 binding in C2BBe1 co-cultures compared with that observed in monocultures

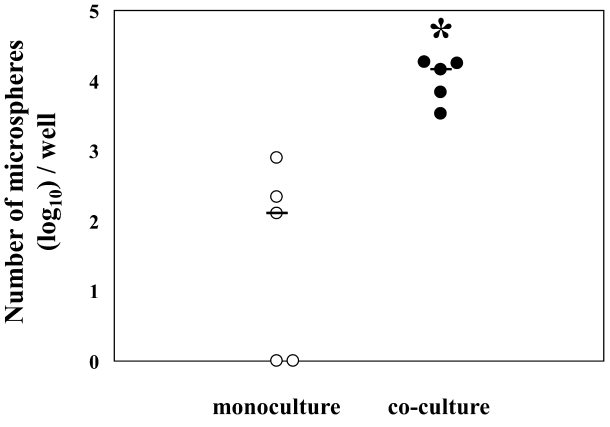

Fig. 6. Transport of microspheres across C2BBe1 monocultures and co-cultures. Mono- and co-cultures were incubated with microspheres for 3 hr at 37°C. The number of transported microspheres was evaluated by FACS.

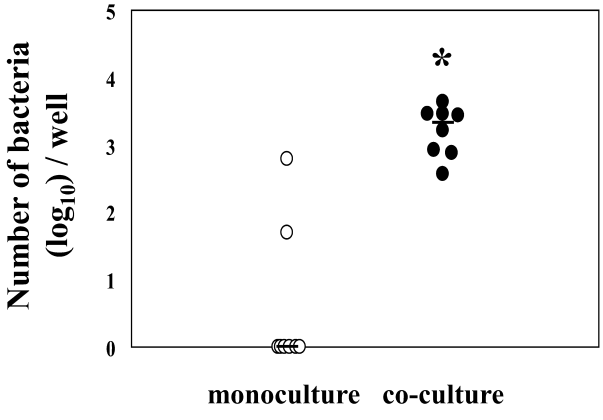

Fig. 7. Transport of *Lactobacillus casei* IGM393 across C2BBe1 monocultures and co-cultures. Mono- and co-cultures were incubated with *L. case*i IGM393 for 3 hr at 37°C. The number of transported bacteria was measured by CFU.

mono cultures.

Furthermore, to investigate the transport function of our model, microsphere transport was examined in both the co-cultures and monocultures. The number of transported particles was significantly increased in the co-cultures (Fig. 6). Before and after the particle transport

assay, the TEER values of each monolayer were not change, indicating that the integrity of the monolayers was maintained during the transport assay. Increased particle transport is a typical feature of M cell models *(20, 31)*. These results suggest that Raji cells induce C2BBe1 cell differentiation.

Finally, to investigate whether the co-cultures are able to transport non-invasive bacteria, we observed *L. casei* IGM393 transport across the monolayers. The number of transported *L. casei* was significantly increased in the basolateral compartments of the co-cultures compared to those of the monocultures (Fig. 7). Consequently, it was shown that the co-cultures were capable of incorporating even non-pathogenic and non-invasive lactic acid bacteria. However, in a few monocultures, a similar level of *L. casei* transport was found. It was reported that the differentiation of Caco-2 cells into M cell like-cells occurred without lymphocyte treatment, and a similar phenomenon was also seen in our experiment *(22)*.

Recently, it has been suggested that the induction of M cell features in Caco-2 monolayers is mediated by direct contact between Caco-2 and Raji cells, soluble factors such as those found in the Raji cell culture supernatant, and/or macrophage migration inhibitory factor (MIF) *(21, 30, 32)*. However, neither the Raji cell culture supernatant nor MIF efficiently induced the differentiation of C2BBe1 monolayers in our experiment. The differences in the results between the above studies and ours might have been due to the different Caco-2 subclones and culture conditions used including differences in the FBS used. At the very least, the presence of Raji cells is important in our C2BBe1 model.

C2BBe1 cells co-cultured with murine Peyer's patch lymphocytes showed M cell-like features such as disordered apical membrane brush borders and bacterial transport *(25)*. That model was constructed with an established human cell culture and primary mouse cells which were isolated from mouse Peyer's patch. On the other hand, our model was based entirely on established human cell lines. Hence, it is thought that our model is a homologous co-culture like the *in vitro* human M cell model compared with previous murine Peyer's patch model reported by Corr et al. in 2006 (*25*).

However, the LAB transport efficiency of our model is lower than that of the murine Peyer's patch model. This difference might be due to the induction efficiency of C2BBe1 differentiation during co-culture because Peyer's patches contain a variety of immunocompetent cells. To obtain an efficient differentiation model, improvements in the culture conditions such as ensuring the close contact of C2BBe1 cells and Raji cells will be necessary *(21)*. Alternatively, there might be differences between the abilities of *L. salivarius* and *L. casei* to adhere to intestinal epithelial cells and Peyer's patch cells *(33, 34)*. However, it remains to be determined whether the uptake of LAB by M cells is a specific or non-specific response.

In this study, to establish a more homologous co-culture model using C2BBe1 cells, C2BBe1 cells were co-cultured with Raji B cells. We demonstrated that Raji cells induced C2BBe1 cells to differentiate in a manner similar to Caco-2 cells that had been co-cultured with murine Peyer's patch cells and the cells used in a number of other *in vitro* M cell models. Therefore, we consider that our C2BBe1 co-cultured model is a useful M cell model. As the interactions between M cells and LAB are poorly understood, investigations of these interactions would help to elucidate the mechanisms of immunostimulation by lactic acid bacteria. Furthermore, our M cell model might contribute not only to examinations of the factors that affect the adhesion and uptake of lactic acid bacteria by M cells but also to studies selecting M cell targeted bacterial strains as vehicles for mucosal vaccine delivery.

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