

ORIGINAL PAPER

Conditioned polarized Caco-2 cell monolayers allow to discriminate for the ability of gut-derived microorganisms to modulate permeability and antigen-induced basophil degranulation

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Clinical and Experimental Allergy

Summary

Background Food allergy is a common allergic disorder – especially in early childhood. The avoidance of the allergenic food is the only available method to prevent further reactions in sensitized patients. A better understanding of the immunologic mechanisms involved in this reaction would help to develop therapeutic approaches applicable to the prevention of food allergy.

Objective To establish a multi-cell *in vitro* model of sensitized intestinal epithelium that mimics the intestinal epithelial barrier to study the capacity of probiotic microorganisms to modulate permeability, translocation and immunoreactivity of ovalbumin (OVA) used as a model antigen.

Methods Polarized Caco-2 cell monolayers were conditioned by basolateral basophils and used to examine apical to basolateral transport of OVA by ELISA. Activation of basophils with translocated OVA was measured by β -hexosaminidase release assay. This experimental setting was used to assess how microorganisms added apically affected these parameters. Basolateral secretion of cytokine/chemokines by polarized Caco-2 cell monolayers was analysed by ELISA.

Results Basophils loaded with OVA-specific IgE responded to OVA in a dose-dependent manner. OVA transported across polarized Caco-2 cell monolayers was found to trigger basolateral basophil activation. Microorganisms including lactobacilli and *Escherichia coli* increased transepithelial electrical resistance while promoting OVA passage capable to trigger basophil activation. Non-inflammatory levels of IL-8 and thymic stromal lymphopoietin were produced basolaterally by Caco-2 cells exposed to microorganisms.

Conclusion The complex model designed inhere is adequate to learn about the consequence of the interaction between microorganisms and epithelial cells vis-a-vis the barrier function and antigen translocation, two parameters essential to mucosal homeostasis. It can further serve as a direct tool to search for microorganisms with anti-allergic and anti-inflammatory properties.

Keywords allergenicity, basophil activation, cytokine/chemokines, epithelial permeability, gut-derived bacteria

Submitted 28 August 2008; revised 12 November 2008; accepted 19 November 2008

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Introduction

Various modes of action have been proposed for the beneficial effects of the gut microbiota, ranging from improved mucosal barrier function to direct influences on the immune system. A better understanding of the

underlying mechanisms will provide a strong basis for the use of probiotics in the nutritional management of inflammatory [1–3] and atopic diseases [4]. Probiotics are 'live microorganisms, which when administered in adequate amounts confer a health benefit on the host' (WHO/FAO definition, 2001). As probiotics have been suggested

to ameliorate food allergy, the effect of gut-derived bacteria on epithelial cell permeability to antigen is of interest.

In the case of food allergy, the antigen must pass the epithelial cell layer to be capable to cross-link pre-existing IgE on basophils and mast cells and subsequently trigger release of chemical mediators. Epithelial cells represent the primary site of interaction with gut commensals and allochthonous microorganisms. Because of the intrinsic complexity of the intestinal barrier, it is not known whether this cross-talk can alter the transport, processing and reactivity of the antigen. Therefore, the development of novel allergy prevention strategies based on the use of probiotic bacteria will require designing screening systems that facilitate characterization and selection of valuable candidates before initiating demanding and expensive pre-clinical and clinical trials.

To tackle this issue, we thus set up an *in vitro* model combining two cell lines, i.e. polarized Caco-2 epithelial cell monolayers and basolateral RBL-2H3 basophils loaded with antigen-specific IgE that serve, respectively, as filter and sensor of ovalbumin (OVA) used as a model antigen. The model was used to evaluate the effect of apically delivered gut-derived microorganisms with reported probiotic properties, on permeability-related parameters including monolayer integrity, antigen transport and reactivity of target basophils.

Materials and methods

Preparation of biotinylated ovalbumin

Twenty-five milligrams of OVA (albumin from hen egg white; Fluka, Buchs, Switzerland) dissolved in phosphate-buffered saline (PBS) were reacted with 1 mg of sulfo-NHS-LC-biotin (Pierce, Rockford, IL, USA) for 30 min at room temperature under gentle agitation. Unreacted biotin was removed by using Sephadex G-25 prepacked PD-10 desalting columns (Amersham Biosciences, Uppsala, Sweden) in PBS. Fractions of 1 mL were recovered and the presence of biotinylated ovalbumin (OVAbiot) was checked by immunoblot and ELISA using horseradish peroxidase (HRP)-conjugated Extravidin for detection (Sigma, St. Louis, MO, USA). Final concentration of OVAbiot was determined using the bicinchoninic acid assay (BCA Protein Quantitation kit, Uptima-Interchim, France).

Preparation of ovalbumin-specific immunoglobulin E-containing mouse sera

Six-to-eight-week-old female BALB/c mice (Harlan, Ad Horst, the Netherlands) maintained under standard housing conditions were sensitized twice at days 0 and 14 by intraperitoneal injections of 10 µg of OVA (Fluka)

adsorbed on 1 mg Alum (Sigma). On days 24, 26 and 28, animals in a plexiglas chamber were exposed to a single aerosol of OVA (0.25%) in PBS for 20 min (De Vilbiss Nebulizer, Sunrise Medical GmbH, Germany). Blood was obtained 3 days after the last aerosol, and sera were pooled and stored at -20°C . The OVA-specific IgE reactivity of pools of mouse sera was determined using the RBL-2H3 degranulation assay.

RBL-2H3 cell culture conditions and β -hexosaminidase release assay

Rat basophil leukemia RBL-2H3 cells, a gift from Dr U. Wiedermann (Medical University of Vienna, Austria), were maintained in culture as previously published [5]. For degranulation assays, cells were detached using 0.25% trypsin and 0.5 mM EDTA, seeded into a 6-well culture plate (1.2×10^6 cells/well in 2.5 mL C-RPMI) and incubated for 16 h at 37°C . Cells were sensitized for 2 h at 37°C with appropriate dilutions of OVA-specific IgE mouse sera. After two washes in Hank's balanced salt solution (HBSS; Sigma), IgE cross-linking was performed using dilutions of OVAbiot for 2 h at 37°C , and the release of β -hexosaminidase was measured as described [6].

Caco-2 cell culture conditions and transepithelial electrical resistance measurements

The human colonic adenocarcinoma epithelial Caco-2 cell line was obtained from ATCC (Rockville, MD, USA) and was used between passages 40 and 50. The cells were cultured and seeded on 12-mm cell cultured inserts as published [7]. The Caco-2 cell monolayer integrity was checked by measuring the transepithelial electrical resistance (TER) using a Millicell-ERS device (Millipore, Bedford, MA, USA). TER values of well-differentiated monolayers were in a range of $300\text{--}450\ \Omega\ \text{cm}^2$ [7].

Microorganisms and growth conditions

Lactobacillus paracasei ST11 (*L. para*; CNCM I-2116 deposited by Nestlé, Switzerland), *Lactobacillus rhamnosus* LPR (*L. rhamno*; CGMCC 1.37234, deposited by Nestlé) and *Lactobacillus johnsonii* La1 (*L. john*; CNCM I-1225 deposited by Nestlé) were grown in MRS (Difco Laboratories, Detroit, MI, USA) supplemented with 1% glucose at 37°C without shaking. *Escherichia coli* Nissle 1917 (Nissle) was cultured in BHI broth (Difco Laboratories) at 37°C under shaking. *Bifidobacterium longum* strains NCC2705 (*Bifido-1*; CNCM I-2618, deposited by Nestlé) and NCC3001 (*Bifido-2*; ATCC BAA-999, deposited by Morinaga Milk, Japan) were grown in MRS containing 0.05% cysteine, at 37°C , without shaking, under microaerophilic conditions. A non-bacterial control in our assay, *Schizosaccharomyces pombe* 961 (*S. pombe*) was cultured in MV

broth [8] at 30 °C under shaking. For use on Caco-2/RBL-2H3 co-cultures, microorganisms from frozen stocks (−80 °C) were inoculated for 16 h in their strain-specific media. The inocula were adjusted to the OD reached by the strain having grown the least, and then allowed to multiply for 7 additional hours. Microorganisms were harvested by centrifugation at 2000 g for 5 min and washed twice in HBSS. OD were translated in colony-forming unit (CFU) using the following correlations: one OD unit per mL measured at 660 nm corresponds to: 1×10^8 CFU/mL for *Lactobacillus* strains; 1.5×10^8 CFU/mL for *E. coli* Nissle 1917; 2.1×10^7 CFU/mL for *Bifido-1*; 5.4×10^7 CFU/mL for *Bifido-2*; 2.15×10^6 CFU/mL for *S. pombe*. For subsequent addition to Caco-2 cell monolayers, bacterial strains were diluted into C-DMEM without antibiotic to obtain a final concentration of 4×10^7 CFU/mL.

Caco-2/RBL-2H3 co-cultures and exposure to bacteria

Six hours before incubation with Caco-2, RBL-2H3 cells were seeded in 6-well tissue culture plates (1.2×10^6 cells/well) in C-RPMI. Before use, C-RPMI was replaced by 2.5 mL of C-DMEM without antibiotic, and Snapwell™ inserts carrying polarized Caco-2 cell monolayers were placed into the RBL-2H3-containing wells. Co-cultures were maintained for 16 h to allow conditioning of Caco-2 cell monolayers. Apical medium was then replaced by 0.5 mL of bacterial suspensions (2×10^7 CFU) prepared as described above. Before sensitization, 1.5 mL of basolateral compartment was recovered to determine epithelial release of IL-8 (BDOptEIA™ set human IL-8; BD-Pharmingen, San Diego, CA, USA), thymic stromal lymphopoietin (TSLP) (R&D Systems, Minneapolis, MN, USA) and monocyte chemoattractant protein-1 (MCP-1) [7]. Then, RBL-2H3 cells were sensitized for 2 h by incubation with mouse serum containing OVA-specific IgE (1/100). The medium in both the apical and basolateral chambers was replaced by HBSS, and RBL-2H3 cell degranulation was triggered by addition of 100 µg of OVAbiot in the apical chamber for 2 h. Basolateral samples were taken for β-hexosaminidase release assay and OVAbiot quantification. TER values were measured at 0, 18, 20 and 44 h. Cell types, time and media of incubation, as well as parameters measured are depicted in Figs 1a, 2a and 3a for clarity.

Enzyme-linked immunosorbent assay for quantification of biotinylated ovalbumin transported across Caco-2 monolayers

Wells of a MaxiSorp™ ELISA plates (Nunc, Roskilde, Denmark) were coated overnight at 4 °C with 200 µL of cleared basolateral supernatants. After blocking for 1 h at 37 °C with PBS/1% BSA/0.05% Tween-20, wells were washed in PBS/0.05% Tween-20 (PBS/T), and detection was performed with HRP-conjugated Extravidin (1/1000;

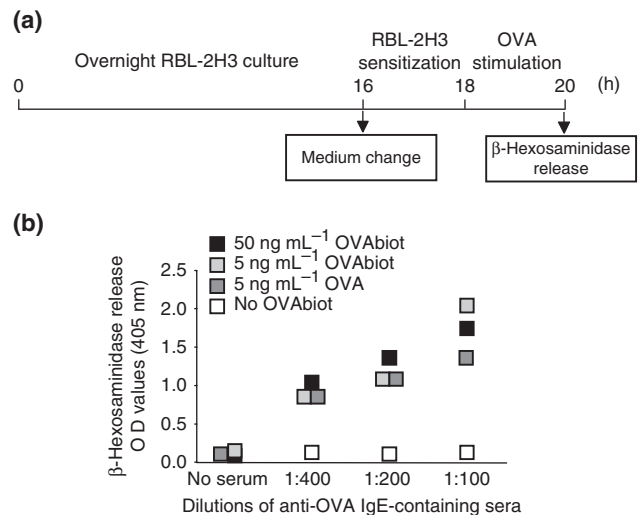


Fig. 1. (a) Schematic representation of the experimental setting designed to test IgE and ovalbumin (OVA)-dependent degranulation of RBL-2H3 as measured by the release of β-hexosaminidase (OD units at 405 nm). Medium change indicates replacement of complete DMEM by HBSS medium. (b) Dose-response obtained as a function of sensitization by serum containing OVA-specific IgE and OVA-mediated stimulation of RBL-2H3 cells. Similar stimulation by OVA was obtained in comparison with biotinylated ovalbumin (OVAbiot). SD below 0.1 OD unit does not show on the plot.

Sigma) in blocking solution for 1 h at 37 °C. Colorimetric reactions were developed with 1,2-benzenediamine as a chromogen, and quantified by using a standard curve established with serial dilutions of OVAbiot in HBSS.

Laser scanning confocal microscopy

Overnight conditioned Caco-2 cell monolayers were washed and pulsed for 1 h with 50 µg of OVAbiot in HBSS at 37 °C. Inserts washed with HBSS, cells were fixed with cold methanol:acetic acid (3:1) for 5 min, washed again with PBS/T, and non-specific binding sites were blocked with 1% fish gelatin (Sigma) in PBS/T for 60 min. After washing with PBS/T, cells were incubated for 45 min at room temperature with 1/100 fluorescein isothiocyanate-conjugated streptavidin (Dako, Glostrup, Denmark) in PBS/T containing 0.1% fish gelatin. Filters were cut out of their holders, and mounted in the presence of Vectashield (Vector Laboratories, Burlingame, CA, USA). Observations were performed using a laser scanning confocal microscope (Zeiss LSM 510 Meta; Zeiss, Jena, Germany) equipped with an argon-krypton laser. All images were identically processed using the LSM 5 Image Browser application (Zeiss).

Evaluation of biotinylated ovalbumin proteolysis by bacterial strains

Fresh culture of bacteria (4×10^7 CFU/mL) were incubated in a final volume of 500 µL of PBS with 100 µg of OVAbiot

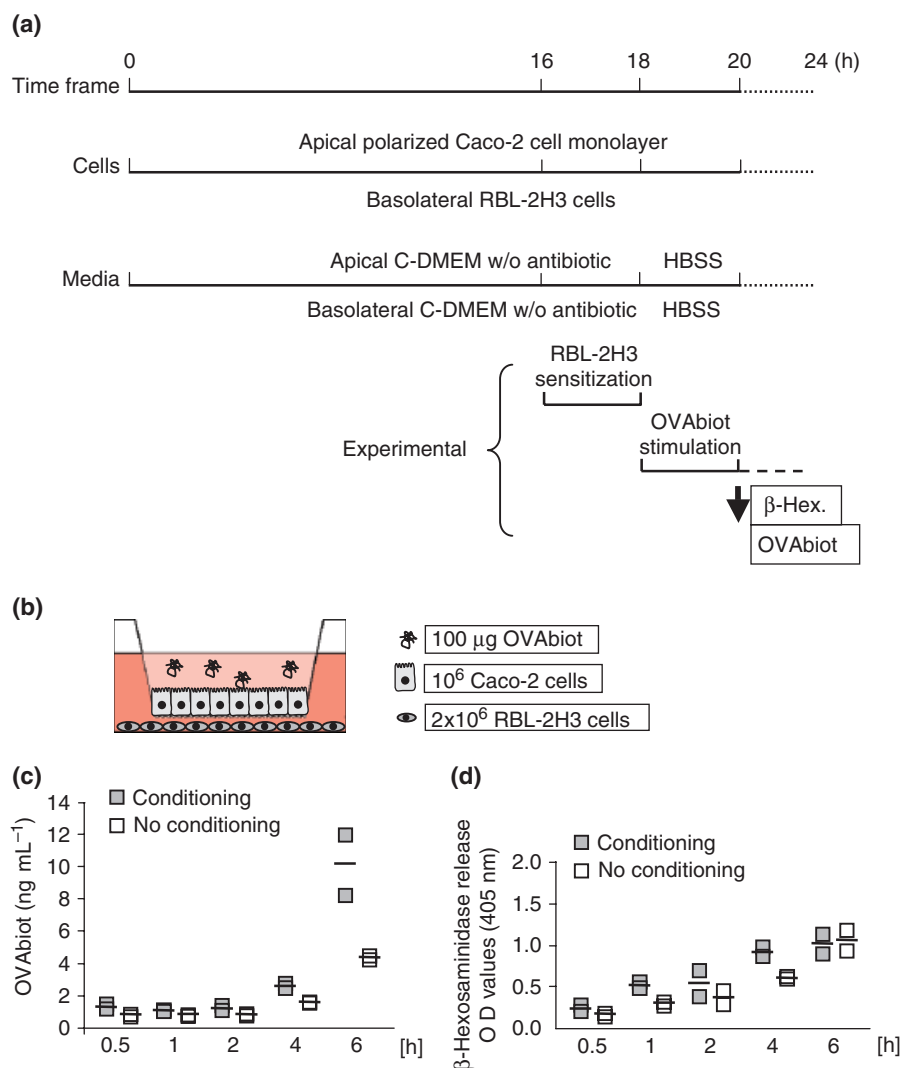


Fig. 2. (a) Time-frame indicating the cell types, media in apical and basolateral chambers, and duration (h) of the successive steps leading to ovalbumin (OVA)-dependent degranulation of RBL-2H3 cells. (b) Scheme depicting the setting used to assess the apical to basolateral passage of biotinylated ovalbumin (OVAbiot) across the polarized Caco-2 cell monolayers/RBL-2H3 cells model. (c) Kinetics of OVAbiot transport across the Caco-2 cell monolayer upon overnight conditioning by RBL-2H3 (grey) or not (open). (d) Time-course of degranulation of sensitized RBL-2H3 mediated by transported OVAbiot in (c). Data are depicted as values of duplicate cultures.

for 2, 4 and 24 h at 37 °C. Incubation was stopped by transfer into PAGE-loading buffer [50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 2% SDS, 10% glycerol, 0.05% bromophenol blue]. Boiled samples (corresponding to 25 ng of OVAbiot) were loaded onto an 8% denaturing polyacrylamide gel [9], transferred onto PVDF membranes (Millipore), and blotted OVAbiot was detected using HRP-conjugated streptavidin (Sigma) and the Uptilight substrate for chemiluminescence (Interchim, Montluçon, France).

Statistical analysis

Standard error means were calculated using the Prism4 application (GraphPad, San Diego, CA, USA). Statistical

significance was determined using the two-tailed non-parametric Mann-Whitney *U*-test. *P*-values were calculated using the Prism4 application.

Results

Characterization of RBL-2H3 responsiveness and the co-culture model

Activation of RBL-2H3 cells (reflected by degranulation and release of β-hexosaminidase) depends on both loading of IgE on the cell Fc receptor and subsequent cross-linking by the antigen across the polarized Caco-2 cell monolayer (Fig. 1a). Degranulation of the rat basophil cells was achieved using various dilutions of mouse serum

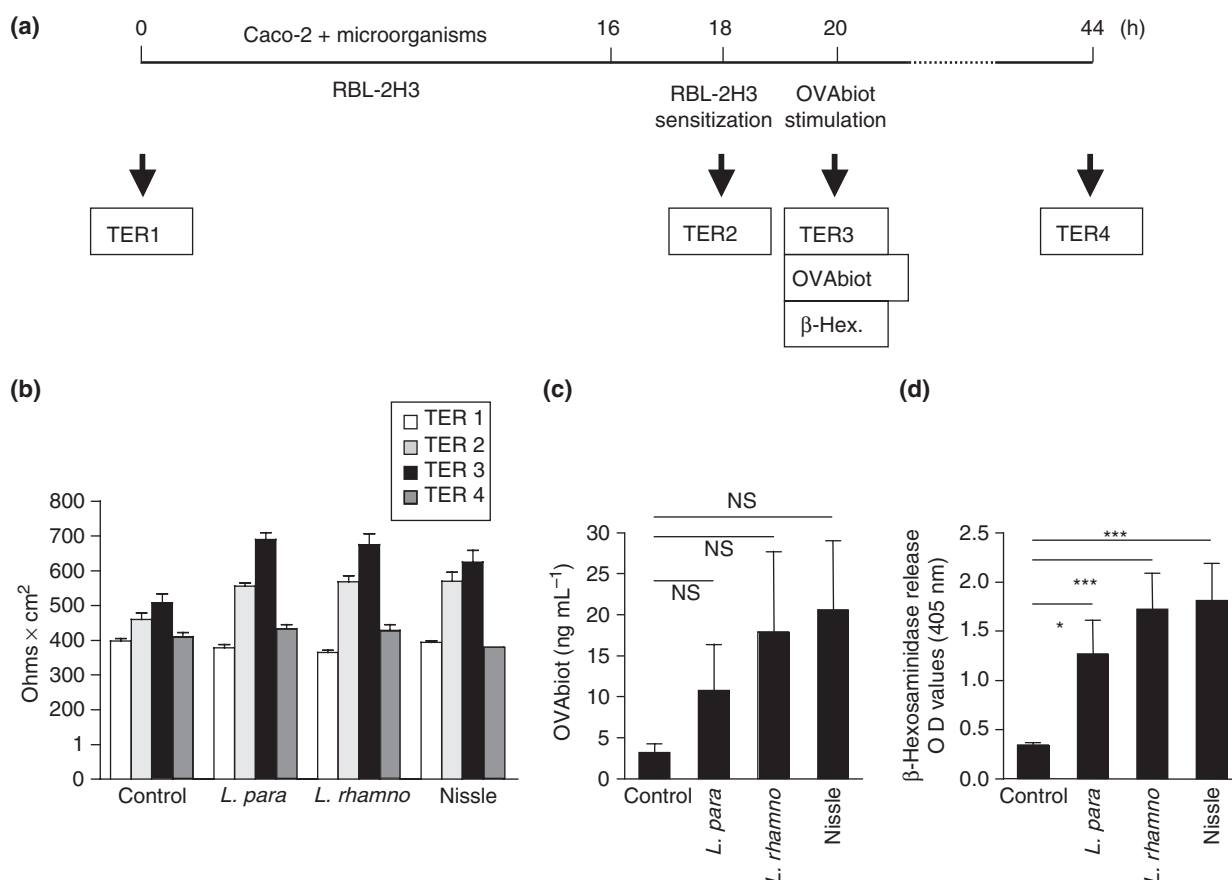


Fig. 3. Mapping the effect of a selection of bacteria tested in the co-culture model. (a) Time-frame and duration (h) of the successive steps allowing for measurements of parameters including transepithelial electrical resistance (TER), biotinylated ovalbumin (OVAbiot) passage across the monolayer, and degranulation of RBL-2H3 cells. (b) TER ($\Omega \text{ cm}^2$) of epithelial Caco-2 cell monolayers exposed to bacterial strains, determined at four time-points as indicated in (a). (c) Differential effect of bacteria on the transport of OVAbiot (ng/mL) across the Caco-2 cell monolayer. (d) Degranulation of sensitized RBL-2H3 cells in the basolateral compartment by transported OVAbiot. *L. para*, *Lactobacillus paracasei* ST11; *L. rhamno*, *Lactobacillus rhamnosus* LPR; Nissle, *Escherichia coli* Nissle 1917. Data are means and SD (panel b) or SEM (panels c and d) of triplicate cultures. NS, non-significant; *, $P < 0.05$; ***, $P < 0.005$. One representative out of three experiments is shown.

containing OVA-specific IgE in the presence of OVAbiot only (Fig. 1b). As little as 5 ng/mL of OVAbiot led to responses proportional to the serum dilutions, and behaved like plain OVA (Fig. 1b). OVAbiot was thus used in all experiments, as this is the form of OVA that ensures specific analysis through streptavidin-mediated detection.

We next examined the reactivity of RBL-2H3 cells toward the OVAbiot antigen which had been transported across the polarized Caco-2 cell monolayer. Cell types in the co-culture model are listed in Fig. 2a, and their physical distribution in the system is depicted in Fig. 2b. C-DMEM lacking antibiotics was used to analyse the effect of replicating bacteria. Tightness of the reconstituted Caco-2 cell monolayer and in the co-culture system was assessed by TER and/or ZO-1 staining as reported [10]. This was further confirmed by controlled, limited passage of OVAbiot from the apical to the basolateral compartment (Fig. 2c). OVAbiot having reached the basolateral compartment was capable to trigger degranulation of

RBL-2H3 cells loaded with OVA-specific IgE (Fig. 2d), indicating that immunoreactive forms of the protein had been transported. Similar results were obtained when conditioning by RBL-2H3 cells was omitted during the first 16 h in the co-culture system (Fig. 2c and d). As we found a correlation between the presence of OVAbiot and release of β -hexosaminidase by RBL-2H3 cells in the 1–4 h range, the effect of incubating polarized Caco-2 cell monolayers with a selection of gut-derived bacteria was therefore evaluated at 2 h.

Incubation with bacterial strains increases the transport rate of biotinylated ovalbumin and triggers RBL-2H3 cell degranulation

The co-culture system was then used to investigate the possible effect of apical incubation of microorganisms on OVA-mediated RBL-2H3 cell degranulation. Figure 3a depicts the steps and read-outs assayed timewise. In trial

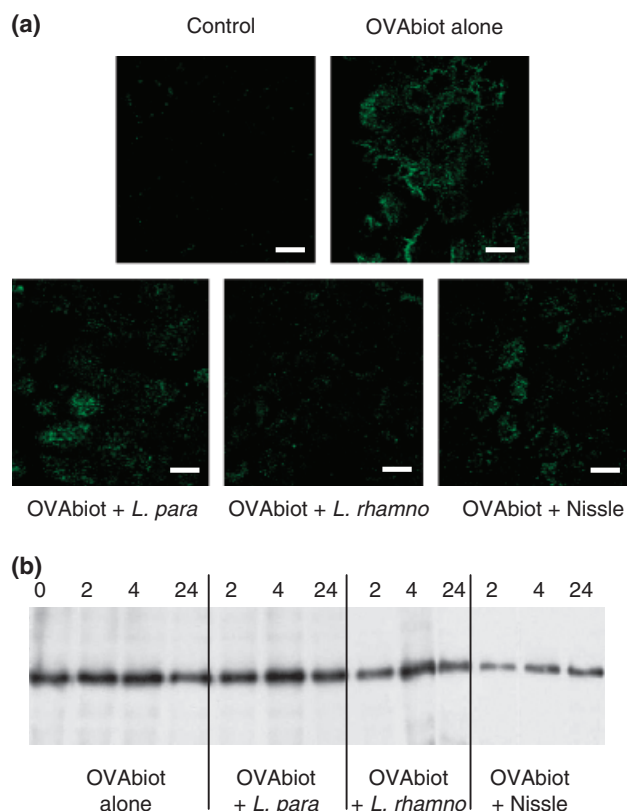


Fig. 4. (a) Cellular localization of biotinylated ovalbumin (OVA_{biot}) in conditioned polarized Caco-2 cell monolayers previously exposed to bacterial strains. Caco-2 cell monolayers were pulsed with OVA_{biot}, and analysed by laser scanning confocal microscopy using FITC-conjugated streptavidin. One representative out of two to three experiments is shown. Bars: 10 μ m. (b) Western blot analysis (signal obtained with 25 ng of loaded OVA_{biot}) demonstrating the stability of OVA_{biot} incubated with various bacterial strains as a function of time (h). Abbreviations for bacteria are the same as in Fig. 3.

assays, we reproducibly observed acidification of the apical compartment after addition of 10^8 bacteria from different species (data not shown). In addition to possibly interfering with the interaction between cells, this translated into drastic drops of TER that turned out to be irreversible, and artifactual diffusion of OVA_{biot} into the basolateral compartment. We determined that addition of 2×10^7 microorganisms per Transwell insert (bacteria/Caco-2 cell ratio of approximately 20) guaranteed the stability of both the medium pH and TER for all microorganisms tested (data not shown).

We first examined the potential of *L. paracasei*, *L. rhamnosus* and Nissle strains, known to modulate immune responses associated with allergy or inflammatory status [11–13], to affect OVA-dependent RBL-2H3 reactivity in the co-culture model. We found that upon incubation with bacteria, TER increased after overnight culture, remained stable during serum sensitization, and increased further during antigen stimulation. Interestingly, TER returned to initial values when Caco-2 cell

monolayers were allowed to recover for 24 h in C-DMEM (Fig. 3b), indicating that the monolayer exhibited transient and reversible modifications arguing for the specificity of the effects measured. This marks the system as adequate to evaluate the consequence of the interaction between multiple cell partners.

L. rhamnosus and Nissle, and to a lesser extent *L. paracasei*, favoured transport of OVA_{biot} from the apical to basolateral compartment in comparison with the control Caco-2 cells alone (Fig. 3c), even though *P*-values were above statistical significance. A proportion of the protein was likely to be transported intact, or at least in an immunogenic conformation, as confirmed by capacity to trigger RBL-2H3 degranulation as compared with control (Fig. 3d). In this sensitive assay, the trend observed for OVA passage is correlated with the release of β -hexosaminidase by RBL-2H3 cells in a statistically significant manner. Because TER were preserved and even increased, this suggests a specific effect of the three strains in promoting transcellular transport in this complex multi-cell type setting.

When the cellular distribution of the protein was tracked through laser scanning confocal microscopy, intracellular fluorescence was observed for the three strains tested, with brighter discrete structures in the central portion of the cell, indicating accumulation in endocytic vesicles along the transcellular route (Fig. 4a). Moreover, absence of signal in the intercellular space at the upper section level further reflected the lack of paracellular transepithelial transport (data not shown).

In order to exclude that increased transport of OVA_{biot} was due to degradation mediated by residual bacteria associated with the Caco-2 cell plasma membrane, the protein was incubated for up to 24 h in the presence of *L. paracasei*, *L. rhamnosus* and Nissle. Immunodetection of a single band co-migrating with undigested OVA_{biot} confirmed that the immunoreactive and intact form of the protein was indeed taken up by Caco-2 cells (Fig. 4b).

Requirement of conditioning of polarized Caco-2 cell monolayer by both RBL-2H3 cells and bacteria

Co-incubation of epithelial cell monolayer and another cellular type(s) has proven to be a prerequisite to allow deciphering of complex mechanisms in reconstituted *in vitro* systems [11, 14, 15]. Figure 2c and d shows that in the absence of bacteria, co-incubation of Caco-2 cell monolayers with RBL-2H3 did not alter OVA_{biot} translocation and subsequent release of β -hexosaminidase. However, in presence of microorganisms, overnight conditioning of polarized Caco-2 cells by RBL-2H3 became crucial to establish differences in TER values (data not shown), transport of OVA_{biot} and release of β -hexosaminidase (Fig. 5b and c, compare filled with open bars). Thus, it appears that for polarized Caco-2 cells to 'sense' the

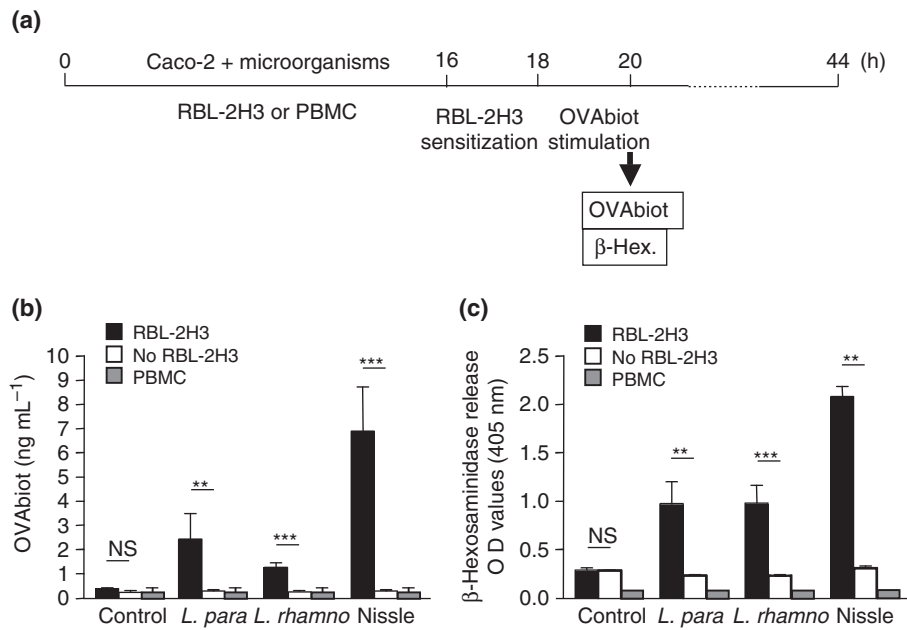


Fig. 5. Differential effect of bacteria on biotinylated ovalbumin (OVA) transport requires conditioning of Caco-2 cells by RBL-2H3. (a) Parameters were measured in the presence or absence of RBL-2H3 cells, or alternatively in the presence of PBMCs in the basolateral compartment during the first 16-h of Caco-2 cells-bacteria co-incubation. (b) Dual effect of bacteria and conditioning by RBL-2H3 cells on OVA passage (filled bars), as compared with unconditioned Caco-2 cell monolayers (open bars) or after conditioning with PBMC (grey bars). (c) Activation of RBL-2H3 cells resulting from the transport of OVA to the basolateral compartment. Abbreviations for bacteria are the same as in Fig. 3. NS, non-significant; **, $P < 0.01$; ***, $P < 0.005$. Data are means and SEM of triplicate cultures.

microorganisms, exposure to RBL-2H3 must occur concomitantly. This results in differential passage of OVA as a function of the microorganism applied, with Nissle inducing higher degranulation levels than *L. paracasei* and *L. rhamnosus* as compared with the control (Fig. 5b and c, filled and open bars). The complexity and specificity of the multi-cell system found another illustration in the observation that incubation with human peripheral blood mononuclear cells (PBMC) did not permit to discriminate between the bacteria tested (Fig. 5b and c, grey bars).

Specific impact of bacteria on the cytokine/chemokine profile of polarized Caco-2 cells

The *in vitro* model was then used to extend the analysis to other microorganisms known for their potential probiotic properties including *L. johnsonii*, *Bifidobacterium-1* and -2, and *S. pombe* as a non-bacterial control. None of the microorganisms assayed increased TER more effectively than *L. paracasei*, which was used as an inter-experiment control (Fig. 6b). The return to initial TER values after 24 h (44-h time-point) confirmed the plasticity of the model already observed in Fig. 3. As far as OVA transport was concerned, only *Bifidobacterium-1* turned out to lead to accumulation in the basolateral compartment at levels close to those induced by *L. paracasei* (Fig. 6c). In support

of this, β -hexosaminidase activity emanating from IgE-sensitized RBL-2H3 strongly correlated with the level of OVA, with statistically significant differences obtained for both *L. paracasei* and *Bifidobacterium-1* (Fig. 6d). This indicates that the system is adequate to examine the differential effect of microorganisms on the permeability of polarized Caco-2 cell monolayers, as reflected by the differential passage of OVA susceptible to trigger IgE-dependent degranulation of target basophils.

Although the bacteria tested express microbe-associated molecular patterns, the latter did not prove to activate pro-inflammatory pathways. Production by Caco-2 cells of IL-8 measured in the basolateral compartment after overnight incubation (Fig. 6e) was limited to a range of concentration well below the level observed after incubation with pro-inflammatory *Helicobacter pylori* (> 120 pg/mL [7]) or *Shigella flexneri* (> 2 ng/mL [16]). With the exception of *S. pombe*, secretion of TSLP by Caco-2 cells varied within a range (47–84 pg/mL; Fig. 6e) reported to trigger maturation of dendritic cells (DCs) toward a tolerogenic phenotype [15]. Production of monocyte chemoattractant protein-1 (MCP-1) was below the detection limit in ELISA. This indicates that the system is appropriate to further address the underlying mechanisms that account for the differences observed upon incubation with microorganisms.

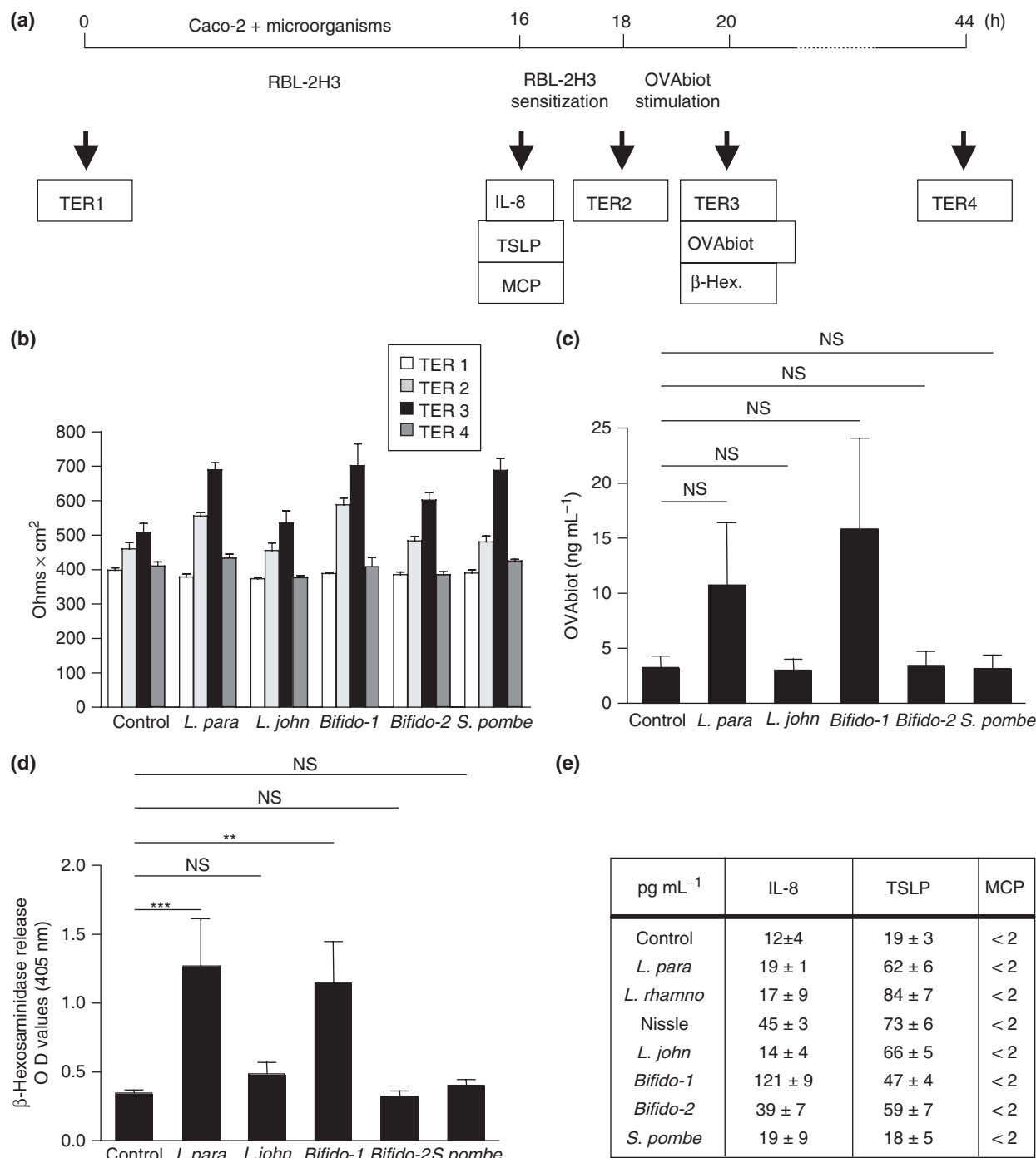


Fig. 6. Testing of additional bacteria and effect on immune mediators. (a) Experimental settings are the same as in Figs 3 and 5. (b) TER of epithelial Caco-2 cell monolayers exposed to *Lactobacillus paracasei* ST11 (*L. para*), *Lactobacillus johnsonii* La1 (*L. john*), *Bifidobacterium longum* NCC2705 (*Bifido-1*), *Bifidobacterium longum* NCC3001 (*Bifido-2*) and *Schizosaccharomyces pombe* strain 961 (*S. pombe*) used as a non-bacterial control. (c) Effect of microorganisms on the transport of biotinylated ovalbumin (OVAbiot) across the Caco-2 cell monolayer and (d) subsequent degranulation of sensitized RBL-2H3 cells. NS, non-significant; **, $P < 0.01$; ***, $P < 0.005$. (e) Analysis of basolateral cytokines/chemokine released by Caco-2 cell monolayer as a function of co-incubation with microorganisms and conditioning by RBL-2H3 cells. Because of changes of medium for β -hexosaminidase assay, cytokines could not be determined again at 20 h (post-stimulation). IL-8, interleukin-8; TSLP, thymic stromal lymphopoietin; MCP, monocyte chemoattractant protein-1. All experimental groups were performed in triplicates and data are expressed as means and SD (panels b and e) or SEM (panels c and d).

Discussion

The *in vitro* model reported herein exhibits the striking feature that exposure of polarized Caco-2 cell monolayers to microorganisms leads to differential translocation of immunoreactive OVA. This is consistent with the observation that polarized Caco-2 cell monolayers take up soluble OVA via tightly regulated fluid-phase endocytosis [17]. Proportional degranulation of basophils as a function of transported OVA indicates that the filter properties of Caco-2 cells are essential to avoid saturation of basophil-bound IgE by OVA in the basolateral compartment. This also suggests that transport across the reconstituted epithelium leaves all or part of OVA in an immunoreactive conformation adequate to trigger IgE-loaded RBL-2H3 degranulation. The prerequisite of co-incubating Caco-2 cell monolayers with RBL-2H3 cells finds an explanation in the need for mediators, which are naturally found in the lamina propria. These mediators contribute to condition Caco-2 cells to respond differentially to the microorganisms both in terms of transport efficacy and production of IL-8 and TSLP.

Interestingly, microorganisms that lead to increased endocytosis and transcellular transport of OVA all resulted in enhanced epithelial tightness and maintenance of permeability. Bacteria with probiotic properties may display such an activity as reflected by increased TER [18–20], with relative TER increments averaging around 30%, as observed in our study. Markedly increased transport of OVA_{biot} occurred upon exposure of Caco-2 cells to *L. paracasei*, *L. rhamnosus*, Nissle and *Bifidobacterium-1*, but not the two other bacteria tested, thus indicating a specific effect in promoting translocation. Of note, Nissle and *L. rhamnosus* facilitating translocation of immunoreactive OVA were reported to exhibit anti-inflammatory or anti-allergic profile [21–23]. In this respect, the production by Caco-2 cells of TSLP, which generates a non-inflammatory environment [14], might prevent exaggerated production of basophil-derived mediators, which would otherwise alter permeability and fine-tuning by microorganisms. Furthermore, the almost complete absence of IL-8 and MCP-1 argues in favour of the role of the studied gut-derived microorganisms in maintaining a homeostatic type of responses in the *in vitro* system.

In vivo, small amounts of intact proteins including HRP, ferritin, lactoglobulin and OVA are known to be taken up by intestinal epithelial cells [24–27]. In the context of established food allergy, mucosal delivery of intact antigen might conduct to exacerbation of clinical symptoms. Conversely, limited entry of intact antigens in the context of a healthy epithelial barrier might be instrumental for the development of antigen-specific tolerance [28–30].

This would argue in favour of the hypothesis that the initial exposure to intact antigen under conditions preventing both inflammation and onset of allergic reactions

can play a role in shaping the epithelium-associated immune responses. Furthermore, because the model displays marked differences between the bacteria tested, it offers the potential to address mechanistic questions such as the identification of the bacterial factor(s) responsible for the modulation of the barrier integrity, the role of epithelial cell Toll-like receptor signalling pathways (Myd88 vs. Tollip [31]), as well as the function of secreted epithelial cell factors.

While the consequences of the cross-talk between epithelial cells and pathogenic microorganisms are well dissected [32, 33], the mode of action of 'health-promoting' microorganisms remains elusive. The mere presence of microbe-associated molecular patterns has to be backed-up by other cellular and/or molecular partners (namely basophil conditioning in our model) to study the fine-tuned reactivity of epithelial cells. The model reported herein presents the advantage that the conditioning basophil cell line is used to analyse how microorganisms can affect epithelial permeability, translocation and allergenicity.

Acknowledgements

We thank Drs Clémentine Perrier and Sophie Nutten for critical and thorough reading of the manuscript, and together with Drs Rodolphe Fritsché and Adrian Zuercher, for invaluable discussions. This work was supported by research grants from Nestec, Vers-chez-les-Blanc and the Swiss Science Research Foundation (3200-109545).

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