Old Herborn University Seminar Monograph 16:
Host microflora crosstalk.
Editors: Peter J. Heidt, Tore Midtvedt, Volker Rusch, and Dirk van der Waaij.
Herborn Litterae, Herborn-Dill, Germany: 83-89 (2003).

DEVELOPING AN *IN VITRO* MODEL ON THE INVESTIGATION OF THE CROSSTALK AMONG BACTERIA, ENTEROCYTES AND LEUKOCYTES NEAR THE INTESTINAL MUCOSA

ALEXANDR PARLESAK and CHRISTIANE BODE

Department of Physiology of Nutrition, Hohenheim University, Stuttgart, Germany

SUMMARY

In vitro models comprising human cells are potent tools to improve our understanding on the crosstalk between enterocytes and bacteria on the one hand and between leukocytes and enterocytes on the other. The present manuscript deals with the possibility to develop a co-culture model for the investigation of inflammatory processes near the intestinal mucosa. One important demand on such a model is the spatial separation of bacteria and leukocytes mimicking the morphologic conditions in the intestine. This is achieved by the separation of the apical compartment with non-pathogenic bacteria from the basolateral compartment with human leukocytes by a confluent monolayer of a differentiated cell line of intestinal origin (Caco-2).

The gut-associated lymphoid tissue (GALT) comprises Peyer's patches, mesenteric lymph nodes, intra-epithelial lymphocytes, and leukocytes of the lamina propria. The complex interaction of these four compartments cannot be simulated by an *in vitro* model, but inflammatory processes are likely to be initiated and controlled by alpha-beta T cells in the lamina propria, the action of which is likely to be simulated most closely by the present model.

Preliminary results on the kinetics of cytokines in the basolateral compartment revealed a temporary occurrence of tumour-necrosis factor alpha, a steady increase in the concentration of interleukin (IL)-8 and a stable concentration of IL-6 and IL-10 after 24 hours. Cytokines associated with the activation of Th1-cells (IL-2 and interferon gamma) occurred after 24 h and steadily increased from that time. Th2-type cytokines (IL-4, IL-5) were not detectable in the basal medium.

The present model offers chances in the investigation of drugs and food ingredients on the enhancement or impairment of inflammatory processes in the intestine avoiding differences between the immune responses of man and animals in e.g. feeding models with rodents.

INTRODUCTION

Background

To improve our understanding of mechanisms inducing processes in the intestine makes sense from different points of view. First, events leading to inflammatory bowel disease (IBD), namely Crohn's disease and ulcerative colitis, are not completely understood. Second, the need for developing new strategies to take influence on the effi-

cacy of the immune response by compounds or nutrients will increase. Especially as the percentage of elderly people increases in industrialised countries, problems associated with the impaired immune response in these people ('immunosenescence') have to be met.

The systemic immune response in terms of resistance to pathogens can highly be improved by the application of probiotic organisms. In mice, the application of *Lactobacillus casei* prior to infection with *Salmonella typhimurium* (dose: 20 x LD_{50}) prevented completely the occurrence of pathogens in the liver and spleen (*Perdigon* et al., 1991). As evident from these experiments, the effect of e.g. probiotics can be immense but further investigations are necessary to understand the mechanisms standing behind these effects. Although insights can be achieved by using experiments with animals, considerable differences can occur in parameters of immune function between animals and man (*Lebrec* et al., 1995). Therefore, *in vitro* models using cells of human origin have become more important during the last two decades, but efforts in the development of such models mimicking the immune response in the intestine were very limited so far. Furthermore, at least at the primary stage of investigating immunomodulating properties of foodstuff, screening methods will be needed enabling first statements on their effectiveness.

An important mediator of the systemic immune response is the gut-associated lymphoid tissue (GALT). This complex system, which is part of the mucosa-associated lymphoid tissue (MALT), comprises four different parts: The Peyer's patches, mesenteric lymph nodes, intra-epithelial lymphocytes (IEL), and various leukocytes located in the *lamina propria*. Despite the fact that the latter two compartments are separated only by the thin basal membrane, they have to be distinguished clearly due to their immunological functionality. Developing an *in vitro*-model for the modulation of inflammatory processes in the intestine, one has to be aware which parts of this immune system *should* and *can* be simulated.

Which part of the GALT action can be mimicked in an *in vitro* system?

The initiation of the specific immunity acquired within the GALT takes place in the Peyer's patches (PP), which can be considered as classical secondary lymphoid organs. After controlled translocation of single bacteria by specialised M-cells, interdigitating dendritic cells (IDC) present antigens to T lymphocytes and macrophages mainly to B lymphocytes. During the maturation process, the primed cells leave the PP via the mesenteric lymph nodes to relocate after maturation in the lamina propria or other sites such as the mammary gland, the lacrimal gland and beneath other mucosa surfaces of the body. The expression 'homing' termed for this process is not completely exact, as the cells never return to the site of their initiation but to places where they can fulfil their efferent immunological tasks. The main function of the cells having undergone this fate is the predominant production of IgA. Evidently, this process involving differentiation processes in other tissues than near the intestinal mucosa cannot be mimicked in an *in vitro* model dealing with the immune response in the intestine. Therefore, investigations within such a model can only focus on actions of cells other than naïve T/B cells in PP and activated memory B lymphocytes and $CD45RO^{+}/CD45RB^{low}$ lymphocytes, which have lost their ability for being stimulated in terms of acute inflammation (*Abreu-Martin* et *Targan*, 1996).

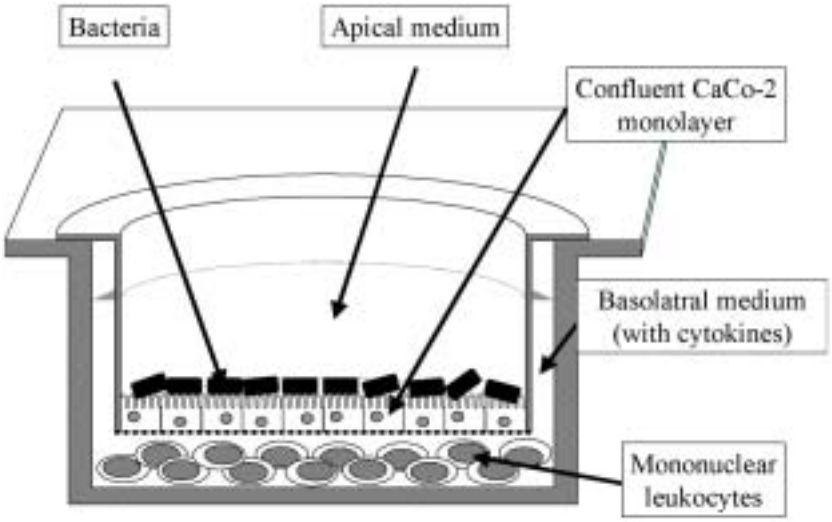

Figure 1: Arrangement of bacteria, Caco-2 cells and peripheral blood mononuclear cells (PBMC) for the investigation of immune responses near the intestinal surface

Although an important part of the GALT, intra-epithelial lymphocytes (IEL), which comprise a considerable percentage of CD8-positive cells (>80%) expressing frequently the CD8αα+ receptor (*Latthe* et al., 1994), are also considered as being practically not being able to respond to TcR stimuli (*Ebert*, 1989). The low, but quite constant percentage of γδ-TcR cells (in humans: ~10% of total T cells; *Viney* et al., 1990) seems also to be of inferior importance for intestinal inflammation, as αβ-TcR cell depleted mice failed to develop inflammation after *Salmonella* infection but not γδ-TcR cell depleted animals (*Weintraub* et al., 1997). Hence, an *in vitro* model focussing on inflammatory processes within the intestine should mainly be oriented on the interaction among naïve LPL (and macrophages), enterocytes and the intestinal flora.

Which kind of cells can be used in the co-culture model?

To achieve the best possible results in experiments concerning reproducibility and similarity to conditions in the intestine, the following demands should be met by cells representing the three parts of the system mentioned above:

Leukocytes should mainly comprise human naïve αβ-T cells and cells of the monocyte/macrophage lineage being susceptible for an inflammatory challenge. An acceptable source for these leukocytes is peripheral blood mononuclear cells (PBMC), which can easily be obtained in large amounts from cell-enriched plasma ('buffy coats').

Enterocytes are only appropriate for an *in vitro* model of intestinal inflammation if they can maintain the separation of two compartments: the 'outer' (apical) compartment with bacteria and the 'inner' (basolateral) compartment with leukocytes. This will only succeed with cells that keep their proliferation only until confluence and are able to develop a polar differentiation. Caco-2 cells meet these requirements and are able to form desmosomes, microvilli, and tight junctions (*Hidalgo* et al., 1989). Additionally, this extremely well-defined cell line (*Pinto* et al., 1989) keeps its viability also in media suitable for incubation of leukocytes such as DME medium.

The Caco-2 cells have to be kept for growth until confluence and differentiation on a semi-permeable membrane until the end of incubation to enable signalling between leukocytes and the enterocyte-like cell line by soluble factors. To guarantee that bacteria cannot transmigrate through the membrane, a pore diameter below the average size of bacteria (e.g. 0.4 μm) is suitable for this purpose.

Bacteria found in the intestine are very inhomogeneous and their composition can differ significantly between single hosts. Apart from studies where differences among specific rods are to be investigated, the application of one non-pathogenic, well-characterised bacteria species that is able to evoke an immunological response within the described system seems appropriate. The immunomodulating effect of drugs or nutrients to be investigated can than be assessed by enhancement or impairment of the immunological response to the standardised bacterial challenge. Evidently, the germs have to be impaired in their growth to avoid destruction of the Caco-2 cell monolayer. A major problem in this context is the question in how far the growth of the applied bacteria can be reduced without changing the induced immune response. Antibiotics damaging the integrity of the bacterial membrane should be avoided, as the bacterial lysate is likely to cause modifications of the induced immuno-activation. A sketch of the co-culture model is given in Figure 1.

How to measure the extent of the immune response?

Concerning the readout of the experiments, the middle-termed immune response can be assessed by measurement of cytokines in the medium. First experiments with this model by *Haller* et al. (2000) revealed that practically all cytokines are secreted into the basolateral but not the apical compartment. Out of the numerous cytokines/chemokines, those indicating acute inflammation [tumour necrosis factor alpha (TNF-α), Interleukin 1 beta (IL-1β), IL-8)], immunomodulation [IL-10, transforming growth factor beta (TGF-β), IL-6], activation of Th1 cells (IL-2, interferon gamma (IFN-γ)], and activation of Th2 cells (IL-4, IL-5, IL-13) might be of special importance for the assessment of changes in the immune response. For a profound understanding of the interaction between these cytokines and the eukaryotic cells of the system, the recording of a time kinetic of each cytokine is necessary. The involvement of the enterocyte-like cell line can be estimated by (semi-)quantitative measurement of mRNA expression for some of the cytokines mentioned above (i. E. IL-8 and TNF-α, *Haller* et al., 2000).

Further important information can be obtained by measuring the release of reactive oxygen species (ROS). The activation of phagocytotic cells, which are also part of the PBMC (monocytes, ~20-35%), results in the pentose phosphate shunt, NADPH production, and the massive release of ROS. As ROS are known as potent reagents being able to modify DNA, especially mechanisms leading to the high coincidence of chronic inflammatory bowel disease (IBD) and malignant neoplasiae in the colon might be elucidated by investigations with the present model. The release of ROS can easily be measured online by the application of chemiluminescence-enhancing techniques (*Parlesak* et al., 1998).

MATERIAL AND METHODS

Cell culture

The cell line Caco-2 was obtained from the 'Deutsche Sammlung von Mikroorganismen und Zellkulturen' (Braunschweig, Germany: DSMZ No. ACC169). Cells were cultured in inserts on a semi-permeable PET membrane (Falcon, Becton-Dickinson, Le Pont De Claix, France). Inserts were placed into cavities of six-well plates (Greiner, Frickenhausen, Germany), which were filled with cell culture medium that consisted of Dulbecco's Modified Eagle Medium (DMEM) and heat-inactivated (56°C for 30 min) foetal calf serum (FCS) (Gibco BRL/Life Technologies, Karlsruhe, Germany). FCS was controlled for endotoxin content (<0.2 ng/ml: LAL Test, Chromogenix, Mölndal, Sweden). Confluency was checked by measurement of transepithelial electrical resistance (TEER; *Haller* et al., 2000) and visual control of cell layer integrity under the microscope. After complete differentiation, which lasted for further 10 days, co-incubation experiments were performed.

As a stimulus, the non-pathogenic rod *Escherichia coli* K12 was used at the stationary growth phase. Gentamicin (120 μg/ml; Gibco BRL/Life Technologies) was added to the medium to avoid uncontrolled growth that would lead to destruction of the Caco-2 cell monolayer (*Haller* et al., 2000).

Mononuclear leukocytes from peripheral venous blood (PBMC) of healthy donors (n=3) were isolated from buffy coats (cell-enriched plasma) by density gradient centrifugation (Ficoll® solution, Seromed/Biochrom, Berlin, Germany). After centrifugation, mononuclear leukocytes were taken from the interface and washed three times with cell culture medium.

Each experiment was performed as a triplicate. Incubations with non-pathogenic bacteria (*E. coli* K12, 2.0×10^7 CFU) were applied either apically or basolaterally with PBMC (4.0×10^6). The release of cytokines into the basolateral compartment was measured by commercially available ELISA kits (BD Pharmingen, Heidelberg, Germany). The resulting values were compared to the corresponding control experiments.

FIRST RESULTS AND CONCLUSION

First results from experiments with this model revealed that findings obtained in this model cannot be compared with those achieved by bacteria-leukocyte or bacteria-enterocyte co-incubations. *Haller* et al. (2000) stimulated the differentiated Caco-2 cell monolayer in the described trans-membrane system with different bacteria (*Lactobacillus johnsonii, L. sakei,* and *E. coli*) with and without leukocytes in the basolateral compartment. Messenger RNA for IL-8 and monocyte chemoattractant protein 1 (MCP-1) was expressed in Caco-2 cells only if leukocytes were present in the basal compartment and only by *E. coli* and *L. sakei* but not by *L. johnsonii*. Hence, the Caco-2 cells can distinguish between single bacteria species only if mononuclear leukocytes are present in the basolateral compartment. Further actual findings with this model demonstrated that IL-2, which cannot be produced by the enterocyte-like cell line (*Jung* et al., 1995), is produced in the basolateral compartment only if the Caco-2 cell monolayer separates bacteria and leukocytes. If bacteria have cell-cell contact to the leukocytes, practically no IL-2 production occurs within the first 68 h after challenge.

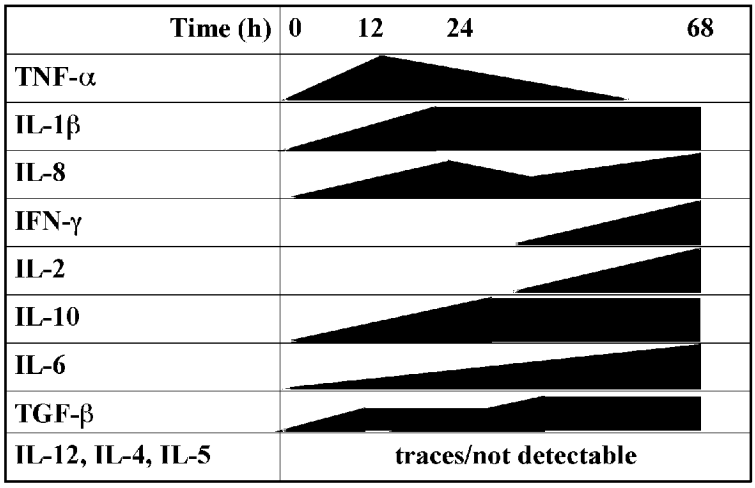

Figure 2: Kinetics of cytokine release into the basolateral compartment of the co-culture model after apical stimulation with E. coli K12 (4.0 x 10^7 bacteria); the absolute concentrations of the single cytokines are not identical.

These findings underscore the diversity of the present model compared to models where the spatial separation between bacteria and leukocytes by a cell monolayer comparable to the intestinal mucosa does not occur. Furthermore, from the results motioned above a three-step mechanism can be concluded in the activation of the eucaryotic cell 'crosstalk'. First, the leukocytes secrete unidentified soluble factors sensitising the Caco-2 cells for selective activation by bacteria. Second, leukocytes are activated by soluble factors from Caco-2 cells, enhancing the production of cytokines already produced by the enterocyte-like cell line (TNF-α, IL-8, IL-6 and others) and initiating the production of cytokines that cannot be synthesised by the Caco-2 cells (IL-2, IL-10, IFN-γ and others). In the third step, changes in the concentration of cytokines in the basolateral compartment regulate the activation status of enterocytes.

Using a non-pathogenic rod as a standard, a characteristic pattern of cytokine concentrations that depends on the incubation time will be evoked. The single cytokines differ significantly in some orders of magnitude regarding their absolute concentration. As can be expected, out of the measured cytokines (TNF-α, IL-1β, IL-12, IL-8, IL-6, IL-2, IL-10, IFN-γ, IL-4, IL-5, and TGF-β) those having a systemic effect (IL-6) or being important for chemotaxis (IL-8) contribute a major part of the produced cytokine mass (ng/ml range; >90%) while cytokines with local efficacy occur only at clearly inferior concentrations (pg/ml-range). Cytokines associated to the activation of Th2 cells (IL-4 and IL-5) were not detected in the basolateral medium at all. A schematic overview of the time kinetics of the investigated cytokines is given in Figure 2.

To further elucidate the differences of the present model as compared to models of leukocyte challenge by direct contact with bacteria, further experiments were performed. Keeping the number of leukocytes constant, the direct cell-cell challenge with bacteria (*E. coli* K12) without Caco-2 cells resulted in the production of about 12,000 to 20,000 pg/ml TNF-α after 16 h. The

presence of soluble factors produced by the differentiated Caco-2 cells reduced this production by about 45%. The spatial separation of the bacteria from the leukocytes by the semi-permeable membrane only reduced the TNF-α production by about 80%. Separation of leukocytes and bacteria by the semi-permeable membrane and the differentiated, confluent Caco-2 cell monolayer reduced the TNF-α synthesis to about 80 to 300 pg/ml (~1 to 2%), which was still significantly higher than the value resulting from the incubation of Caco-2 cells and leukocytes solely (2-30 pg/ml). Evidently, investigations on effects of e.g. nutrients on immunomodulation in the intestine with the present model are performed in a completely different inflammatory 'range' than those where leukocytes are challenged directly by bacteria or bacterial products.

In conclusion, from the primary experimental results obtained with the described co-culture model it can be seen suitable for the assessment of immunomodulation near the intestinal mucosa by food ingredients, drugs, and different bacteria species.

LITERATURE

Abreu-Martin, M.T. and Targan, S.R.: Regulation of immune responses of the intestinal mucosa. Crit. Rev. Immunol. 16, 277-309 (1996).

Ebert, E.C.: Proliferative responses of human intraepithelial lymphocytes to various T-cell stimuli. Gastroenterology 97, 1372-1381 (1989).

Haller, D., Bode, C., Hammes, W.P., Pfeifer, A.M., Schiffrin, E.J., and Blum, S.: Non-pathogenic bacteria elicit a differential cytokine response by intestinal epithelial cell/leucocyte co-cultures. Gut 47, 79-87 (2000).

Hidalgo, I.J., Raub, T.J., and Borchardt, R.T.: Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. Gastroenterology 96, 736-749 (1989).

Jung, H.C., Eckmann, L., Yang, S.K., Panja, A., Fierer, J., Morzycka-Wroblewska, E., and Kagnoff, M.F.: A distinct array of proinflammatory cytokines is expressed in human colon epithelial cells in response to bacterial invasion. J. Clin. Invest. 95, 55-65 (1995).

Latthe, M., Terry, L., and MacDonald, T.T.: High frequency of CD8 alpha alpha homodimer-bearing T cells in human fetal intestine. Eur. J. Immunol. 24, 1703-1705 (1994).

Lebrec, H., Roger, R., Blot, C., Burleson, G.R., Bohuon, C., and Pallardy, M.: Immunotoxicological investigation using pharmaceutical drugs. *In vitro* evaluation of immune effects using rodent or human immune cells. Toxicology 96, 147-156 (1995).

Parlesak, A., Diedrich, J.P., Schäfer, C., and Bode, C.: A low concentration of ethanol reduces the chemiluminescence of human granulocytes and monocytes but not the tumor necrosis factor alpha production by monocytes after endotoxin stimulation. Infect. Immun. 66, 2809-2813 (1998).

Perdigon, G., Alvarez, S., Pesce de Ruiz Holgado, A.: 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).

Pinto, M., Robine-Leon, S., Appay, M.D., Kedinger, M., Triadou, N., Dussaulx, E., Lacroix, B., Simon-Assmann, P., Haffen, K., Fogh, J., and Zweibaum, A.: Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. Biol. Cell 47, 323-376 (1983).

Viney, J., MacDonald, T.T., and Spencer, J.: Gamma/delta T cells in the gut epithelium. Gut 31, 841-844 (1990).

Weintraub, B.C., Eckmann, L., Okamoto, S., Hense, M., Hedrick, S.M., and Fierer, J.: Role of alphabeta and gammadelta T cells in the host response to *Salmonella* infection as demonstrated in T-cell-receptor-deficient mice of defined Ity genotypes. Infect. Immun. 65, 2306-2312 (1997).