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MOLECULAR BASIS FOR THE CROSS TALK BETWEEN PATHOGENS, INTESTINAL CELLS, AND THE GUT MICROFLORA

GERMAIN TRUGNAN, MIGUEL FREITAS, AND CATHERINE SAPIN

INSERM unit 538, Université Pierre et Marie Curie,
Faculty of Medicine Saint-Antoine, Paris, France

SUMMARY

A rational and logical use of probiotic for therapeutic or nutritional tasks require a better understanding of the language used by bacteria and host cells to communicate. This cross-talk is mainly based on the exchange of molecules in both directions that control crucial metabolic steps. To start to learn this language we analysed, as an example, the glycosylation process in intestinal cells living in the presence of soluble factors produced by a bacterial species from the microflora, namely *Bacteroides thetaiotaomicron*. *In vitro* experiments carried out on the intestinal cell line HT-29 indicated that these bacteria communicate with their target cells through a remote control process that was able to modulate surface galactosylation. We demonstrated that this control was at a post-translational level. *In vivo* experiments confirmed that a similar process should be at work in mice. Germfree (GF) mice feed with the soluble factor, or mono-contaminated with the corresponding live bacteria or with a complete microflora were shown to change the surface glycosylation pattern of intestinal cells in a species-specific, segment-specific and cell-type specific manner. These data open a new window to analyse the molecular bases for the cross talk between eucaryotic and prokaryotic worlds.

INTRODUCTION

Bacteria that reside in the gut are well know to exert several effects that are good for health, either in maintaining a favourable balance or in helping to fight against diseases. Although these beneficial effects have been recognised for a long time, their mechanisms remain mainly unknown. Numerous studies have attempted to understand how gut microflora works. Based on the research of some groups, including our Unit, we will review here some of the lines of research that are currently followed to use microflora more rationally and in a more efficient way. We will first summarise the available data that accurately describe the cross talk between host cells and microflora. We will then more precisely focus on one topic where microflora is expected to play an important role, namely infectious diseases.

The cross talk between the intestinal barrier and gut microflora seems to involve various levels of regulation. During these “discussions”, either some

individual bacterial species and/or microflora as whole and/or specific cell types from the gut may change their "point de vue". Physical contact between host cells and bacteria is not obligatory and it is now recognised that a new family of molecules, the modulins, should play a key role by mediating a remote control on the cross talk. Recent data suggest that the remote control of host cells by bacteria or of bacteria by intestinal cells may concern the whole range of biologically active molecules. This means that transcriptional, post-transcriptional, translational and/or post-translational control of bacteria or cell functions may be involved.

We have been interested for several years in the regulation of intestinal cell functions. We have developed several cell models that have allowed us to start to understand some of the molecular mechanisms that stay behind the cross talk. Human intestinal cultured cells represent a vast repertoire of phenotypes in which it is possible to select a set of specific intestinal functions. We have also developed experimental systems in which we have been able to check *in vitro* the effects of whole bacteria or of specific components of the microflora. In vivo experiments, using the huge potential of gnotobiotic animals, have been also developed.

The ways by which pathogens invade intestinal cells are numerous and pathogens have learned a lot on mammalian cell biology to overcome the natural defence of the host. Microflora also interfere with pathogen-host cells cross talk. Although a number of distinct processes are here at work, it is clear that some common themes emerge in the complex cross talk between pathogens, microflora and the gut. We were especially interested by one of these themes, namely the role of host glycosylation processes in the modulation of pathogen entry. Using first an *in vitro* approach, we identified a new "modulin", produced by a major component of the microflora, namely *Bacteroides thetaiotaomicron* (BETIM) that was able to specifically modulate a galactosylation process in a model of goblet cells. We have also shown that the modulin was active through a post-translational event in which the activity of galactosyltransferase was up-regulated with no change at the trancriptional level. Further we demonstrated that this up-regulation was associated with an increased capacity to resist rotavirus infection. *In vivo* experiments have demonstrated that modulin, individual bacteria or the whole microflora were also able to modulate intestinal cell glycosylation with a species-specific, cell type-specific and tissue-specific pattern.

THE CROSS TALK BETWEEN THE INTESTINAL BARRIER AND GUT MICROFLORA INVOLVES VARIOUS LEVELS OF REGULATION

Since Metschnikoff discovered that bacteria might have beneficial effects, the mechanisms by which microflora and probiotics exert their effects are still a matter of intense research and generate a huge amount of debate. For example it is still unclear whether some effects require that bacteria remain live or not and which fraction contains the activity. Another still unresolved question is to know whether the bacteria must maintain a tight contact with the mucosa or not. These questions are however highly relevant to define a logical approach for a clinical and/or a nutritional use of microflora-derived components.

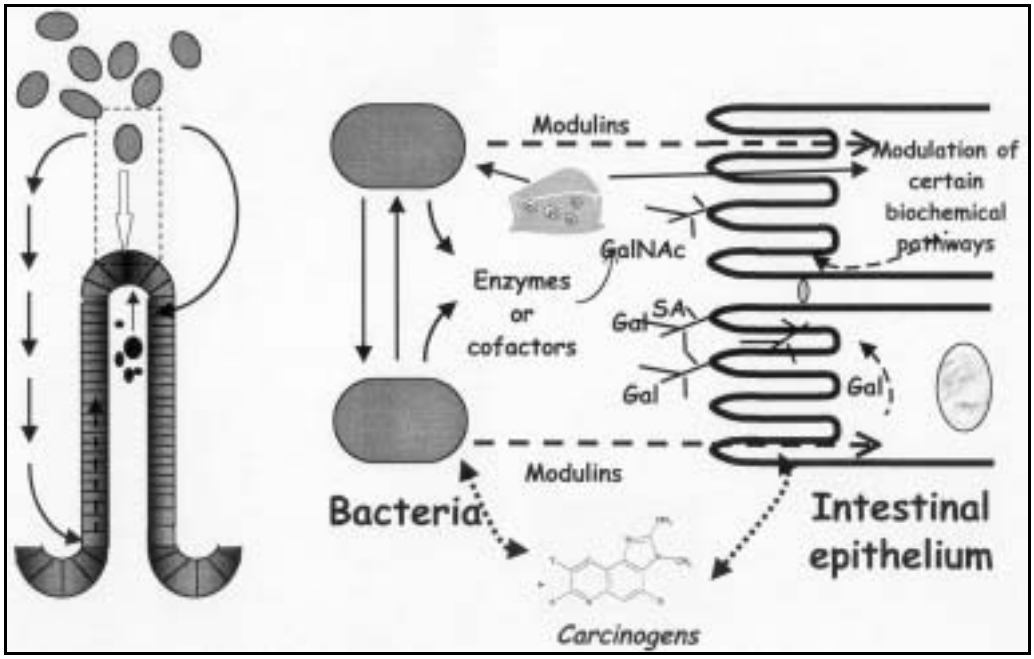

Figure 1: Host-microflora cross-talk, an overview.

Since few years, however, some works have started to exploit the revolution of genomics, proteomics and cell biology. These studies strongly suggest that besides effects mediated by the "mechanical" contact between bacteria and their host cells, the cross talk between the eukaryotic and prokaryotic worlds may be mediated through a remote control process that use soluble molecules produced by either of or the two partners (Figure 1). The concept of modulins emerges from these studies and should be central in the next few years to understand the molecular bases of the cross talk. Modulins should define those molecules produced by resident bacteria able to modulate the host cell function or to promote their cross-talk with bacteria. This functional definition doesn't tell us anything on the molecular nature of these modulins. Efforts have to be made in the next future to carefully define the chemical composition of these molecules and to know whether they belong to one or more families.

From these first studies it appears that several levels of control may be involved in the cross-talk control. As summarised in Figure 2, if we only consider the way by which bacteria may communicate with intestinal cells, three main mechanisms have to be discussed. Bacteria produce numerous compounds, including enzymes that may act directly on the surface of epithelial cells to modify their biochemical composition and/or biophysical properties. The knowledge of the full genome of an increasing number of bacteria will certainly help to define and classify these compounds. Among the bacterial enzymes, some are known for a long time and have been used to characterise the bacterial species. One should now consider how these enzymes might modulate the host cell surface. It is also clear now that bacteria produce other compounds that enter target cells or are incorporated in their membranes where they will exert at least two types of

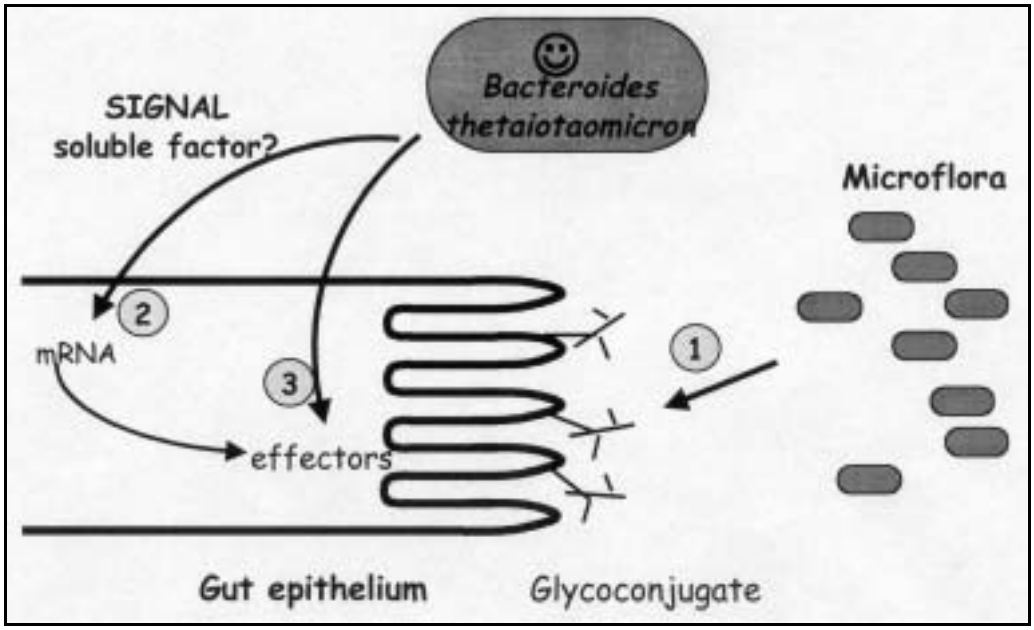

Figure 2: Putative regulation levels of the cross-talk.

effects. One is a transcriptional effect that will perturb protein biosynthesis of the host cells. In a recent paper the micro-array technology has been used to start to define the pathways and molecules whose biosynthesis may be transcriptionaly controlled. Interestingly this concern almost all the mammalian functions thus indicating that the final phenotype of mammalian cells strongly depends on the presence of the microflora. The other type of effect is a post-translational effect that will interfere with the maturation and the intracellular trafficking of another set of glycoproteins. This point is less documented and we have started to focus our research on a particular cell function, namely protein and lipid glycosylation to know more about this regulation level.

NEW EXPERIMENTAL MODELS EXPLOIT THE PROPERTIES OF CULTURED CELL LINES AND GNOTOBIOTIC ANIMALS

The cellular glycosylation process is the main post-translational event. It is of crucial importance because it concerns more than 80% of the proteins synthesised by a cell. Glycosylation will add to the complexity of the molecule, will change the capacities of the modified proteins to interact with other molecules, with cellular structures and with pathogens or microflora. For example, mucus is mostly composed of highly glycosylated proteins and mucus biophysical properties will depend on the nature of the glycans.

A survey of the literature recently published on the interactions between pathogens, microflora and intestinal cells surprisingly indicate that data derived from *in vitro* and *in vivo* experiments do not provide concordant results. These discrepancies concern both the nature of the sugars involved in the interactions and the effect of the bacteria on the sugar composition of the target

Table 1: Carbohydrates specificity of several pathogenic and non-pathogenic bacteria and viruses

Bacterial species	Carbohydrate	Reference
Clostridium difficile	Galα(1-3), Galβ(1-4)	*Krivan* et al., 1986
Entamoeba histolytica	Galactose	*Chadre* et al., 1988
Escherichia coli	Galactose, NeuNAc	*Mouricout*, 1987
Fusobacterium nucleatum FN-2	Galactose	*Shanitzki* et al., 1997
Helicobacter pylori	Fucose	*Guruge* et al., 1998
Lactobacillus acidophilus	Carbohydrates	*Greene* and *Klae*, 1994
Lactobacillus casei	galactose	*Yamamoto* et al., 1996
Lactobacillus fermentum	Carbohydrates	*Conway* and *Kjelleberg*, 1989
Lactobacillus plantarum	Mannose	*Adlerberth* et al., 1996
Lactobacillus reuteri	β-Galactose	*Mukai* et al., 1998
Listeria monocytogenes	NeuNAc	*Maganti* et al., 1998
Listeria monocytogenes	Galactose	*Cowart* et al., 1990
Propionibacterium	Gal(β1-4)Glc	*Pulverer* et al., 1994
Pseudomonas aeruginosa	Galactose, Fucose	*Gilboa-Garber* et al., 1994
Salmonella	Mannose	*Lee* et al., 1996
Streptococcus pyogenes	Galactose	*Kumar* et al., 1996
Vibrio cholerae	Fucose	*Gardel* and *Mekalanos*, 1996
Rotavirus	Sialic acid	*Dai* et al., 2000

cells. Therefore it is now needed to develop model systems both *in vitro* and *in vivo* that will allow to directly compare the effect of a given species or of a mix of endogenous bacteria or probiotics.

We have been involved since several years in the production and characterisation of *in vitro* models that reproduce part of the differentiated phenotypes of normal intestinal cells. Several cultured intestinal cell lines may be used to mimic a given phenotype such as goblet cells or enterocytes. For example the HT-29 cell line which display a mostly undifferentiated phenotype when grown in standard conditions may differentiate either in enterocytes or in goblet cells depending on the culture conditions. Other cell lines will develop a well differentiated enterocytic phenotype when grown in standard conditions, whereas some others will display a phenotype that resemble colonocytes in that they will be polarised but they will not express brush border enzyme activities. Interestingly, it has not been possible until now to induce a cell line to differentiate into a Paneth cell phenotype. It should be noted however that all cell lines used in these studies derived from human colon cancer cells, since until now experiments using normal intestinal cells over long period of time have been unsuccessful.

In the meantime, several laboratories have developed relevant *in vivo* models. Gnotobiotic animals, especially mice represent the most used model for studies on the interactions between bacteria and intestinal cells. Several studies have been published using these models but it should be mentioned that a full characterisation of the properties of these animals is still needed. For example the glycosylation pattern of the gut of germ-free mice was unknown since a very recent period. Several questions remain to know whether gnotobiotic animal may mimic all the functions of normal animals.

MICROFLORA, PROBIOTICS AND INFECTIOUS DISEASES: *IN VITRO* AND *IN VIVO* EXPERIMENTS POINTS TO A MAJOR ROLE FOR HOST CELL GLYCOSYLATION

Numerous pathogens as well as bacterial species from the microflora interfere with the host cells through specific glycans. It is also thought that bacteria may modify the glycosylation pattern of the host cells. We therefore elaborate a strategy to know whether it will be possible to manipulate the composition of intestinal cell glycosylation by using specific bacterial species in order to obtain a specific effect. To this end we used the mucus producing intestinal cell line, HT-29 MTX which have been previously characterised. We make this choice because these cells do express at reasonable levels a large repertoire of glycosyltransferases. We grow these cells in the presence of bacterial products. We selected *Bacteroides thetaiotaomicron* (BETIM) as a bacterial species for two reasons. The first one derived from the knowledge of the composition of the microflora that indicates that this bacterium is one of the most representative species in the colon. The second one is that this bacterium was previously used in *in vivo* experiments and we wanted to make comparison between the two systems. We also decided to study the effect of the products secreted by this bacteria rather than the whole bacteria because our task was to demonstrate a kind of remote control and also because we previously experienced that growing mammalian cells together with bacteria is possible but difficult and do not allow to check various conditions. We set up an experimental system in which HT-29 cells were grown for 2 weeks in the presence of a medium previously conditioned with BETIM. To explore the glycosylation pattern we used a panel of 10 lectins that recognise most of the peripheral sugars founds in mammalian glycoconjugates. These lectins were labelled with fluorescein in order to follow their distribution using immunofluorescence or to quantify their surface expression by using flow cytometry. We also used biotinylated lectins to identify the proteins that will express the different glycans by western blot. To summarise, we found that BETIM was able to specifically increase the level of expression of galactose with no modification of the other peripheral sugars. We further demonstrated that the soluble factor produced by BETIM was not a neuraminidase that may remove the peripheral sialic acid and expose more galactose. We have also shown that the soluble factor was unable to change the level of mRNA of the main galactosyltransferases, indicating that it was not acting at a transcriptional level. We finally demonstrated that the soluble factor was able to significantly increase the activity of galactosyltransferases. Together these results are the first to directly demonstrate that a soluble factor is able to modulate the activity of a glycosylation enzyme of the host cell through a remote control process. The exact nature of the soluble factor is currently under investigation. Preliminary results indicate that it is a small molecule (< 8kD), it is thermosensitive, and it is not a lipid.

Whether this soluble factor may also be active *in vivo* was investigated in a second series of experiments. We first established a baseline for the glycosylation pattern of GF mice, which was unknown. This was done by using the above-described strategy using fluorescent lectins. Each segment of the gut (duodenum, jejunum, caecum, ileum, colon) was analysed and within each segment we analysed the label of each

cell type (absorbing cells, mucus secreting cells, Paneth cells, crypt cells) both from a quantitative and qualitative point of view. We found that each lectin has a restricted distribution and that this distribution was dependent of the segment and the cell type considered. For example, Paneth cells were characterised by an intense label with WGA and DSA but the other lectins were not detected within this cell type. We also found that most of the lectins used labelled a well-defined compartment within each cell type. For example, WGA that mostly recognised α-GlcNac was essentially localised in the Golgi apparatus of enterocytes and labelled mature mucus vesicles, whereas GSI that mostly recognise Gal β1-3 labelled the Golgi apparatus and the membrane of immature mucus vesicles. Interestingly we found that this distribution of glycan expression was different in GF and conventional (CV) animals. However the differences were again restricted to some lectins. For example, WGA expression in enterocytes was reduced in CV mice compared to GF mice and the localisation of this lectin was also modified from a Golgi label in GF mice to a brush border label in CV mice. According to the lectin considered, we observed switch-on or switch-off of given sugar labels and also localisation changes.

Feeding animals with either the soluble factor of BETIM of live BETIM also promoted specific changes in lectin labelling. Interestingly in all the situations analysed the soluble factor and the live bacteria resulted in similar changes, except in two cases (expression of UEA fucose specific lectin in small intestine goblet cells and expression of RCA-I galactose specific lectin in large intestine goblet cells) where the soluble factor was unable to reproduce the effect of the live bacteria. This is interesting since in the work made by Bry in Gordon's lab, UEA was shown to be up-regulated in BETIM feeding animals (as in our hands) and since in our *in vitro* work we showed that RCA was up-regulated by the soluble factor, which is not the case *in vivo*. It is not clear however whether the active molecules present in the soluble factor may pass the stomach without being partially degraded. This also point to the fact that it should not be possible to extrapolate data obtained in the mice to human since it is well known that they do not display the same glycosylation capacities.

CONCLUSION AND PERSPECTIVES

Our results, together with those recently published by other groups indicate that we are just at the beginning of a new era where one may start to think to manipulate either the host or the microflora to interfere with their cross talk in a therapeutic or nutritional perspective. However, before being able to do so, several questions have to be answered.

As mentioned earlier in this review, the definition of the content of the new modulin family remains to be done. Are there related molecules, how many members, are there species specificities, are the compounds released constantly or in a regulated manner? Our *in vivo* experiments that demonstrated the induction of specific changes argue for the presence of a small number of factors involved in the control of host cell glycosylation, but this remain to be clearly established. The biochemical nature of the soluble factor also remained to be refined in order to further consider the possibility to use it as a

prebiotic. It is not know if there is a signalling pathway for the post-translational regulation we have identified similar to the one demonstrated for the fucose regulation at the transcriptional level as described by L. Hooper et al. Other studies will have to study the bioavailability of the soluble factor and particularly its resistance during the transit through the stomach. There is no data on the dose-response curves for these effects. There is no indication on the amount of bacteria needed to produce an active concentration of soluble factor, nor whether this is modulated by intra-intestinal process, such as the quorum sensing for example. Finally it is of crucial importance to develop challenge tests in order to clearly demonstrate that change in host cell glycosylation may be instrumental.

Much more work is now needed to answer these questions but it is now clear that we have a strategy to study the cross talk in a more logical way. Whether this will help us to define a new therapeutic or nutritional strategy remain to be clarified.

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