TITLE : INTERACTIONS BETWEEN INSECTICIDAL CRY TOXINS AND THE DIGESTIVE TRACT MUCUS

Programme : Risk OGM 2010

Synthesis

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State of the art and objectives

Scientific data and the public concerns showed (in 2010-2011) the need for the evaluation of new risks associated with consumption of insecticidal Cry toxin from *Bacillus thuringiensis*, expressed in certain genetically modified plants (PGM). This project is specifically dealing with case of the Cry1Ab toxin expressed in the Corn BtMon 810 event. The project was based on results from the partners (Nielsen-Leroux and al. INRA-Micalis) of this study. We had indeed shown, in an unexpected way, that these toxins could bind and degrade the peritrophic matrix (PM) (a tube like structure rich in mucus, which protects the insect intestine). Being given the strong homology between certain components of the mucus of invertebrates and vertebrate, in particular the glycosylated mucins proteins, it was important to acquire precise knowledge on this capacity of the Cry toxins to fix and degrade the mucus of the digestive tract of the insect and to analyze if this interaction is also effective with mucus from vertebrate ones (mammals). The objective was, on the one hand, to understand if the analyses of risk must be adjusted to take into account the new properties of the Cry toxins, on the other hand, to identify the new projects to be realized to look further into the study of the interactions between the Cry toxins and the components of the mucus of various origins. The challenge of this research is thus to evaluate if these insecticidal toxins get an effect (positive or negative) on the cells with mucus and/or mucus itself, in order to get information new on the impact of these plants GMO on the target and non-target organisms.

Methodologies:

We characterized the Cry-Mucus interactions using the purified and proteolysis activated 66 kDa Cry1Ab toxin, (similar to that expressed in Bt Mon810). The toxin is produced either by at *E. coli* or *Bacillus thuringiensis*. Monoclonal Cry1Ab antibodies and different lectines and sugars were used to measure the interaction and specificity by immuno test (ELISA). The search for target molecules was made by many biochemical techniques measuring protein-protein interactions (Ligand blot, pull-down, cross-country race-linking) by using toxin labelled by the biotin, by fluorophores or by immuno-detection). The Cry1Ab toxin was tested on various mucus: peritrophic matrices (PM) of the larval stage of the hive pest insect *Galleria mellonella* (the greater wax moth)), mucins of pig stomach (commercial), from human mucus producing epithelial cells and on the mucus isolated from intestines of mouse. Analyses on the effect of toxin were made *in vitro*: enzymatic tests, sugar release, measurements of rheology and *in vivo*: Degradation of the peritrophic matrix, responses of toxicity/stress on cell-culture, histo-pathological effects on intestinal cells of mouse. Oral gavages' of toxin were carried out in the BalbC mouse over 28 days in order to analyze this interaction on the total and specific immunizing responses to the Cry toxins. Finally larvae of *G.mellonella* having consumed MON810 leaves were analyzed on the level of the PM.

Major results, their impact and continuation:

1. Effects Cry1Ab on the peritrophic matrix of the G.melonella insect

The insect *G. mellonella* is used as model infection to analyze the role of bacterium (BT) because this insect is not sensitive to the Cry1Ca toxin. Indeed, in strong amounts, the caterpillars do not die, on the other hand one observes the degradation of the peritrophic matrix. We had tested if that is the case for Cry1Ab. This toxin in high concentrations is not either toxic alone, and it also weakens the PM as well *in vivo* as *in vitro*. The toxin is detectable in the PM by a very sensitive system of immuno-detection. That shows that Cry1Ab can also recognize the mucus rich structures of the insect, it remains to see whether that is the case for other types of mucus too (see below). In addition, our results showing the capacity of Cry toxins to fragile the peritrophic matrix is of novelty.

2. Analysis with Corn leaves BT Mon810 in the insect

In the project it was planned to purify the Cry1Ab toxin of the Corn sheets My 810: that was not possible because the quantity of toxin present in the plants is very weak (about 10 µg/g of fresh sheet). Nevertheless we tested the leaves of Mon810 and the parental Iso-génique line on the larvae of *G. mellonella* in order to visualize the effect on the PM. No difference on the PM, or the health of the caterpillars, was observed with the Mon810 leaves. For further studies It would be interesting and certainly easier to produce the form present in the plants (Mon810) in another eucaryote expression system in order to work with a pure protein nearest possible to that presents in the plant.

3. The Cry1Ab toxin binds to all the types of mucus's tested:

In vitro, the Cry1Ab toxin interacts with the peritrophic matrix, the intestinal mucus of mouse and gastric mucins of pig. The signal (immuno-detection) increases with the concentration of mucus's (protein) until reaching saturation towards 5µg/ml samples immobilized on multititer plates. These results suggest the existence of specific sites of interaction of toxin within these mucus's. The parallelism observed between the data of the interactions mucus-Cry1Ab and those of the highly specific interaction of positive control mAb120-Cry1Ab confirms this assumption. Under these conditions, Cry1Ab thus has certain affinity for components of all studied mucus's.

4. Cry1AB domain involved in mucus binding :

Several anti Cry 1Ab mAb could be identified as inhibiting the bond toxin – mucus in particular the mAb104, which almost completely inhibits this binding, with the various mucus's tested. Others mAb have an effect depending on the type of mucus. It is the case of the mAb23, which partly inhibits the binding of toxin to the mucus of mouse (and to a lesser extent with mucins of pig) but not with the PM of insect. This result suggests the existence of particular interaction domains, which differs slightly according to the type of mucus considered. Several approaches were used in order to determine the part of toxin implied in the binding. The domain recognized by the mAb104 is probably localised in the C-final part of the Cry1Ab toxin between amino acid I466 and L617, but of other analyses will be necessary to confirm these data.

5. Roles of the binding to the glycosylated components

Mucuses are rich in glycoproteins (eg. the mucins) and in the insect the peritrophine proteins (components of the PM) are also glycosylated. In order to check if this part would be envolved in toxin binding, competition/inhibition experiments were set up. However, with the toxin concentrations used, it was not possible to inhibit the binding by lectines nor by amino sugars. Even the deglycosylation of mucus's did not affect the binding of Cry1Ab. These results are different from those observed previously for Cry1Ac (Valaitis et al.; 2013). In addition we did not observe any structure change of pig stomach mucins in the presence of Cry1Ab toxin.

6. Potential targets of Cry1Ab in mucus

Various approaches (protein-protein interaction by ligand-blot, Pull-down followed by mass spectrometry analyses) were used in order to identify molecules present in the mucus involved in the binding of the Cry1Ab toxin. The results highlighted several candidates indicating that not only the mucus glycoproteines are target molecules but also molecules which are adsorbed there. In order to determine the relative importance of these molecules other analyses are needed.

7. Effect of Cry1Ab on mammal intestinal cell-cultures

By using mammal mucus producing intestinal cell lines which also were transformed with repoter systems, we measured the effect of two Cry toxins on the cellular viability and the production of mucins, and used cell responding signal pathways namely the NF-KB and and immune sensors TGFβ1 and IDO-1. We showed that Cry1Ab and Cry1Ca did not affect the viability of the cells, nor the immunizing responses or production of mucins of the epithelial HT29 cell line .

8. Effects Cry1Ab *in vivo* in the Mouse

After 28 days of intra-gastric gavage (100 µg per day) no histo-pathological differences (effects on cells, layer of mucus) were observed between the sections of mice intestines having received toxin and those of the controls. On the other hand a specific humoral respone was obvious in the mice having received toxin. Indeed, a production of specific IgG1 and IgG2a to Cry1Ab is observed. On the other hand, no specific IgE (allergenic) is detected under these conditions. These *in vivo* results highlight a certain immunogenicity of Cry1Ab in the mouse (an already reported effect) and thus no negative effect on these BalbC mice.

Perspective conclusions:
The results of this project progress to the knowledge on the specificity of the Cry toxins of *B. thuringiensis* and also answer questions related to the risk assessments' to the PGM. Our approaches are innovative; to our knowledge, it is the first project, which focused specifically on the interactions between Cry toxin and the mucus of the digestive tract of various organisms (Invertebrate and mammals). The results show that the Cry1Ab toxin does not bind only to the insect mucus but of vertebrate also, thus questioning the known specificity of these toxins.
However, it is necessary to distinguish the effect of lysis and intestinal cell death of the target insects and the binding of Cry1Ab to various mucus's tested during the project. Therefore, on the basis of our analysis, one cannot regard the mucus binding as a risk. In order to understand these results, more research it is necessary in several directions. It would be for example interesting to analyze the effect of a Cry-toxin food on the intestinal microbiote of a mammalian model and on target and non-target insects. It seems also important to understand the relative importance of the glycosyl "caps" of these glycoproteines on a larger part of Cry toxins. Our results also suggest that it would be appropriated to take into account these new properties of the Cry toxins to bind to mucus rich substances during the evaluation of the risks related on the PGM expressing a larger number of cry Toxins (known as pyramidal) in plants GMO and in particular with respect to the possible combinative effects. In conclusions, similar studies and other approaches are necessary to evaluate whether the Cry-Mucus binding is a risk or to the contrary a positive effect on non-target species. Finally, it is important to recall that the approaches (eg. Toxin doses and tissue) used in this study are different from those found in real Cry-food containing ingestions by man and animals.

Presentations & publications:
The project has been presented to the international congress of the "Society for Invertebrate Pathology" (SIP 2014) in Mainz (Germany) and during a special meeting at the INRA to Jouy-in-Josas in December 2014. It was presented, on invitation, at the biological control conference with (SICONBIOL 2015) in Rio, in Brazil, in June 2015 and the subject was selected for oral presentation at the time of the international Congress Bacillus-ACT with New Delhi, India, in October 2015. An article based on the most bearing results on the capacity of Cry1Ab to bind to several types of mucus is in the course of drafting and it will be submitted to a good level of impact journal. A second articlewill focus on the studies on the insect with Cry1Ca is also in the course of drafting, and finally we program a more descriptive article concerning all the analyses (biochemical) in relation to the peritrophic matrix of *Galleria mellonella* in order to use many data gathered at the time of the project but which will not appear in the principal publication.