Vitenskapskomiteen for mattrygghet
Norwegian Scientific Committee for Food Safety

Summary of the health risk assessment of the adjuvant effects of Cry proteins from genetically modified plants used in food and fodder

Opinion of the Panel on Genetically Modified Organism of the Norwegian Scientific Committee for Food Safety

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Persons working for VKM, either as appointed members of the Committee or as ad hoc experts, do this by virtue of their scientific expertise, not as representatives for their employers. The Civil Services Act instructions on legal competence apply for all work prepared by VKM.

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Summary

Background

In 2000, the "novel food" group of the former Norwegian Food Control Authority, pointed out a possible allergenic risk from foods based on genetically modified plants (GM plants) which had been inserted with genes from *Bacillus thuringiensis* (*Bt*) that express Cry proteins (Bt toxins). These Cry proteins might act as immunological adjuvants (see chapter "Adjuvant effects"), thereby promoting immune responses to other components (allergens) in the food. In a letter dated 06.12.2011, the Norwegian Food Safety Authority commissioned VKM to conduct a brief health risk assessment of food and feed based on GM plants in which genes from *Bt* that express Cry proteins have been inserted. The risk assessment is enclosed.

Cry proteins

Cry proteins (Bt toxins) are crystalline toxins that are synthesised by the Gram-positive spore forming bacterium *Bt*. More than 500 different *cry* gene sequences have been identified to date, and these can be classified into 67 different groups of Cry proteins. At the amino acid level, these proteins can differ considerably but related active domains exist. Pesticides containing *Bt* are used in both organic and conventional farming. Several GM food plants and fodder plants have one or more genes encoding Bt toxins inserted into their genomes to make the plants resistant to insect attack. Cry proteins currently used in GM plants have been investigated in a range of standardised (OECD) tests and have not been found to be acutely toxic to mammals.

Maize MON810 containing the Cry protein Cry1Ab has been used in Norwegian feeding trials of Atlantic salmon (Sanden et al. 2005, 2006; Hemre et al. 2007; Sagstad et al. 2007; Bakke-McKellep et al. 2008; Frøystad-Saugen et al. 2009; Sissener et al. 2010). Small alterations between fish fed with GM maize and fed with unmodified maize were demonstrated, but the changes detected were not dose related (Sissener et al. 2011a). In one study, more granulocytes were detected in the blood of salmon fed with GM maize than salmon fed with unmodified maize. The changes were related to mild cellular stress response (Frøystad-Saugen et al. 2009). In a recent study conducted by Sissener et al. (2011b), the mycotoxin dioxynivalenol (DON) was detected in MON810 in quantities of 90 µg/kg, but was not detected in the unmodified maize (that is, occurred below 2.5 µg/ kg). The effects seen in salmon fed with MON810 seem to be more related to the content of DON than to demonstrated differences for other metabolites (Sissener et al. 2011b).

Adjuvant effects

An adjuvant is defined as a substance that stimulates the immune response against an antigen/allergen that is administered simultaneously ("bystander effect"), and can sometimes even act as an antigen itself. The biological mechanisms underlying the adjuvant effect are only partially understood and can be outlined as follows:

(a) Transport effect (adjuvants can prevent degradation of the antigen and transport it to a suitable location for immune stimulation). This includes mechanisms that allow the antigen to cross the epithelial barrier.

(b) Depot effect (adjuvants may prevent the rapid removal of antigen, bind it, and release it slowly, so that the immune system is stimulated over a prolonged period).

(c) Irritation and stimulation effect (increase the influx of immune cells to the immunisation site and activate the cells to respond, both antigen-presenting cells (APCs) and lymphocytes). These properties will provide the necessary additional signal ("danger signal") that is required by the immune system to react against an antigen/allergen (rather than being down-regulated).

In this assessment, we have specifically considered the possible adjuvant effects of Cry proteins in connection with the mucosa (mucosal adjuvant), as increased permeability of the epithelium may be very important.

Only two of the 10 Cry proteins that are currently used in genetically modified plants, Cry1Ab and Cry1Ac, have been studied experimentally regarding adjuvant effects. Therefore, this risk assessment is based upon immunological observations from these studies. To the knowledge of the Panel, adjuvant effects have not been investigated for the other eight Cry proteins used in GM plants, or for other groups of Cry proteins.

Animal studies have shown that the Cry1Ac protein binds to the surface of the mouse gut and induces immunological reactions against itself and against proteins administered simultaneously (Vázquez-Padrón et al. 2000 a, b, Vázquez-Padrón et al. 1999; Moreno-Fierros et al. 2003; Rojas-Hernandez et al. 2004).

Furthermore, immunological mapping of the systemic and mucosal immune responses to Cry1Ac has shown that mice produce both systemic IgM and IgG and secretory IgA following intraperitonal and intragastric immunisation. In a mouse study, the adjuvant effect of Cry1Ac was found to be as strong as the effect of cholera toxin (CT) (Vazquez et al. 1999). The adjuvant effect of CT is thus a relevant basis for comparison in a risk assessment of Cry1Ac. It is uncertain whether this likewise applies to Cry1Ab.

Cry1Ab studies in animals have been examined with regard to allergic sensitization against peanut extract. In these experiments, IgE antibody was not induced by oral sensitization, whereas CT provided effective stimulation of the IgE response. However, Cry1Ab had a significant effect on the production/release of leukotrienes C4 and E4 and on the influx of eosinophils, indicating that an immune response had been triggered (Guimaraes et al. 2008).

It is important to emphasise that only a limited number of publications from a few research groups are dealing with the adjuvant effects of these two Cry proteins.

"Bystander" sensitization

"Bystander" sensitization can occur when an adjuvant in food, or an immune response against a food antigen, results in an increase in the permeability of the intestinal epithelium for other components in food. Previously it was assumed that the epithelial cells of the intestine were permanently "glued together" by the so-called "tight junctions". More recent knowledge shows that these complex protein structures are dynamic and can be opened up by different stimuli.

Both *in vitro* and *in vivo* experiments have demonstrated that when a potentially proinflammatory IgG response (which for instance may result in complement activation) is not balanced by a non-inflammatory IgA response, the epithelial barrier becomes leaky; unwanted proteins are then able to enter the body ("bystander" penetration) and cause immunological sensitization (Brandtzaeg & Tolo 1977, Lim & Rowley 1982).

In 2005, an Australian study in mice demonstrated that feeding with pea seed meal containing a transgenic bean protein, alpha-amylase inhibitor, induced an IgG response that opened up the barrier in the intestinal lining – thereby allowing a "bystander" protein (ovalbumin) to leak in, with subsequent indications of airway hypersensitivity (Prescott et al. 2005). This effect was considered to be caused by post-translational modification of the non-native transgenic alpha-amylase inhibitor, including glycosylation, in the new host plant. Induction of IgG antibodies to the relevant food proteins, and possible cross-sensitization to food antigens presented simultaneously at mucosal surfaces, could thus result in adverse biological effects.

Risk characterisation

The hazard associated with immunological sensitization to antigens in food products is the development of food allergies. This is a serious health problem in industrialised countries. In Europe it may affect up to 2% of the adult population and 6-8% of the children (Sicherer 2003; Wuthrich 2000). Food allergies may manifest themselves in a variety of ways. The diagnosis is not always clear and it may be difficult to identify causalities. Acute and severe allergic reactions are IgE mediated and it may be necessary that affected persons always carry an "adrenaline pen" for injection in case of an anaphylactic shock. Allergies to milk and eggs often become apparent at a young age, and approximately 40% of cow's milk allergies do not seem to be mediated by IgE. Other kinds of food allergies may appear both in childhood and adulthood, such as the IgE-mediated reactions to nuts and fish. Such allergic reactions may be quite severe and in some individuals the symptoms are triggered just by the smell of fish or from the dust of nuts or flour. IgE-mediated allergy to fish and nuts is primarily considered as a persistent problem, while children often grow out of an allergy to cow's milk. One serious complication is that about 15% of the IgE-mediated food allergies develop into a so-called "allergic march" - that is, patients develop respiratory allergies and may end up as asthmatics.

Exposure via the digestive system

The concentrations of Cry proteins in GM plants are much higher than in conventional foods, even if these have been sprayed with *Bt* pesticide for insect control. Sources of human exposure to Cry proteins are via naturally occurring *Bt* in soil residues on foods, via inhalation from the use of *Bt*-based pesticides, from residues of such products on plant-based foods such as tomatoes, and also from GM plants, mainly maize and soya, expressing Cry proteins. Exposure level, type, and duration will vary with these sources. Furthermore, exposure may vary according to the extent of processing and other treatments of various plant products before consumption. As maize is the most likely source of exposure, it is used as an example in the following text.

All maize is cooked or processed before it is used as food, and therefore VKM's Panel on Genetically Modified Organisms has not assessed the risk following ingestion of raw maize. Estimates of human consumption of maize products in Norway (Vikse 2008) provide a good basis for understanding the extent of possible exposure to plant-produced Cry proteins. By using theoretical calculations it can be shown that if the intake of sweet maize in Europe comes from GM maize with a content of approx. 2 ppm Cry protein (i.e. 2 µg/g) (Hicks 2008) then the intake of the general population can be estimated to about 14 µg Cry protein/kg body weight/day, and for children of six years or less, to approximately 22 µg/kg body weight/day, assuming that the Cry proteins are not denatured.

Due to the denaturation and degradation of Cry proteins during different types of processing, exposure to Cry proteins in processed maize is lower than in raw maize. For example, experiments have demonstrated that denatured Cry1Ab is rapidly digested in simulated intestinal juices (Okunuki et al. 2002).

Likewise, it has been documented that Cry1Ab protein is not stable above 100 °C (de Luis et al. 2009). However, the Panel has been unable to find documentation on the extent to which Cry1Ab is broken down by, for example, boiling of sweet maize for five minutes.

In some experiments with simulated gastric juice, native Cry1Ab was found to be more stable against degradation than previously thought (Guimaraes et al. 2010). However, in feeding experiments with pigs fed with Bt11 maize containing Cry1Ab, the level of Cry1Ab in the gut was found to be low (<3-300 ppb) (Walsh et al. 2011, Chowdhury et al. 2003).

Exposure via airways and skin

To the Panel's knowledge there are no data available on the importance of possible exposure to Cry proteins via the airways in individuals handling GM feed containing Cry proteins. However, there is information on the effects of exposure to pesticides containing *Bt* in greenhouses. These studies concluded that this route of exposure was not associated with respiratory symptoms, but that it could cause skin reactions (Bernstein et al. 1999). Food allergies can be induced both via the airways and the skin by exposure to allergens present in house dust, but a possible adjuvant effect of Cry proteins in this context has not been investigated.

Risk assessment

Risk is defined as the probability that an event will occur multiplied by the consequences of that event if it occurs.

Few studies have examined the possible adjuvant effects of the two Cry proteins investigated. As food in the human intestine will have a low content of Cry proteins, the likelihood is small that food based on GM plants containing Cry proteins will result in sensitization of a person with subsequent development of food allergy. In general, Cry proteins will be unstable because of processing, heat treatment, and the low pH (pH <2.0) in the stomach. It is therefore

difficult to quantify the risk. However, the possible consequence (that is, food allergy) may potentially be quite serious for the individuals affected.

Knowledge gaps

There are many knowledge gaps related to assessment of adjuvants. Most of the immunologic adjuvant experiments have been performed using Cry1Ac. Whether the other Cry proteins have similar adjuvant properties is unknown.

The quantities of Cry proteins in genetically modified maize and soya are marginal compared with the amounts of other adjuvants that are natural components of food. However, the extent to which these naturally occurring adjuvants and Cry proteins contribute to the development of allergies is largely unknown. Determination of their importance is hampered by the lack of validated methods for measuring adjuvant effects.

The possibility that Cry proteins might increase the permeability of the intestinal epithelium and thereby lead to "bystander" sensitization to strong allergens in the diet of genetically susceptible individuals cannot be completely excluded. This possibility could be explored in a relevant animal model.

One element of uncertainty in exposure assessment is the lack of knowledge concerning exposure via the respiratory tract and the skin, and also the lack of quantitative understanding of the relationship between the extent of exposure to an adjuvant and its effects in terms of development of allergies.

Conclusion

The sale and use of genetically modified food and feed products in Norway requires approval. The Panel has assessed the health risks associated with food and feed with genes encoding Cry proteins inserted into its DNA from a general perspective.

The risk assessment does not include genetically modified raw vegetables containing Cry proteins, because in Norway such plants (maize and soya) are processed before consumption, and unprosessed food and feed are therefore of no current relevance to our market. Thus, this assessment addresses only processed foods and feeds from plants containing Cry proteins.

Despite several uncertainties, the Panel concludes on the basis of current knowledge that it is very unlikely that the Cry proteins in food pose an increased health risk in the amounts that would be ingested by eating processed GM maize or soya, compared with eating food based on isogenic non-modified plants.

For animals, it would be reasonable to assume that a possible adjuvant effect is unlikely to affect an animal's health. It is also very unlikely that consumption of food from animals that have eaten feed containing Cry proteins, will exert adverse effects on human health.

The Panel has found no documented evidence for any effect of exposure to Cry proteins in dust from GM material by inhalation or via the skin, nor of any possible adjuvant effects from such exposure.

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